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REMEDIATION OF BIOFILMS IN BIODIESEL STORAGE TANKS

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14. ABSTRACT The efforts described in this final report were initiated under Task Order 0005 of contract number FA8650-13-D-6405 and then completed under Task Order 0001 of contract number FA8650-19-F-6990. The technical efforts reported herein were aimed at remediation of microbial growth in biodiesel storage systems. METSS evaluated processes for decontaminating fuel storage systems to dramatically slow the regrowth of biofilms; thereby, extending the time between tank cleanings and reducing the need for fuel system maintenance and repairs caused by biofilm formation and biofouling. Two processes were initially considered for bioremediation: thermal decontamination and chlorine dioxide (ClO ₂) fumigation. Although both methods were demonstrated to be effective in laboratory experiments, ClO ₂ was ultimately chosen as the best candidate based on the ease of implementation in a field environment. A field demonstration using a novel ClO ₂ fumigation system in a 25,000-gal fuel tank reduced biofilm concentrations by >99.999%. The decontamination system was also shown to be easy to set up, operate, and perform within 8-hours or less and, therefore, could be incorporated as part of the standard fuel tank cleaning and maintenance procedure as deemed necessary.					
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1.0 EXECUTIVE SUMMARY

Biodiesel is promoted in the United States and around the world as a more environmentally friendly alternative to traditional diesel fuel. The Air Force was an early adaptor of biodiesel among the Services; however, fuel managers found that biodiesel storage tanks required more frequent cleaning due to excessive microbial growth in the tanks that clogs filters and fouls sensors. While the environmental impact may be positive, the increased cost to maintain biodiesel storage system is undesirable and comes with increased risks of degraded engine performance downstream.

The technical efforts reported herein were aimed at remediation of microbial growth in biodiesel fuel storage tanks that adversely affects fuel systems in ground support equipment. The goal of this project was to develop a decontamination process that would enhance existing cleaning procedures by dramatically slowing the regrowth of biofilms in biodiesel fuel storage systems to extend the time between tank cleanings and reduce the need for fuel system maintenance and repairs caused by biofilm formation and biofouling. The current method used to remediate contaminated fuel tanks is to empty the tank, manually remove the bulk of the bioaccumulation (sometimes requiring shovels), then clean the tank with a combination of pressure washing and hand-scrubbing. This method is time consuming, labor intensive, generates large waste streams, and exposes personnel to hazardous materials in confined spaces. Although the tank may appear clean following this process, there are enough microorganisms present in the tank to re-seed the next batch of fuel when the tank is refilled. Within a matter of months, biodiesel storage tanks can re-accumulate enough biomass to negatively affect water and fuel probes, cause corrosion in the storage tank, clog filters downstream, and degrade vehicle fuel systems that receive contaminated fuel.

Two processes were initially considered for bioremediation: thermal decontamination and chlorine dioxide (ClO₂) fumigation. Thermal decontamination options included Hot Air Decontamination (HAD, elevated temperature at low humidity) and Biothermal Decontamination (BTD; elevated temperature and high humidity). BTD was developed to address decontamination of biological warfare agents (BWAs) on aircraft surfaces that are not compatible with more reactive decontaminants. ClO₂ is currently used in the food, agriculture, water, and medical industries for disinfection. These vapor-phase methods are particularly well suited to the problem at hand because they can fill any volume – the tank, valves, piping, transfer lines, etc., and do not leave a residue – resulting in maximum surface treatment with minimal labor. During this project, METSS worked with collaborators within AFRL/RXAS to bring together the best solution for bioremediation in fuel storage tanks based on existing decontamination processes and equipment.

The specific objectives of this study were met and included the following:

- Assessing the use of novel and developing thermal decontamination technologies as well as ClO₂ fumigation for the inactivation of microbial organisms that contribute to biofouling in biodiesel fuel storage systems,
- Developing, testing, and evaluating a decontamination system designed for full-scale remediation,
- Demonstrating the ability to employ a novel remediation technology on an operational fuel tank storage system.

Through this research, ClO₂ was chosen as the best option for remediation of biofilms in fuel storage tanks. Although thermal decontamination methods were effective, maintaining precise temperature and humidity parameters in a variety of fuel tank configurations (above ground/below ground, insulated/uninsulated, etc.) proved impractical. ClO₂, on the other hand, can be generated onsite in varied conditions and readily scaled to any volume. A prototype ClO₂ delivery system was demonstrated on a 25,000-gallon fuel storage tank at Wright Patterson Air Force Base (WPAFB). The novel ClO₂ fumigation system was shown to reduce biofilm concentrations by greater than (>) 99.999 percent (%), and there were no visual signs of degradation to the materials used in the fuel system. The decontamination system was also shown to be easy to set up, operate, and perform within 8 hours or less, and could be incorporated as part of the standard fuel tank cleaning and maintenance procedure as deemed necessary. The process can be performed by one person within an 8-hour work shift. Long-term benefits of the decontamination process (i.e., effects of preventing, limiting, or slowing the regrowth of biofilm in the decontaminated fuel system) could not be assessed as the fuel system was scheduled for demolition following the decontamination demonstration.

2.0 INTRODUCTION

The efforts described in this report were initiated under Task Order 0005 of contract number FA8650-13-D-6405 and then completed under Task Order 0001 of contract number FA8650-19-F-6990. Efforts under these task orders expanded upon the level of research provided to AFRL/711th HPW/RHB to evaluate methods of mitigating environmental hazards associated with aircraft sustainment. Specifically, the technical efforts reported herein were aimed at remediation of microbial growth in biodiesel storage systems that adversely affects fuel systems in ground support equipment. METSS evaluated processes for decontaminating fuel storage systems to significantly reduce the reoccurrence of heavy microbial growth, which is detrimental to fuel systems and dramatically increases the frequency of tank cleanings. The goal was to develop a decontamination process that would enhance existing cleaning procedures by dramatically slowing the regrowth of biofilms in biodiesel fuel storage systems to extend the time between tank cleanings and reduce the need for fuel system maintenance and repairs caused by biofilm formation and biofouling.

Microbial infestations impact the functionality of the fuel systems (clogged filters, fouled fuel probes, degraded fuel, accelerated corrosion) and the vehicles downstream (poor combustion, clogged fuel injectors, corrosion). Extended exposure to microbial contamination has even greater costs since multi-year exposure causes corrosion of tank materials. A study performed by AFRL/RXAS at multiple US Air Force bases has shown that current tank remediation procedures are insufficient at controlling microbial growth in fuel systems, with rapid and intrusive regrowth of biofilms occurring within 6 months after cleaning.¹ Traditionally, ground fuel storage tanks are maintained on a 7-8-year cleaning schedule to remove any accumulation of biomass or other debris in the tank. Even on that schedule, the accumulation after several years is usually not severe enough to interfere with fueling operations. In contrast, tanks containing 20% biodiesel in diesel (B20) must be cleaned as often as every 6-12 months. Within a matter of months, biodiesel storage tanks can accumulate enough biomass to negatively affect water and fuel probes and to cause corrosion. When pumped from the tanks, the biomass clogs filters in dispensing systems and is passed into ground vehicles where it can clog fuel filters, degrade the performance of the engine, and foster new growth within the vehicles themselves.

The primary bioremediation method used today requires manual cleaning with a combination of pressure washing and hand-scrubbing within the confined space of the fuel tank, which is time consuming, labor intensive, generates large waste streams, and exposes personnel to hazardous materials in confined spaces. The combined effect is a significant obstacle to maintaining fuel quality and security within the US Air Force. Most importantly, however, the current method of bioremediation has little effect on the rate of biomass regrowth in the future. Removing obvious biomass may remove the immediate threat to fuel quality, but current methods are incapable of removing 100% of the microbiology within the tank, leaving millions of microbes behind to repopulate the next batch of biodiesel. Even in areas where biodiesel is not directly implemented, the same infrastructure is often used to manipulate diesel and biodiesel fuels.

¹ B. Stamps, C. Bojanowski, C. Drake, H. Nunn, P. Lloyd, J. Floyd, K. Emmerich, A. Neal, W. Crookes-Goodson, and B. Stevenson. *In situ Linkage of Fungal and Bacterial Proliferation to Microbiologically Influenced Corrosion in B20 Biodiesel Storage Tanks*. Frontiers in Microbiology. Vol 111, Article 167, February 2020.

Therefore, biodiesel is being introduced into conventional fuel systems (up to 5% is acceptable), and higher levels of microbial growth in those systems will eventually follow.

Recent advancements for the decontamination of BWAs and common bioburdens like mold were considered under this program to prevent the rapid re-accumulation of biomass in biodiesel fuel tanks. Processes considered for bioremediation were HAD and/or BTD, and ClO₂ fumigation. Both HAD and BTD involve elevating the temperature of an environment for a prolonged period of time to kill microbes. They differ in that HAD uses low relative humidity (RH, less than (<) 10%) and BTD uses high RH (80-90%). BTD was developed to address decontamination of BWA on aircraft surfaces and can be adapted for other more innocuous biological contaminants like mold. An alternative technology for bioremediation is ClO₂ fumigation, which is currently used in the food, agriculture, water, and medical industries for disinfection. These vapor-phase methods are particularly well suited to the problem at hand because they can fill any volume – the tank, valves, piping, transfer lines, etc. – resulting in maximum surface treatment with minimal labor. During this project, METSS worked with collaborators within RXAS to bring together the best solution for bioremediation in fuel storage tanks based on existing decontamination processes and equipment.

The specific objectives of this study were to:

- Assess the use of thermal decontamination technologies and ClO₂ fumigation for the inactivation of microbial organisms that contribute to biofouling in biodiesel fuel storage systems
- Develop, test, and evaluate a decontamination system designed for full-scale remediation
- Demonstrate the ability to employ a novel remediation technology on an operational fuel tank storage system
- Assess long-term effects of the remediation process on biofilm regrowth

3.0 TECHNICAL EFFORTS

To meet the objectives of this study, the following technical efforts were accomplished. These efforts are described in detail in the subsequent sections.

1) Field Assessments

Biodiesel, diesel, and jet fuel samples were collected from multiple fuel storage systems at several military installations including locations in the Southeast (Florida, Mississippi, and Georgia) and Pacific Northwest (Washington and Oregon). Biofilm collected from the fuel systems was cultivated in the laboratory and used to study growth characteristics in biodiesel fuel. Fungal isolates predominant in contaminated fuel samples were isolated and subsequently used in a series of ClO₂ and HAD efficacy tests to identify a process to slow the accumulation of biomass growth in biodiesel fuel storage systems.

2) Fuel System Characterization

Fuel storage tank design and construction standards were reviewed to identify potential limitations of remediation technologies with respect to logistics, size, and material compatibility.

3) Remediation Technology Assessment

Disinfection technologies, HAD and ClO₂, were compared for their ability to be utilized for fuel tank remediation.

4) System Development and Mock-up Evaluations

Bench- and pilot-scale characterization and efficacy tests were conducted with down-selected technologies against common fuel tank microbial contaminants to ultimately identify a single remediation technology for further development and testing. This task resulted in the construction of a remediation system that could be used in the field demonstration.

A series of full-scale mock remediation tests were conducted to further assess the function and effectiveness of the remediation technology and make refinements in preparation for a field demonstration.

5) Field Demonstration

A field demonstration of the developed remediation technology was conducted on a full-scale, operational fuel storage tank system.

3.1 Site Visits and Fuel Sample Collection/Analysis

Over a one-year period, 36 samples of both B20 biodiesel (BDI) and Ultra Low Sulfur Diesel (ULSD) were obtained from the following locations: Keesler, Moody, Hurlburt, Eglin, Tyndall, Fairchild, Mountain Home, and McChord Air Force Bases; Portland and Gowen Field Air National Guard Bases; Fort Lewis; Naval Air Station Whidbey Island; and Naval Base Kitsap (Manchester Fuel Depot and Bangor Base). The sampling events were conducted over three site visits during which fuel samples (examples of fuel samples collected are shown in Figure 1) were collected from multiple fuel storage tanks and subsequently analyzed to characterize the

biofilm from various regions of the country. The goal was to determine if there were common microbes (bacteria or fungi) responsible for biofouling in the fuel storage systems and, subsequently, evaluate their resistance to various decontamination process. Efforts were primarily focused on biodiesel fuel systems; however, samples from diesel tanks and a limited number of Jet A fuel tanks were collected and analyzed as well.

During each visit, the fuel system maintenance manager and supporting maintenance personnel were interviewed to gather information about their fuel storage systems such as age, size, type of fuel system, fuel use rate, tank cleaning history, frequency of fuel filter changes, and other miscellaneous information pertaining to routine and non-routine maintenance performed on the fuel system, including the tank, associated plumbing, and fuel dispensers. AFRL and METSS were also afforded the opportunity to observe a tank cleaning event performed by a contractor, Jones Bros. Company, at Barksdale AFB on 20 September 2017. Biofilm samples from within the tank were collected before and after cleaning to assess how well the cleaning process removed the biofilm. In addition, bottom samples were acquired from each tank using a bottom sampler (i.e., “Bacon bomb” or “sample thief”). At least four liters of fuel were collected from each tank. Two liters were used to evaluate the level of adenosine triphosphate (ATP) within the fuel sample (an indication of microbial activity), one liter was used for microbial community analysis via next generation sequencing, and one liter was used for microbial cultivation. All onsite and cultivation analyses were conducted by RXAS, but a summary is provided here for reference.

Summary of Results

No clear relationship was found between geographic location and the presence of microbial contamination. Samples were taken from each geographic region that contained high microbial loads. Both ULSD and BDI were found to be contaminated with significant microbial loads ($>10^4$ colony forming units / milliliter (cfu)/mL). Contamination also varied from tank to tank within a base. For example, Tank 10 at Mountain Home AFB had microbial loads up to 10^8 cfu/mL while Tank 15 had no cultivable organisms present, and Tank 10 was filled by transfer from Tank 15. This suggests that it is the individual history of each tank or internal environment that leads to contamination, not general practices. After isolating the recovered cultivable microorganisms from the installations in Florida, 145 isolates were submitted for sequencing. The most abundant isolates identified were *Byssochlamys*, *Yarrowia lipolytica*, *Gluconacetobacter*, *Acetobacter*, *Candida*, and *Stenotrophomonas*. *Byssochlamys* was isolated from fuel samples at several bases across the US, including 4 out of the 5 bases in the Florida panhandle, as well as Davis-Monthan (Arizona) and Barksdale (Louisiana) AFBs. Therefore, *Byssochlamys* was used as the primary organism for disinfection process development, testing, and evaluation performed on this project.

Next generation sequencing and standard cultivation methods identified similar organisms within fuel samples across the US. This suggests that a mitigation approach that is effective against the most prevalent organisms may be effective at universally remediating contaminated tanks across the US. These organisms have been isolated and preserved, and now serve as an important resource for the test and evaluation of microbial decontamination methods.

The key observations and sample analysis results from these site visits are listed below:

- The appearance of biodiesel fuel samples ranged dramatically in color and clarity; however, all fuels passed routine specification analysis. Colors ranged from light green or tan to dark

brown, and turbidity ranged from transparent to very turbid. Figure 1 illustrates the varied appearance of the fuel samples.

- The amount of water recovered in tank bottom samples varied significantly ranging from nearly none to several inches.
- Manual cleaning of an empty tank via power washing removes a significant portion of the visible biomass accumulated as sludge on the tank bottom and biofilm on the interior walls of the tank; however, biological sampling demonstrated that a substantial level of viable microbes remained in the tank. This was to be expected as the spray washing process is only meant to clean the tank, not disinfect the tank.
- Fuel tank systems come in a variety of sizes, shapes, and configurations, including above ground storage tank (AST) and underground storage tanks (UST). Accessibility to the tanks can vary greatly with varying sizes and numbers of manways, vents, and gauge hatches in each tank. Understanding fuel tank system design and specification and seeing various AST and UST systems first-hand proved invaluable in the development of a fuel tank remediation process that is robust and adaptable to all fuel systems.



Figure 1. Examples of BDI fuel samples collected from different tanks at a single AFB

3.2 Fuel System Characterization

Industry fuel tank design specifications were reviewed to aid in development of the remediation process that would be universally acceptable for most (if not all) fuel storage systems.

Documents reviewed included:

- American Petroleum Institute Standard 650: Welded Steel Tanks for Oil Storage, Code of Federal Regulations (CFR) Section 195.132(b)(3); Eleventh Edition, June 2007 and Addendums, February 1, 2012.
- Coordinating Research Council, Inc. Report No. 667. Diesel Fuel Storage and Handling Guide, September 2014.
- Unified Facilities Criteria Doc 3-460-01. Design: Petroleum Fuel Facilities. June 17, 2015.
- Underwriters Laboratories Inc, UL 142 - Standard for Safety for Steel Aboveground Tanks for Flammable and Combustible Liquids. Nine Edition, December 28, 2006.
- Containment Solutions. Petroleum Storage: Fiberglass Underground Tank Specification. September 2017.
- Containment Solutions. Specifications: Hoover Vault Tank UL Listed 2085 Protected Secondary Containment Tank (rectangular design). February 2002.
- Containment Solutions. Double-wall Fiberglass Tanks for Underground Petroleum Storage. October 2005.
- Highland Tank. Design specification drawings for single and double walled ASTs and USTs. Accessed April 18, 2018.
- Xerves. Single-wall and double-walled fiberglass underground storage tanks design specifications. Accessed April 18, 2019.
- Wyoming Storage Tank Program. Chapter 17: Storage Tanks. Accessed May 23, 2018.

Although the overall design (mode of operation, function, and utilities) of fuel systems are generally the same, the materials used in their construction can vary. Common materials used for the tank, plumbing, and dispensers are listed in Table 1. Some key design aspects of note include:

- All above-ground tanks are steel (per building codes)
- Underground tanks can be steel or fiberglass, with fiberglass becoming more prevalent
- Piping can be steel or fiberglass
- Many types of materials are used for tank, pipes, and dispensers including 8 metals, 12 plastics, and 13 elastomers (Table 1)
- Tanks and pipes may or may not be epoxy-lined for corrosion protection.

Table 1. Common Materials Used for Fuel Storage Systems

Metals	Elastomers
Aluminum 1100	Teflon
Stainless steel (304, 316)	Nylon 6/6
Carbon steel 1020 or alike	Nitrile
Hastelloy	Viton A401-C
Halar ECTFE Coated S.S.	Viton GFLT
	Viton fluorocarbon
Plastics	Dyneon fluorocarbon
Rigid fiberglass reinforced plastic (RFP_	Acrylonitrile butadiene rubber
Flexible plastic piping	Fluorosilicone
Polyvinyl Chloride (PVC)	Polyurethane
Nylon	Polypropylene
Carbon-fill acetal	Rubberized cork
Polypropylene	Hifluoer
High density polyethylene	Sealants
Polyvinylidene fluoride	Polytetrafluroethylene-based sealants
Isophthalic ester resin	
Terephthalic ester resin	
Vinyl ester resin	
Epoxy resin	

The materials of construction are variable across the market; likewise, temperature and pressure limitations vary significantly from vendor to vendor and tank to tank. However, fiberglass and steel tanks generally have a recommended maximum operating temperature of 150 degrees Fahrenheit (°F) and 200 °F, respectively. These temperature limits were used to guide thermal decontamination methods to inactivate the microbes prevalent in the contaminated fuel systems.

3.3 Remediation Technology Assessment

There are a number of disinfection technologies with advantages and disadvantages depending on their intended use. Table 2 lists disinfection technologies that were assessed in this program based on their key attributes, including efficacy against bacteria and fungi, physical and chemical properties, ease of use (applicability), scalability, material compatibility, cost, and safety. No single method is ideal for all fuel storage tank configurations; however, HAD and ClO₂ gas fumigation were the best candidates. Therefore, these two technologies were selected for further investigation in this project.

Table 2. General Assessment of Disinfection Technologies

Disinfection Technology	General Remarks	Selected for Further Evaluation
ClO ₂ Fumigation	Can be generated easily and safely on-site using chemical precursors; pure, stable gas generated is efficacious to many microbial agents including bacterial and fungal spores; no post-treatment residue and decays quickly via exposure to sunlight or adsorbed to activated carbon; compatible with most materials having a much lower redox potential than corrosive disinfectants such as bleach, hydrogen peroxide, and formaldehyde	Yes
HAD	Utilizes elevated heat under low relative humidity; proven effective against bacterial spores and utility to disinfect operational aircraft (interior and exterior) has been demonstrated; leaves no residue; high-capacity heater required to achieve necessary temperatures that can effectively and efficiently inactivate microbial contamination	Yes
Ultraviolet (UV) Light	Line-of-sight required; effectiveness proportional to distance between UV source and surface; ineffective against biofilms	No
Hydrogen Peroxide Fumigation	Commercially available gas dispersion equipment is expensive, requires extensive training to operate, is heavy, and is not easily transportable; process requires humidity control; treatment is not effective on biofilms	No
Ethylene bromide fumigation	Significant safety concerns as the gas is explosive; commercially available gas dispersion equipment is expensive, requires extensive training to operate, is heavy, and is not easily transportable	No
Formaldehyde fumigation	Leaves a residue which would require post-treatment rinse and drying	No
Liquid disinfectants	Many commercially available products are available; however, excessive water rinse would be required to ensure tank is disinfectant free; incompatibility with fuel tank materials is a concern as well	No

3.4 Microbiological Analysis Methods

The following analytical methods were used to support testing efforts.

3.4.1 Cultivation

Predominant strains of fungal isolates recovered from biodiesel fuel samples that were collected from the on-site field inspections are listed in Table 3. These strains, identified via genomic sequencing conducted by ARFL and/or Oklahoma University, were provided to METSS and used for in-house biofilm growth, characterization, and efficacy testing. A strain of *Aspergillus niger*, a fungus that is very common in the environment, was also tested. These isolates were cultivated on Potato Dextrose Agar (PDA) and generally incubated at 26-28 degrees Celsius (°C). Spore stocks of each strain were prepared by inoculating multiple agar plates and incubating them for 1-2 weeks to allow fungal growth to mature and reach sporulation, after which the spores were harvested from the agar surface, suspended in pure phosphate buffered saline (without calcium chloride or magnesium chloride), and stored refrigerated. The stocks were enumerated via standard spread plating and used as needed throughout the project as seed cultures. Genomic sequencing indicated that *Byssoschlamys* was the most prevalent strain found

in fuel samples; therefore, most of the efficacy testing was performed against this strain. The appearance of these strains grown on agar are illustrated in Figure 2.

Table 3. Fungal Isolates from Fuel Samples

Fungal Strain Identification	Isolate I.D. *	Internal Reference I.D.
<i>Aspergillus niger</i>	Not applicable	AN**
<i>Aspergillus terreus</i>	KDS45P4D	AT
<i>Byssoschlamys nivea</i>	TDS30P4E	B
	B13; BAFB4P2 DFSP2 71	B13
	BAFB3P3 DFSP2 64	B21
<i>Fusarium sp.</i>	KDS45P4E	F
<i>Penicillium citrinum</i>	TDS20P3A	P
<i>Thyridium vestidium</i>	D216	T

*Assigned by AFRL

**METSS in-house strain

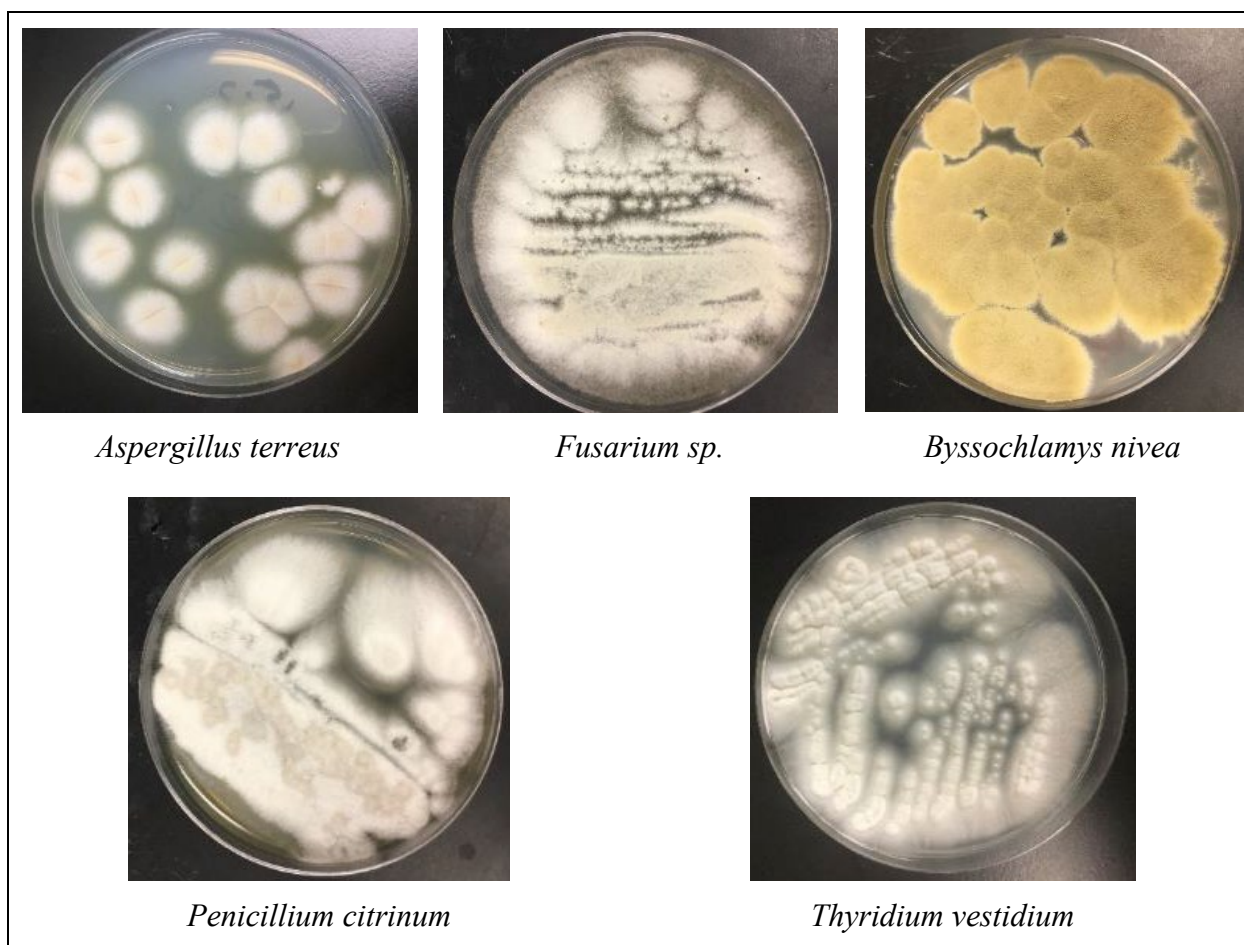


Figure 2. Predominant fungal isolates recovered from contaminated fuel systems

3.4.2 Coupon Preparation

Efficacy tests against fungal spores or biofilms were performed by inoculating $\frac{1}{2}$ " x $\frac{3}{4}$ " coupons of a non-porous substrate and then exposing the inoculated coupons to a specific decontamination treatment. The two substrates (i.e., materials) used were glass and steel. Glass coupons were prepared from standard glass microscope slides and were sterilized via autoclaving. Steel coupons were prepared from sheets of R46 mild steel (Q-Labs) having a composition of 0.15% carbon, 0.6% manganese, 0.03% potassium, and 0.04% sulfur. This composition is comparable to other common mild steels including A36, C1010, and other steel grades used in the construction of fuel tanks. Steel coupons were soaked in acetone for at least 15 minutes and then flamed to remove any corrosion prevention fluid that may have been applied by the manufacturer during the production process. R46 steel coupons cut to $\frac{3}{4}$ " x 3" were also prepared and used for biofilm growth studies and biofilm efficacy tests. A $\frac{1}{4}$ " hole was drilled at one end and used to suspend the coupon in a specific test system, chamber, or apparatus.

3.4.3 Biofilm Formation

Biofilms in fuel tank systems are predominately located at the bottom of tank where a layer of water (several inches deep) accumulates. The water layer forms from condensation of humidity

within the tank and/or ingress of rainwater, and the fuel/water interface is an ideal environment to support microbial growth. Biofilms are also found in diesel storage tanks, but the growth in biodiesel tanks is much greater due to the high nutrient value of the fuel and the lower turnover of fuel in the storage tanks. To mimic these conditions in the lab, microcosm test systems were established using a Mason jars or glass beakers as small-scale test vessels. The vessels were partially filled with a 1:1 or 1:3 ratio of B20 to Artificial Sump Water (ASW). ASW is created to mimic the elemental analysis of sump water samples.² Steel coupons (¾" x 3") were suspended vertically within the vessel by attaching a paper clip to the coupon and fastening the paper clip to a wire secured across the opening of the vessel. The coupons were positioned with the middle of the vessel at the level of the fuel/water interface. Examples of a 3-L beaker microcosm systems are shown in Figure 3. Microcosms were then inoculated with a suspension of fungal mycelium and spores prepared from one or more strains of fungi. The microcosms were then incubated statically at 26-28 °C for several weeks to allow biofilms to establish on the coupons. The contaminated coupons, covered in the biofilm, were then used as needed for efficacy testing and assayed as described in Section 3.4.

An alternative, more robust method was also used to generate biofilm-laden coupons. The ¾" x 3" steel coupons were placed in individual 50-mL conical tubes containing 20 mL of Potato Dextrose Broth (PDB), a culture medium that supports the growth of many fungi. After inoculating with a fungal suspension, the tubes were incubated for 7-14 days to allow a biofilm to form on the coupons.

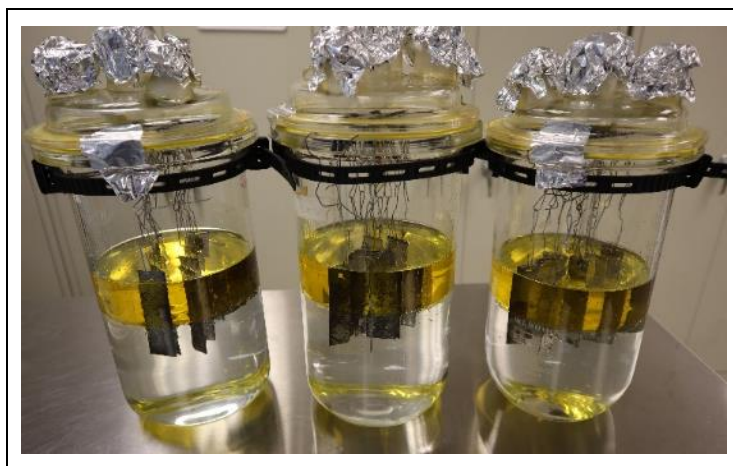


Figure 3. 3-L beaker microcosm systems

3.4.4 Coupon Extraction and Analysis

To determine the quantity of culturable organisms per coupon, either pre- or post-decontamination treatment, replicates coupons (2 or 3) were extracted in phosphate buffered saline supplemented with 0.05% Tween 20 (PBST) (10 mL for small coupons and 15 mL for larger coupons) within individual 50 mL conical tubes. Coupons were vortexed vigorously for

² McNamara, C.J., T.D. Perry, L. Ryan, K. Bearce, J. Dante, and R. Mitchell. Corrosion of aluminum alloy 2024 by microorganisms isolated from aircraft fuel tanks. *Biofouling*, 2005; 21 (5/6): 257-265.

30 seconds, sonicated for 5 minutes in a sonicating water bath, and then vortexed again for 30 seconds. The extracts were then serially diluted in PBST. Replicate 100 microliters (μL) aliquots of various dilutions were spread plated, allowed to dry, and then incubated at 26-28 °C until colonies reached a distinguishable size (generally 3-5 days for fungal strains used for this study). The colony counts per plate, reported as cfu, were then used to calculate the concentration (cfu/mL) of the extract. This value was multiplied by the extraction volume to calculate total cfu recovered from the coupon. This value was then converted to a log_(base 10) value. The delta log between untreated controls and treated samples represented the efficacy of treatment, expressed as log reduction (LR).

3.4.5 Biofilm Stability

An experiment was performed to assess the stability of fungal spore inoculum following rapid and slow drying techniques. The objective of the test is to demonstrate that either drying method could be used when conducting efficacy tests and that the spores remain stable for up to two days which represents, in general, the maximum amount of time that a tank remediation process would take to complete. It was important to demonstrate the spores remained stable during this time and did not simply die from desiccation. Replicate steel coupons were inoculated with 100 μL of *Byssoschlamys* and *Aspergillus* spores and then dried either passively in a biosafety cabinet (approximately 4 hours) or rapidly using a hot air gun set to 140 °F. The coupons were then incubated statically at ambient conditions. After 19 and 43 hours, replicate samples were extracted and enumerated. In summary, the results (Figure 4) indicated that either drying method could be used and the spores remained stable for at least two days (i.e., 43 hours).

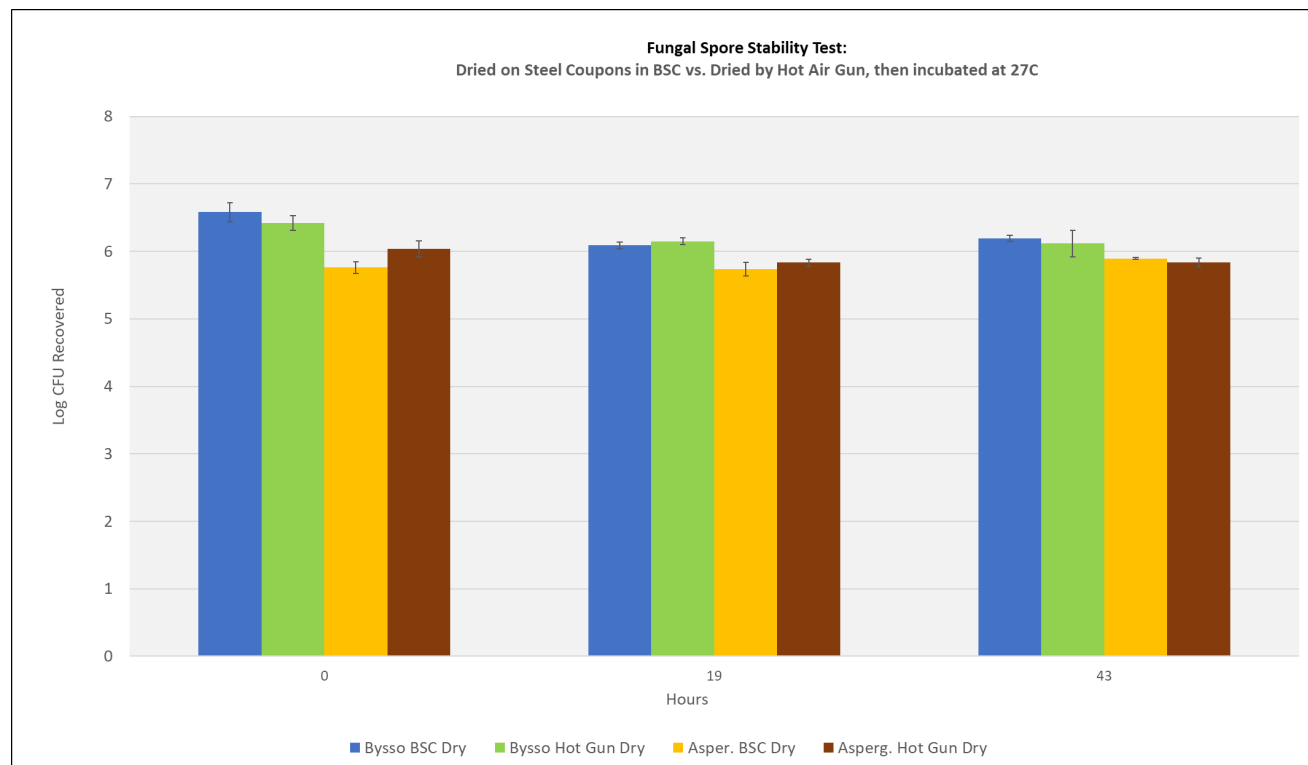


Figure 4. Stability test results

3.4.6 Biological Indicators

Biological indicators (BIs) are used in the sterilization industry to verify or validate decontamination processes. BIs are strips of Tyvek (or a like material) that are inoculated with a known quantity of bacterial spores (generally $>1 \times 10^6$ spores or >6 logs) and packaged dry in individual heat-resistant or gas-permeable envelopes. The envelopes are then exposed to the treatment process, after which the inoculated strip is aseptically transferred from the envelope into tubes containing sterile growth medium. The samples are then incubated at the appropriate temperature and duration and inspected for color change as compared to untreated controls. The results are reported as Positive (signifying growth and incomplete kill of all spores) or Negative (signifying complete kill and >6 LR was achieved). In this study, BIs were used during efficacy testing as a simple qualitative tool to assess the decontamination process. BIs prepared with *Geobacillus stearothermophilus* (Gs) spores (Crosstex) or *Bacillus atrophaeus* (Bg) spores (Mesa Labs) were used.

3.5 Efficacy Testing

3.5.1 HAD Efficacy Testing

Table 4 describes the series of HAD efficacy tests performed. Bench-scale efficacy testing was performed using an environmental chamber in which a desired temperature and relative humidity could be reached and maintained. The tests were performed at elevated temperatures with low, medium, and high RH conditions, replicating various options for thermal decontamination. Low carbon steel and/or glass coupons were inoculated with a known concentration of fungal spores, allowed to dry, and then exposed at thermal conditions for specified time periods (generally from 4 hours to up to 48 hours). At each time point, replicate coupons were removed from the environmental chamber, extracted, and analyzed as described previously (Section 3.4.4). The concentration of culturable (i.e., viable) spores was plotted versus time. Details and results of all tests are described in chronological order in Appendix A. Select experiments are summarized in this section to demonstrate the efficacy of hot air.

Table 4. Description of Bench-Scale Hot Air Decontamination Tests

Test ID	Treatment	Description
H1	140 °F @ 10%RH for 4 and 24 hours	<i>Byss</i> -biofilms on steel coupons established for 3 weeks in 50-mL microcosm tubes
H2	140 °F @ 10%RH for 4 and 24 hours	<i>Byss</i> -biofilms on steel coupons established for 4 weeks in 3-L microcosm
H3	140 °F @ 10%RH for 4, 24 and 48 hours	<i>Byss</i> -biofilm and spores on glass coupons
H4	180 °F @ 10%RH for 4 hours	<i>Byss</i> and <i>Asperg.</i> spores on glass and steel coupons
H5	190 °F @ 10%RH for 4 hours	<i>Byss</i> and <i>Asperg.</i> spores on glass and steel coupons
H6	140 °F @ 90%RH for 4, 24 and 48 hours	<i>Byss</i> spores on steel coupons
H7	140 °F @ 50%RH for 4, 8 and 24 hours	<i>Byss</i> spores on steel coupons
H8	140 °F @ 50%RH for 6 hours	<i>Byss</i> , <i>A. niger</i> , and biofilm on steel coupons
H9	140 °F @ 50%RH for 8, 12 and 24 hours	2 <i>Byss</i> and 1 <i>Asperg</i> on steel coupons
H10	140 °F @ 5%RH for 4, 8 and 24 hours	<i>A. niger</i> on steel coupons
H11	140 °F @ 80%RH for 4, 8 and 24 hours	<i>A. niger</i> on steel coupons
H12	140 °F @ 80%RH for 4, 8 and 24 hours	<i>A. terreus</i> on steel coupons
H13	140 °F @ 80%RH for 4, 8 and 24 hours	<i>A. terreus</i> on steel coupons
H14	140 °F @ 80%RH for 4, 8 and 24 hours	<i>A. niger</i> on steel coupons
H15	140 °F @ 80%RH for 4, 8 and 24 hours	<i>T. vesticles</i> on steel coupons
H16	140 °F @ 80%RH for 4, 8 and 24 hours	<i>Byss</i> and biofilm on steel coupons
H17	140 °F @ 10%RH for 2 and 5 days	<i>Byss</i> spores, 3-L biofilm and PDA biofilm

3.5.1.1 Hot Dry Air

The efficacy of HAD was demonstrated first at 180 °F for 4 hours against spores of *Byss* and *Aspergillus* inoculated onto glass and steel coupons. The results (Figure 5) illustrated this treatment was effective on both substrates as >4 log reduction was observed for *Byss* on steel and glass as well as *Aspergillus* on glass. A reduction of nearly 3-logs was observed for *Aspergillus* on steel.

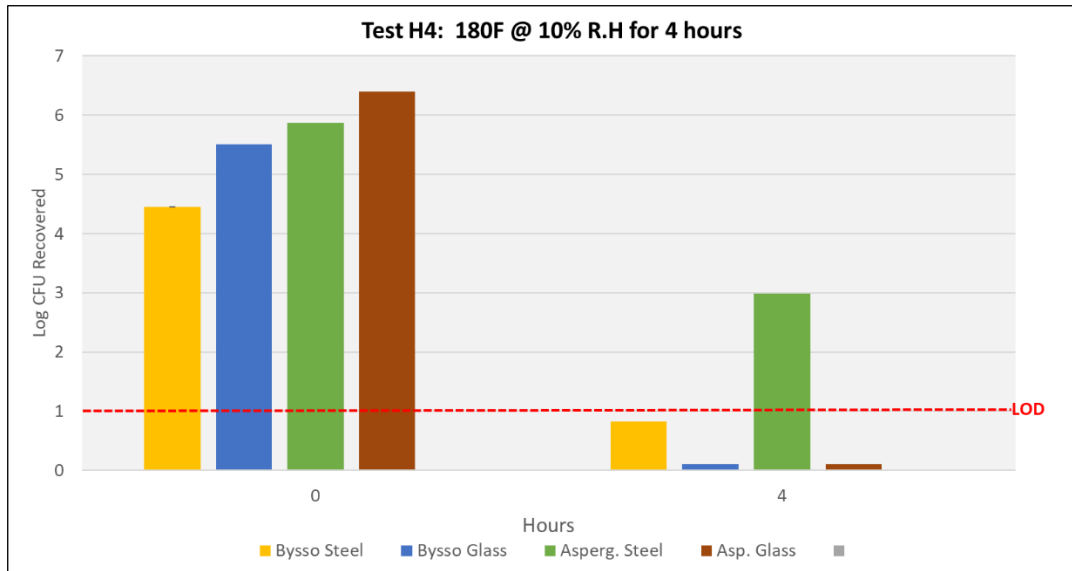


Figure 5. Results of HAD - 180 °F and 10% RH for 4 hours

Based on these encouraging efficacy results, the temperature of HAD was increased to 190 °F to assess whether this slight increase would achieve complete kill. The results (Figure 6), illustrated this was the case, as only a few colonies of *Aspergillus* were recovered after a 4-hour treatment period.

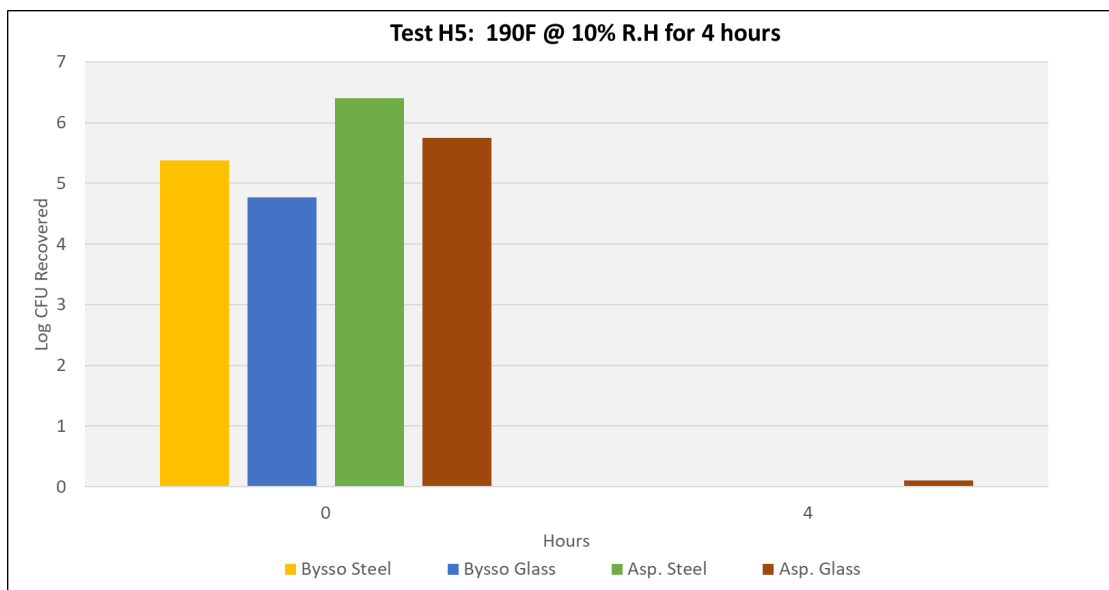


Figure 6. Results of HAD - 190 °F and 10% RH for 4 hours

3.5.1.2 Hot Humid Air / BTD

While hot/dry air was effective, the temperatures required are too high for fiberglass tanks. Therefore, METSS demonstrated the efficacy of lower temperatures combined with high humidity. Since *Aspergillus niger* was more resilient to thermal decontamination than *Byssoschlamys* in previous HAD testing, a test was conducted to assess efficacy at high and low RH conditions with elevated temperature against *Aspergillus* spores. Spore-inoculated coupons were treated for 4, 8, and 24 hours at 140 °F at 80% and 5% RH. The results, shown in Figure 7, indicated *Aspergillus* was far more sensitive to high-RH conditions than low-RH conditions. These results align with the premise that elevated temperature and high RH is more efficacious against biological agents than elevated temperature and low RH conditions.

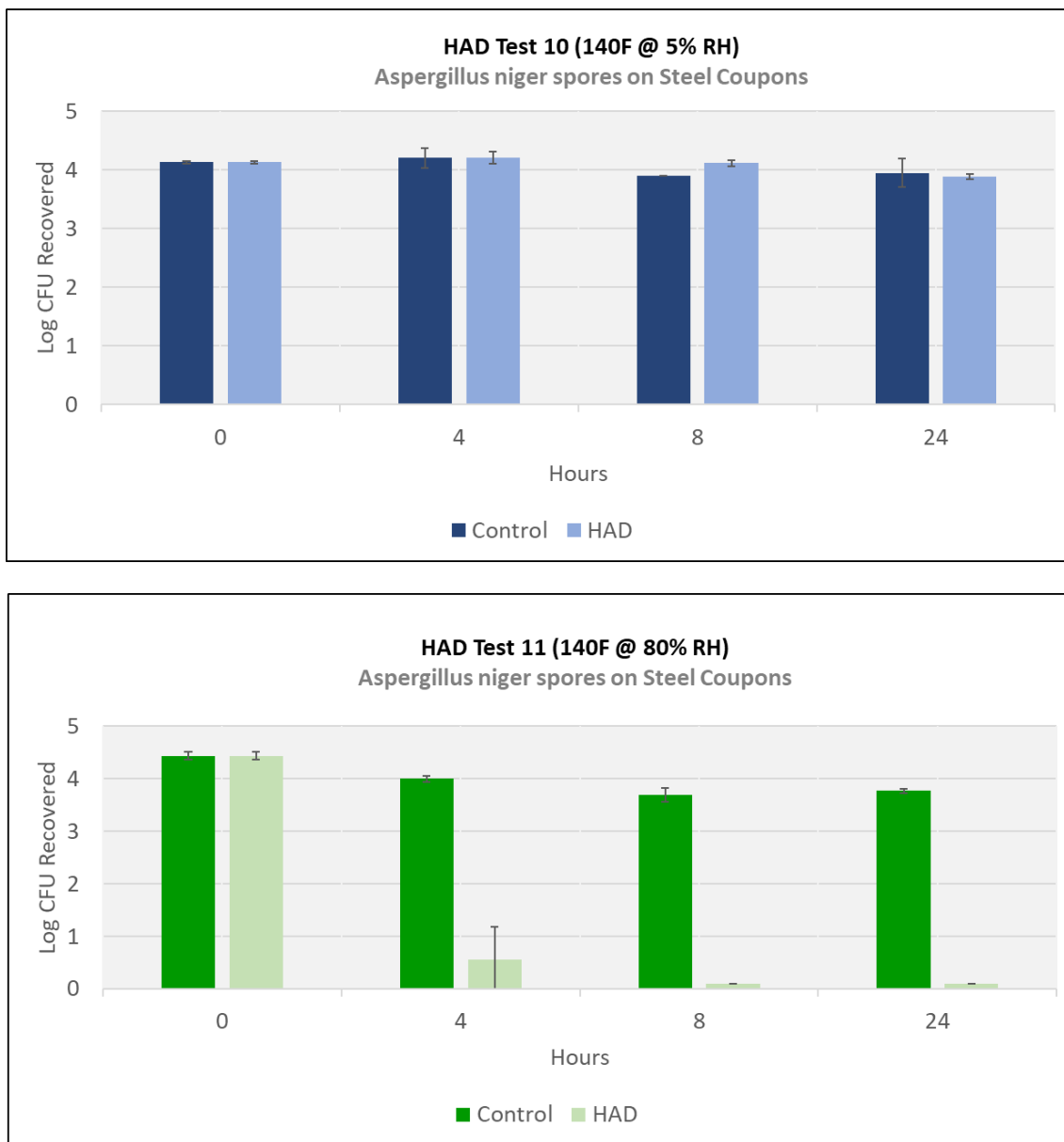


Figure 7. Test H10 and H11 results

Having demonstrated efficacy against a robust spore, an efficacy test was designed to evaluate 140 °F and 80% RH against *Byssoschlamys* spores and *Byssoschlamys* biofilm (the dominant contributors to biofilms in biodiesel storage tanks). The results (Figure 8) indicate the levels of kill between these two stages of growth were comparable, as no viable organisms were recovered at 4, 8, and 24 hours. These results suggest that if these conditions can be achieved in the field within an empty, contaminated fuel storage tank, effective remediation would likely be achieved in under 4 hours.

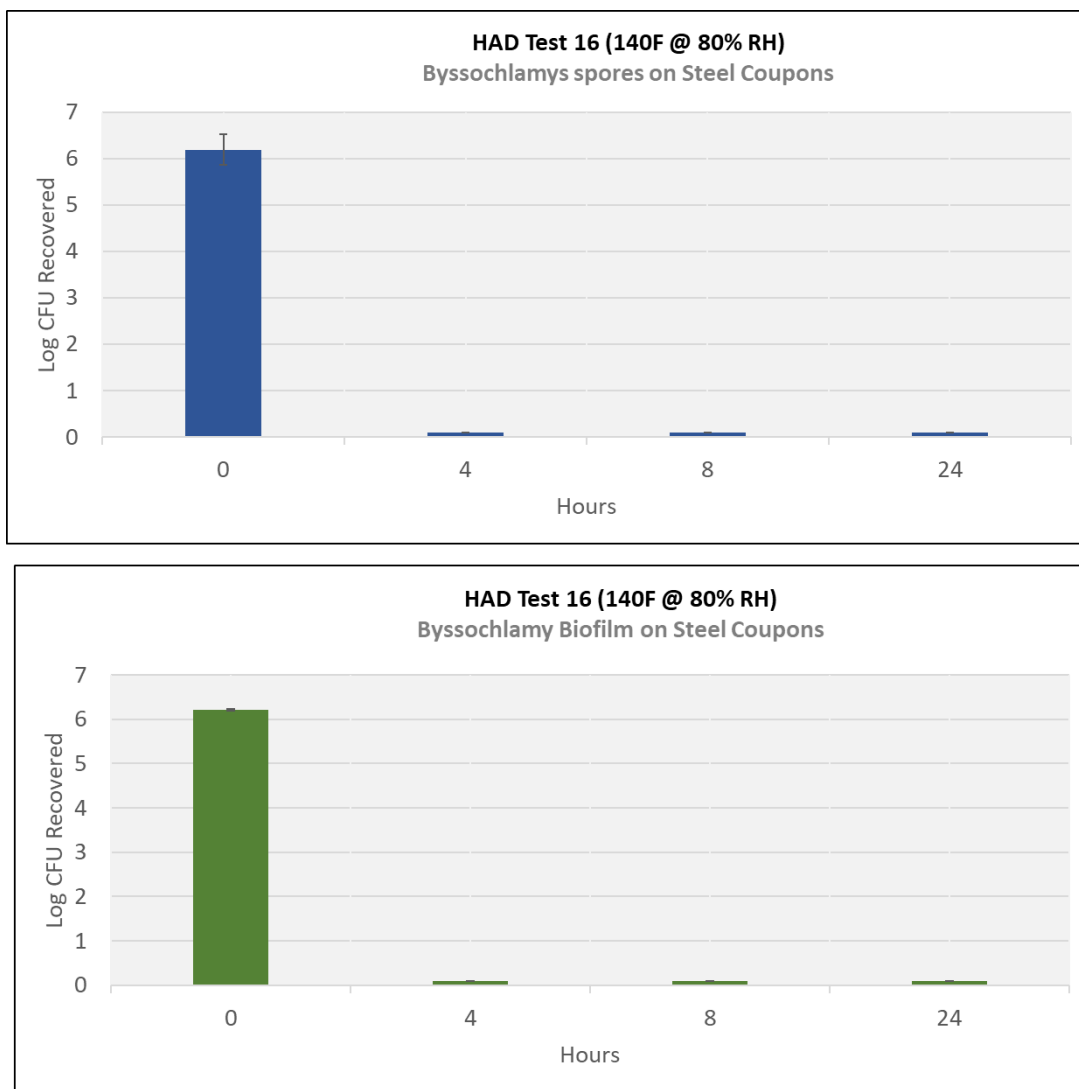


Figure 8. Test H16 results

This test was repeated against *Thyridium vestidium*, another fungus recovered in BDI fuel samples by AFRL. Samples were treated at this condition for 4, 8, and 24 hours. The results (Figure 9), illustrate this strain is also susceptible to thermal decontamination like *Byssoschlamys* and *Aspergillus* (a common environmental fungus).

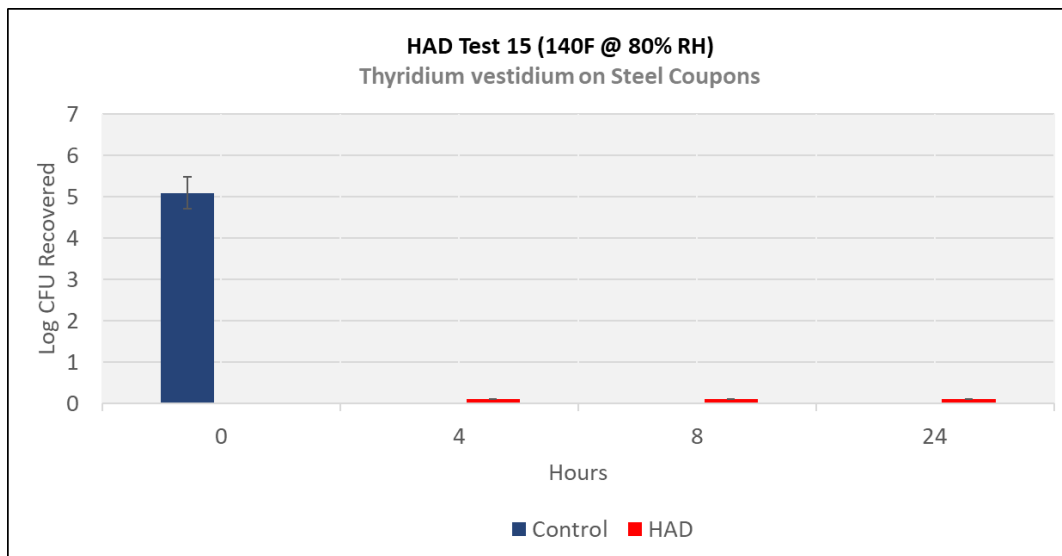


Figure 9. Test H15 results

3.5.2 ClO₂ Efficacy Testing

A series of ClO₂ tests (denoted C1...C14) were performed as described in Table 5. Details and results of all tests are described in chronological order in Appendix B. Select experiments are summarized in this section to demonstrate the efficacy of ClO₂. The key parameters monitored were gas concentration (expressed as parts per million volume (ppmv)), relative humidity, and time. The total gas treatment was expressed as ppmv-hours (ppmvh); representing the average parts per million (ppm) concentration of the entire treatment duration multiplied by the total treatment time. The test systems and methods used to perform tests were described previously in Section 3.4.

A portable PortaSens II gas detector (Analytical Technology, Inc.) with a ClO₂ gas sensor was used to monitor the gas concentration (ppmv) during testing. This hand-held device is commonly used to detect leaks of toxic or otherwise dangerous/hazardous gas, as well as oxygen deprivation in the workspace. Chlorine dioxide gas indicator strips (CD Checkstrips™ from ClorDiSys) were also used as deemed appropriate during ClO₂ gas testing. According to the manufacturer, the tip of the indicator changes from a light pink to light blue/grey when exposed to ≥ 300 ppm ClO₂ gas. The PortaSens and indicator strips were used to assess whether ClO₂ gas concentrations reached this concentration at various locations within the enclosed space used for full-scale efficacy testing.

Table 5. ClO₂ Efficacy Testing

Test ID	Treatment	Description
C1	50 ppm ClO ₂ aq. and off-gas for 2 hours	<i>Byss</i> -biofilms on steel coupons established for 3 weeks in 50 mL microcosm tubes
C2	46 ppm ClO ₂ aq. and off-gas for 4 and 24 hours	<i>Byss</i> -spore laden glass coupons
C3	gas from 50 ppm ClO ₂ solution for 4 hours	Glass and steel coupons inoculated with spores of <i>Byss</i> , <i>Penicillium</i> , <i>Aspergillus</i> , and <i>Fusarium</i>
C4	gas from 100 ppm ClO ₂ solution for 2 hours	<i>Byss</i> biofilm, dry <i>Byss</i> and <i>Asp.</i> spores
C5	gas from 100 ppm ClO ₂ solution for 3 hours	<i>Byss</i> biofilm, wet and dry spores of <i>Byss</i> and <i>Asp.</i>
C6	Two 12G Yellow Jacket w/ RH	<i>Byss</i> PDB biofilms, <i>Byss</i> and <i>Asp.</i> wet spores
C7	Two 12G Yellow Jacket w/ RH	<i>Byss</i> PDB biofilms
C8	Two 12G Yellow Jacket w/ RH & temp	<i>Byss</i> PDB biofilms
C9	Two 12G Yellow Jacket w/ RH	<i>Byss</i> PDB biofilms and Ba spore strips
C10	One 12G Yellow Jacket w/RH	<i>Byss</i> PDB biofilms and Ba spore strips
C11	One 12G Yellow Jacket w/RH	UV light neutralization
C12	Two 12G Yellow Jacket w/ RH	<i>Byss</i> spores, 3-L biofilm, and PDA biofilm
C13	Two 12G Yellow Jacket w/ RH	Gas dispersion test 1: CD Check Strips and BIs in ISO
C14	Two 12G Yellow Jacket w/ RH	Gas Dispersion Test 2: CD Check Strips and BIs in ISO

Testing was performed using either low carbon steel coupons (representing the interior of uncoated steel fuel tanks) or glass coupons (representing another non-porous substrate that is non-reactive to ClO₂ gas). Steel and glass coupons were used interchangeably throughout the study as both represented non-porous substrates.

In early ClO₂ testing, concentrated ClO₂ solutions were generated using commercially available ClO₂ generating micro-reactors (Selectroside® 2L500 or 12G), which utilize a proprietary gas water/gas permeable membrane to encase two chemical precursors. The type and number of micro-reactors used was based on the concentration of gas needed in the volume of the test system. The micro-reactors are easy to use; the micro-reactor is immersed in tap water and allowed to generate for two hours. However, the cost of the micro-reactors along with a two-hour generation period made them less appealing for this application. Therefore, METSS utilized experience and knowledge from internal research and development efforts of generating ClO₂ from chemical precursors to perform controlled ClO₂ gas exposure tests in later stages of the project.

3.5.2.1 Bench-scale Testing

Initial ClO₂ efficacy tests were performed at bench-scale using a retrofitted 80-gallon storage tote as the test chamber to identify the approximate gas treatment (concentration and duration) needed to achieve at least a 3-log reduction in fungal spores or biofilm viability. A ClO₂ solution (varying volume and concentration) contained in a rectangular plastic box was placed in the bottom of the tote. To start a test, BIs and/or non-porous coupons inoculated with fungal spores or biofilm were placed in the tote. A battery-powered fan, directed at a 45° angle downward at the surface of the ClO₂ solution, was used to promote off-gassing (Figure 10). The tote was promptly sealed, denoting the start time. A PortaSens gas detector attached to the tote was set to record gas concentrations every 1 to 5 minutes. To end a given test, the lid of the tote was removed, the fan was turned off, and the coupons were aseptically transferred to a biosafety cabinet for analysis. The PortaSens data was downloaded and then plotted as gas concentration (ppmv and ppmvh) versus time. The total gas treatment was then calculated based on the average ppm concentration multiplied by the total treatment time and expressed as ppmvh.

Note: Testing performed on another study using the bench-scale system showed the RH in the tote quickly reaches >90% within the first 5 minutes; therefore, all tests within the bench-scale tote are assumed to have been conducted under high RH conditions.



Figure 10. Tote ClO₂ gas treatment test system used for bench-scale ClO₂ efficacy testing

Testing was performed with *Byssoschlamys* biofilm, as well as wet and dry 100-μL droplets of *Byssoschlamys* and *A. terreus* spores. *Aspergillus* species are prevalent in the environment, and *A. terreus* was recovered by AFRL from field collected BDI samples. The intent of this study was, in part, to assess whether the efficacy of gas was affected by whether the contamination was either dry or wet. Both conditions would be found in a contaminated fuel tank that has been prepared for gas fumigation via pressure washing, after which the tank will be immediately fumigated (still moist inside) or fumigated a day or two after pressure washing (and therefore dryer). In this bench-scale test, the gas treatment was 1109 ppmvh. The results (Figure 11) illustrated the treatment was very effective against *Byssoschlamys* and *A. terreus* dried spores, but mildly or moderately efficacious against the wet inoculum.

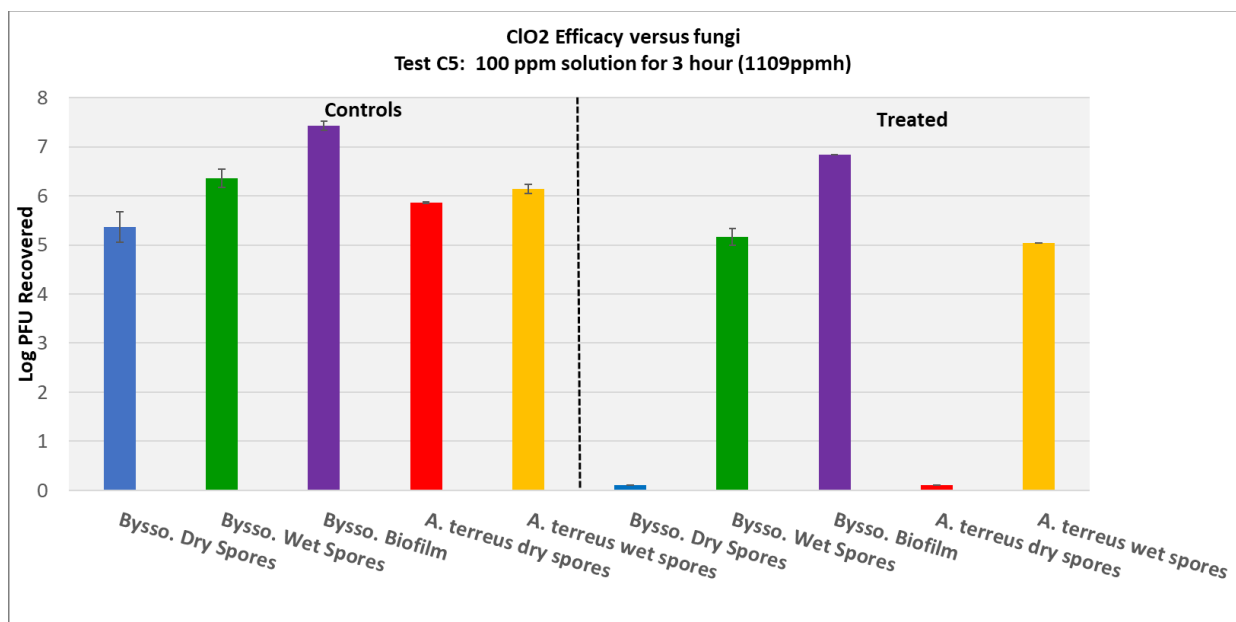


Figure 11. Test C5 results

The bench-scale results provided a first-hand look at the efficacy levels achieved at relatively low to moderate gas treatment level (<1200 ppmvh) against fungal biofilms and spores, and indicated that greater treatment levels were needed to achieve complete or near-complete kill (defined herein as >3 log reduction or a 99.9% reduction in viability). Subsequent tests were transitioned from bench-scale to full-scale.

3.5.2.2 Full Scale Testing

METSS utilized a 1,169 cubic ft. shipping container to simulate an 8,800-gallon fuel storage tank in full-scale decontamination trials. These tests were performed to identify the parameters needed to generate gas concentrations that would achieve complete or near complete kill in an 8,000-gallon tank. ClO₂ gas was dispersed throughout the full-scale test system using a gas dispersion unit referred to as the Yellow Jacket (Figure 12). This device can be used with one or more types of Selectroicide® micro-reactors, including models 2L500, 750A, and 12G, that are submerged in 5 to 6 liters of water within the unit. As the micro-reactor generates dissolved ClO₂ gas in solution, integrated fans blow air over the surface of the solution to distribute the gas generated by the solution into the room. Since the micro-reactors float, a plastic mesh and a beaker of glass beads and stainless-steel nuts was placed overtop the micro-reactor to keep it submerged. The manufacturer instructions recommend allowing the micro-reactors to generate ClO₂ for two hours in water in a sealed container prior to use. In later stages of the project, the micro-reactors were replaced with pre-generated solutions of dissolved ClO₂. To generate these solutions, chemical precursors were combined in a polypropylene carboy to make aqueous ClO₂ at a desired concentration. This solution was stored at ambient conditions in the dark until needed for fumigation. To conduct a fumigation test, an appropriate volume of aqueous ClO₂ was added to the Yellow Jacket.

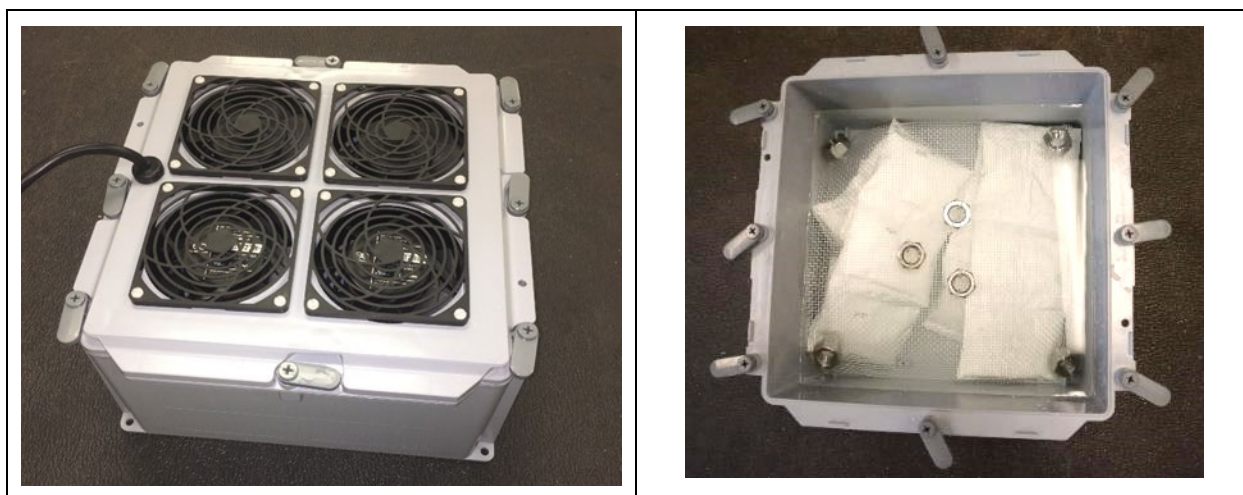


Figure 12. Yellow Jacket gas dispersion unit: (left) complete unit with lid retrofitted with fans and (right) view of unit interior with two weighted-down 12G micro-reactors in water.

The first full-scale tests were conducted with the Yellow Jacket inside the ISO container; however, this may not always be practical for fuel tanks with limited accessibility. Therefore, a unique Gas Recirculation System (GRS) was assembled, and functionality tested as described in Section 3.7.2.1. This system was designed to generate the ClO_2 gas and humidification outside of the tank within a contained, recirculating system that could be easily connected to the manway or gauge hatches. After the system was shown to operate as expected, a series of efficacy tests were performed. The objectives of the full-scale tests were to (1) assess the functionality of the recirculation system, (2) determine the appropriate concentration of ClO_2 solution to achieve gas concentrations identified from bench-scale testing to achieve complete kill of fungal biofilm commonly found in contaminated BDI fuel storage tanks, and (3) gain experience in using the recirculation equipment.

Coupons inoculated with *Byssochlamys* spores and *Byssochlamys* biofilms established in both fuel/water microcosms and in PDB were exposed to gas generated from two 12G micro-reactor under elevated RH conditions. The results (Figure 13) indicated such a treatment (3173 ppmvh) was very efficacious (>3 log reduction) against all three types of contaminants. The gas profile is shown in Figure 14.

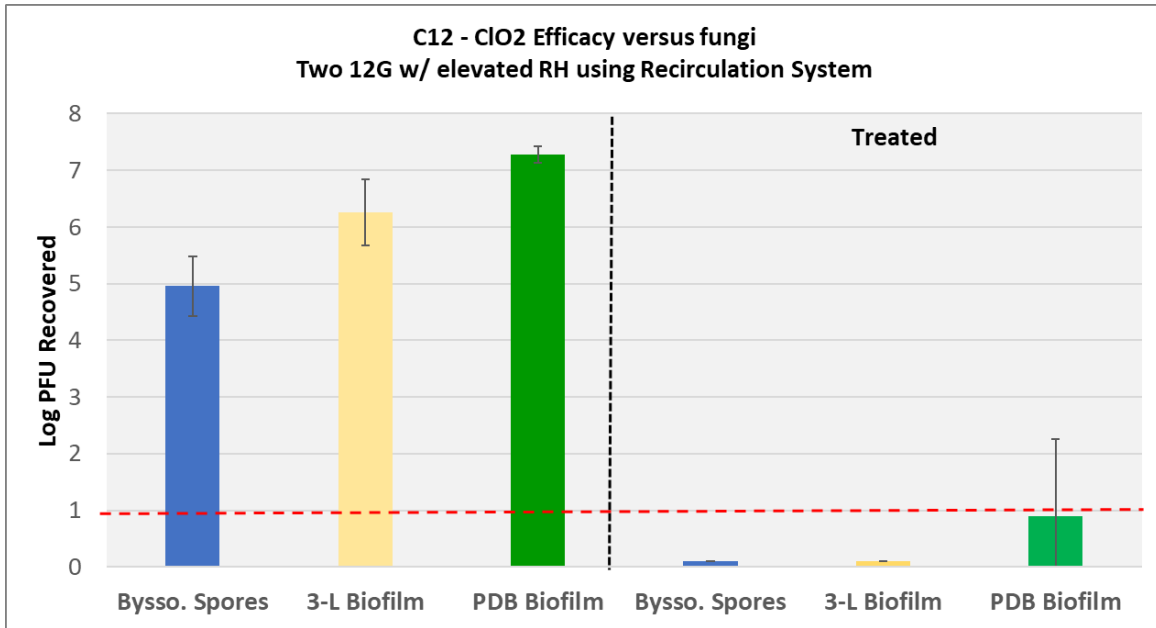


Figure 13. Test C12 results

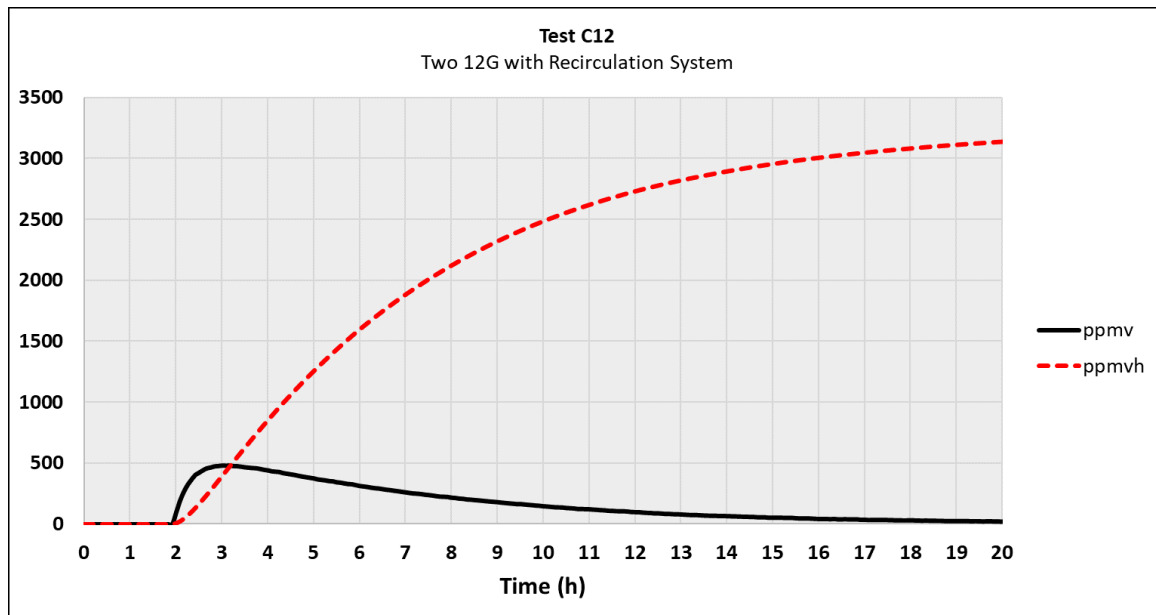


Figure 14. Gas profile from Test C12

A second test with the same parameters was performed to show repeatability of the treatment process while demonstrating efficacy against *Geobacillus stearothermophilus* and *Bacillus atrophaeus* BIs that were placed throughout the test system. CD Check Strips (ClorDiSys) that change from pink to light blue when exposed to at least 300 ppm gas were also placed throughout the test system. The results of the CD strips and BIs are shown in Figure 15 and Figure 16, respectively. The strips indicated the gas concentration did exceed 300 ppm

throughout the shipping container, which is consistent with the gas profile shown in Figure 14. The results of the BIs indicated the treatment was very efficacious, as all Gs BIs tested negative for growth and five of six Ba BIs tested negative for growth.

Sample Type	Placement in Conex	Sample ID	Result (Pink or Blue)
Treated	Front Right Floor	1	Blue
	Front Left Floor	2	Blue
	Front Right elevated	3	Blue
	Front Left Elevated	4	Blue
	Mid Front Right Floor	5	Blue
	Mid Front Left Floor	6	Blue
	Mid Front Right elevated	7	Blue
	Mid Front Left Elevated	8	Blue
	Mid Back Right Floor	9	Blue
	Mid Back Left Floor	10	Blue
	Mid Back Right elevated	11	Blue
	Mid Back Left Elevated	12	Blue
	Back Right Floor	13	Blue
	Back Left Floor	14	Blue
	Back Right elevated	15	Blue
	Back Left Elevated	16	Blue
Controls	Outside front door	17	Pink
	Outside rear door	18	Pink
	Warehouse (at least 20 ft. from ISO)	19	Pink
	Laboratory bench-top	20	Pink
	Unexposed	21	Pink



Figure 15. Test C13 CD Check strip results

Sample Type	Placement in Conex	Sample ID	BI Organism	Results (Pos. or Neg.)
Treated	Front bottom	1	Gs	Neg.
	Middle bottom	2	Gs	Neg.
	Rear Bottom	3	Gs	Neg.
	Front Top	4	Gs	Neg.
	Middle Top	5	Gs	Neg.
	Rear Top	6	Gs	Neg.
	Front bottom	7	Bg	Neg.
	Middle bottom	8	Bg	Neg.
	Rear Bottom	9	Bg	Neg.
	Front Top	10	Bg	Neg.
	Middle Top	11	Bg	Pos.
	Rear Top	12	Bg	Neg.
Neg. BI Controls	n/a	13	Gs	Pos.
	n/a	14	Bg	Pos.

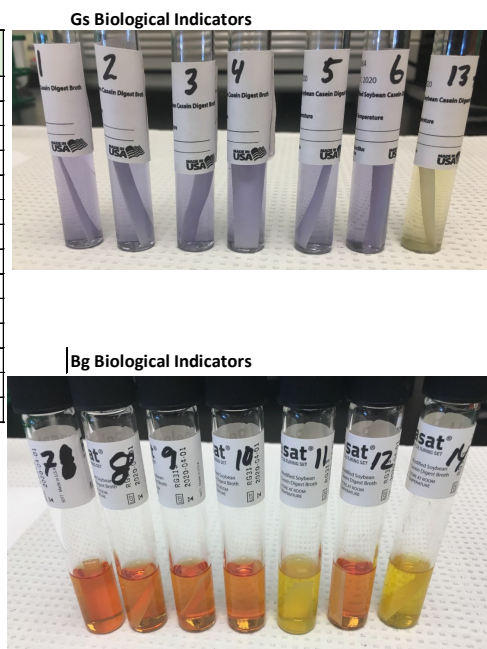


Figure 16. Test C13 BI efficacy results

When the test was repeated, all Gs BIs tested negative, and ten of twelve Ba BIs tested negative. The two Ba BIs that tested positive were located on the floor at one end of shipping container. These tests demonstrated that using the recirculation system with two 12G micro-reactors with elevated RH (ranging 65-90%) would achieve the desired level of kill (>3 logs) to successfully remediate an empty, biofilm contaminated fuel storage tank.

3.6 Supporting Laboratory Tests

A series of supporting laboratory tests were conducted to assess various aspects of the test and analytical methodologies including:

- Materials Compatibility - Assessment of corrosion by ClO₂ on various metals
- Re-growth Analysis After Decon - Assessment of long-term effects on microbial growth following disinfection treatment
- Biodiesel vs. Diesel Biofilm Growth Rates - Comparison of growth rates of biofilm in B5 vs B20.

3.6.1 Materials Compatibility

One advantage of thermal decontamination methodologies is that they are generally compatible with a wide range of materials. However, it was important to assess the compatibility of the ClO₂ decontamination method to determine if it would be a viable option for tank cleaning without compromising materials within the tank. As such, METSS selected four metals, two stainless steels (SS) and two carbon steels, along with four common elastomers for materials compatibility testing with ClO₂. Plastics were omitted in this first round of testing because they are generally compatible with ClO₂, based on previous test reports. The metals and elastomers were tested under two conditions: complete immersion in a ClO₂ solution and exposure to the vapor-rich headspace above the ClO₂ solution. The results from this study are presented in Table 6 and Table 7. Elastomers were generally compatible with ClO₂ at the tested concentrations, and they did not show any significant change in volume (ASTM D471), mass (ASTM D471), or hardness (ASTM D2240) when exposed to a 500-ppm solution of ClO₂ or the vapor-rich headspace above the solution. However, the metals exposed to the same conditions did not fare as well. There were no compatibility issues with SS 316 or 304, but the carbon steels, SA-36 and SA-516, were heavily corroded in both liquid and vapor exposure conditions. The tests were repeated in a more dilute solution of 50 ppm ClO₂, and while the level of corrosion was less severe, the test coupons were still heavily corroded. In practice, structural, carbon steels like these should have passivation coatings or barrier coatings (e.g., epoxy, paint, etc.) to prevent excessive corrosion from water infiltration in the tank and/or direct exposure to precipitation. However, there is no guarantee that the coatings will be pristine when decontamination is required, and the underlying metal in those areas could be compromised.

Table 6. Materials Compatibility with 500 ppm ClO₂ Solution and Headspace

Method	Title	Material Category	Material	Results (pass/fail)
ASTM F483	<i>Standard Test Method for Total Immersion Corrosion Test for Aircraft Maintenance Chemicals</i>	Metals	SS 316	Pass
			SS 304	Pass
			SA-36	Fail
			SA-516 Grade 70	Fail
ASTM D471	<i>Standard Test Method for Rubber Property – Effect of Liquids</i>	Elastomers	Viton	Pass
			Butadiene (Buna-N)	Pass
			Fluorosilicon	Pass
			Neocork	Pass
ASTM D2240	<i>Standard Test Method for Rubber Property – Durometer Hardness</i>	Elastomers	Viton	Pass
			Butadiene (Buna-N)	Pass
			Fluorosilicon	Pass
			Neocork	Pass

Table 7. Materials Compatibility with 50 ppm ClO₂ Solution and Headspace

Method	Title	Material Category	Material	Results (pass/fail)
ASTM F483	<i>Standard Test Method for Total Immersion Corrosion Test for Aircraft Maintenance Chemicals</i>	Metals	SS 316	Pass
			SS 304	Pass
			SA-36	Fail
			SA-516 Grade 70	Fail

Additional testing was performed to evaluate the extent of corrosion that occurs after exposing mild steel to ClO₂ gas. Various types of low carbon steel including A516, A36, R46, and C1010 along with 304 and 316 stainless steel were exposed to repeated ClO₂ gas treatments. The composition of these substrates is provided in Table 8 (*Note: API 650 substrate was not tested but its composition is presented to illustrate how the various low carbon steels are very comparable.*) The dry weights of coupons (1"x 3") or panels (3"x 5") were measured before and after the treatments. Mild steel corrodes rapidly in the environment without passivating treatment or coatings to protect the metal. Therefore, coupons or panels were soaked in water prior to treatment to simulate the level of corrosion that would be apparent in uncoated mild steel tanks simply due to environmental factors. This base layer of oxidation/corrosion could passivate the surface of the metal, reducing the rate of further corrosion compared to pristine steel. This condition was thought to be more indicative of how operational fuel tanks would be impacted by ClO₂ treatment.

Mild steel and stainless steel coupons or panels were first soaked in acetone to remove manufacturer-applied anticorrosion oils. Once dry, they were suspended in tap water for 9 days to induce corrosion. The samples were then dried and weighed. Samples were exposed to ClO₂ gas in a sealed shipping container using the GRS operated with two 12G micro-reactors and with elevated RH. The samples were then weighed. The samples were exposed a second time to ClO₂ gas and weighed again. Photos the samples were taken before and after the water soak and after each ClO₂ treatment (Figure 17 through Figure 20). One set of R46 panels that had been previously exposed to multiple gas treatments were also included in this test, representing pre-corroded samples. The appearances of these pre-corroded samples are shown in Figure 21. The mean dry weights of the replicate samples of each type of steel substrate at the onset of the test and following each treatment are shown in Figure 22.

Table 8. Composition of Various Types of Steel Used in Construction of Fuel Tanks

Matrix	Carbon	Manganese	Potassium	Sulfur
304 SS	0.03-0.08%	2%	0.05%	0.03%
316 SS	0.03-0.08%	2%	0.05%	0.03%
A516, grade 70 mild steel	0.27-0.31%	0.9-1.2%	0.03%	0.03%
A36 mild steel ^a	0.26%	0.8%	0.04%	0.05%
R46 mild steel ^b	0.15%	0.6%	0.03%	0.04%
C1010 mild steel ^c	0.13%	0.6%	0.04%	0.05%
API Standard 650 (plate) Grades 235-275	0.24-0.29%	--	0.05%	0.05%

- a) Structural steel accepted by American Petroleum Institute per API standard 650
- b) Used at METSS for efficacy testing
- c) Used at AFRL for microcosm growth/corrosion studies

Photos of the substrates are shown in Figure 17, Figure 18, Figure 19, and Figure 20. As expected, the low carbon steel substrates all showed significant corrosion after being immersed in water. Some additional corrosion appeared on these substrates following each ClO₂ treatment. The stainless steel samples did not appear affected.

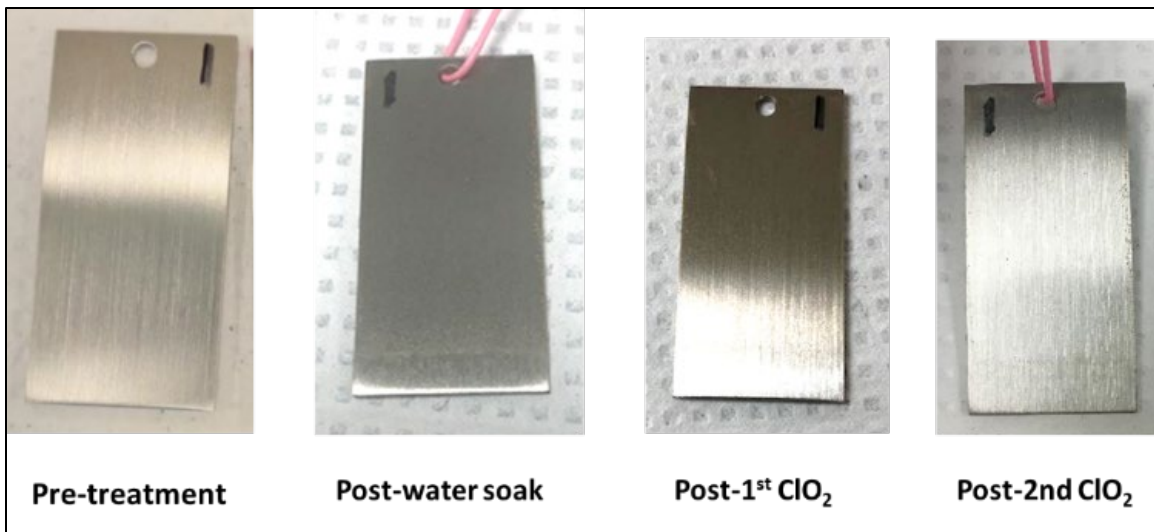


Figure 17. 304 stainless steel coupons

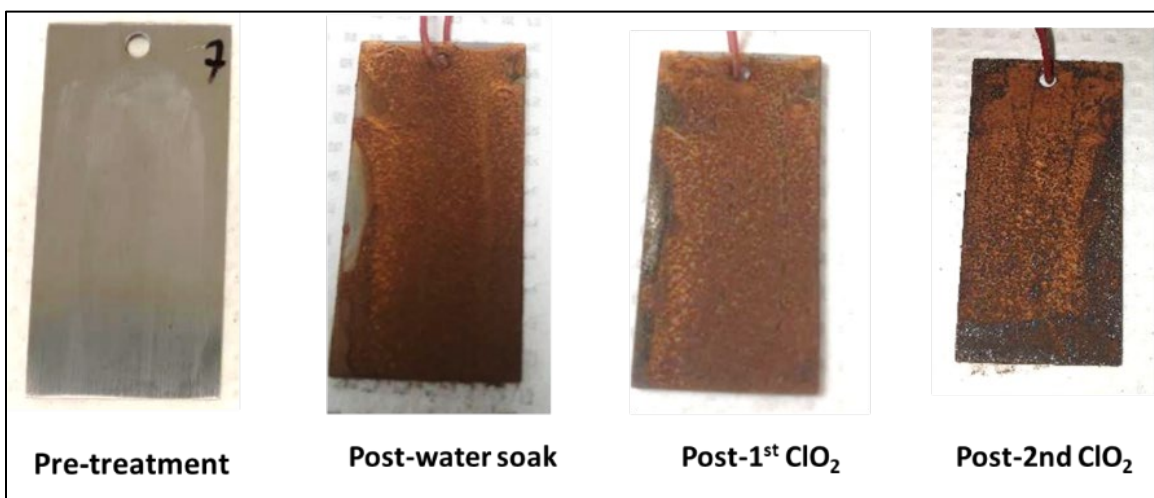


Figure 18. A516 coupons

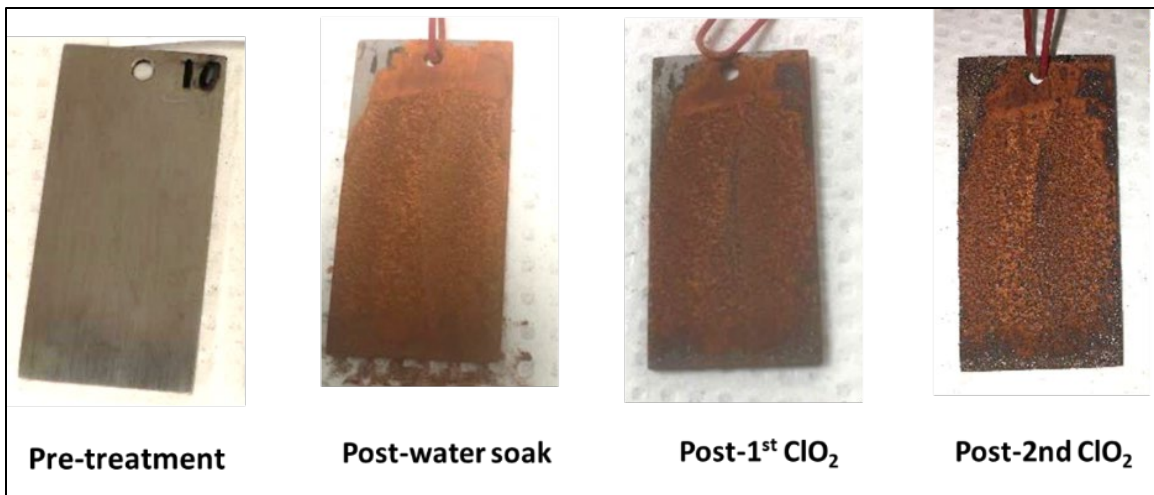


Figure 19. A36 mild steel coupons

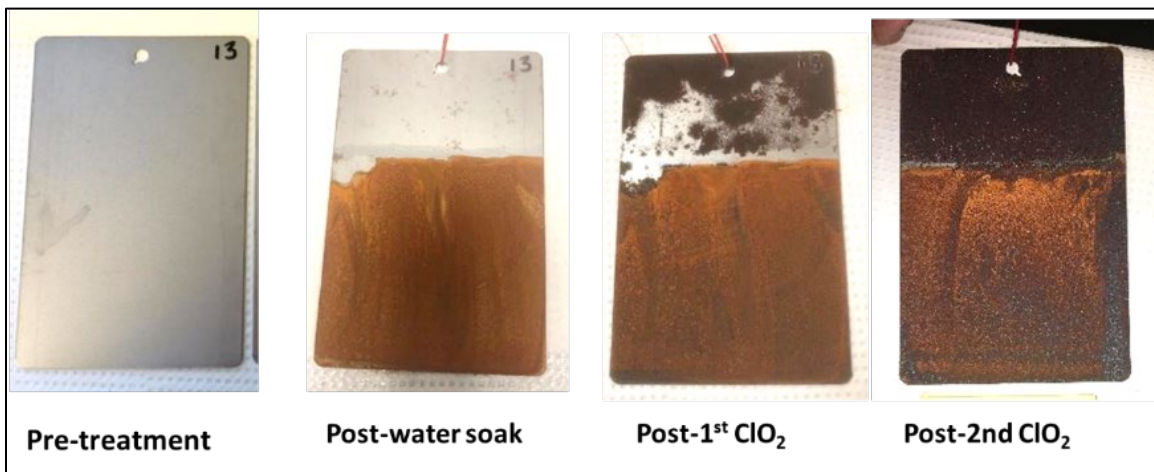


Figure 20. R46 mild steel panels

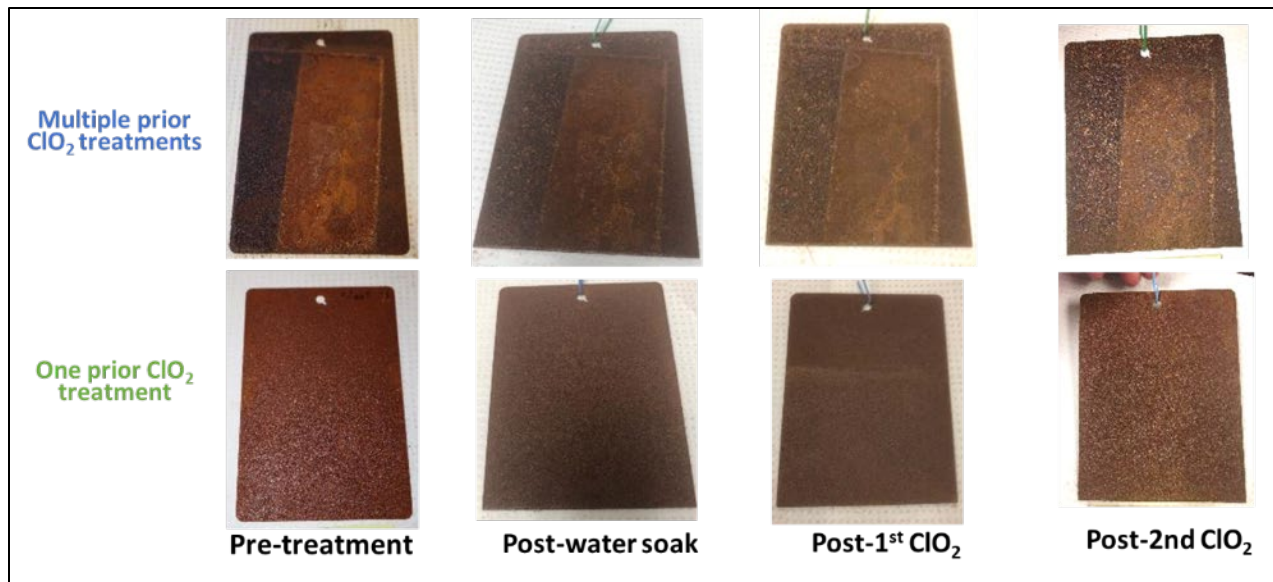


Figure 21. Pre-CIO₂ treated R46 mild steel panels

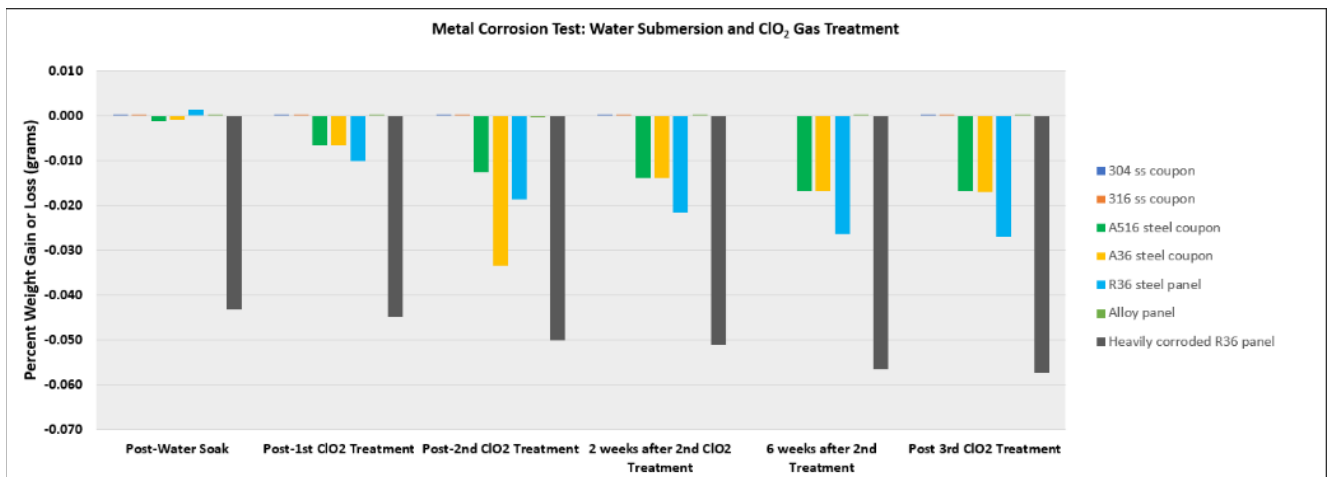


Figure 22. Corrosion test results

The key observations from this test were:

- No macroscopic corrosion observed on stainless steel
- Significant visual corrosion on mild steel (A516, A36, and R36) from water, then slightly more corrosion observed from CIO₂ treatment
- Perhaps the most important finding from this test is that the amount of corrosion of pre-corroded substrates did not change significantly following each treatment, suggesting a CIO₂ gas treatment of an aged steel tank would not significantly increase corrosion
- The amount of corrosion did not change 2-6 weeks after exposure, indicating that corrosion mechanisms are limited to the duration of CIO₂ exposure and not beyond.

3.6.2 Growth Rates in B5 vs. B20

Two 1-L microcosms were established in which one contained B5 (5% biodiesel in diesel) and ASW and the other contained B20 and ASW. Diesel fuel (D2) and B20 were acquired from local gas stations Meijer, Westerville, OH and Heritage Cooperative, Urbana, OH, respectively. B5 was prepared by diluting B20 accordingly with D2. Ten steel coupons were suspended in each microcosm and each microcosm was inoculated with mixture of *Byssochlamys* mycelium and spores. The microcosms were incubated statically at 27 °C. Replicate coupons were assayed (i.e., extracted and enumerated) on days 13, 26, 42 and 60. The results (Figure 23) indicated the level of biofilm growth remained steady over time with the level of biofilm growth on the B20 coupons being only slightly higher (<1 log). These results suggest that *Byssochlamys* will grow equally well in either B20 or B5. This is significant because up to 5% biodiesel is deemed “acceptable” in most D2 fuel systems. Therefore, the biofouling that is most common in biodiesel storage tanks will become common in D2 storage tanks as levels of biodiesel or fatty acid methyl ester (FAME) contamination increase over time.

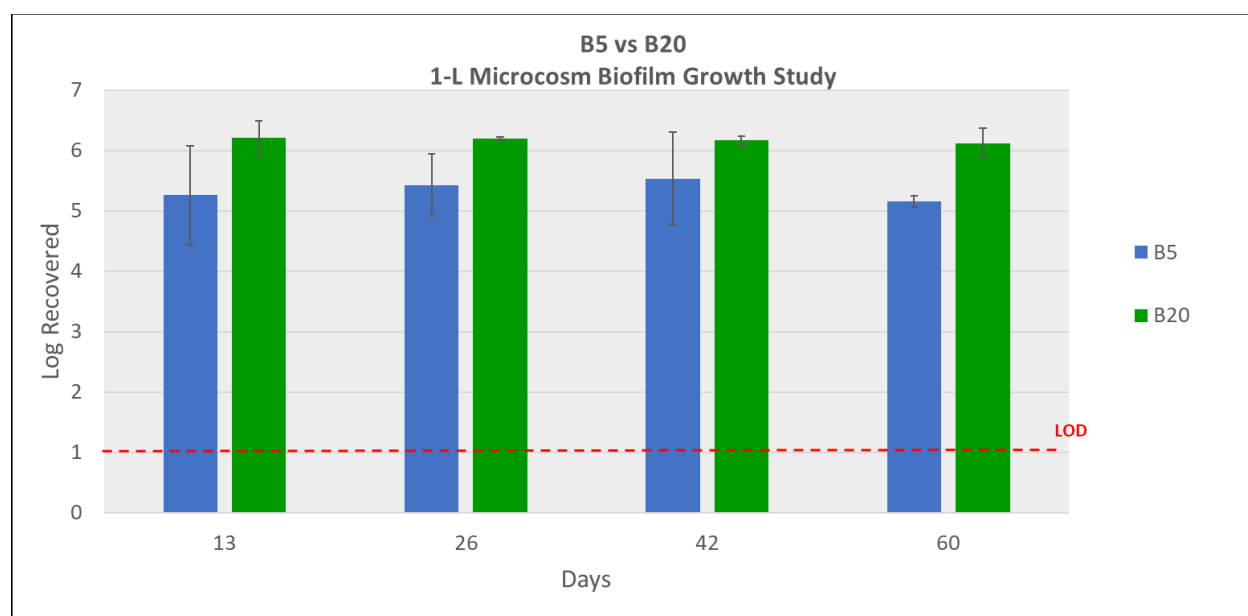


Figure 23. Growth rates of biofilm in B5 vs. B20

3.7 Decontamination System Design and Mock-up Evaluations

METSS utilized a 1,169 cubic ft. shipping container (Figure 24) to simulate an 8,800-gallon fuel storage tank in full scale HAD and ClO₂ decontamination trials. Several types of heaters and humidifiers were used to perform a series of full-scale HAD characterization trials to assess whether the surface and air temperatures of 140 °F (60 °C) and elevated RH conditions (>80%) could be reached and maintained. The HAD mock trials provided the opportunity to gain experience in controlling the rate of heating and humidification in a manner that minimizes condensation build up within the shipping container, which would be undesirable if

decontaminating an operational fuel storage tank. The shipping container is generally similar in shape (rectangular) and size to large fuel tanks common to many AFB fuel storage facilities. A series of ClO₂ mock-up trials were also performed using the shipping container to assess if gas concentrations needed to achieve complete or near complete kill could be achieved at this scale. These mock-up trials afforded METSS the opportunity to develop and evaluate full-scale HAD and ClO₂ decontamination processes in-house. Such testing also afforded the opportunity to refine the processes such that either or both processes could be rapidly deployed for a field demonstration when a fuel system requiring cleaning would come available.



Figure 24. 20-ft. shipping container (often referred to as an ISO or Conex container)

3.7.1 HAD Mock-up Trials

Table 9 describes the series of mock-up HAD trials that were performed. Trials 1-3 used a 250K BTU government-furnished equipment (GFE) forced air heater (rated with 120-150 cfm air circulation output), a 325K BTU indirect fired space heater (Flagro; Model FVO0400) was rented from a local heating and cooling equipment rental company to perform Trials 4-7, and household heaters were used to perform Trial 8. These heaters were generally comparable in capacity to GFE New Generation 400K BTH NGH portable heaters commonly used at many Air Force bases. A Carel humiSteam Xplus steam generator was used in Trials 3, and 5-7 to assess if the RH could be elevated and controlled during heating. A household humidifier was used during Trial 8. These trials are described in detail in Appendix C; select trials are presented in this section to illustrate the best options for HAD.

Table 9. List of HAD Mock-up Trials

HAD Mock Trial Number	Description
1	200K BTU GFE heater; no humidification added
2	200K BTU GFE heater; no humidification added
3	200K BTU GFE heater with humidification
4	325K BTU heater; no humidification added
5	325K BTU heater; humidification added after 3 hours
6	325K BTU heater; humidification added after 1 hour
7	325K BTU heater; with intermittent humidification
8	Two space heaters, household humidifier, and fan

The shipping container was retrofitted to accommodate heating and humidification ducts, and numerous thermocouples and RH sensors were positioned throughout the interior of the container and wired to an exterior data logger. One door of the shipping container was replaced with a 2-inch thick insulation panel, in which ports were cut to accommodate heating and humidifying ducts that were connected to heaters and humidifiers positioned outside the storage container. The heating and humidifying devices used for Trial 1-3 aligned with the retrofitted door of the shipping container and thermocouples are shown in Figure 25. Thermocouples and RH probes were positioned throughout the interior of the shipping container to monitor environmental conditions of the air and at the surface (Figure 26).



Figure 25. Test system used for HAD Mock-up Trials 1-3: 250K BTU heater and Carel steam generator shown in left image; steam diffuser placed inside the shipping container and connected to an exterior steam generator (middle image); and thermocouples and RH sensors connected to data loggers (right image).

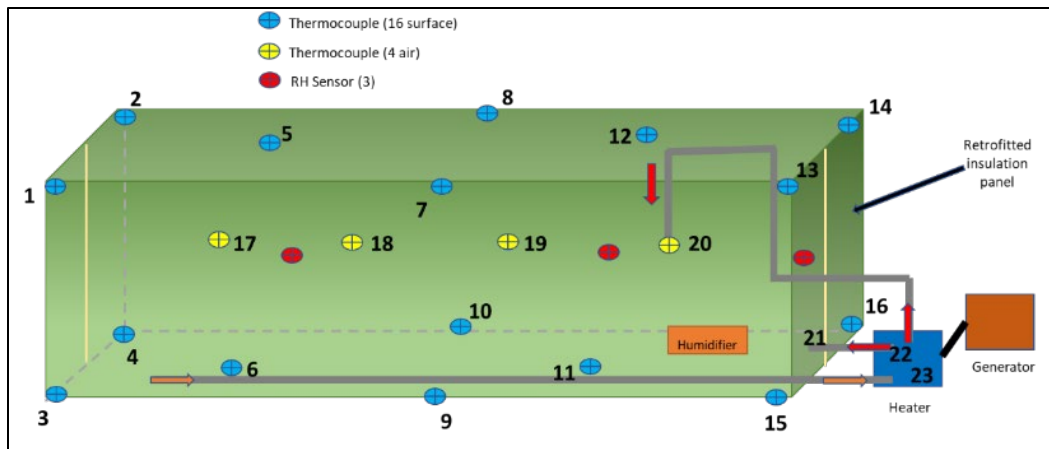


Figure 26. Placement of thermocouples and RH sensors

METSS also identified commercially available ducting and duct adaptors (Figure 27) that could be used to easily connect a decontamination system to fuel storage tanks using manways and gauge hatches as ports of incoming and outgoing heated air or fumigant. All fuel tanks have a combination of gauge hatches, vents, or manways in various standardized sizes. METSS also planned to manufacture custom adaptors from heavy plastic, aluminum, or steel (such as the one shown in the lower right corner of Figure 27) in the event commercial adaptors were not available.



Figure 27. Commercially available ducting and duct adaptors that could be used to attach a mobile decontamination system to any style of fuel storage tank.

In the most successful trial, the 325K BTU heater was run for 7 hours; the steam generator was used after three hours to assess the ability of this test set up to elevate RH. Figure 28 and Figure 29 show the thermal profiles on the surfaces and in the air, respectively. The surface temperatures appeared to reach a steady state at about 1.5 hours, averaging approximately 50 °C with one location near the heater inlets reaching 60 °C. The air temperatures did increase rapidly within the first 15 minutes to 60 °C or greater. Two thermocouples held steady around 60 °C

while the two other couples held fairly steady between 80-90 °C. Due to the high air capacity of the heater, it was observed around 6 hours that air pressure within the shipping container started dislodging the retrofitted door. To prevent this from worsening, the door at the opposite end of the shipping container was cracked open to allow for more efficient venting. By doing so, the surface and air temperatures increased; however, the RH profile (Figure 30) indicates the steam generator did not have the capacity to elevate humidity over 20% as the air flow through the shipping container was simply too high.

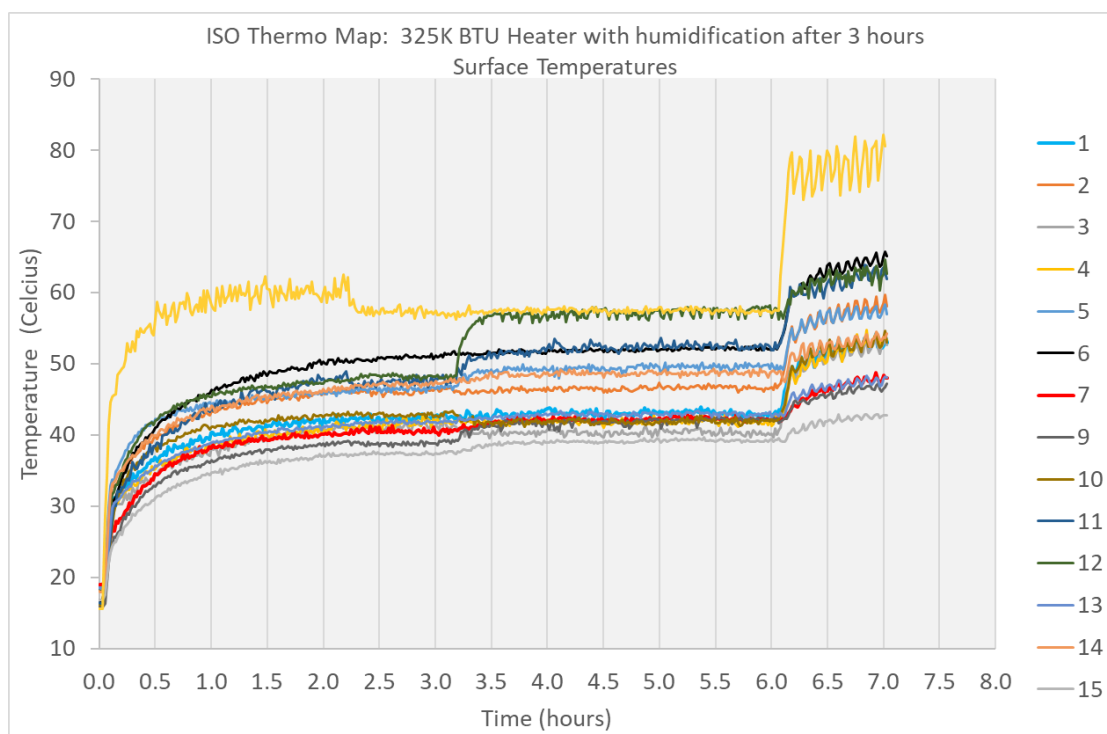


Figure 28. HAD Mock-up Trial 5 thermal profiles on surfaces

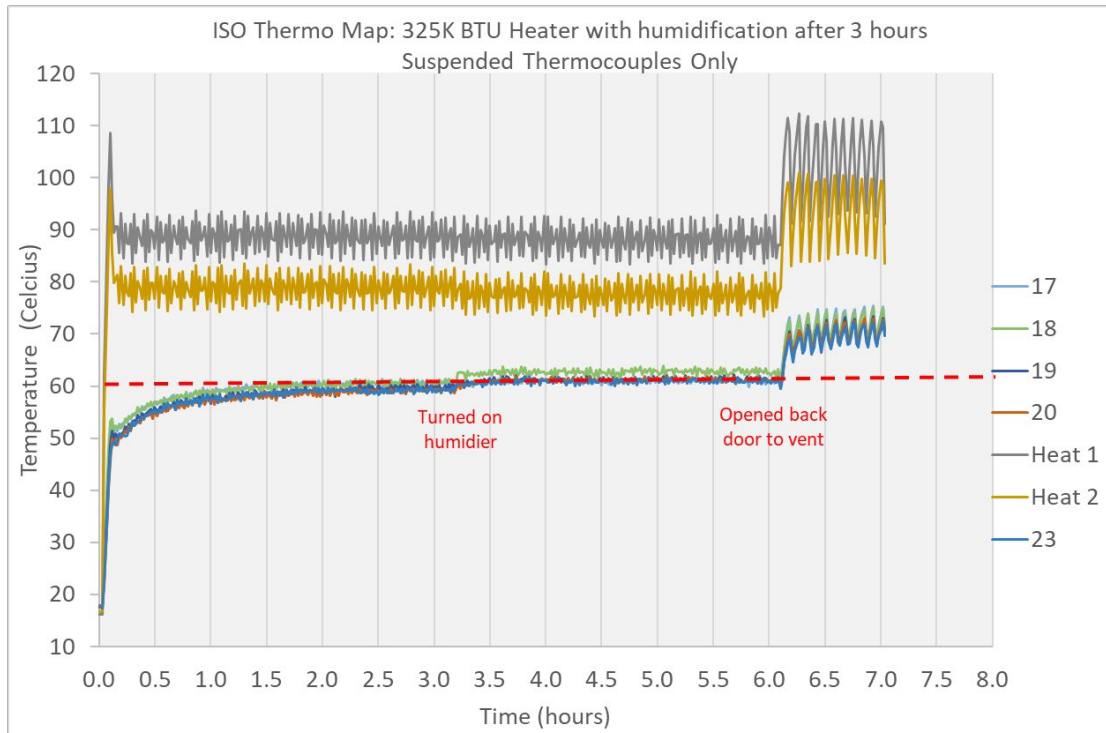


Figure 29. HAD Mock-up Trial 5 thermal profiles in air

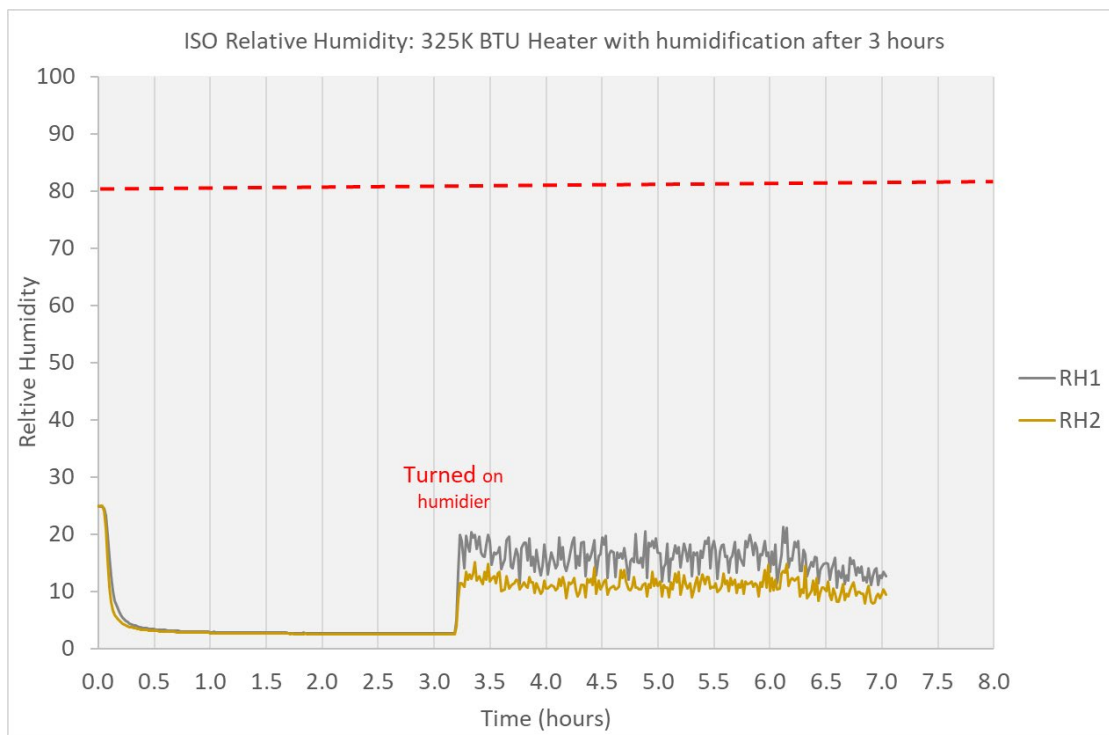


Figure 30. HAD Mock-up Trial 5 RH profiles

In a subsequent test, a combination of heating and humidification scenarios were assessed in which the heater and steam generator were turned on and off intermittently. In addition, the heater was initially operated using both outputs; after approximately 1 hour and 20 minutes, only one output was used. The sequence of events is depicted in Figure 31, Figure 32, and Figure 33, showing surface temperature, air temperature, and RH profiles, respectively.

The temperature and RH profiles tracked as expected. Higher temperatures were reached when both heater outputs were used, and the RH increased slightly to approximately 30% when only one heater output was used. When the heater was turned off, the RH quickly approached 100%.

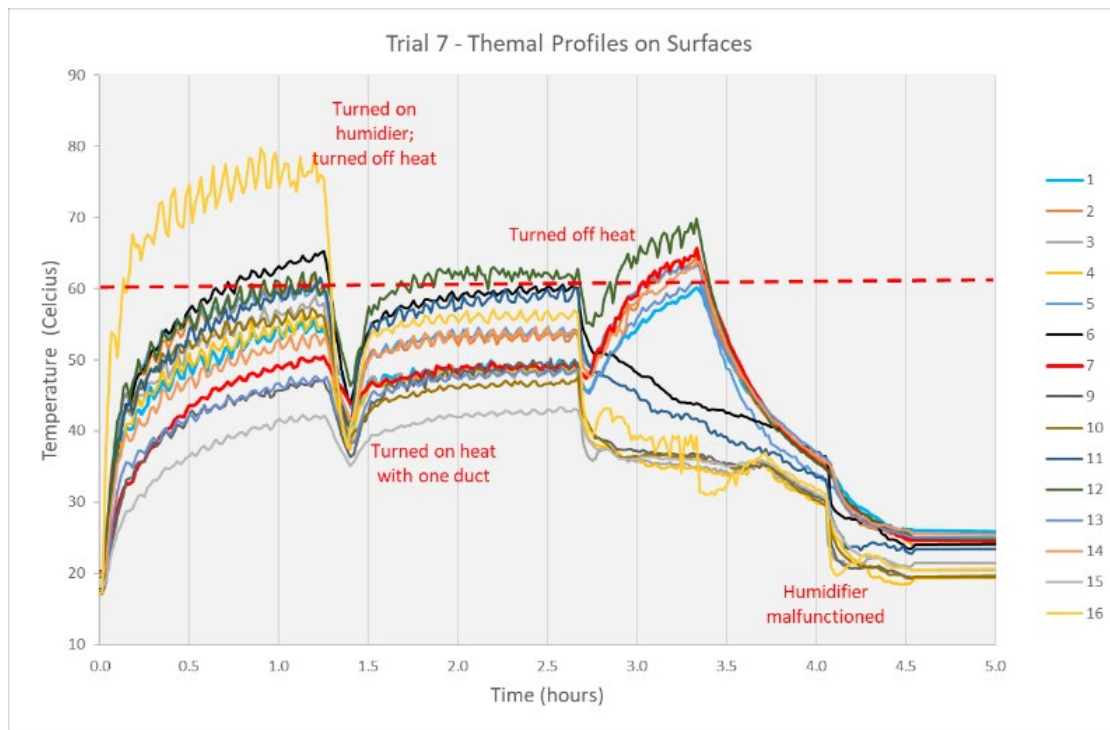


Figure 31. HAD Mock-up Trial 7 thermal profiles on surfaces

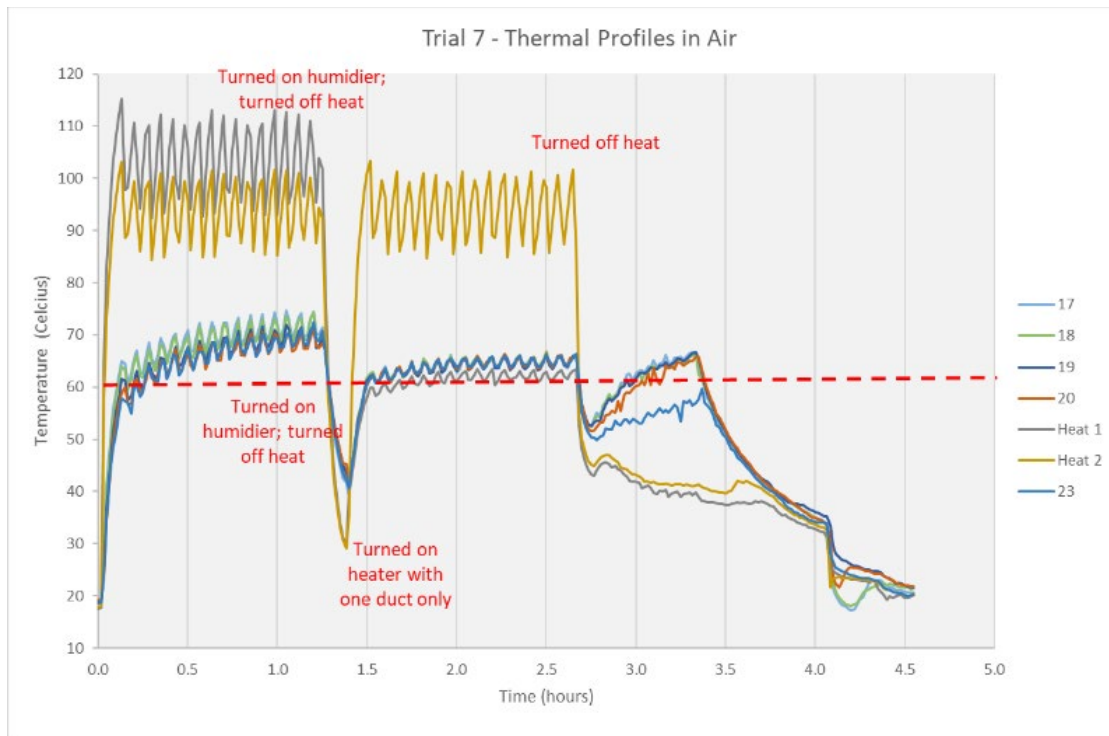


Figure 32. HAD Mock-up Trial 7 thermal profiles in air

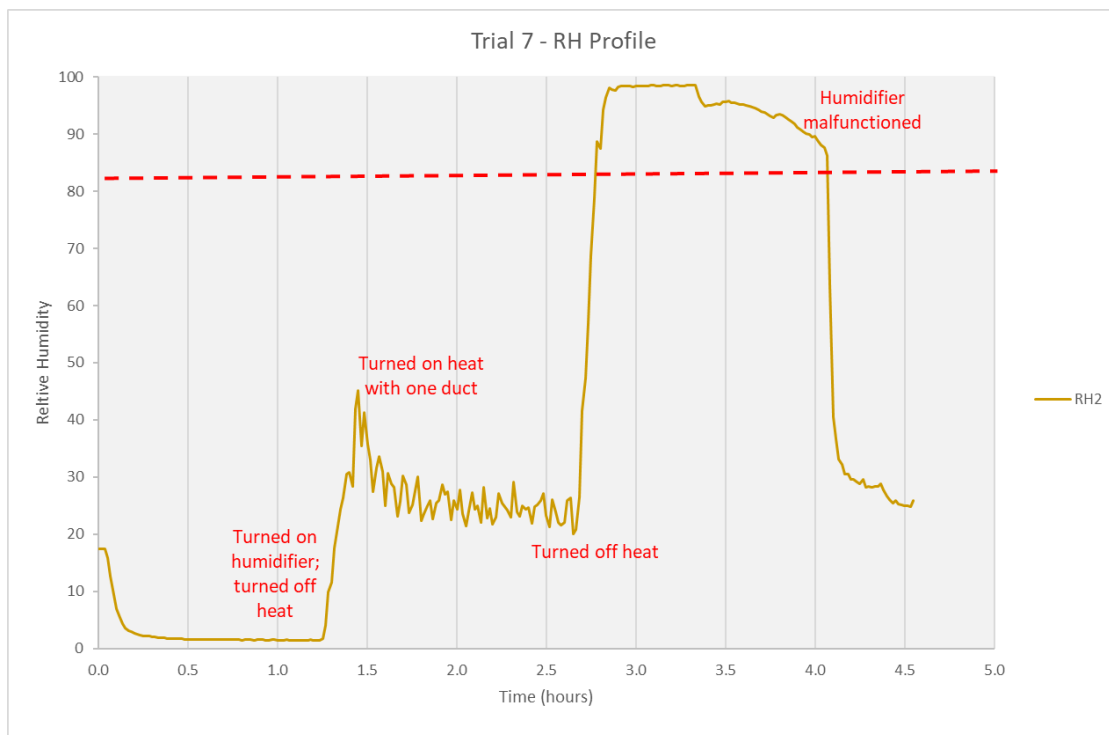


Figure 33. HAD Mock-up Trial 7 RH profile

The HAD Mock-up trials demonstrated that it would be very difficult to reach interior surface temperatures of 140 °F (60 °C) in a large volume (range of 8,000 gallons) and achieve elevated RH conditions using commercially available heaters and humidifiers; a custom heating and humidification system would be needed. Designing, constructing, and testing such a system was outside the scope of this study. Based on the results of these HAD mock-up trials and the successful results shown from the ClO₂ efficacy testing, full-scale testing using a ClO₂ gas fumigation method to remediate contaminated, empty fuel storage tanks was investigated further. This work is summarized in following section.

3.7.2 ClO₂ Mock-up Trials

After completing the HAD mock-up trials, a series of ClO₂ efficacy tests were performed which demonstrated ClO₂ gas could kill biofilm (described in Section 3.5.2.1). Efficacy testing continued at full-scale using a gas dispersion unit called the Yellow Jacket which utilized commercially available ClO₂ gas generating micro-reactors to disperse ClO₂ gas into a confined space. Full-scale testing (described in Section 3.5.2.2 and Appendix B) confirmed this gas dispersion methodology could be used to achieve efficacious gas concentrations. Thereafter, a unique gas dispersion recirculation system was then designed and tested using the ISO shipping container as the test bed for mock-up trails.

3.7.2.1 Gas Recirculation System (GRS)

The GRS was constructed using two rugged, sealable totes, PVC piping and associated adaptors, flexible dryer hose, tubing clamps, and an electrical recirculation air blower (Figure 34). One of the totes housed the Yellow Jacket gas dispersion system and the other tote housed a common household ultrasonic humidifier (Boneco Ultrasonic Humidifier 7135). The totes were connected via PVC piping. Flexible tubing was connected from the outlet of the second tote (housing the humidifier) and connected to a 4-inch diameter port located in the retrofitted side-door panel of the shipping container. This port represented a manway or gauge hatch port that would generally be accessible on any fuel tank system to introduce the ClO₂ treatment. Another section of flexible dryer hose was connected from a second port in the retrofitted side-door panel of the shipping container, presenting a second gauge hatch port on a fuel tank system, from which gas would be returned in a closed loop to an air recirculation blower which, in turn, was connected to the tote housing the Yellow Jacket. Extension cords were fed through the walls of the totes to provide a power source for the Yellow Jacket and the humidifier. A short section of silicone tubing was run through the first tote and into the reservoir of the Yellow Jacket, and a 60-mL syringe was then connected to the external end of the tubing. This line was used to inject a concentrated solution of sodium thiosulfate into the Yellow Jacket to neutralize the ClO₂ solution as desired, subsequently stopping the fumigation process.

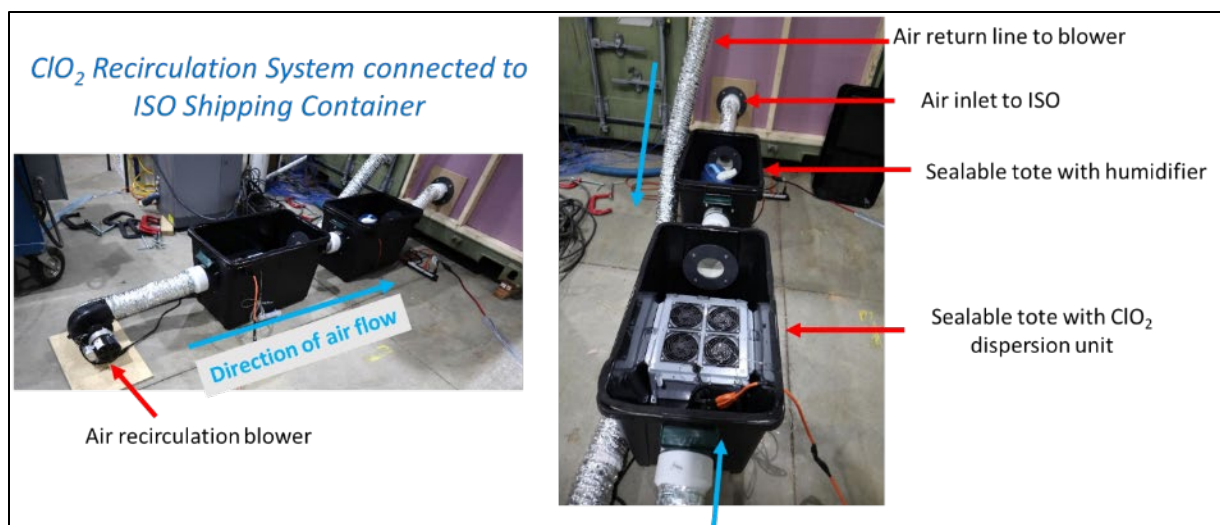


Figure 34. ClO₂ Gas Recirculation System

Once assembled and connected to the fuel tank, the operator would perform the following procedure to operate the GRS:

- 1) Fill humidifier with water, turn on, and set to desired set point of 70% RH. Seal lid of tote.
- 2) Add 6-liters of 20-25 °C tap water to Yellow Jacket. Add appropriate number of Selectroside 12G micro-reactors to the water. Fasten lid to Yellow Jacket and seal lid of tote.
- 3) Attach PortaSens gas detector installed with a 200-2000 ppm gas sensor to either tote. Set PortaSens to record gas readings at least every 5 minutes for the duration of the test.
- 4) Ensure shipping container is closed (or, if a fuel tank is used, ensure all ports and manways are closed and sealed).
- 5) Plug in air recirculation blower to initiate the fumigation.
- 6) After 2 hours, plug in extension cord that runs to the tote housing the Yellow Jacket. This activates the fans of the Yellow Jacket, which begins the off-gassing process.
- 7) Allow system to run for desired length of time.
- 8) Manually inject approximately 50 mL of a 10% sodium thiosulfate solution into the Yellow Jacket using the syringe/silicone tubing installed in the first tote to neutralize the ClO₂ solution and terminate the fumigation process.
- 9) Once the gas in the recirculation system decreases to below 1 ppm (based on readings from the PortaSens unit), turn off air blower and unplug extension cords running to the Yellow Jacket and humidifier.

Two functionality tests were performed. The first was performed with one 12G micro-reactor and the second was performed with two 12G micro-reactors. The gas profiles from these tests are shown in Figure 35. The system performed well in that expected gas profiles and treatment levels were generated and were reproducible. In addition, the dual-tote recirculation system was

shown to be safe to operate during each of the functionality tests (Figure 36). The occupation exposure limit (OEL) of ClO_2 is 0.1 ppm over an 8-hour work period, and no gas was detected within close proximity of the recirculation system testing during testing. The recirculation system was then used for all subsequent efficacy tests (Tests C12 to C14) including a full-scale, simulated field demonstration activity.

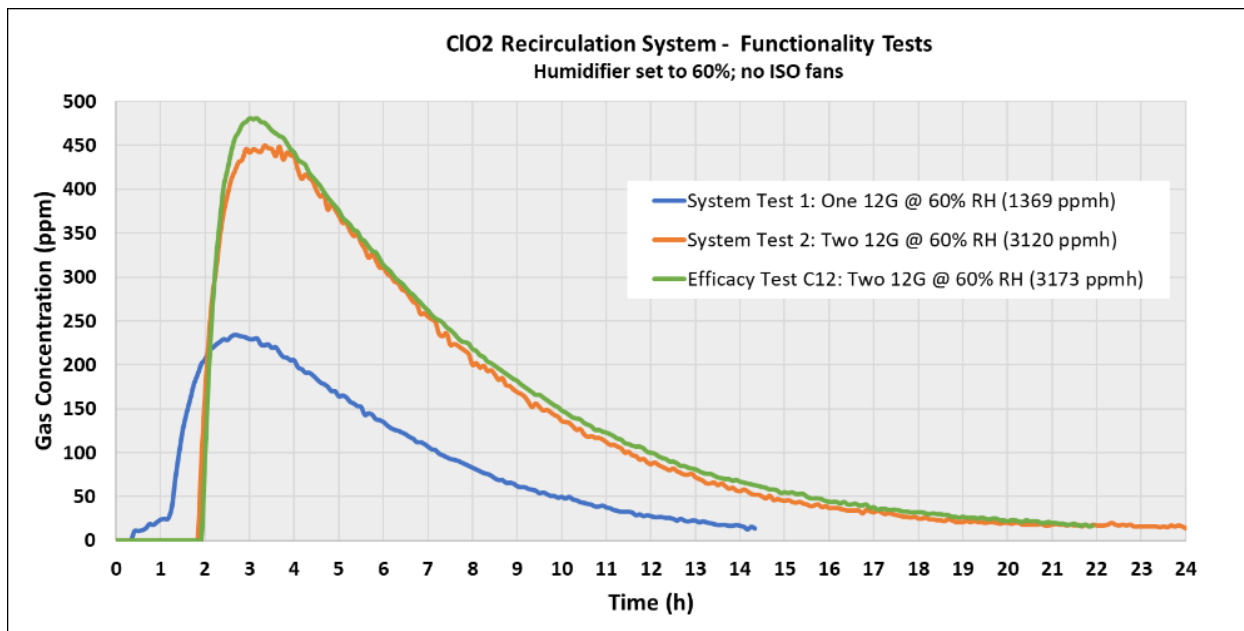


Figure 35. Gas recirculation system functionality tests

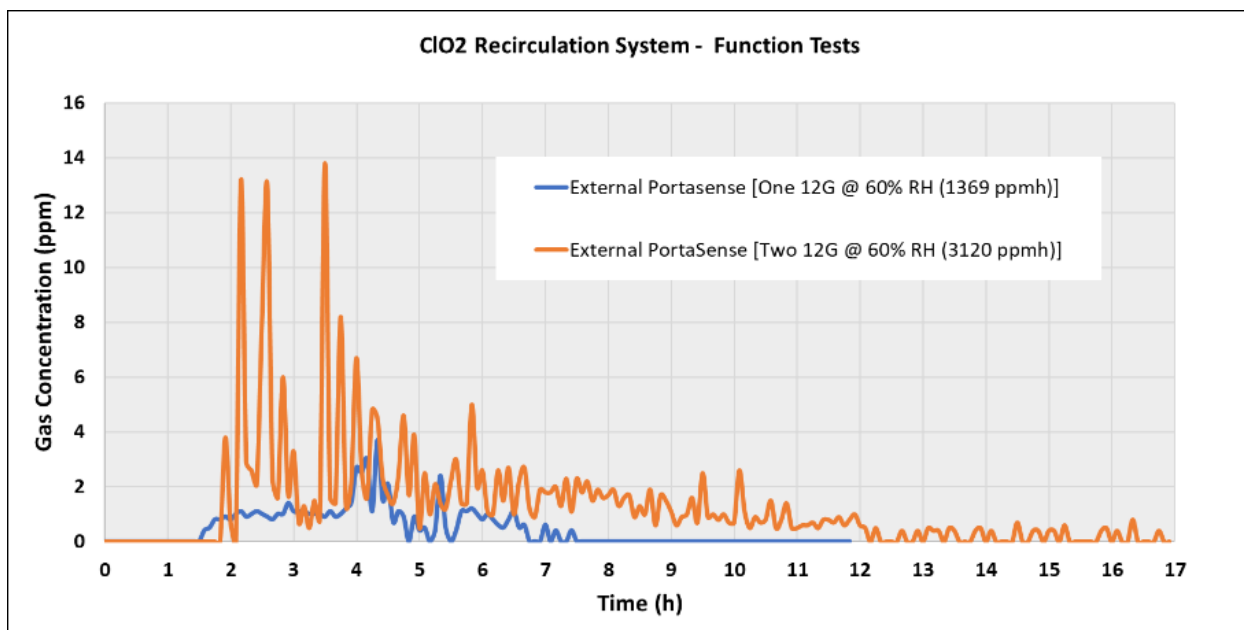


Figure 36. Gas profiles from PortaSens gas detector positioned adjacent to gas recirculation system during functionality testing.

3.7.2.2 Efficacy Trials

Prior to these mock field trials, ClO₂ gas was generated in the ISO container using two 12G micro-reactors which resulted in >3-log kill. These previous tests were all carried out overnight and allowed to run for 14-18 hours. To shorten the treatment time to a single work day (a time that would be viewed as more favorable to the Air Force and crew that would be conducting process), three 12G micro-reactors were used in each of two mock field tests in which the process was run for only 8 hours (this includes the two-hour gas generation period).

Two mock field tests were conducted to assess the rate of regrowth of biofilm in a BDI/water microcosm following a simulated tank cleaning/ClO₂ gas decontamination treatment. Contaminated empty glass microcosm vessels containing biofilm-laden steel coupons were treated with ClO₂ gas in the ISO container, then the vessels were filled with fuel and water and incubated at room temperature. Coupons suspended in the fuel-water environment were analyzed for biofilm growth over time. Untreated vessels, prepared in the same manner, represented the control group.

For each mock field test, seven 3-L beakers, each containing ten 1"x3" steel coupons, were filled with approximately 1 L ASW and 0.5 L B20 (Figure 37). Low carbon steel coupons were suspended at the fuel/water interface. The beakers were then contaminated with *Byssochlamys* biomass/spore mixture and incubated for several weeks to allow biofilm to establish within the vessel and on the coupons (Figure 38). Thereafter, the fuel and water were removed from each beaker, leaving empty, contaminated beakers. The interior surface and coupons were gently rinsed with sterile water to mimic a fuel tank cleaning operation (Figure 38). Four beakers (denoted D, E, F, and G) were then placed in the shipping container with one beaker on the floor in each of the four corners. Two coupons from these beakers were suspended from the ceiling at the respective corners. *Geobacillus stearothermophilus* and *B. atrophaeus* BIs and ClO₂ Check strips were also placed in all eight corners of the test chamber, as well as eight other locations (Figure 39). The test system was sealed and then decontaminated using the GRS with three 12G micro-reactors. The three remaining 3-L microcosm beakers (denoted A, B, and C) were left on the benchtop, representing untreated control beakers. Duplicate coupons from each beaker were assayed just prior to emptying the beakers, and just after the ClO₂ treatment. The coupons suspended from the ceiling were also assayed after treatment. The locations of the test samples are shown in Figure 40.

Following the decontamination treatment, all beakers were aseptically refilled with ASW and B20, then incubated statically at room temperature for several months. Every 2-4 weeks, replicate coupons from each beaker were assayed for biofilm to assess and compare the rate of regrowth between treated and untreated beakers. Photographs of each beaker were taken periodically throughout the incubation period.

The only difference between the two tests is that foil was placed loosely over the 3-L vessels during the gas treatment in Test 1, and the microcosm vessels were not covered in Test 2. The microcosms were covered in Test 1 to minimize the potential for cross-contamination from airborne contaminants. Although loosely fitted, the foil may have had adverse effects on the gas treatment inside the microcosm vessel during Test 1; therefore, the vessels were left uncovered during treatment in Test 2.

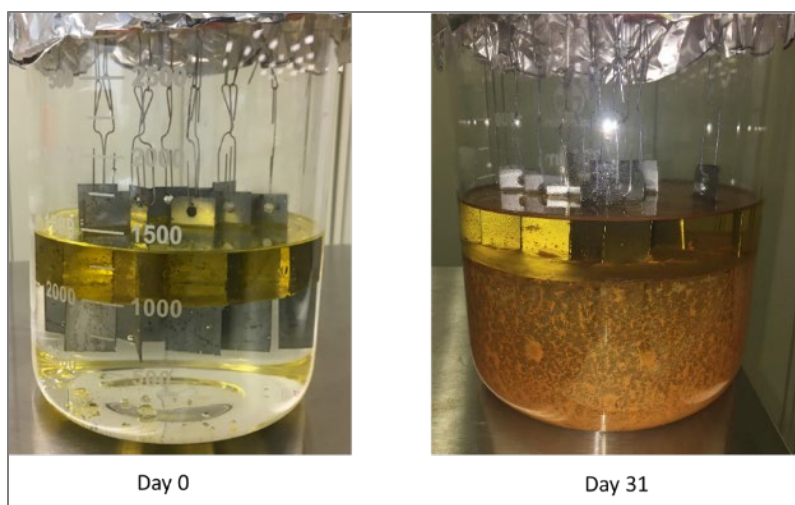


Figure 37. Establishment of biofilm-laden coupons and contaminated 3-L fuel/water microcosms

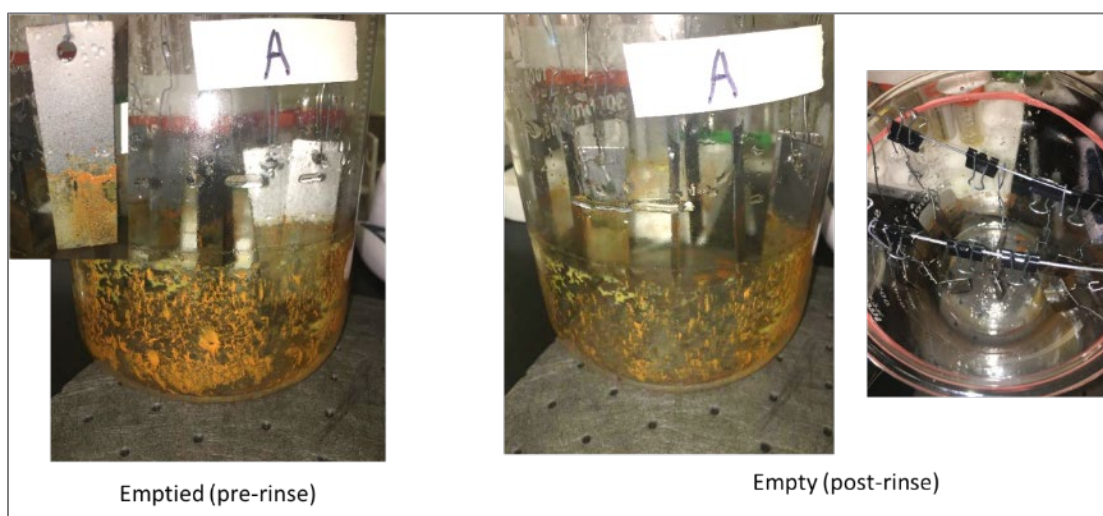


Figure 38. Appearance of microcosms after being emptied and gently rinsed



Figure 39. Interior of shipping container (i.e., full-scale test system) showing location of microcosm vessels, BIs, and gas indicator strips

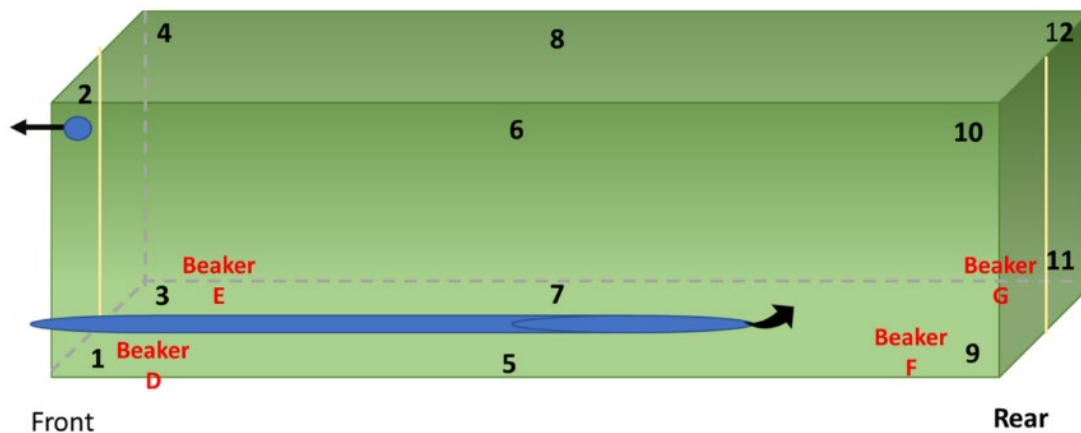


Figure 40. Location IDs for BIs, CD Check strips, and microcosm 3-L beakers

The gas and RH profiles from these tests are shown in Figure 41 and Figure 42. The treatment levels achieved for Tests 1 and 2 were 3500 and 3152 ppmvh, respectively. The reason for the slightly lower treatment level for Test 2 is unclear, but may be attributed in part to lower RH levels and perhaps undetected gas leaks. In any event, the treatments were deemed comparable.

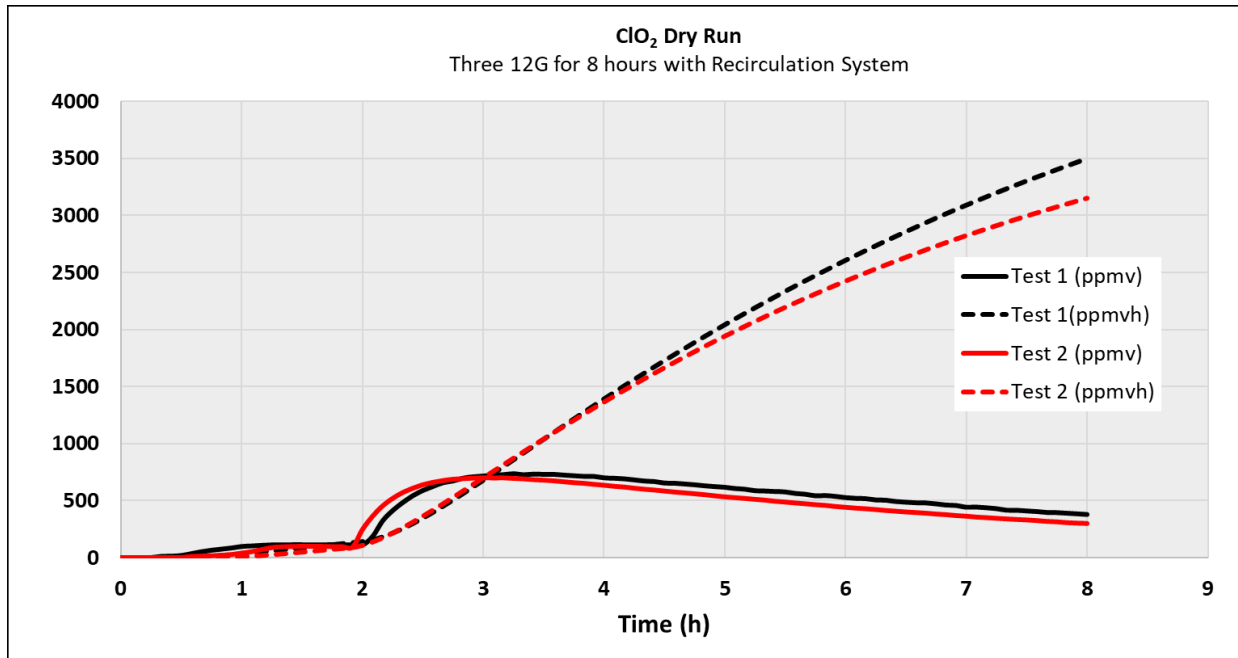


Figure 41. Gas profiles from Dry Run tests

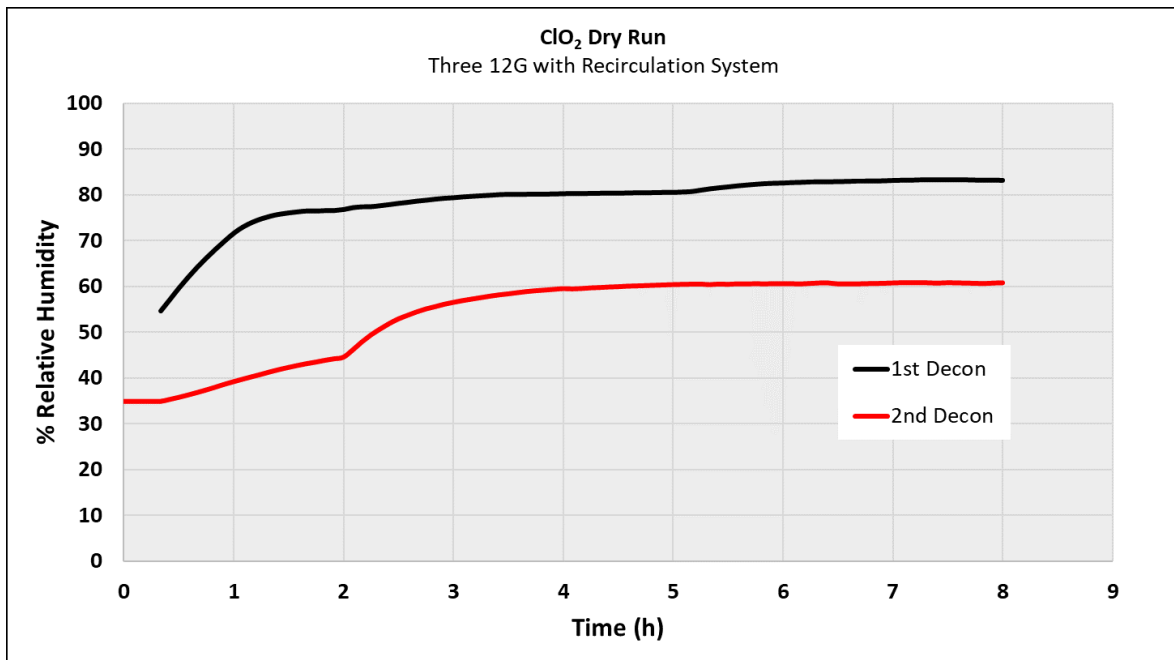


Figure 42. Relative humidity profiles from Dry Run Tests 1 and 2

The results of the CD Check strips and BIs for Tests 1 and 2 are shown in Table 10 and Table 11, respectively. In both tests, all exposed CD Check strips tested positive, indicating the gas concentration reached >300 ppm throughout the test system. As shown in Table 12, the majority of the BIs tested negative for growth (22 of 24 Gs BIs and 19 of 24 Bg BIs tested negative). This

demonstrates the treatment was very effective against bacterial spores. Each BI contains $>1 \times 10^6$ spores, therefore a no growth result indicates a 6-log reduction was achieved.

Table 10. Efficacy Results from Dry Run Test 1

Sample Type	Placement in Conex	Sample ID	CD Check*	BI's (Pos. or Neg.)	
				Gs	Ba
Treated	Front Right Bottom Corner	1	Pos.	Neg.	Neg.
	Front Right Top Corner	2	Pos.	Neg.	Neg.
	Front Left Bottom Corner	3	Pos.	Neg.	Neg.
	Front Left Top Corner	4	Pos.	Neg.	Neg.
	Mid Right Bottom Corner	5	Pos.	Neg.	Neg.
	Mid Right Top Corner	6	Pos.	Neg.	Neg.
	Mid Left Bottom Corner	7	Pos.	Neg.	Neg.
	Mid Left Top Corner	8	Pos.	Neg.	Neg.
	Back Right Bottom Corner	9	Pos.	Pos.	Neg.
	Back Right Top Corner	10	Pos.	Neg.	Neg.
	Back Left Bottom Corner	11	Pos.	Neg.	Neg.
	Back Left Top Corner	12	Pos.	Neg.	Neg.
Controls	Neg. Control	13	Neg.	Neg.	Neg.
	Pos.. Control	14	n/a	Pos.	Pos.
*Lt. Blue= Positive; Pink= Negative					

Table 11. Efficacy Results from Dry Run Test 2

Sample Type	Placement in Conex	Sample ID	CD Check*	BI's (Pos. or Neg.)	
				Gs	Ba
Treated	Front Right Bottom Corner	1	Pos.	Neg.	Pos.
	Front Right Top Corner	2	Pos.	Neg.	Neg.
	Front Left Bottom Corner	3	Pos.	Neg.	Neg.
	Front Left Top Corner	4	Pos.	Neg.	Pos.
	Mid Right Bottom Corner	5	Pos.	Neg.	Pos.
	Mid Right Top Corner	6	Pos.	Neg.	Neg.
	Mid Left Bottom Corner	7	Pos.	Neg.	Pos.
	Mid Left Top Corner	8	Pos.	Pos.	Neg.
	Back Right Bottom Corner	9	Pos.	Neg.	Neg.
	Back Right Top Corner	10	Pos.	Neg.	Neg.
	Back Left Bottom Corner	11	Pos.	Neg.	Neg.
	Back Left Top Corner	12	Pos.	Neg.	Pos.
Controls	Neg. Control	13	Neg.	Neg.	Neg.
	Pos.. Control	14	n/a	Pos.	Pos.
*Lt. Blue= Positive; Pink= Negative					

Table 12. Results of BIs from Dry Run Tests

Test	Number of BIs that tested negative (no growth)	Location of BIs that tested positive
1	11 of 12 Gs BIs	Back right bottom corner (location 9)
	12 of 12 Bg BIs	Not applicable
2	11 of 12 Gs BIs	Back right bottom corner (location 9)
	7 of 12 Bg BIs	Front right bottom corner (location 1) Front top left corner (location 4) Mid-right bottom corner (location 5) Mid-left bottom corner (location 7) Back-left bottom corner (location 12)

Table 13 shows the enumeration results of the biofilm-laden coupons before and after the treatment from Test 1. The results indicate the biofilms developed consistently on the coupons at 6.4 ± 0.8 logs per coupon. The treatment did reduce the biofilm concentration by approximately 4 logs to 2.3 ± 2.7 logs. After the microcosms (beakers A-G) were refilled with fresh sterile ASW and fuel and incubated for 10 days, the concentration of biofilm on the untreated and treated microcosms differed by approximately 2 logs, and in two instances (beakers D and F) the concentrations were the same as the untreated controls. Based on these results, it was suspected the foil lids placed loosely over the microcosms D-G may have adversely affected the efficacy of the gas treatment. Therefore, Test 1 was discontinued and a new set of seven microcosms were established. Test 2 was then repeated in the same manner as Test 1 with the exception of the treated microcosms were left uncovered during the fumigation process.

Table 13. Biofilm Growth Results of Dry Run Test 1

Group	Type	Beaker	Sample ID	Log CFU	Mean Log	Std. Dev.
Post-Rinse/Pre-Dry	Untreated	A	A1	5.3	6.4	0.8
		B	B1	7.4		
		C	C1	7.1		
		D	D1	6.2		
		E	E1	7.0		
		F	F1	5.4		
		G	G1	6.3		
Post-rinse/Post Dry	Untreated	A	A2	5.9	6.1	0.9
		B	B2	5.4		
		C	C2	4.8		
		D	D2	7.1		
		E	E2	5.9		
		F	F2	7.3		
		G	G2	6.0		
Post-CIO2	Treated	Front Right Bottom Corner (Beaker D)	1	5.9	2.3	2.7
		Front Right Top Corner	2	0.0		
		Front Left Bottom Corner (Beaker E)	3	6.0		
		Front Left Top Corner	4	0.0		
		Back Right Bottom Corner (Beaker F)	9	4.2		
		Back Right Top Corner	10	0.0		
		Back Left Bottom Corner (Beaker G)	11	2.3		
		Back Left Top Corner	12	0.0		
Refill Day 10	Control	A	A1	6.2	6.2	0.0
		B	B1	6.2		
		C	C1	6.2		
	Treated	D	D1	6.1	4.1	2.2
		E	E1	2.2		
		F	F1	6.0		
		G	G1	2.2		

The biofilm growth results from Test 2 are shown in Table 14. The results show that biofilms were established consistently from coupon to coupon at 5.6 ± 0.8 logs per coupon. Following treatment, the mean biofilm concentration was 0.8 ± 1.0 logs per coupon and ranged from 0 to 2.7 logs per coupon. This result suggests that removing the foil lids (although placed loosely) improved the efficacy of the gas treatment over Test 1. After refilling the microcosms and incubating for 7 days, the biofilm concentrations remained much lower on the treated coupons at 1.5 ± 2.7 logs per coupon compared to the controls at 6.2 ± 0.2 . After 27 days, the biofilm levels on the treated coupons increased to 4.6 ± 0.4 logs, approximately 1.5 logs lower than the untreated at 6.0 ± 0.6 logs. The rate of regrowth is illustrated in Figure 43. This result suggested that although the treatment significantly reduced the biofilm load, the rate of regrowth was rapid (weeks). Although the test was discontinued, it was anticipated the biofilm concentrations on the treated and untreated coupons would be comparable within 6-8 weeks.

Table 14. Biofilm Growth Results from Dry Run Test 2

Group	Type	Beaker	Sample ID	Log CFU Recovered	Mean Log	Std. Dev.
Post-rinse/Post Dry	Untreated	A	A2	5.4	5.6	0.8
		B	B2	6.2		
		C	C2	6.4		
		D	D2	6.3		
		E	E2	5.5		
		F	F2	4.9		
		G	G2	4.3		
Post-CIO2	Treated	Front Right Bottom Corner (Beaker D)	1	2.7	0.8	1.0
		Front Right Top Corner	2	1.5		
		Front Left Bottom Corner (Beaker E)	3	0.0		
		Front Left Top Corner	4	1.2		
		Back Right Bottom Corner (Beaker F)	9	0.0		
		Back Right Top Corner	10	0.0		
		Back Left Bottom Corner (Beaker G)	11	0.0		
Refill Day 7	Control	A	A1	6.1	6.2	0.2
		B	B1	6.2		
		C	C1	6.5		
	Treated	D	D1	4.7	1.5	2.7
		E	E1	0.0		
		F	F1	0*		
		G	G1	0.0		
Refill Day 27	Control	A	A1	5.4	6.0	0.6
		B	B1	6.4		
		C	C1	6.4		
	Treated	D	D1	4.2	4.6	0.4
		E	E1	5.0		
		F	F1	4.8		
		G	G1	4.4		

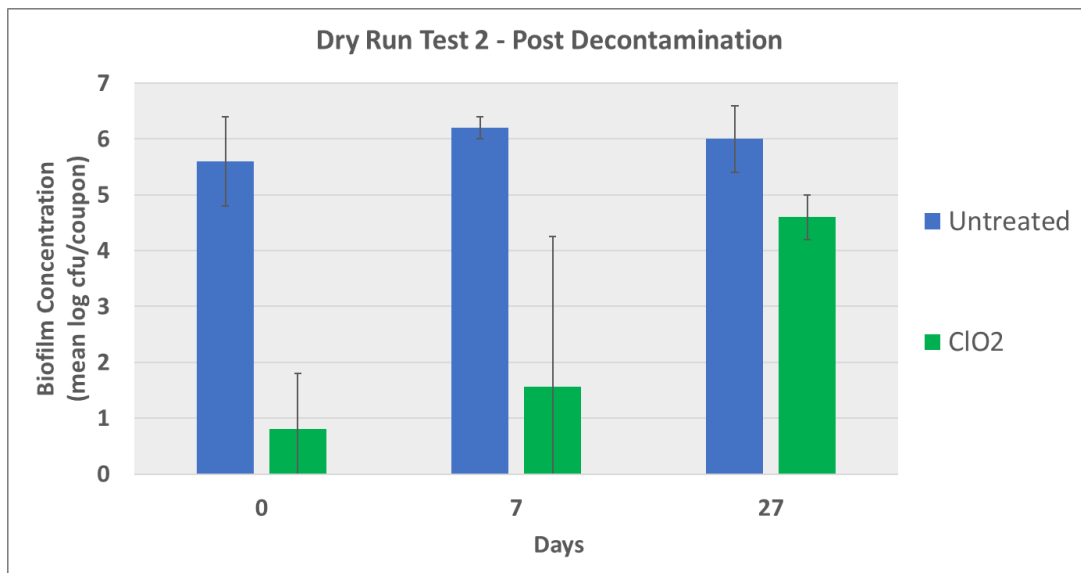


Figure 43. Biofilm regrowth results from Dry Run Test 2

3.8 Field Demonstration – ClO₂

Several months after the mock-field trials were completed, an operational fuel tank became available for testing, and a true field demonstration was arranged by AFRL with support from Wright Patterson AFB (WPAFB) Fuel Systems and Safety personnel. Based on the challenges achieving and maintaining HAD conditions in the mock-up trials compared to the success of ClO₂ mock-up trials, the test team decided to conduct the field trial solely using ClO₂. In the months separating the two demonstrations, an alternate ClO₂ delivery system was developed in-house at METSS under a separate project. This new system uses liquid chemical precursors that generate ClO₂ much faster and in a more controlled manner (i.e., the gas concentration could be accurately controlled) than the micro-reactor technology and was, therefore, deemed better suited for this application.

The goal of the field demonstration was to demonstrate the utility of a novel fuel tank decontamination technology to dramatically reduce the level of microbial contamination in an empty fuel storage tank prior to refueling. During the demonstration, an empty 25,000-gallon fuel storage tank at WPAFB was fumigated using a ClO₂ gas dispersion unit (GDU) developed by METSS. The gas was maintained at approximately 500 ppmvh for several hours such that the cumulative gas treatment was >1800 ppmvh. This level of gas treatment had been shown in preliminary laboratory tests to achieve > 3 LR of biofilms typically found in fuel systems. The efficacy performance of the fumigation was determined by suspending BIs, fiberglass coupons coated with a biofilm commonly associated with contaminated B20 fuel system, in the tank during the fumigation process. The concentration of biofilm on the BIs pre- and post- treatment was used to measure the level of disinfection, expressed as LR, achieved by the fumigation.

One demonstration trial was conducted over two days. The day prior to the demonstration (Friday), a system check was performed by connecting the decontamination system to the fuel tank and operating without ClO₂. The demonstration trial was then performed the following day (Saturday) with the decontamination cycle initiated early in the morning, run throughout the day, and completed by late afternoon.

3.8.1 Demonstration Objectives

The main objective of this demonstration was to assess the robustness, safety, and effectiveness of this novel tank decontamination method. The observations and results from this demonstration will inform stakeholders tasked with deciding whether this method should be incorporated when fuel systems are emptied and cleaned for routine and non-routine maintenance. While there were no formal requirements for this demonstration, the following objectives were established:

- Objective 1: Demonstrate ease of set up and operation
- Objective 2: Demonstrate decontamination system can achieve and maintain gas concentrations of 300 to 500 ppmvh for several hours in an empty 25,000-gallon fuel tank
- Objective 3: Demonstrate the fumigation process is effective at killing the biofilm commonly associated with contaminated B20 fuel storage tanks by achieving a 3-log reduction (equivalent to 99.9% decrease in viability)

- Objective 4: Demonstrate the decontamination method is operationally and environmentally safe

3.8.2 Demonstration Location

The demonstration took place at WPAFB using a 25,000-gallon, underground, fiberglass fuel storage tank located behind Building 490 within a fenced, gated area. The tank, which had been used for research purposes and contained Jet A fuel, had recently been emptied and decommissioned in preparation for demolition. The tank lied north to south and was approximately 34 ft. long, 14 ft. wide, and 14 ft. deep. The tank had a 40-inch manway and three accessible gauge hatches (referred to as Hatch A, B, and C) located along the top of the tank that extended several inches above the ground (Figure 44). Hatch A was used to suspend BIs, Hatch B was used for gas monitoring lines, and Hatch C as a gas exhaust/return port in which 4-inch flexible ducting was connected to an air recirculation blower which was connected to the GDU installed in the retrofitted manway cover. A schematic depicting the general position of the GDU, BIs, and gas monitoring lines in relation to one another as well as the tank dimensions are provided in Figure 45. An outdoor 120V/20Amp outlet located approximately 100 ft. from the tank was used to power the decontamination system.

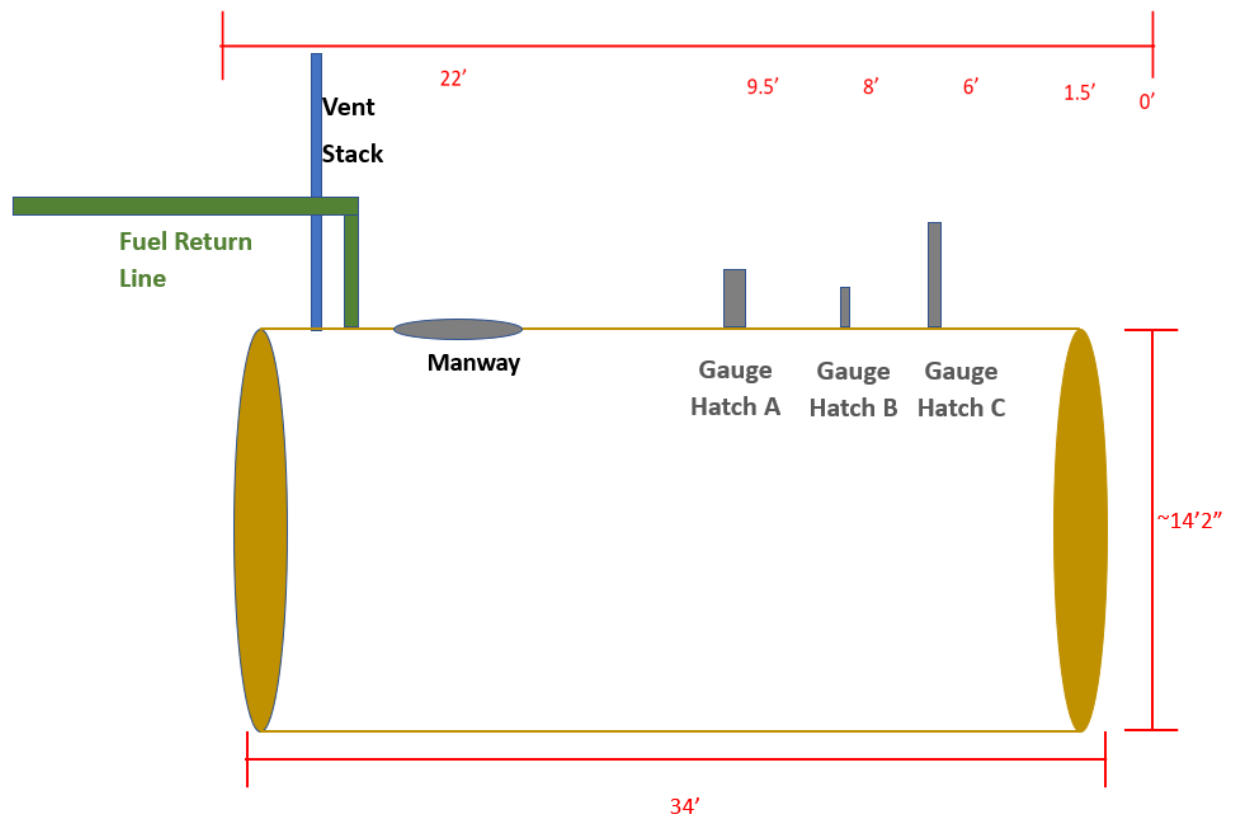


Figure 44. Schematic of fuel tank and location of access points

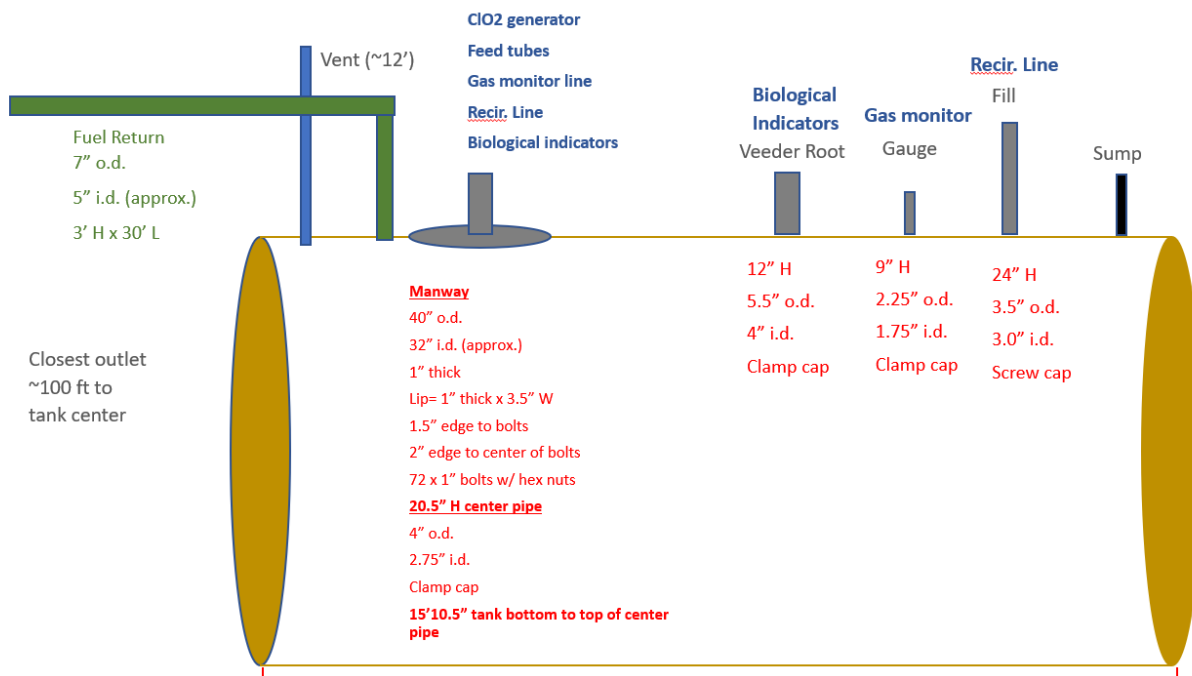


Figure 45. Tank dimensions

3.8.3 Demonstration Procedures

The flow diagram in Figure 46 provides a summary of the decontamination process. The procedures used for the field demonstration are described in more detail in the following subsections.

INSTALLATION

- Remove manway cover and replace with retrofitted manway cover
- Insert GDU into manway cover
- Connect 4" duct from Gauge Hatch to air recirculation blower position next to manway; connect 4" duct to inlet of GDU
- Install biological samples and gas sampling lines
- Seal vent

DECONTAMINATION

- Turn on air recirculation blower and air sparging pump
- Add ClO_2 generation liquid persurors into GDU through fill lines
- Monitor gas concentration inside tank
- Add more precursors as needed to maintain gas at approx. 500 ppmv. Continue until cumulative gas treatment is >1800 ppmv

COMPLETION

- Add neutralizer to GDU
- Turn off blower, and connect two retrofitted totes each containing activated carbon filters to the recirculation loop
- Turn on blowers and allow activated carbon filters to capture ClO_2 ; continue until ClO_2 in tank measures <50 ppmv
- Turn off blowers and retrieve BIs
- Disconnect 4" flexible duct between totes and blower
- Turn on blowers to sparge tank with fresh air until gas in tank measures 0 ppmv

Figure 46. Flow diagram of decontamination process

3.8.3.1 Decontamination System Setup and Operation

METSS constructed a ClO_2 gas decontamination system scaled to decontaminate the 25,000-gallon tank. The decontamination system included the GDU, an air recirculation blower, and flexible 4-inch duct. The GDU, constructed primarily of PVC, was approximately 7 inches wide and 7 feet long and weighed 37 lbs. (Figure 47). The system was designed to generate, disperse, and maintain the ClO_2 gas within the tank at approximately 500 ppmvh for up to 8 hours. An aqueous solution of dissolved ClO_2 gas was generated in the bottom section of the GDU, which extended into the tank through a retrofitted manway cover (Figure 48). ClO_2 was generated by combining solutions of sodium chlorite and sodium persulfate. An air recirculation blower, positioned outside the tank, was connected to the inlet port of the GDU via flexible 4-inch ducting and was used to blow an air stream into the GDU and over the surface of the ClO_2 solution to promote ClO_2 off-gassing. The air/gas stream was then directed out of the GDU through vent holes in the sides of the GDU and into the tank. To promote uniform gas distribution within the tank, 4-inch flexible ducting was attached from a tank gauge hatch to the inlet port of the air blower. This created a closed-loop GRS as illustrated in Figure 48. A schematic of the tank indicating locations of GDU, and gas monitoring tubes and biological samples is shown in Figure 49. The recirculation system then remained on until the decontamination process was complete. After the treatment was complete, residual ClO_2 gas in the tank was removed using in-line activated carbon filters.



Figure 47. METSS Gas Dispersion Unit

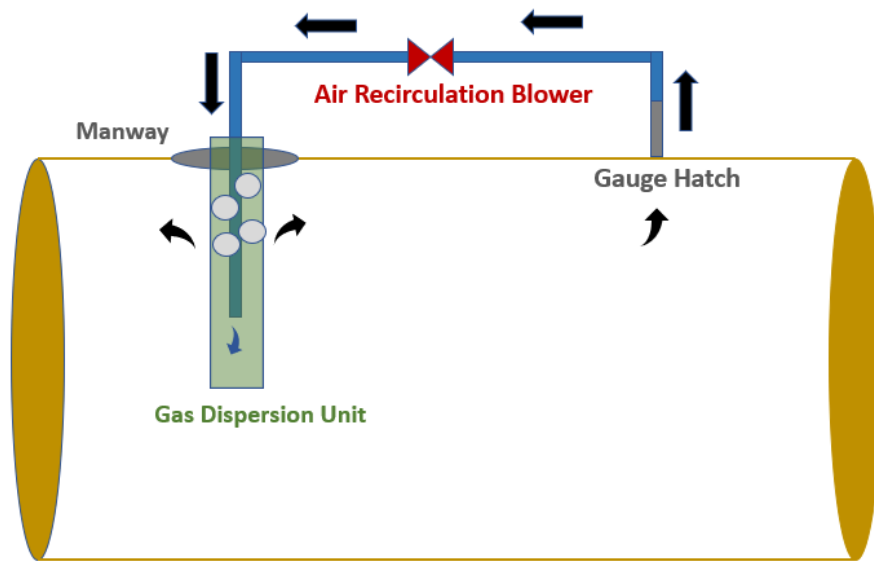


Figure 48. Empty fuel tank with CIO₂ Decontamination System (not to scale). *Note: Gas generated inside GDU and dispersed into tank. A closed-loop recirculation system circulated the gas out of a gauge hatch to a recirculation blower which returned the gas to the tank.*

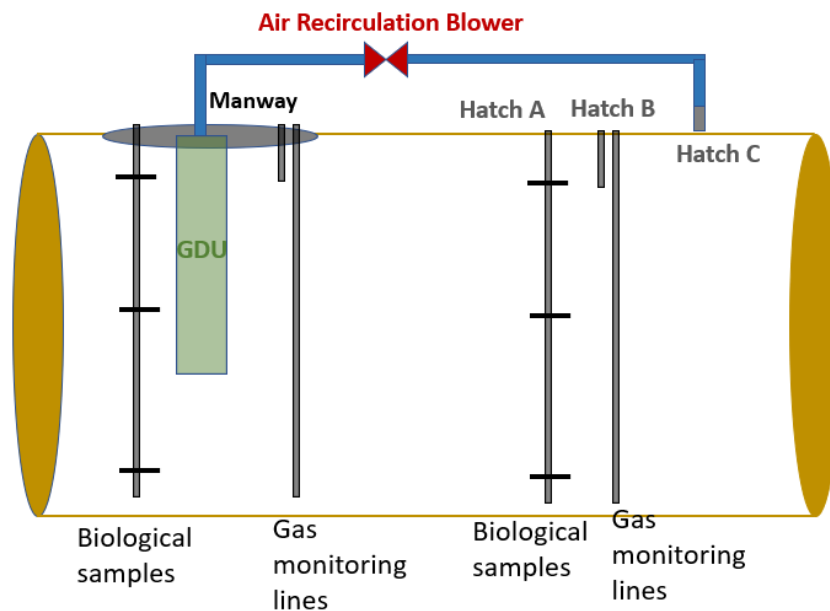


Figure 49. Schematic of tank indicating locations of GDU, gas monitoring tubes, and biological samples

The decontamination system and accessories were installed as follows:

The manway cover was removed and immediately replaced with a painted wood manway cover. The cover, manufactured by METSS, acted as a support structure through which the decontamination system was partially lowered into the tank. The cover also had access ports through which BIs, gas sampling lines, thermocouples, and a humidity probe were extended into the tank. The cover had been designed to provide a complete seal of the manway opening to prevent gas from escaping during the decontamination process and was fastened to the manway flange using multiple C-clamps. The GDU, placed through a hole in the middle of the manway cover, was held in place by a flange such that the lower 2/3rds of the GDU was extended into the tank (Figure 50). The outlet of an air recirculation blower, positioned on the ground next to the manway, was attached via 4-inch flexible ducting to the inlet of the GDU. Flexible 4-inch duct was then attached from Hatch C to the return port of the blower (Figure 51). An electric air pump was connected to an air sparging line built into the GDU. The pump was used to sparge air through the aqueous solution of ClO_2 at the bottom of the GDU to promote off-gassing. Hatches A, B, and C were used for the BIs, gas sampling, and recirculation system, respectively, as shown in Figure 52. Once the decontamination system had been installed, BIs (1"x3" biofilm-laden fiberglass coupons, described in Section 3.4.5) were installed by attaching them to chains suspended into the tank (Figure 53). One set of BIs were suspended through a port in the manway cover and a second set was suspended through Hatch A. The chains were attached to stoppers that connected to the manway cover and hatch. Triplicate BIs were positioned at three depths (approximately 2 ft. from top of tank, 7 ft. into the tank, and approx. 2 ft. from the bottom of the tank) representing Top, Middle, and Bottom positions. To monitor the gas concentration in the tank during decontamination, two gas sampling lines (Nylon tubing) were installed through the manway cover and Hatch A and held in place by 2-hole stoppers. At each location, the sampling lines were extended approximately 2 ft. and 12 ft. into the tank. To complete the setup, the vent stack was sealed with a plastic bag and duct taped, and an extension cord was run from a standard outlet located approximately 100 ft. from the tank to provide power to the air blower and air pump.

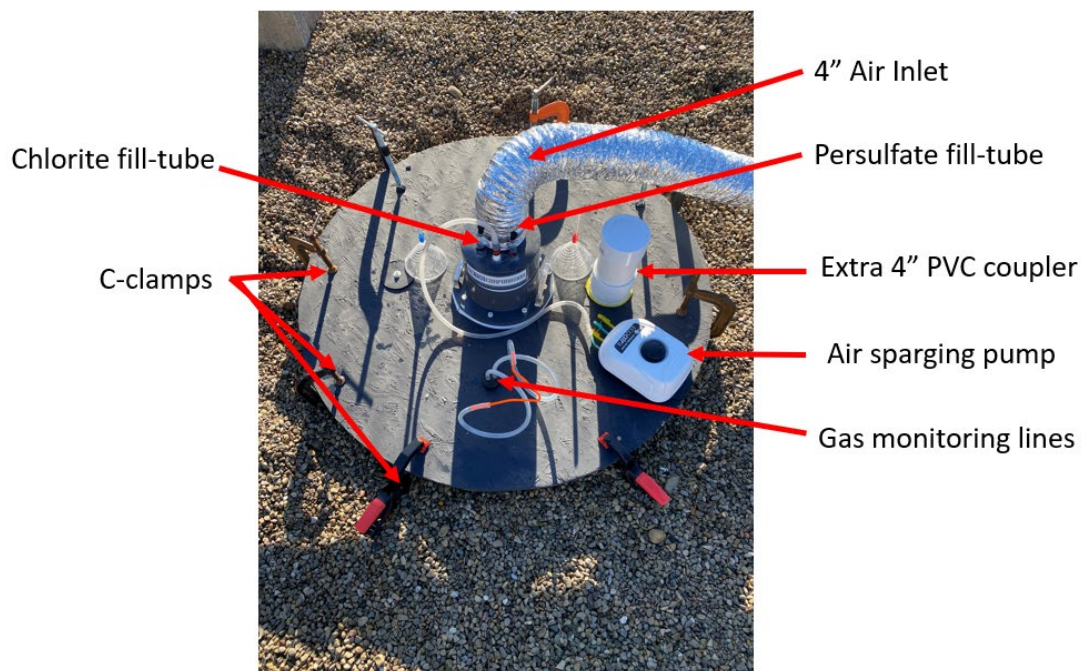


Figure 50. GDU installed in retrofitted manway cover and attached to accessory equipment



Figure 51. Flexible duct connected from Hatch C (upper right corner) to air recirculation blower to GDU



Hatch A
(for BI's)

Hatch B
(for gas
sampling)

Hatch C
(gas outlet;
return to
recirculation
blower)

Figure 52. Hatch A, B, C of fuel tank



Figure 53. Biological indicator (biofilm-laden fiberglass coupon) attached to chain which was suspended into tank

The decontamination process was then performed as follows:

Wearing a full-face respirator, non-latex gloves, and lab coat, a METSS analyst added 425 milliliters of 50% sodium persulfate solution and 2,750-milliliters of 75% sodium chlorite solution into the GDU through separate fill ports (Figure 54). The air blower and air pump were then turned on to initiate off-gassing. A range of 400 to 700 ppmv inside the tank

was targeted and maintained until the cumulative gas treatment was at least 1800 ppmvh. The gas concentration within the tank was monitored via a PortaSens gas monitor with a 5/200 ppm gas sensor. To measure the gas, the PortaSens was connected to a gas-sampling lines for 2-3 minutes to allow the measurement to stabilize after which the gas concentration (ppmv) was recorded manually in a logbook. The gas concentration data was plotted in real-time to monitor the cumulative gas treatment (expressed as ppmvh) throughout the process. Just over three hours into the decontamination process, the gas treatment reached the minimum treatment level of 1800 ppmvh. Since this level was achieved so rapidly, the process was allowed to continue for another hour, at which time the treatment reached approximately 2600 ppmvh.



Figure 54. Chemical precursor being added to GDU to initiate ClO₂ generation

To complete the decontamination process, the ClO₂-generating solution in the bottom of the GDU was neutralized by adding 150 mL of 1% sodium thiosulfate solution through one of the GDU fill lines. The blower was then turned off momentarily to allow two activated carbon filters (Vivosun; 4" x 22") to be installed in the recirculation loop. Each filter was placed inside separate weather-proof totes retrofitted with 4-inch inlet and outlet ports (Figure 55). The blower was then turned back to recirculate the gas through the activated carbon filters. The gas concentration in the tank was monitored during neutralization. After approximately one hour, the rate of gas neutralization appeared to slow; therefore, the used filters were replaced with new filters. The neutralization step continued for another two hours until the gas concentration in the tank was below 50 ppmv. At that time, the blower was briefly turned off to allow the BIs to be retrieved. As the chains were raised from the tank, the BIs were aseptically removed from the chain and placed into individual, pre-labeled conical tubes containing 20 mL PBS. The BIs were placed in coolers with ice packs and transported to METSS for analysis. Once the BIs were collected, the 4-inch flexible ducts between the exhaust ports from the totes containing the activated carbon filters and the

recirculation blowers were removed. The total gas treatment at the time the BIs were removed was 3298 ppmvh. The blowers were then turned on to sparge the tank with fresh air. The air exiting the tank was passed through the activated carbon filters until the gas concentration in the tank measured “0 ppmv”. The blower was then turned off, and the neutralized ClO_2 solution in the GDU was siphoned through the air sparging line using a bilge pump and collected in a leak-proof container. Several liters of water were then added to the GDU as a rinse and then siphoned into another container. The GDU was then removed from the tank, and the retrofitted manway cover was replaced with the original cover. The vent stack was unsealed, and the gauge hatches were closed with their original caps.



Figure 55. Dual activated carbon filter/tote system for in-line gas neutralization

3.8.3.2 Environmental Monitoring

Two PortaSens gas detectors were used to monitor the gas concentration in and around the tank. One unit had a 200-2000 ppm sensor and was used to monitor the gas concentration inside the tank. The other unit had a 1-5 ppm sensor and was used to monitor the gas concentration outside to ensure operators and observers were not exposed ClO_2 from any leaks in the system. The temperature and RH inside the tank were measured using a HOBO datalogger attached to the chain used to suspend BIs from the gauge hatch.

3.8.3.3 Efficacy Testing

A fungus of the *Byssochlamys* genus was used to assess the efficacy of the decontamination process in this demonstration. This organism was identified by AFRL to be the predominant contaminant in the numerous B20 fuel tank systems investigated during this project. For this demonstration, a biofilm of *Byssochlamys* was grown on 24 replicate fiberglass coupons ($\frac{7}{8}$ " x 3") over a three-week period leading up to the demonstration. Each coupon was placed in 50-mL conical tubes containing 15 mL of ASW and 5 mL of B20. The tubes were then inoculated with 0.2 mL of *Byssochlamys* biomass (mycelium and spores derived from a one-week old culture grown in PDB and allowed to incubate statically for three weeks. During this incubation period, a biofilm formed on the surface of the fiberglass, predominately at the ASW/B20 interface. The biofilm appears mucoidal, as illustrated in Figure 53.

The day prior to the demonstration, 21 BIs were transported to the test site in coolers with ice packs. On the day of the demonstration, 18 of the BIs were suspended in the tank and exposed to gas as previously described (section 3.8.3.1). Representing site controls, the biofilm-laden coupons from the three remaining BIs were removed from their original tubes containing ASW and B20, and placed in individual, empty conical tubes with vented caps and stored at ambient temperature in the dark until the decontamination process was completed. The gas-treated BIs and site controls were then placed in individual, pre-labeled tubes containing 15 mL PBS at the completion of decontamination process and transported to METSS with ice packs for analysis. Three additional BIs, designated as lab controls, remained at METSS where they were stored at 27 °C until the site controls and test samples were returned to the lab for analysis.

To analyze the coupons, the lab controls were transferred to individual tubes containing 15 mL PBS. An additional 5 mL of PBS with 0.05% Tween 20 was then added to all BIs. Tween 20, a surfactant, helps detach the biofilm from the coupons. The BIs were then vortexed for 30 seconds, sonicated for five minutes in a sonicating water bath, vortexed again for 30 seconds. The extract of each BI was then serially diluted with PBST and spread plated onto PDA. Control BIs were plated at dilutions 10^{-2} , 10^{-3} , and 10^{-4} . Five replicate 0.2-mL aliquots of undiluted extract of each test BI were spread plated. The plates were then incubated for several days at 27 °C until colonies reached a distinguishable size. For test samples in which no colonies were recovered, the replicate samples at each location were pooled and then membrane filtered. The filter was overlaid onto PDA and incubated for at least four days at 27 °C. Colonies were counted and used to calculate cfu/sample and log cfu/sample. The concentration between treated sample and site controls were used to assess the efficacy of the decontamination process and expressed as LR.

3.8.4 Environmental/Safety Concerns

The test team went to great lengths to ensure the demonstration was conducted as safely as possible. A few risks were identified prior to the demonstration. These risks and the mitigation strategies implemented during the demonstration are provided below:

- **Impact to the fuel storage tank.** METSS conducted numerous materials compatibility studies to understand the impact of ClO_2 on materials that are common in fuel storage tanks. No impact on the physical or operational integrity of the fiberglass tank was anticipated. However, any risk to the fuel supply chain was mitigated by conducting the demonstration on a retired fuel tank that was scheduled for demolition. Any lessons learned will be used to update the prototype design prior to use on any functional fuel storage tanks.
- **Minor gas leaks within close proximity of the decontamination system.** The Occupational Safety and Health Administration/National Institute for Occupational Safety and Health (OSHA/NIOSH) permissible exposure limit (PEL) for ClO_2 is 0.1 ppm over an 8-hour time working average. The short-term exposure limit (STEL) is 0.3 ppm over a 15-minute period. Exposure may cause irritation to the eyes, nose, throat, and lungs. To prevent incidental or accidental gas exposure to observers and bystanders, METSS instructed people within the vicinity of the tank while the decontamination was in progress to maintain a safe distance and stay upwind. The odor of ClO_2 is detectable at 0.1 ppm (similar to chlorine odor); therefore, observers were instructed to increase their distance from the tank if they smelled ClO_2 or if the PortaSens detected ClO_2 outside the tank. METSS personnel operated the decontamination system. During initial set up, periodic monitoring of gas concentration within the tank, and tear down, operators donned full-face respirators with Class I OV/SD/HC/HF/HS filters. A PortaSens gas detector was also used to monitor the gas concentration within the tank. A second PortaSens was used to monitor the gas concentration at the test site and within close vicinity of observers. The demonstration was also conducted during a weekend, limiting the number of observers and bystanders.
- **Safe handling, transport, and disposal of chemicals.** Chemical solutions needed for testing, including 75% sodium chlorite, 50% sodium persulfate, 1% sodium thiosulfate, and 0.1% sodium hydroxide, were transported in plastic, break-resistant containers and in secondary containers (coolers). Chemicals were handled by METSS personnel wearing non-latex gloves, safety glasses, respirator, and lab coat. Neutralized ClO_2 solution was placed in a leak-proof container and returned with any unused chemicals to METSS for proper disposal. Safety Data Sheets for ClO_2 , sodium chlorite, sodium persulfate, and sodium thiosulfate were made available.

3.8.5 Field Test Results

3.8.5.1 Gas Generation and Monitoring

The decontamination process took eight hours (480 minutes) to conduct, starting at 0730 and finishing at 1530. The maximum gas concentration in the tank (688 ppmv) was achieved within the first hour and held steady until the neutralization step was initiated approximately 4.5 hours later. The gas profiles expressed as ppmv and ppmvh (cumulative gas treatment) are shown in Figure 56. The minimum cumulative treatment of 1800 ppmv was reached around 1100, when

the process could have been stopped; however, since this level was rapidly achieved, the process was allowed to continue for another 1.5 hours, at which time the neutralization process was initiated (approximately 1240). The BIs were removed from the tank at 1445 when the gas concentration in the tank was below 50 ppmv. The BIs were removed with the recirculation blowers turned off. After the BIs were collected, the tank was sparged with outdoor air to fully evacuate the gas from the tank. The approximate time required to reduce the gas concentration in the tank from approximately 600 ppmv to below 50 ppmv was two hours. An additional hour of neutralization and air sparging reduced the gas concentration to “0” ppmv (undetectable). The timeline and observations of key activities during the demonstration are summarized in Table 15.

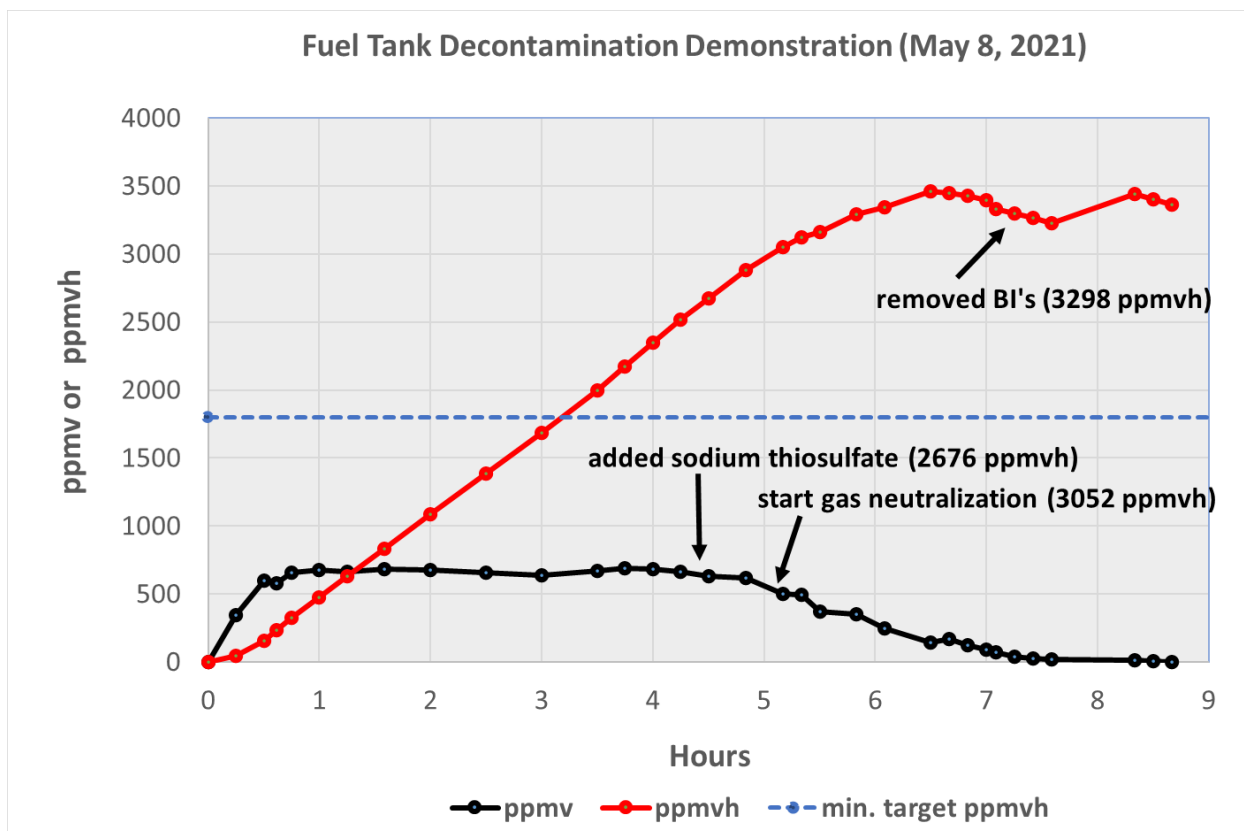


Figure 56. Gas profile during tank decontamination demonstration

Table 15. Key Activities or Observations During Demonstration

Time of Day	Minutes	Hours	ppmv	ppmvh	Comments
0730	0	0	0	0	Started treatment
1100	210	3.5	673	1997	Approximate time at which minimum cumulative treatment of 1800 ppmvh was reached
1200	270	4.5	630	2676	Added sodium thiosulfate to GDU to neutralize solution
1240	310	5.2	500	3052	Installed dual activated carbon filters in recirculation loop and started gas neutralization
1320	350	5.8	350	3291	Replaced activated carbon filters; continued gas neutralization
1445	435	7.3	42	3298	Removed BIs
1510	460	7.7	14	3439	Stopped gas recirculation; began tank sparge with outdoor air
1530	480	8.0	0	3361	Stopped blowers, disconnected decon. system

The temperature and RH inside the tank during the decontamination cycle are illustrated in Figure 57. The mean temperature and RH were 14 °C and 70%, respectively. Elevated RH (generally 65-80%) is known to increase the efficacy properties of ClO₂ gas. Therefore, the ambient RH conditions inside the tank were ideally suited for the ClO₂ fumigation without additional humidification.

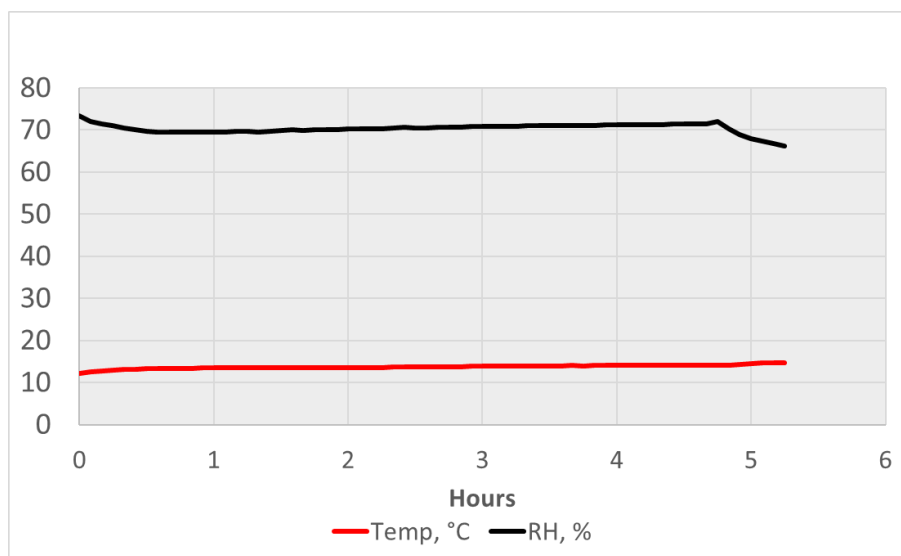


Figure 57. Air temperature and relative humidity inside tank during decontamination cycle

3.8.5.2 Efficacy

The ClO₂ treatment resulted in nearly complete kill of the biofilm on the custom BIs. The mean LR when comparing recoveries from site controls (those shipped to test site but not gas treated) versus the treated BIs was 5.6 ± 0.2 . The mean log cfu recovered of the lab controls, site controls, and test samples along with the log reduction observed of the test samples as compared to the site controls are shown in Table 16. The total cfu recovered from each of the pooled triplicate test BIs per each of the six locations within the tank was very minimal to none as shown in Table 17. The mean log cfu/coupon of biofilm recovered from lab and site control BIs were essentially the same at 6.0 ± 0.1 and 5.8 ± 0.1 , respectively, demonstrating the biofilms remained stable during transit to and from the test site.

Table 16. Biological Indicator Efficacy Results

Sample Type	Log Recovered	Log Reduction (LR) ^c
Lab Controls ^a (n=3)	6.0 ± 0.1	n/a
Site Controls ^b (n=3)	5.8 ± 0.1	n/a
Test (n=18)	0.1 ± 0.3	5.6 ± 0.2

n/a not applicable

^a Stored at ambient conditions in the lab and assayed the same day as site controls and treated samples.

^b Transported to and from test site along with treated samples.

^c LR= Based on recovery of site controls vs. treated samples.

Table 17. Efficacy Results from Test Biological Indicators

Suspended From	Height in Tank	Mean Log ₁₀ Recovered	Mean cfu* Recovered
Manway Cover	Top	0.1	2
	Middle	0.0	0
	Bottom	0.2	5
Gauge Hatch	Top	0.0	2
	Middle	0.0	0
	Bottom	0.5	520

3.8.6 Field Test Discussion

The four objectives of the demonstration were met.

Objective 1: Demonstrate ease of set up and operation.

The decontamination system (including the GDU, retrofitted manway cover, air blowers, 4-inch flexible ducting, and retrofitted totes containing activated carbon filters) was easy to transport and install. All equipment and supplies needed to conduct the demonstration were transported in a mid-size SUV. Although two people set up the system the day prior to the demonstration to perform a system check, the set up could have been performed by one person. It is estimated a typical set up time would be one hour. The retrofitted manway cover fit perfectly on the manway flange and a tight seal was easily established by placing window/door weather stripping between the cover and flange and fastening the cover to the flange with multiple C-clamps. The C-clamps were likely not necessary as the weight of the 37 pounds (lb). GDU appeared to compress the weather stripping and hold the cover in place. Commercially and readily available 4-inch PVC adaptors, PVC transition pieces, and 4-inch flexible ducting also made it simple to connect the decontamination system to the tank.

Operating the decontamination system was very easy as well. The ClO₂-generating chemicals were easy to add to the GDU through dedicated fill lines. The air sparging pump and air blower worked very well at off-gassing and dispersing the ClO₂ into the tank, as the maximum gas concentration in the tank was reached within one hour. Once the decontamination cycle was initiated, the system was self-sustaining, other than making periodic gas concentration measurements in the tank using the PortaSens. The procedure for initiating the neutralization process with a pair of activated carbon filters was simple and took just a few minutes to complete.

Objective 2: Demonstrate system can achieve and maintain gas concentrations of approximately 500 ppmv in an empty 25,000-gallon fuel tank.

Only 3.2-liters of chemical precursor solutions were used to achieve the desired gas concentration in the 25,000-gallon tank. METSS estimates the maximum liquid capacity of the GDU is 11 liters, able to accommodate larger tanks or higher gas concentrations if desired. The GDU generated the gas rapidly, reaching the maximum concentration in the range of 680 ppmv within one hour. The gas concentration held steady for approximately four hours, indicating that

the closed-loop recirculation system contained the gas. At no time during the decontamination process was ClO₂ gas smelled by observers nor detected by the PortaSens outside the tank. After the treatment was complete, the gas concentration within the tank was rapidly reduced to below detectable limits over a span of approximately three hours using a pair of in-line activated carbon filters.

The overall process required eight hours, including the decontamination cycle and gas neutralization cycle. The process time could be reduced, if desired, in a variety of ways including the following:

- The minimum cumulative gas exposure of 1800 ppmvh was reached within just over three hours. The neutralization process could have been initiated then, but the decontamination cycle was intentionally run for additional 1.5 hours to create the greatest probability of success in this one-of-a-kind demonstration.
- The gas concentration could be increased to reduce the decontamination cycle time. A range of 500 ppmv was selected for this demonstration as it allowed the decontamination to be completed within eight hours based on estimates of ClO₂ generation rate (1-2 hours), duration of decon cycle (3-5 hours) and time to neutralize the gas (2-4 hours). Based on the estimated ClO₂ generation capacity of the GDU of 11 liters of chemical precursors, the maximum gas concentration in the 25,000-gallon tank could have been approximately 2500 ppmv. At this concentration, the estimated decontamination cycle time would have been 1.5 hours.
- The neutralization cycle required approximately three hours to perform using a pair of in-line activated carbon filters. This time could have been reduced if additional filters were used; however, this would slightly increase the complexity of the system.

Objective 3: Demonstrate the fumigation process is effective at killing the biofilm commonly associated with contaminated B20 fuel storage tanks by achieving a 3-log reduction (LR; equivalent to 99.9% decrease in viability)

The fumigation process was shown to be highly efficacious as the BIs containing nearly 6 log cfu (equivalent to 1,000,000 cfu) exposed to the gas in the tank had nearly no viable organism post-disinfection. Less than 1000 cfu were recovered from all 18 test BIs, resulting a mean LR of 5.9 or >99.999%.

Objective 4: Demonstrate the decontamination method is operationally and environmentally safe.

All safety precautions to prevent occupational ClO₂ exposure were followed, and there were no safety incidents. The gas was maintained within the tank at all times during the decontamination cycle and was safely neutralized in the closed-loop system. Although the interior of the tank was not inspected pre- or post-decontamination, the appearance of the tank interior as viewed from the open manway appeared unaffected by the gas treatment (i.e., no indications of scaling, corrosion, or color change). The inner walls of the gauge hatches also appeared unaffected. The majority of the ClO₂ was captured by the activated carbon filters. As such, the amount of ClO₂ released to atmosphere was negligible.

4.0 CONCLUSIONS

Decontamination processes for treating fuel storage systems to significantly reduce the occurrence of heavy microbial growth were identified and tested in laboratory and field environments. After assessing decontamination processes available today – such as liquid disinfectants, fumigants, and UV light – two processes came to the forefront: thermal decontamination (HAD and/or BTD) and ClO₂ fumigation. HAD/BTD was developed to address decontamination of BWA on aircraft surfaces and is now being adapted for other more innocuous biological contaminants like mold. ClO₂ fumigation is currently used in the food, agriculture, water, and medical industries for disinfection. These vapor-phase methods are particularly well suited to the problem at hand because they can fill any volume – the tank, valves, piping, transfer lines, etc. – resulting in maximum surface treatment with minimal labor. During this study, METSS worked with collaborators within AFRL to bring together the best solution for bioremediation in fuel storage tanks based on existing decontamination processes and equipment.

The efforts performed during this study resulted in the following key findings or developments:

- Site visits to numerous AFBs around the country revealed the extent of biofilm formation and biofouling that occurs in some fuel storage systems. Bench-top experiments using culture isolates recovered from the fuel samples showed that biofilm formation can visibly be seen after just a few days after inoculating biodiesel fuel and water mixture (referred to as microcosm).
- A series of bench-scale decontamination tests (ClO₂, HAD, and BTD) were performed to identify the process that would kill 99.9% (>3 logs) of the microbes in a fuel tank within eight hours. BTD and HAD conditions were identified to be 140 °F at high humidity (approximately 80%) and 190 °F at low humidity for fiberglass and steel tanks, respectively. ClO₂ treatment of >1800 ppmvh was shown to achieve at least 3-log reduction of biofilm viability.
- Mock-up evaluations of both HAD/BTD and ClO₂ were performed to assess these processes at full scale (representative of common large fuel storage tank). The results demonstrated that it would be difficult to achieve the elevated temperatures and humidity conditions inside fuel tanks needed for successful remediation using commercially available heating systems. Significant investment in design, engineering, and manufacturing of a unique HAD/BTD system would be needed. As such, full-scale decontamination testing was focused on the use of ClO₂ fumigation. Results from these tests were very encouraging. ClO₂ fumigation was very efficacious against fuel system biofilms, and the gas treatments required to achieve greater than 3-log reduction at full-scale could be easily achieved using unique gas generation system.
- Full-scale, mock demonstrations were performed. This activity demonstrated the ClO₂ conditions needed for effective kill of biofilm could be generated and evenly dispersed in a volume that was similar to a fuel storage tank.
- METSS constructed a more refined GDU and tank decontamination system. The system was successfully field tested on an operational (but decommissioned) fuel storage system. The decontamination system was shown to be easy and safe to operate, rapid (process completed within eight hours), and efficacious.

The ultimate goal of the study was to develop a decontamination process that would enhance existing cleaning procedures by dramatically slowing the regrowth of biofilms in biodiesel fuel storage systems to extend the time between tank cleanings and reduce the need for fuel system maintenance and repairs caused by biofilm formation and biofouling. This goal was met using a novel ClO₂ fumigation system that was proven effective at disinfecting a 25,000-gallon operational fuel tank storage system. A field demonstration using a novel ClO₂ fumigation system was shown to reduce biofilm concentrations by >99.999%. The decontamination system was also shown to be easy to set up, operate, and perform within eight-hours or less and, therefore, could be incorporated as part of the standard fuel tank cleaning and maintenance procedure as deemed necessary. The process can be performed by one person within an eight-hour work shift. Long-term benefits of the decontamination process (i.e., effects of preventing, limiting, or slowing the regrowth of biofilm in the decontaminated fuel system) could not be assessed as the fuel system was scheduled for demolition following the decontamination demonstration.

5.0 RECOMMENDATIONS

During the course of this study, the Air Force decided to discontinue the use of BDI and is now converting BDI tanks for storage of other fuels such as D2. Although Air Force fuel maintenance crews have not reported biofouling issues in D2 fuel systems or D2-powered equipment since the elimination of BDI, it may be too early to know if the biofouling issue has been eliminated as D2, by law, can contain up to 5% BDI. In fact, field assessments conducted during this study demonstrated that D2 fuel tanks do contain microbial growth, and biofilms accumulate just as quickly in B5 as B20 (see Section 3.6.2). Therefore, the fuel tank remediation technology using ClO₂ gas developed and tested in this study could still be utilized by the US military, other government agencies, and commercial fuel storage systems to remediate biofouling in tanks that experience high levels of microbial contamination. As such, the following follow-up activities are recommended:

- AFRL and METSS conduct a field assessment by returning to one or more sites visited during this study to assess whether microbial contamination of known contaminated systems has greatly diminished or changed since the conversion from BDI to D2.
- Perform a second ClO₂ decontamination demonstration on a grossly contaminated D2 or B20 fuel storage system. The ClO₂ fumigation process would occur after the tank has been emptied and cleaned following the standard pressure washing procedure. The tank interior would be inspected pre-and post-fumigation for scaling, corrosion, discoloration. Surface swab samples would also be collected pre- and post-fumigation to analyze for biocontamination. Once the tank was refilled with fresh fuel, AFRL and METSS would periodically inspect the fuel system for biofilm and monitor the rate of re-growth. This second demonstration would afford the opportunity to further assess the operation and function of the ClO₂ decontamination system under real-world conditions and to assess the long-term benefits of the treatment. The activity would also allow minor refinements to be made to the system and provide the opportunity to write a concise user manual. This would allow the decontamination system to be deployed and used at a moment's notice.

LIST OF ABBREVIATIONS

°C	degrees Celsius
°F	degrees Fahrenheit
%	percent
AFB	Air Force Base
AFRL	Air Force Research Laboratory
AST	Above Ground Tank
ASW	Artificial Sump Water
B20	20% Biodiesel in diesel
B5	5% Biodiesel in diesel
Bg	Bacillus atrophaeus
BDI	Biodiesel
BI	Biological Indicator
BTD	Biothermal Decontamination
BTU	British Thermal Units
BWA	Biological Warfare Agents
cfu	colony forming units
ClO ₂	Chlorine Dioxide
D2	Diesel fuel
ft	feet
GDU	Gas Dispersion Unit
GFE	Government Furnished Equipment
GRS	Gas Recirculation System
Gs	Geobacillus stearothermophilus
HAD	Hot Air Decontamination
ISO	International Standards Organization
L	Liter
LOD	Limit of Detection
LR	Log Reduction
mL	milliliter
PBST	Phosphate Buffered Saline supplemented with 0.05% Tween 20
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
ppm	Parts per million
ppmv	parts per million volume
ppmvh	parts per million volume hours
PVC	Polyvinyl Chloride
RH	Relative Humidity
SDS	Safety Data Sheet
SS	Stainless Steels
μL	microliter
ULSD	Ultra Low Sulfur Diesel
UST	Underground Tank
UV	Ultraviolet
WPAFB	Wright Patterson Air Force Base

APPENDIX A – HAD EFFICACY TESTING

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Test H1

Biofilms of *Byssochlamys* were established on steel coupons for three weeks in 50 mL microcosm tubes and then exposed to 140 °F and 10% RH for 4 and 24 hours. The results, shown in Figure A-1, indicated that HAD treatment was effective against biofilms; however, the fungal colonies recovered from the ambient coupons (i.e., untreated controls) appeared yeast-like (off-white, non-filamentous) rather than *Byssochlamys*-like (gold and filamentous). Therefore, the results from this test were deemed inconclusive as the inoculum may have been contaminated, but the HAD treatment was effective for the organisms present.

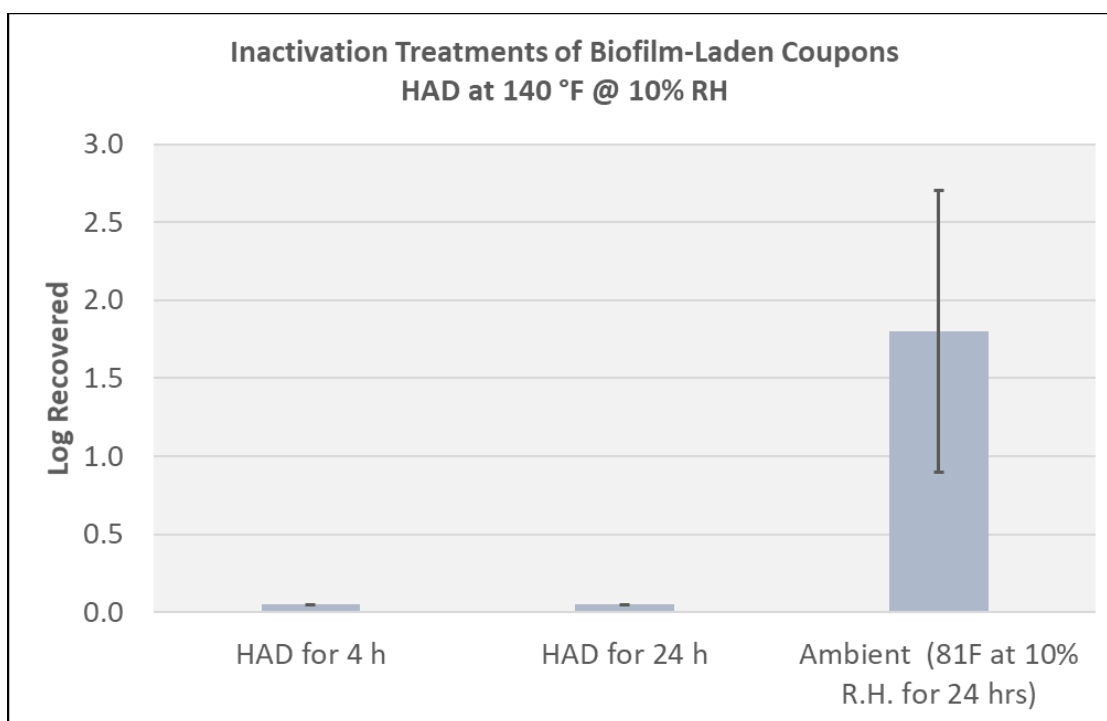


Figure A-1. Test H1 results.

Test H2 – Passive vs. Forced HAD

This test was performed to compare the efficacy of HAD on biofilms using forced hot air versus passive hot air for four hours. If one HAD method would demonstrate to be significantly more effective than the other, the results could drive how HAD would be employed to remediate fuel storage tanks. In this test, biofilms of a mixed culture derived from a Barksdale AFB field sample were grown in three 3-L microcosms. After several weeks of incubation (Figure A-2), the vessels were emptied. One vessel was treated to forced air at 140 °F using a hot air gun (Figure A-3). A second vessel was treated to passive HAD by placing it inside an environmental

chamber set to 140 °F and 10% RH (Figure A-4). The third vessel was used as an untreated control in which replicate coupons were assayed after 4 and 24 hours at ambient conditions.

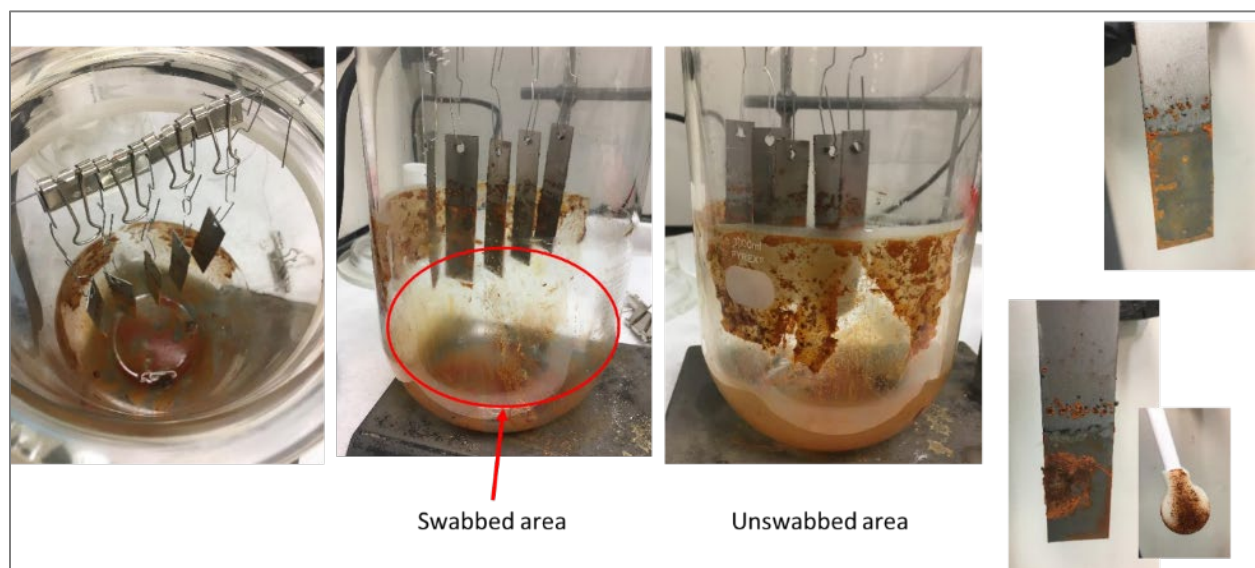


Figure A-2. Appearance of 3-L microcosm after biofilm allowed to establish for 28 days

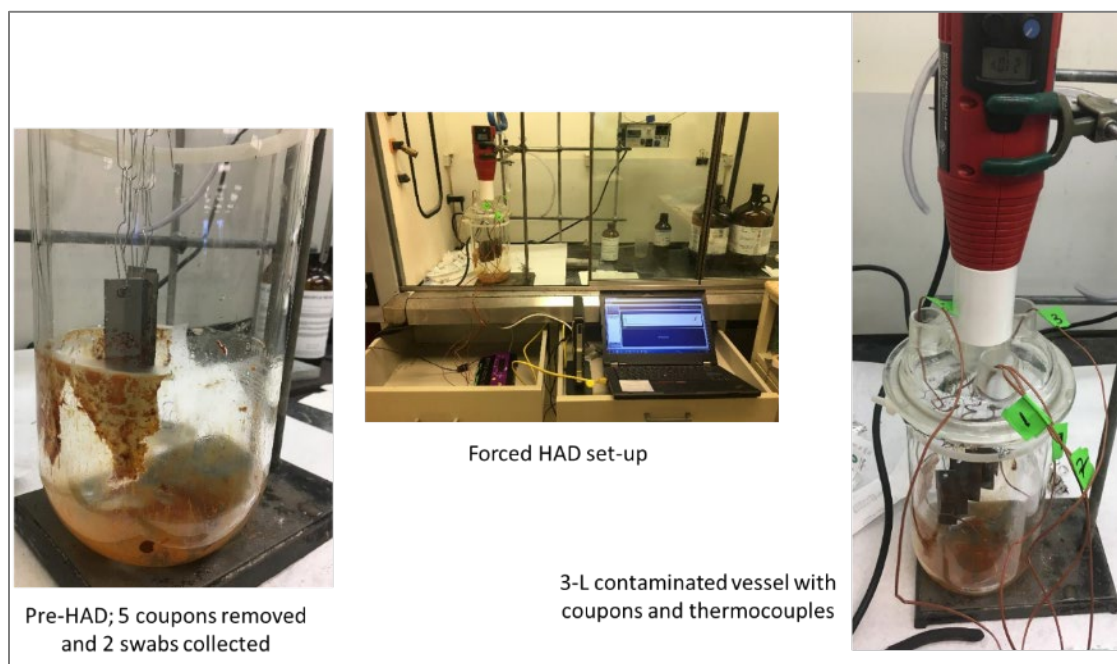


Figure A-3. Empty 3-L vessel being treated with forced HAD via hot air gun



Figure A-4. Empty 3-L contaminated vessel being treated with passive HAD inside an environmental chamber (note: thermocouples were placed inside the vessel with one suspended in air and the other touching the interior wall of the vessel).

The results (Figure A-5) indicated both HAD treatments were comparable, achieving approximately 1-log reduction compared to the controls. A slight decrease in viability, likely attributed to desiccation, was also observed between 4 and 24 hours from the untreated control vessel.

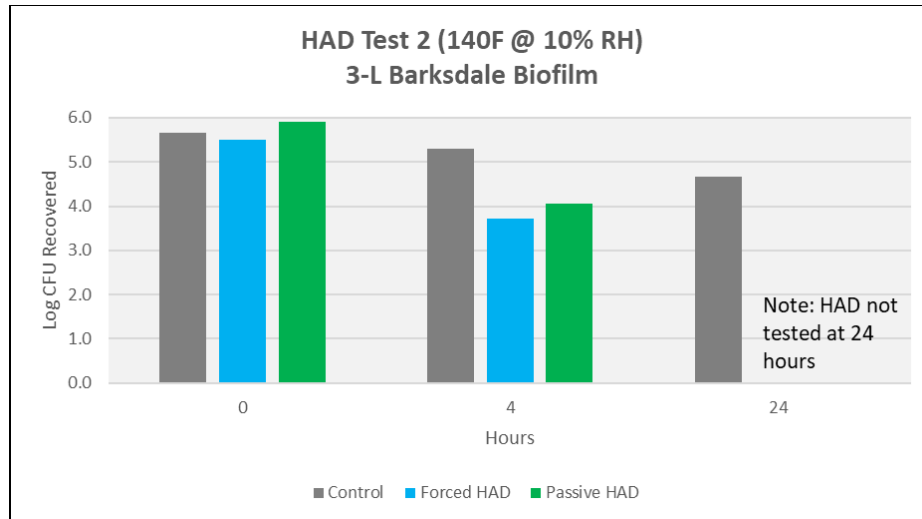


Figure A-5. Recovery of biofilm contaminated coupons after forced and passive HAD conditions

Test H3 – Biofilm Growth Characterization

This test was performed to assess whether a longer HAD treatment at 140 °F and 10% RH would achieve complete or near complete kill of biofilms. Three 3-L microcosms were incubated for 28-days to establish biofilms. Biofilm-contaminated coupons from the microcosms, as well as coupons inoculated with *Byssochlamys* spores, were then treated with HAD within an environmental chamber for 4, 24, and 48 hours (Figure A-6).

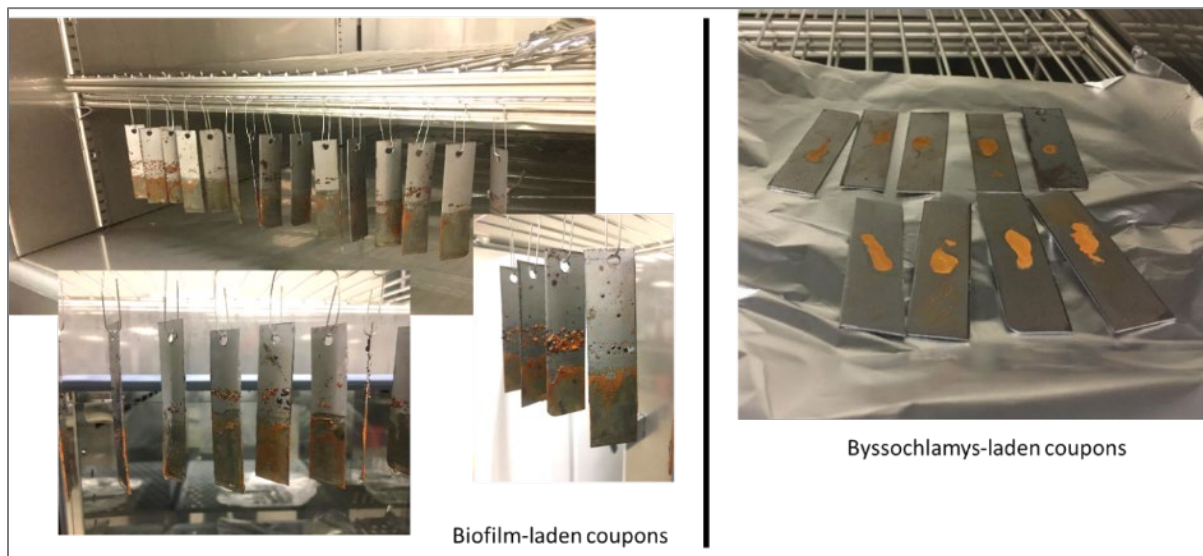


Figure A-6. Biofilm- and spore-contaminated coupons that were HAD treated

The results (Figure A-7) indicated that extending the HAD treatment to 24 and 48 hours did not yield complete kill as desired. Compared to the controls at each time point, the treatments achieved only 1-log more kill, and little difference in kill was observed between 24 and 48 hours. In addition, resistance to HAD was comparable between the biofilm and spores.

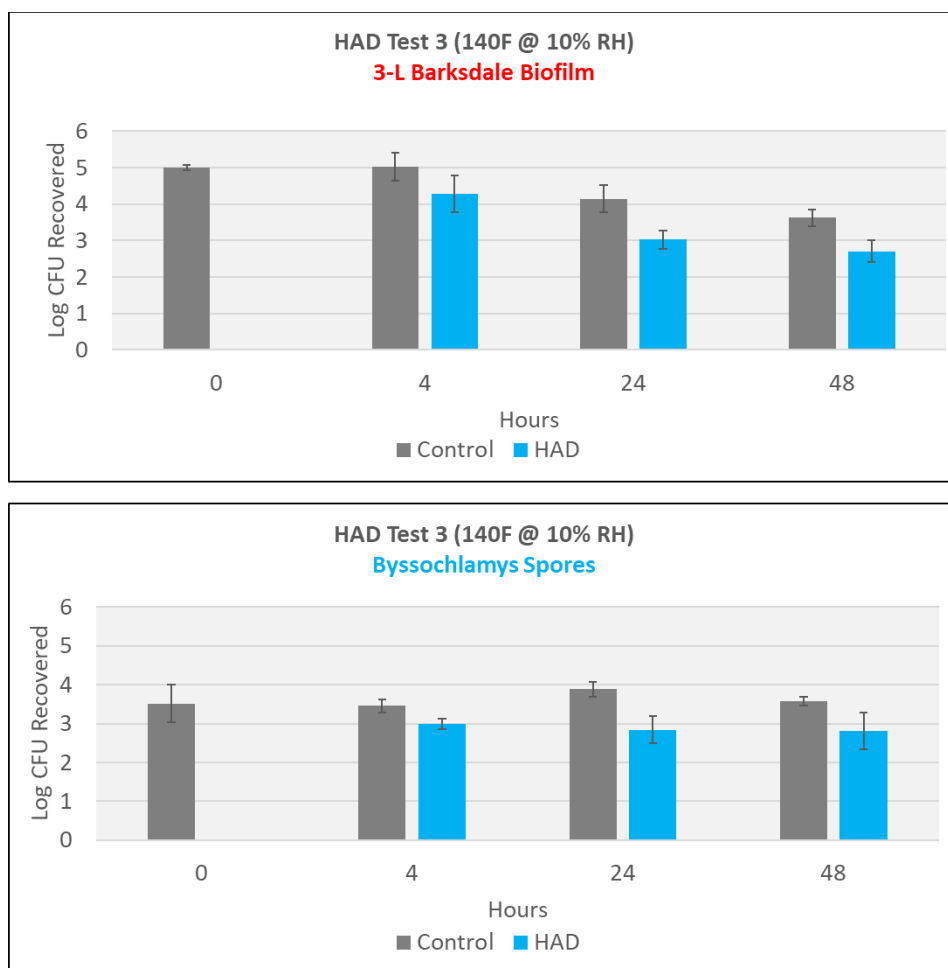


Figure A-7. Test H3 results

Test H4

Since HAD at 140 °F was not highly efficacious as shown in tests H1 to H3, H4 assessed the efficacy of HAD at 180 °F for four hours against spores of *Byssoschlamys* and *Aspergillus* inoculated onto glass and steel coupons. The results (Figure A-8) illustrated this treatment condition did result in favorable kill on both substrates as >4 log reduction was observed for *Byssoschlamys* on steel and glass as well as *Aspergillus* on glass. A reduction of nearly 3 logs was observed for *Aspergillus* on steel. Results from the controls illustrated the spores from both strains on both substrates remained stable under ambient conditions.

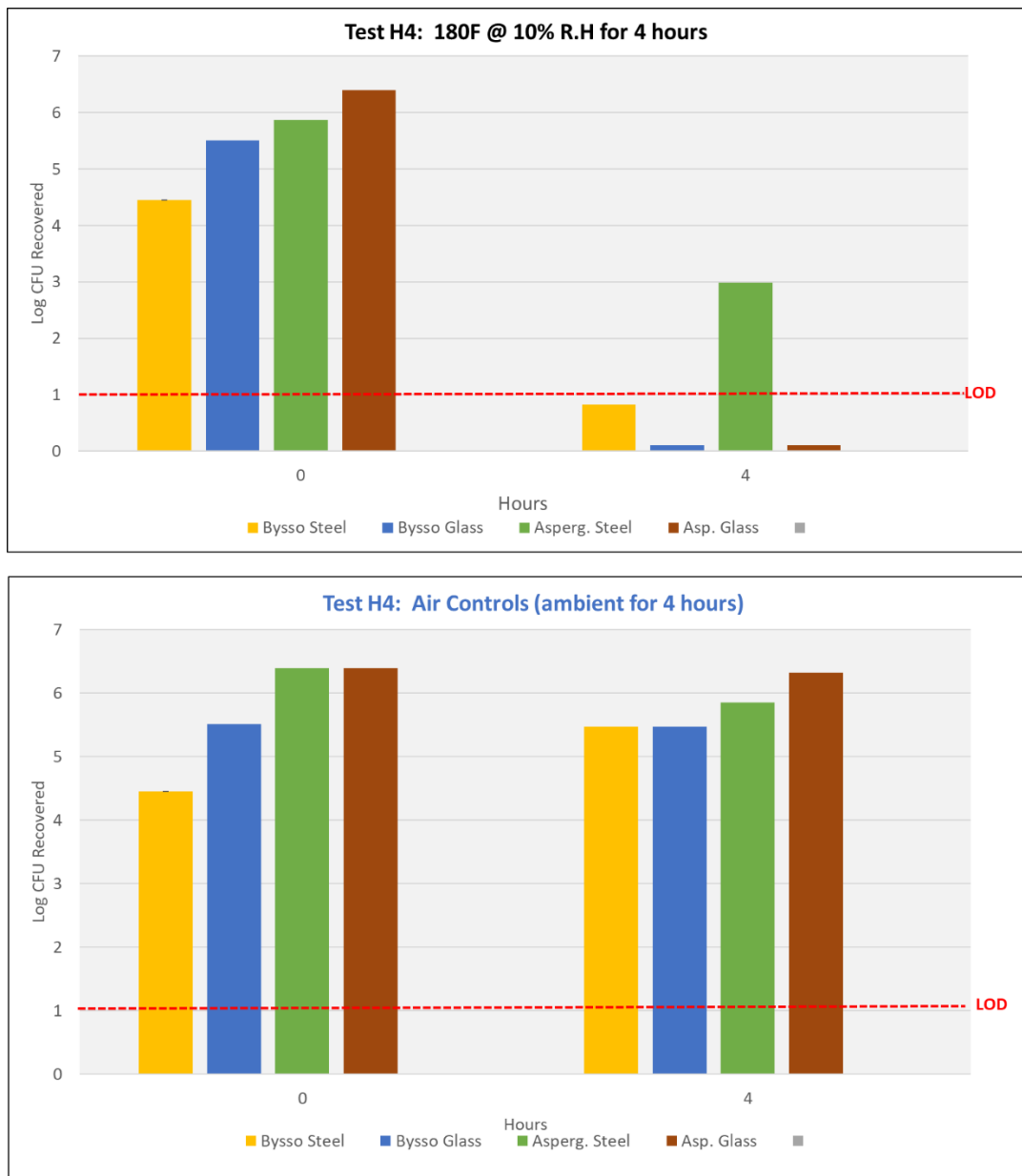


Figure A-8. Test H4 results

Test H5

Based on the encouraging efficacy results from Test H5, the temperature of HAD was increased to 190 °F to assess whether this slight increase would achieve complete kill. The results (Figure A-9), illustrated this was the case as only a few colonies of *Aspergillus* were recovered after a four-hour treatment period.

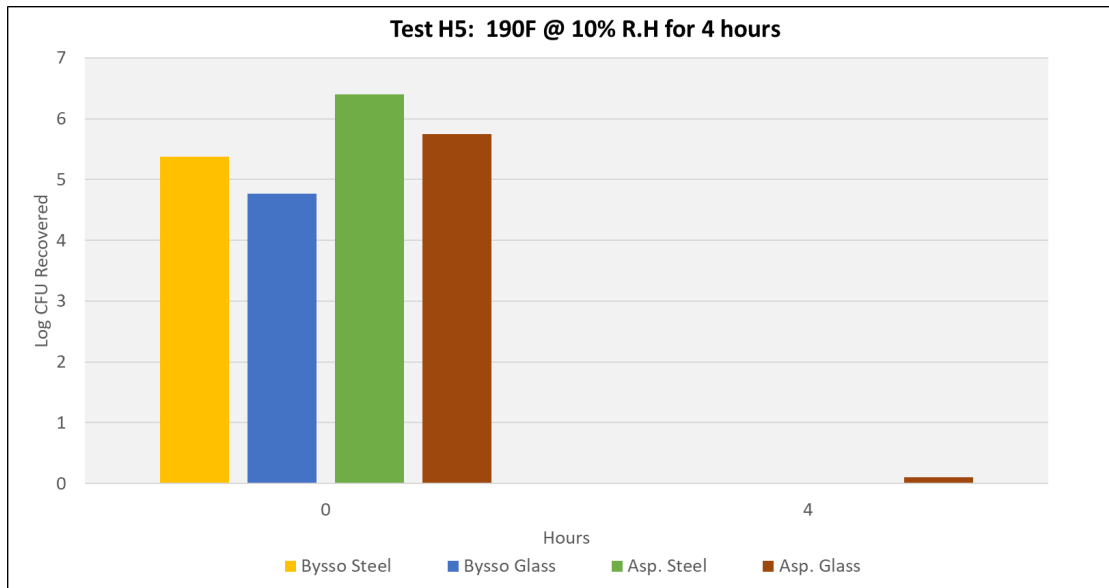


Figure A-9. Test H5 results

Test H6

Although the results from Test H5 were encouraging, it would be very difficult to achieve 190 °F surface temperatures within an empty fuel storage tank, particularly since this temperature would exceed the maximum temperature output of most industrial scale portable heaters and heat loss would be anticipated through the uninsulated tank walls. In addition, this temperature range would only be compatible with steel tanks that have a maximum operating temperature of 200 °F, while the maximum temperature for fiberglass tanks is 150 °F. To gain greater efficacy at lower temperatures, the efficacy of HAD/BTD at 140 °F and 90% RH was evaluated against *Bysssochlamys* spores on steel coupons. The results (Figure A-10) indicated that elevated RH did greatly increase efficacy against *Bysssochlamys* spores, as no viable spores were recovered after two, 24, and 48 hours.

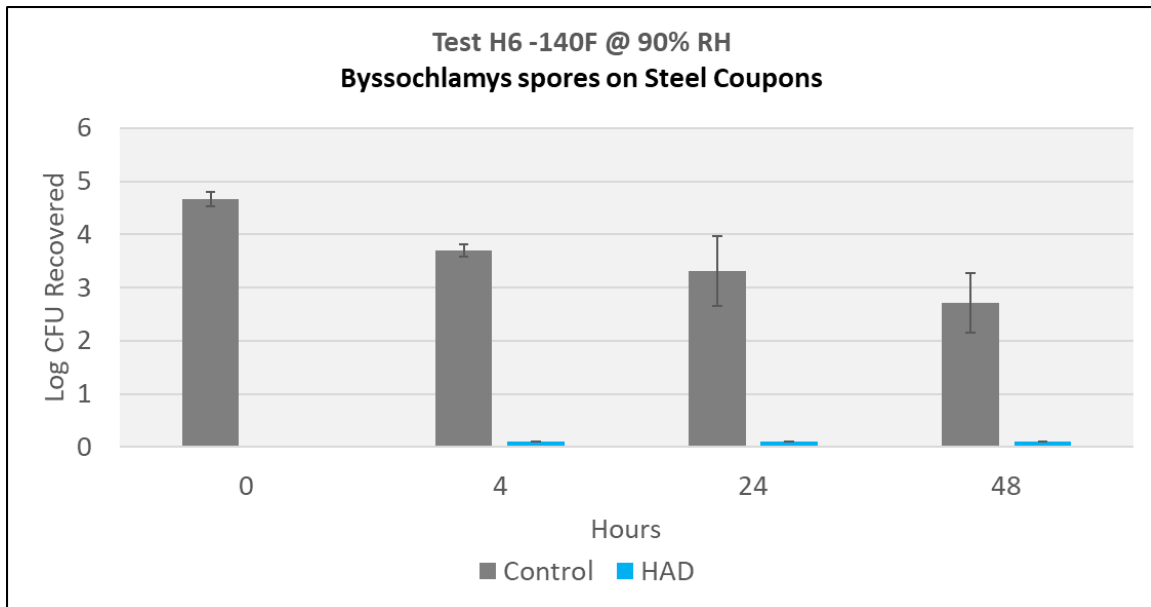


Figure A-10. Test H6 Results

Test H7

Since it may be difficult to achieve and maintain 90% RH conditions in a real-world fuel tank remediation operation, the efficacy of HAD at 50% RH was assessed. The results (Figure A-11) were also encouraging as >3-log reduction was observed after four hours, and no spores were recovered after eight and 24 hours.

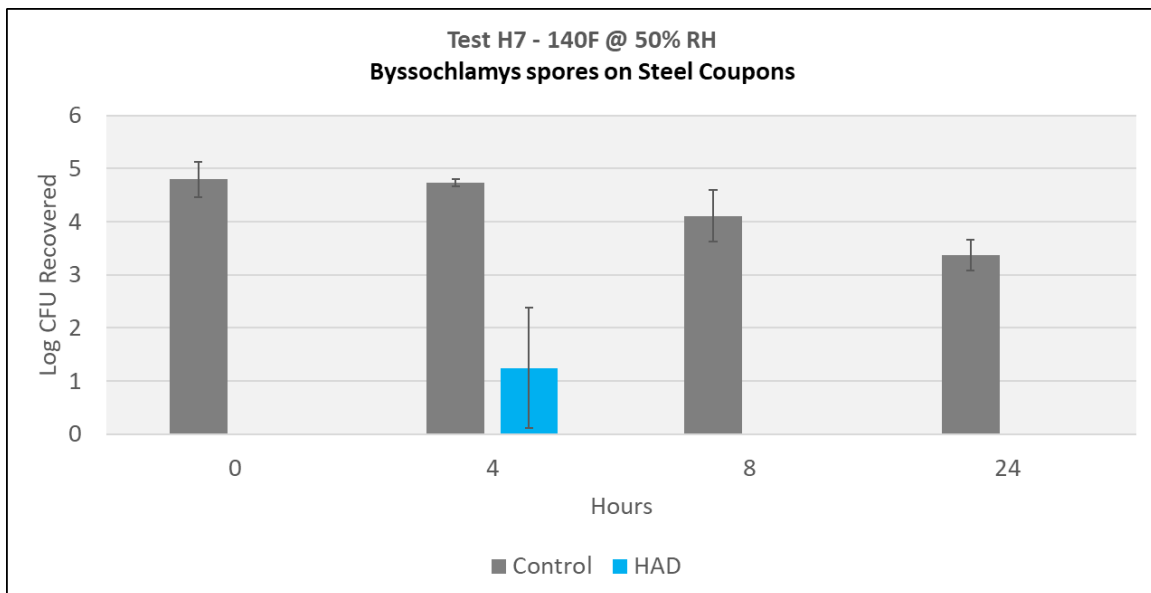


Figure A-11. Test H7 results

Test H8

With the encouraging results from Test H7, treatment at 140 °F and 50% RH was extended slightly to six hours and was performed against a variety of contaminants including biofilms, and spores of *Byssochlamys* and *Aspergillus*. The results (shown in diagrams in Figure A-12), illustrated the treatment was moderately successful against the biofilm and *Byssochlamys* spores but not against *Aspergillus* spores, suggesting longer treatment would be needed to achieve nearly complete or complete kill.

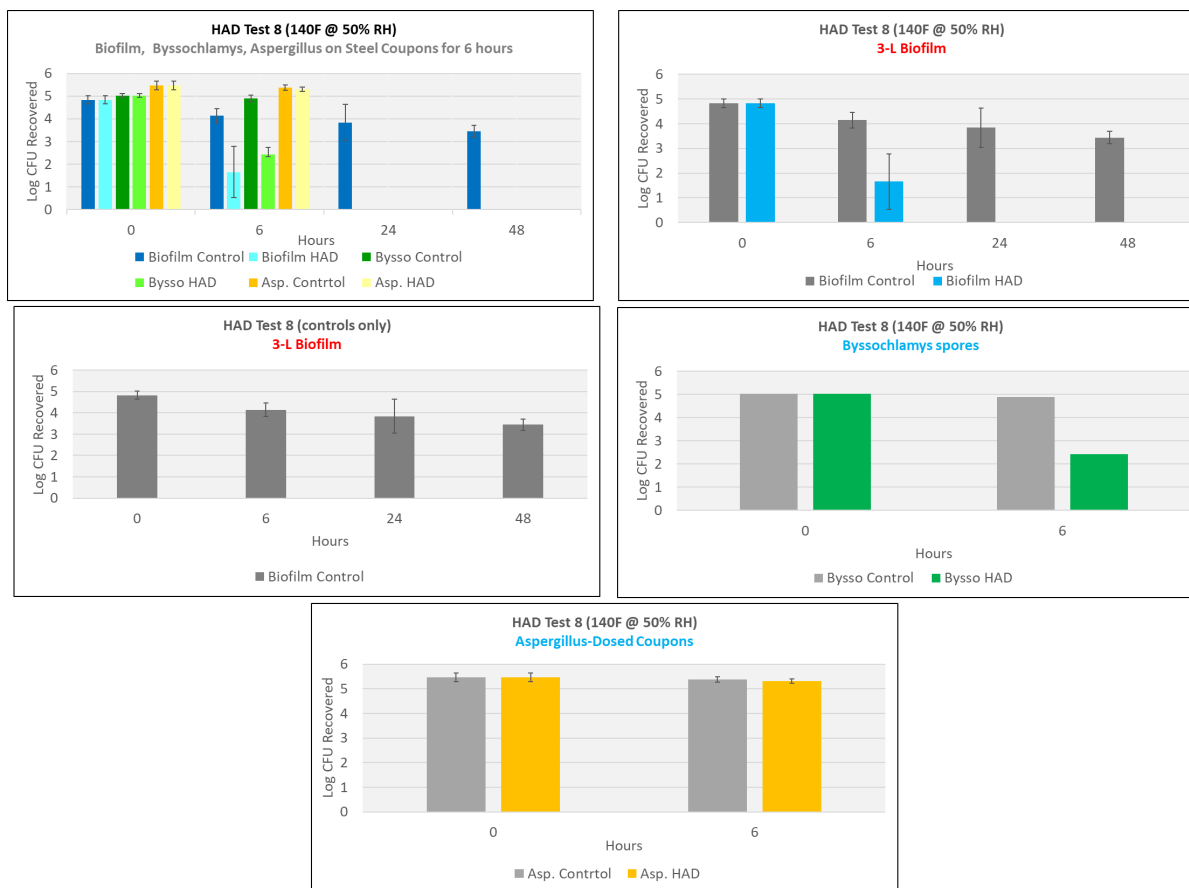


Figure A-12. Test H8 results

Test H9

This test evaluated the efficacy of HAD at 140 °F and 50% RH for eight, 12, and 24 hours against two strains of *Byssochlamys* spores (isolates B13 and B21) and *Aspergillus niger* spores. The log reductions achieved from this treatment (Figure A-13) were promising against *Byssochlamys*, achieving >3 log reduction after eight hours but were not as promising for *Aspergillus*, observing <1 log reduction after 24 hours.

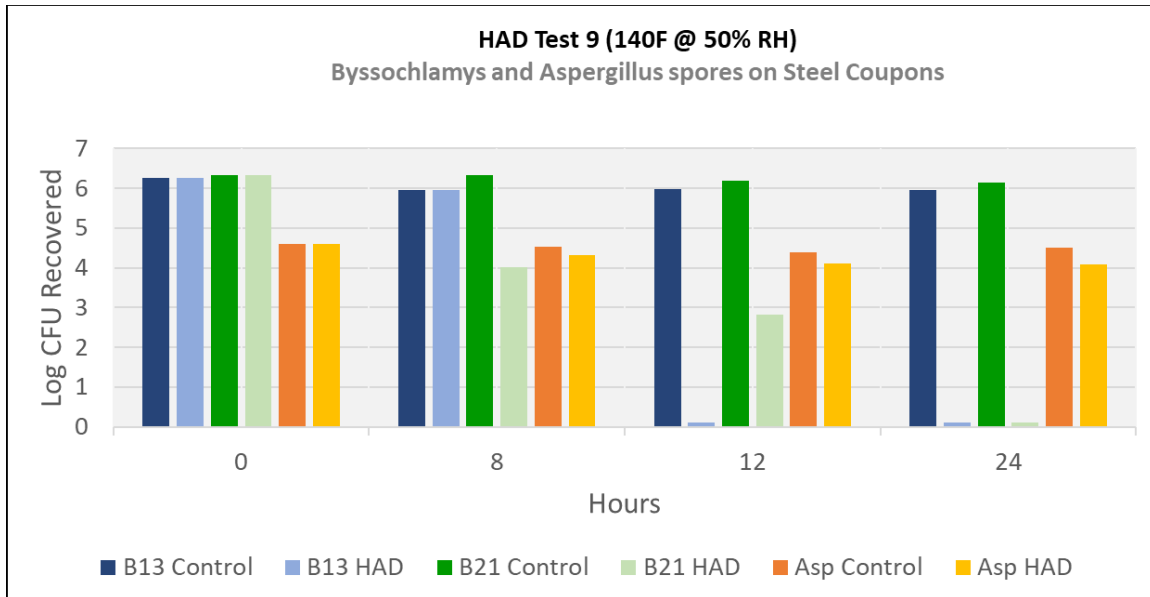


Figure A-13. Test H9 results

Tests H10 and H11 – HAD Treatments on Aspergillus spores

Since *Aspergillus niger* showed to be more resilient to thermal decontamination than *Byssochlamys* (Test H9), this test was conducted to assess efficacy at high and low RH conditions with elevated heat against *Aspergillus* spores. Spore inoculated coupons were treated for four, eight, and 24 hours at 140 °F at 80% and 5% RH. The results (diagrams shown in Figure A-14), indicated *Aspergillus* was far more sensitive to high RH conditions than low RH conditions. These results align with the premise that elevated temperature and high RH is more efficacious against biological agents than elevated temperature and low RH conditions.

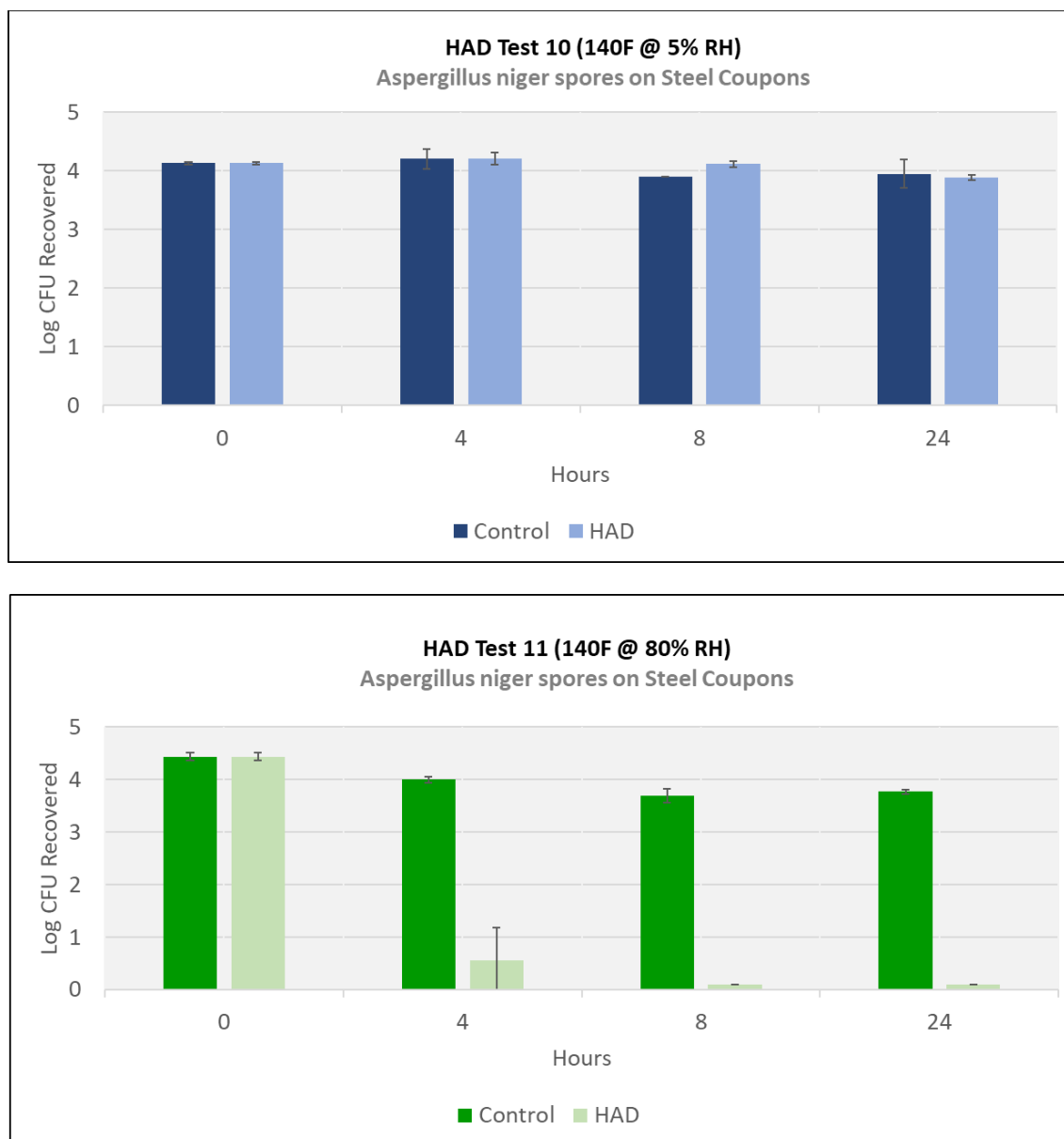


Figure A-14. Test H10 and H11 results

Test H12

This test was performed to assess the resistance of a different strain of *Aspergillus* to 140 °F and 80% RH. Spores of *A. terreus* (derived from a contaminated BDI fuel sample) were inoculated onto steel coupons and treated under these conditions for four, eight, and 24 hours. The results (Figure A-15) indicated this strain was slightly more heat resistant than *A. niger* and further demonstrated there are differences in heat resistance between species within the same genus.

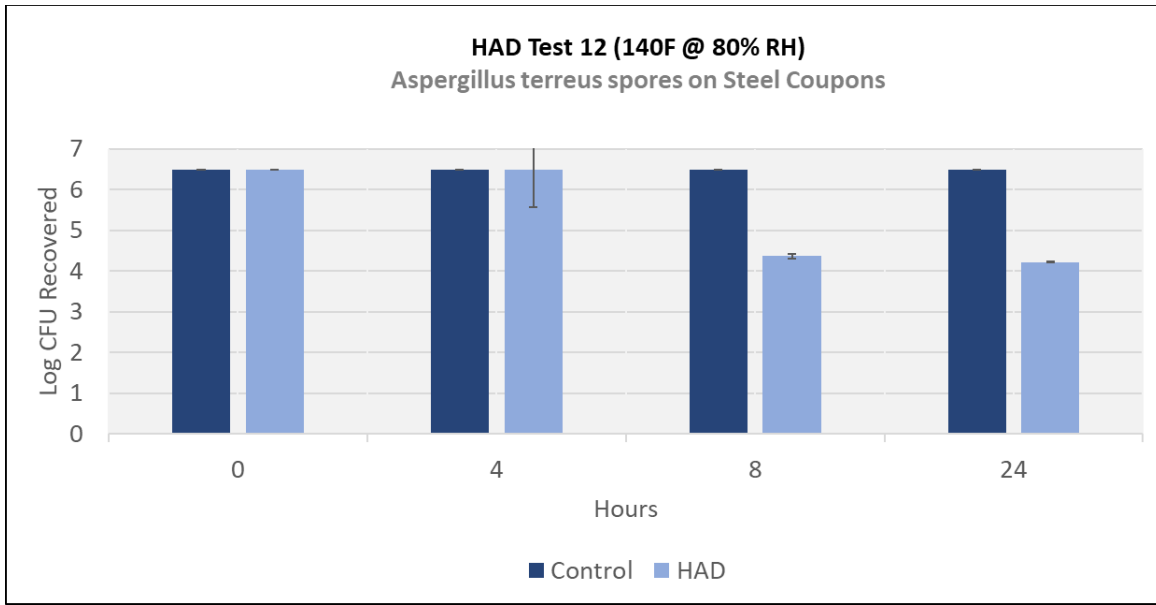


Figure A-15. Test H12 results

Tests H13 and H14

To further assess and compare efficacy of *A. niger* and *A. terreus* at 140 °F and 80% RH, coupons inoculated with spores of these two strains were treated for four, eight, and 24 hours. The results (shown in diagrams in Figure A-16), illustrate the resistance between the two strains are more comparable than shown in previous experiments. The variation in efficacy observed between tests H11 to H14 is not clear, but they do indicate such treatments will be moderately successful at reducing the level of contaminated by 2-3 logs within 24 hours.

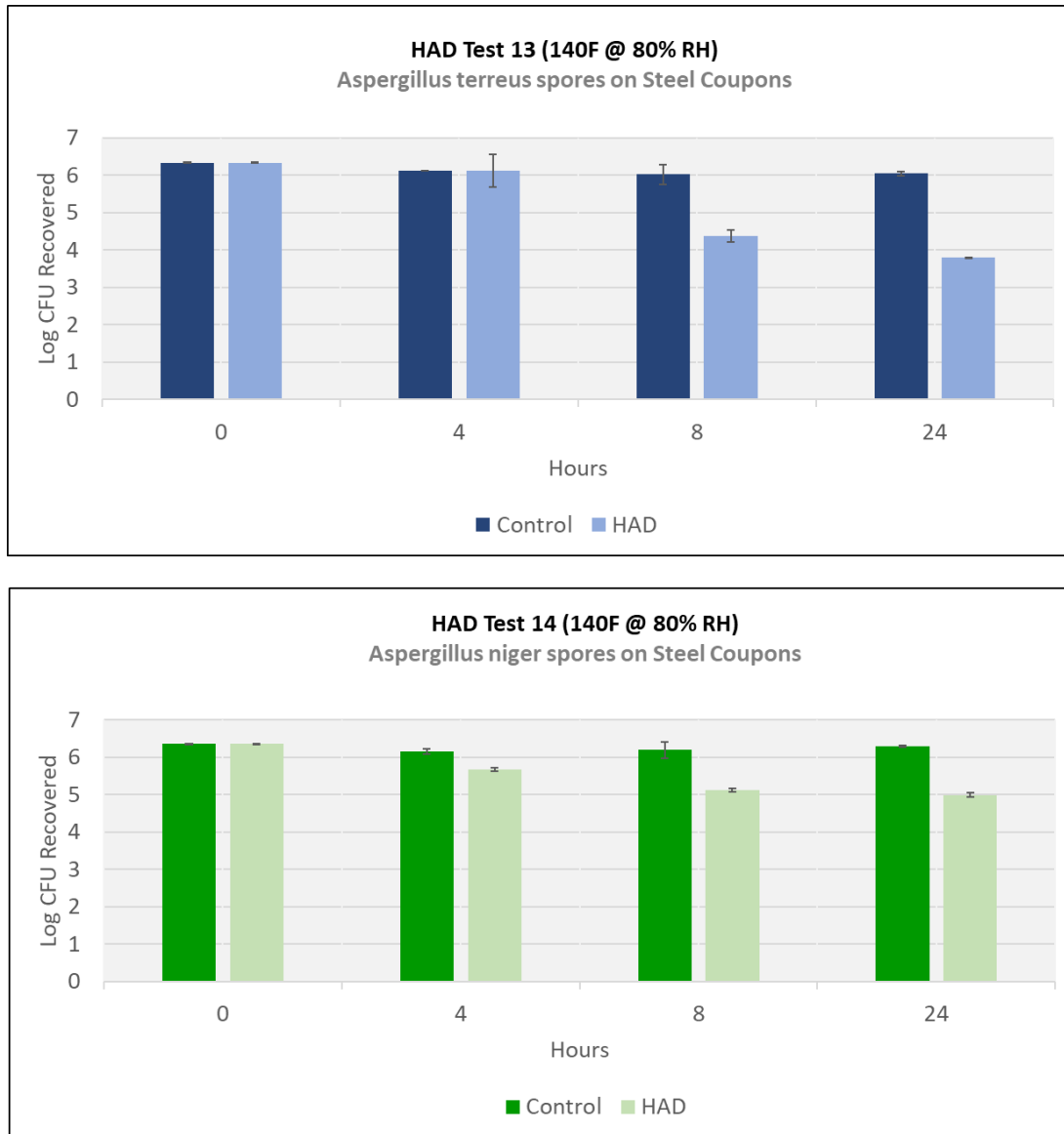


Figure A-16. Tests H13 and H14 results

Test H15

This test evaluated the efficacy of a BTDT treatment of 140 °F and 80% RH against *Thyridium vestidum*, which was another fungus recovered in BDI fuel samples by AFRL. Samples were treated at this condition for four, eight, and 24 hours. The results (Figure A-17), show this strain is far more susceptible to thermal decontamination than *Aspergillus* (a common environmental fungus) and comparable to *Byssoschlamys* (the most predominant strain found in contaminated fuel samples).

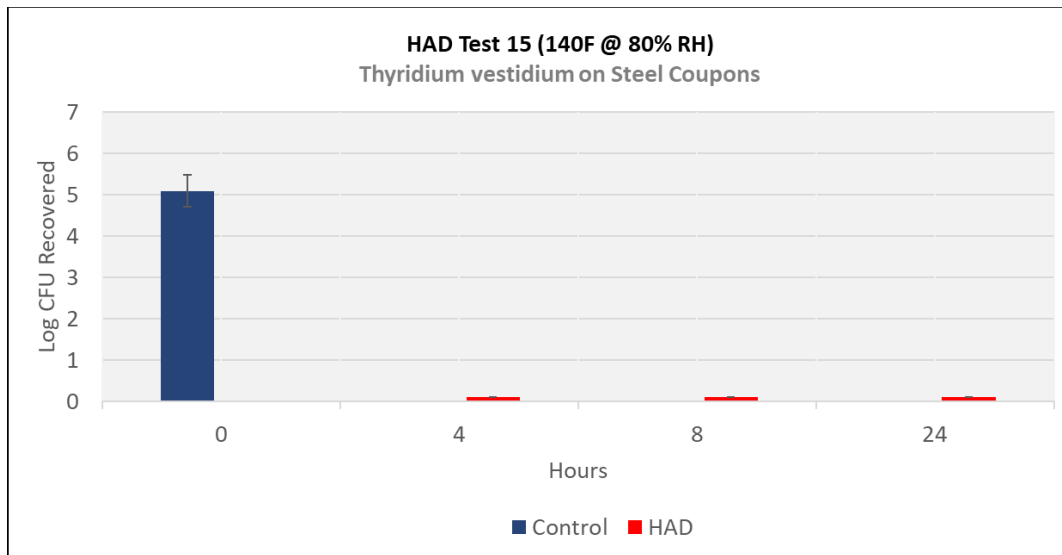


Figure A-17. Test H15 results

Test H16

The final bench-scale HAD efficacy test was designed to evaluate 140 °F and 80% RH against *Byssoschlamys* spores and *Byssoschlamys* biofilm. The results (Figure A-18) indicate the levels of kill between these two stages of growth were comparable as no viable organisms were recovered at four, eight, and 24 hours. These results suggest that if these conditions can be achieved in the field within an empty, contaminated fuel storage tank, effective remediation would likely be achieved.

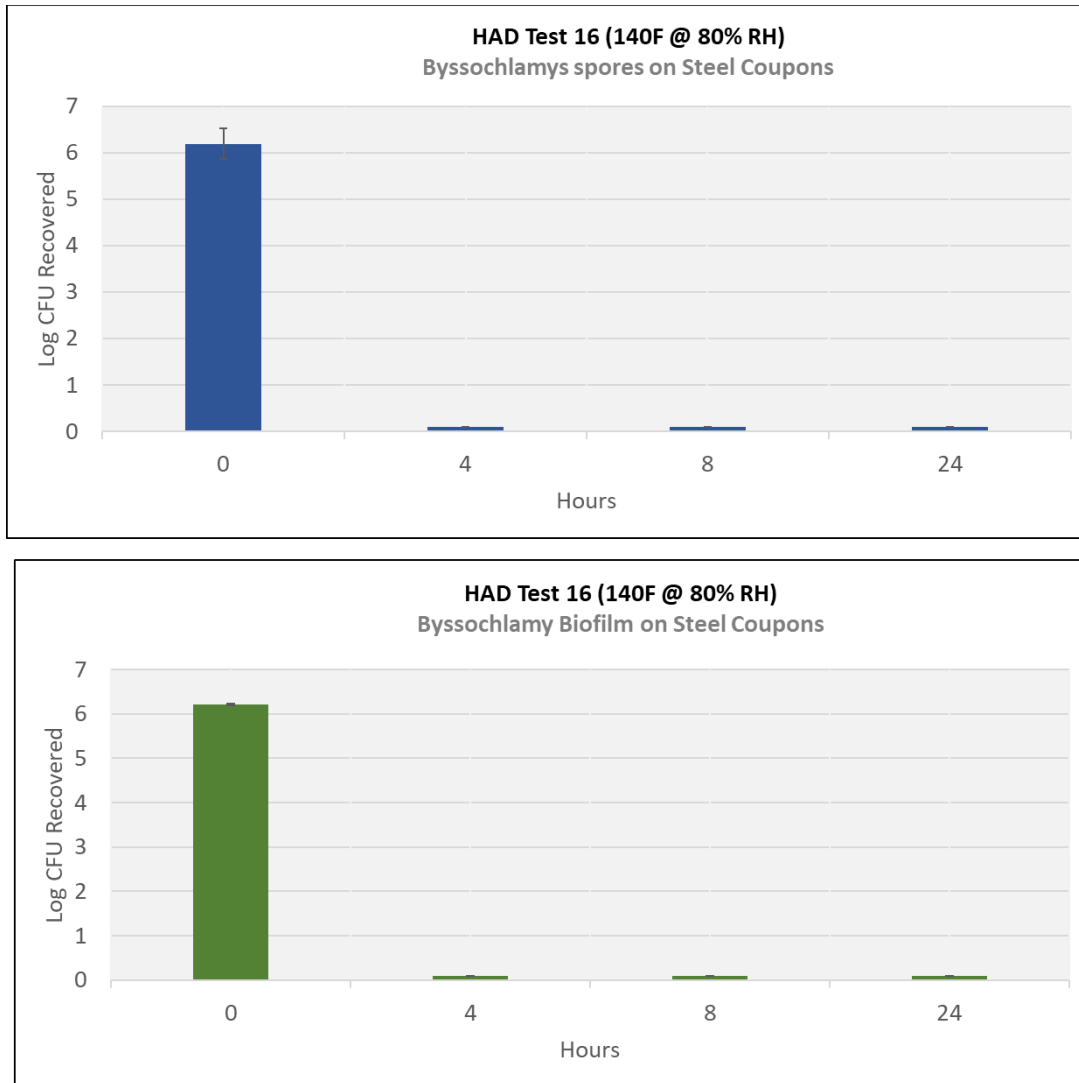


Figure A-18. Test H16 results

HAD Test Summary

The key results and observations from these thermal treatments are provided below:

- 140 °F and 10% RH was not effective within 24 hours
- 140 °F and 5% RH was not effective within 24 hours
- 140 °F and 50% RH resulted in poor-to-moderate effectiveness after several hours
- 140 °F and 80% RH was effective after 4-8 hours
- 140 °F and 90% RH was very effective within four hours

- 180 °F and 10% RH was effective within four hours
- 190 °F and 10% RH achieved nearly complete kill of *Byssoschlamys* and *Aspergillus* within four hours
- Slight differences in log kill were observed with glass versus steel coupons (less kill on steel); however, the difference was negligible and either matrix was deemed acceptable for testing
- Level of kill was comparable between biofilms and pure spores; therefore, the use of spore-laden coupons is suitable for conducting range-finding efficacy tests
- Level of kill was comparable between four fungal strains; most testing was performed with *Byssoschlamys* as genomic analysis indicated this was a predominant species in BDI storage tanks. *Aspergillus* may be more heat resistant; however, it was not a prevalent fuel contaminant species
- Standard deviation between replicate spore-laden coupons was minimal and smaller than biofilm-laden coupons as expected (coupon inoculation with spores is far more accurate and repeatable)
- Biofilms on coupons generally remain stable over two days at 27 °C (~80 % RH)

The results from these bench-scale tests indicated that prevalent fungi found in contaminated fuel systems can be killed within eight hours at 140 to 150 °F at high RH (approx. 80%) or at 180 °F and low RH (<10%). These process parameters were subsequently targeted in the mock-up evaluations described in section 3.7.

APPENDIX B – CLO₂ EFFICACY TESTING

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Test C1

This was the first of fourteen ClO_2 efficacy tests performed. Biofilm-laden coupons of a mixed culture derived from a Barksdale AFB BDI fuel storage tank and generated from a 3-L fuel/water microcosm were exposed to the gas evolving from a 50-ppm aqueous ClO_2 solution and the ClO_2 gas evolving from this solution for two hours. A set of untreated coupons (controls) were assayed as well. The results (Figure B-1) showed the aqueous treatment to be more efficacious than the gas treatment, with log reductions of approximately 4 and 1.5 logs, respectively.

Note: The PortaSens was unavailable for this test so the treatment in terms of ppmvh could not be determined.

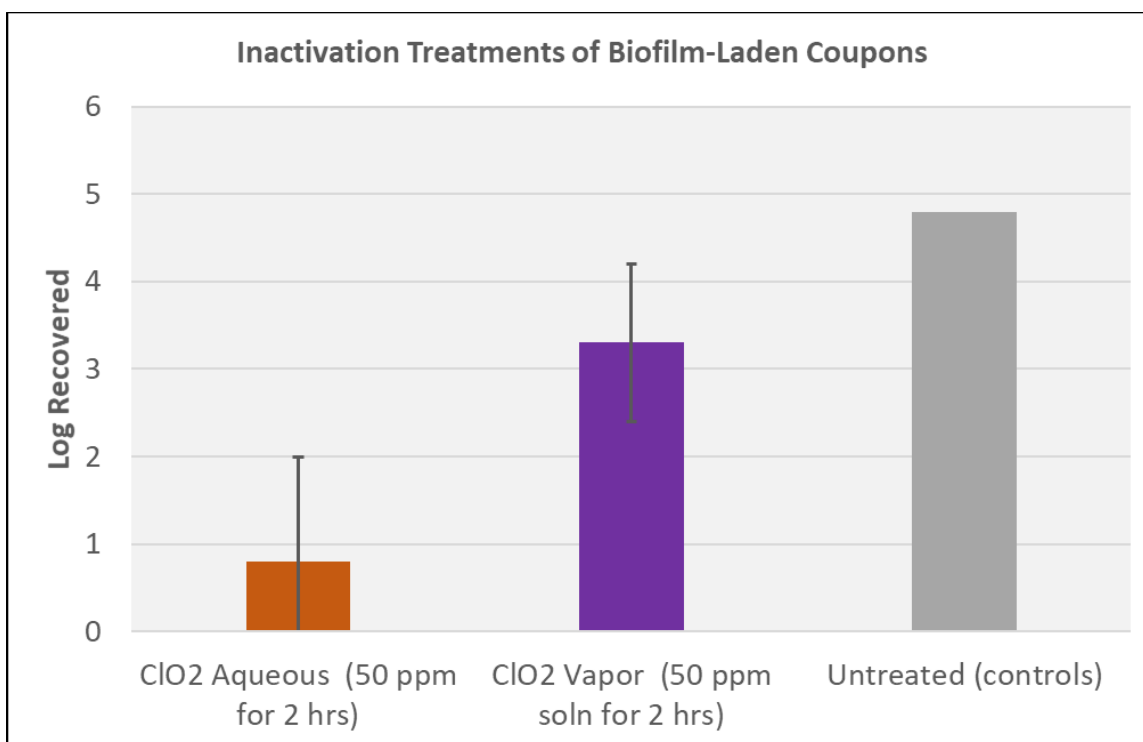


Figure B-1. Test C1 results

Test C2

This test was a repeat of Test C1 with exception that coupons contaminated with *Byssochlamys* spores were used instead of an undefined biofilm. The results (Figure B-2) indicate that aqueous and gas treatments were equally effective. The results of tests C1 and C2 indicated the biofilm is more resistant to either treatment than pure *Byssochlamys* spores.

Note: The PortaSens was unavailable for this test so the treatment in terms of ppmvh could not be determined.

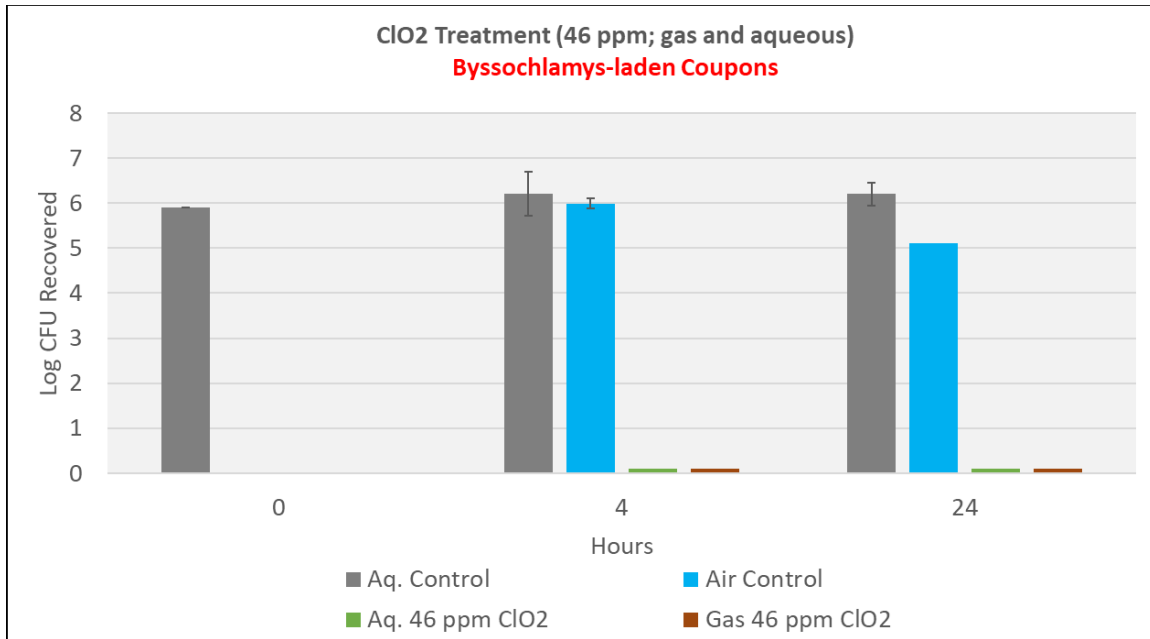


Figure B-2. Test C2 results

Test C3

To gain a better understanding of the efficacy of ClO₂ gas, numerous fungal strains including *Byssoschlamys*, *Aspergillus*, *Fusarium*, and *Penicillium* were tested against the ClO₂ gas evolving from a 50-ppm solution for four hours; good kill was observed (shown in diagrams in Figure B-3). Although only 1-log was observed against *Byssoschlamys* spores on steel coupons, a log reduction of >2 and up to 5 logs was observed for the other samples on glass coupons.

Note: The PortaSens was unavailable for this test so the treatment in terms of ppmvh could not be determined.

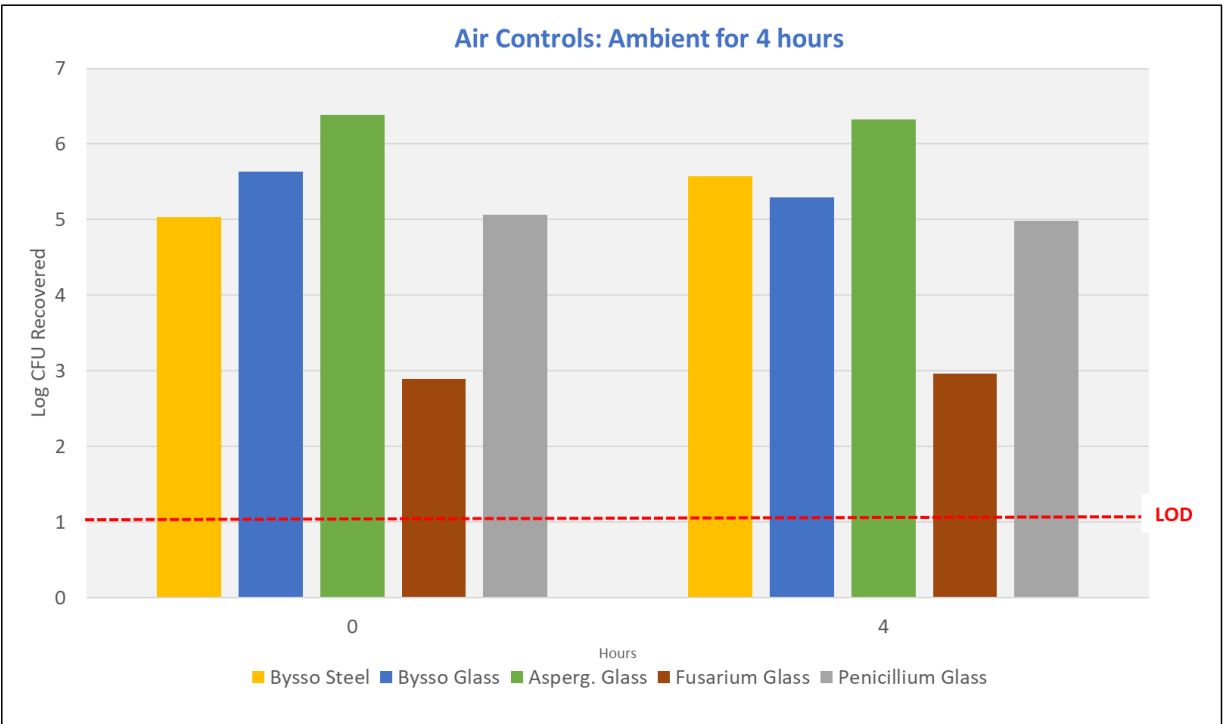
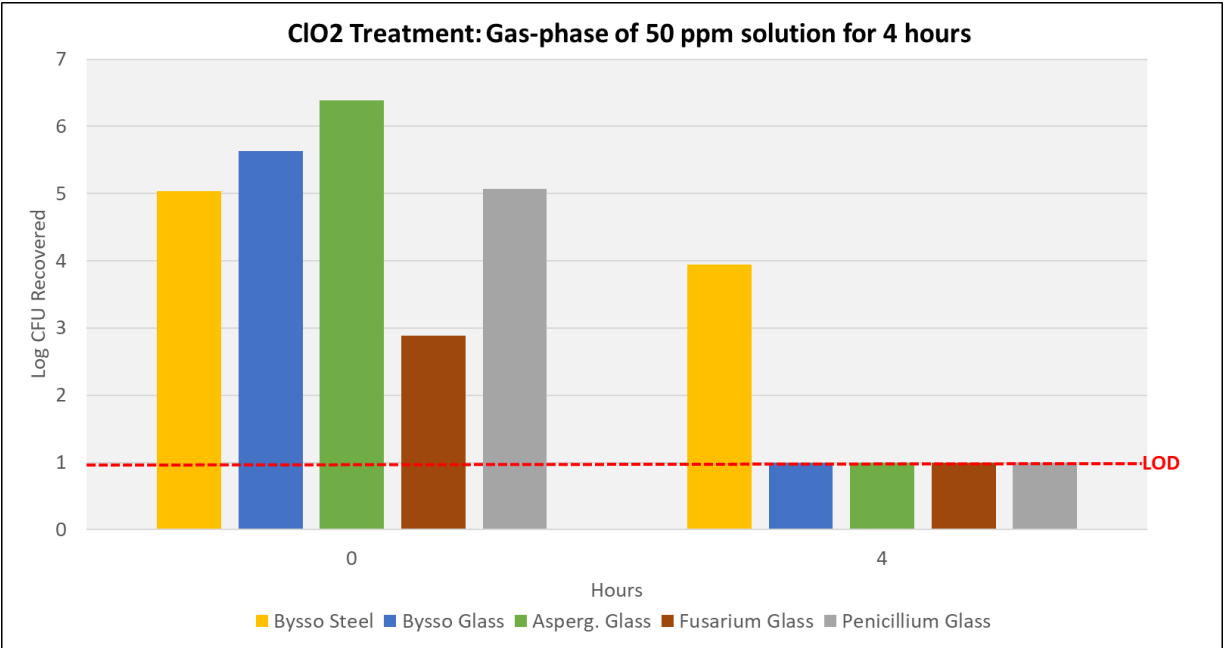


Figure B-3. Test C3 results

Test C4

In this test, the ClO₂ solution was increased to 100 ppm with a gas treatment time of two hours. Based on the PortaSens data, the treatment was calculated to be 1265 ppmvh. The results (Figure B-4), illustrated the gas treatment was very efficacious against *Byssochlamys* spores (> 4

log reduction), mildly efficacious against *Byssoschlamys* biofilm (approximately 1 log reduction), and moderately efficacious against *A. niger* and *A. terreus* spores (>4 log reduction).

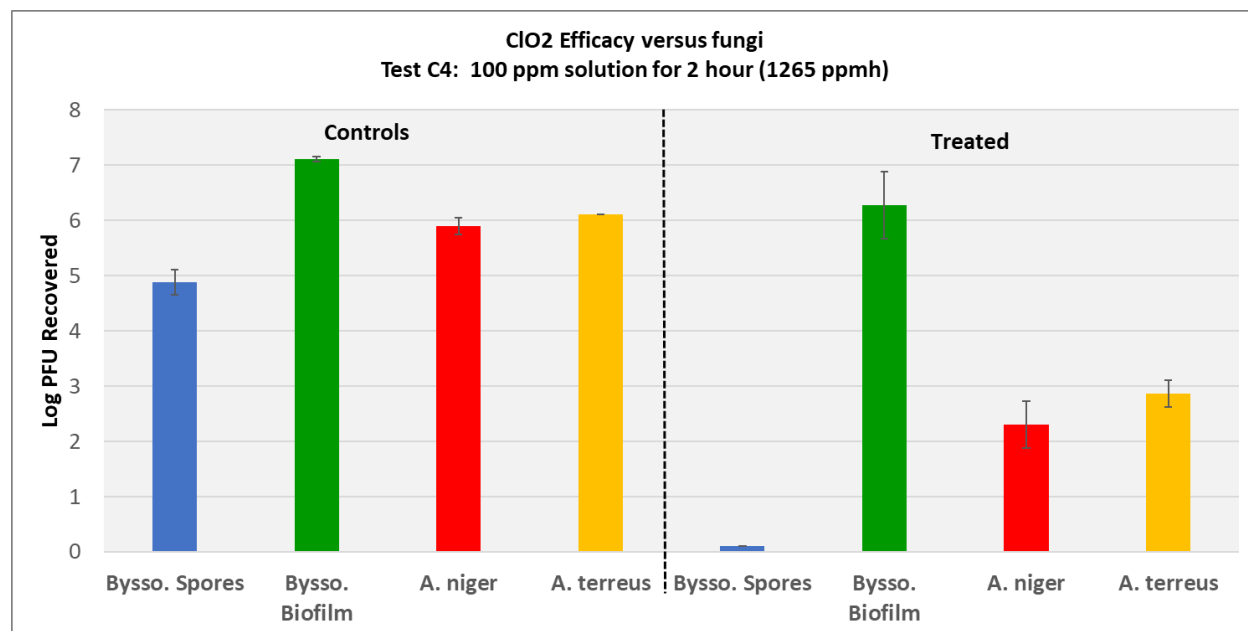


Figure B-4. Test C4 results

Test C5

In this test, the gas treatment was increased by increasing the exposure time from two hours to three hours; however, the measured treatment was slightly lower at 1109 ppmvh. Testing was performed again with *Byssoschlamys* biofilm, but also against wet and dry 100- μ L droplets of *Byssoschlamys* and *A. terreus* spores. *Aspergillus* species are prevalent in the environment, and *A. terreus* was recovered by AFRL from field-collected BDI samples. The intent of this study was, in part, to assess if efficacy of the gas was affected by whether the contamination is wet or dry. Both conditions would be found in a contaminated fuel tank that has been prepared for gas fumigation via pressure washing, after which the tank will be fumigated immediately (still moist inside) or fumigated a day or two after pressure washing (and therefore dryer). The results (Figure B-5) illustrated the treatment was very effective against *Byssoschlamys* and *A. terreus* dried spores, but mildly or moderately efficacious against the wet inoculum.

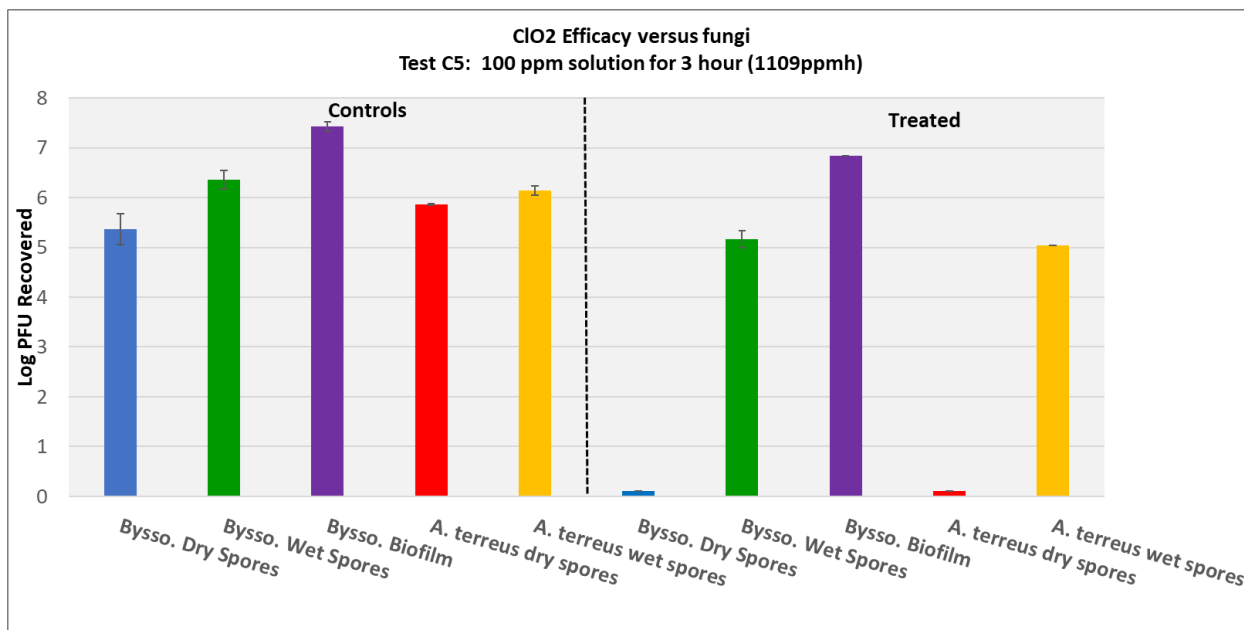


Figure B-5. Test C5 results

The results from tests C1 to C5 provided a first-hand look at the efficacy levels achieved at relatively low to moderate gas treatment level (<1200 ppmvh) against fungal biofilms and spores and indicated that greater treatment levels were needed to achieve complete or near-complete kill (defined herein as >3 log reduction or a 99.9% reduction in viability). Subsequent tests (Tests C6 to C14) were transitioned from bench-scale to full-scale.

Test C6

This was first test conducted using the Yellow Jacket gas dispersion unit with the full-scale system. The Yellow Jacket was used with two 12G micro-reactors and run overnight for total duration of 14 hours. The measured treatment was 2320 ppmvh. Wet spores of *Byssoschlamys* and *Aspergillus terreus* along with *Byssoschlamys* biofilm were treated. The results (Figure B-6) indicated the treatment was very efficacious against the spores, showing > 6-log reduction, but only moderately efficacious to the biofilm, with a log reduction of approximately 3 logs compared to the controls.

Note: The RH was not increased for this test even though it is known that elevated RH in the range of 65-85% increases the efficacy of ClO₂ gas. The intent of this test was to assess efficacy of the gas without added humidification.

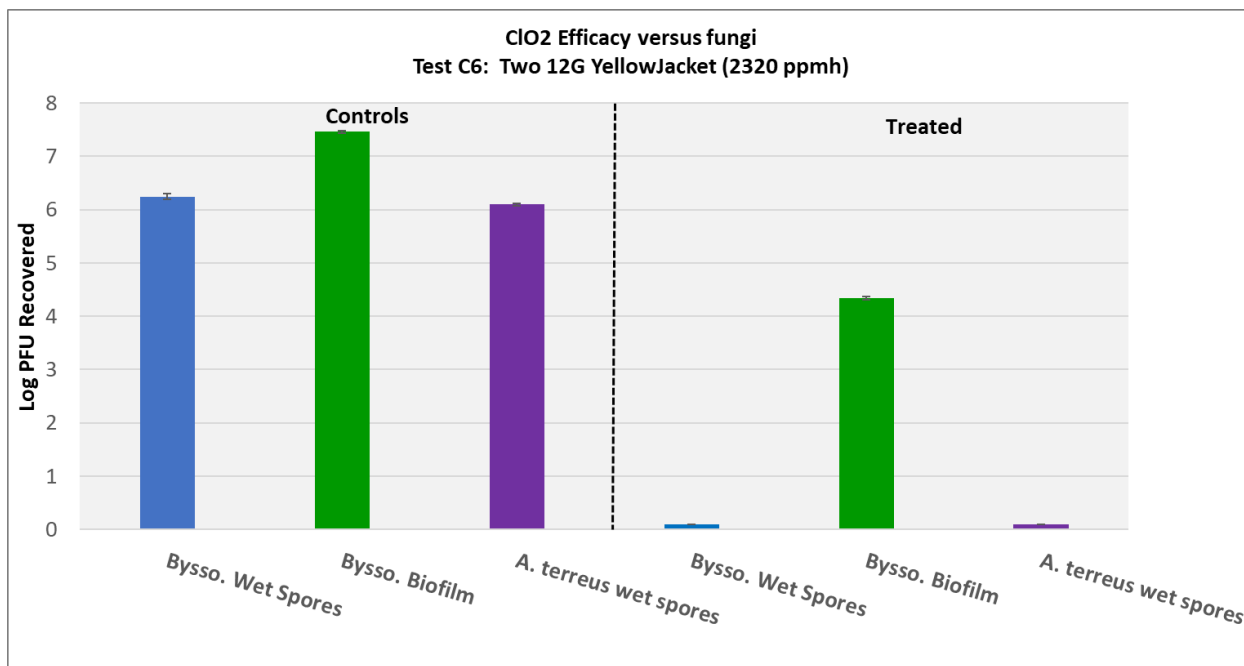


Figure B-6. Test C6 results

Test C7

In this second full-scale test, two 12G micro-reactors were again used; however, the RH was elevated to >65% using a household humidifier. Eight *Byssoschlamys*-biofilm laden coupons were positioned throughout the test system on the floor. The treatment was measured to be 3076 ppmvh. The results (Figure B-7) illustrated that >3-log reduction was achieved including >6-log reduction for three of the eight samples. The gas profile is illustrated in Figure B-8. This test indicated that a treatment of approximately 3000 ppmvh was within the range to achieve the desired level of biofilm inactivation.

Note: The mean air temperature during the treatment was 25 °C and the RH ranged from 75-90% (data not shown).

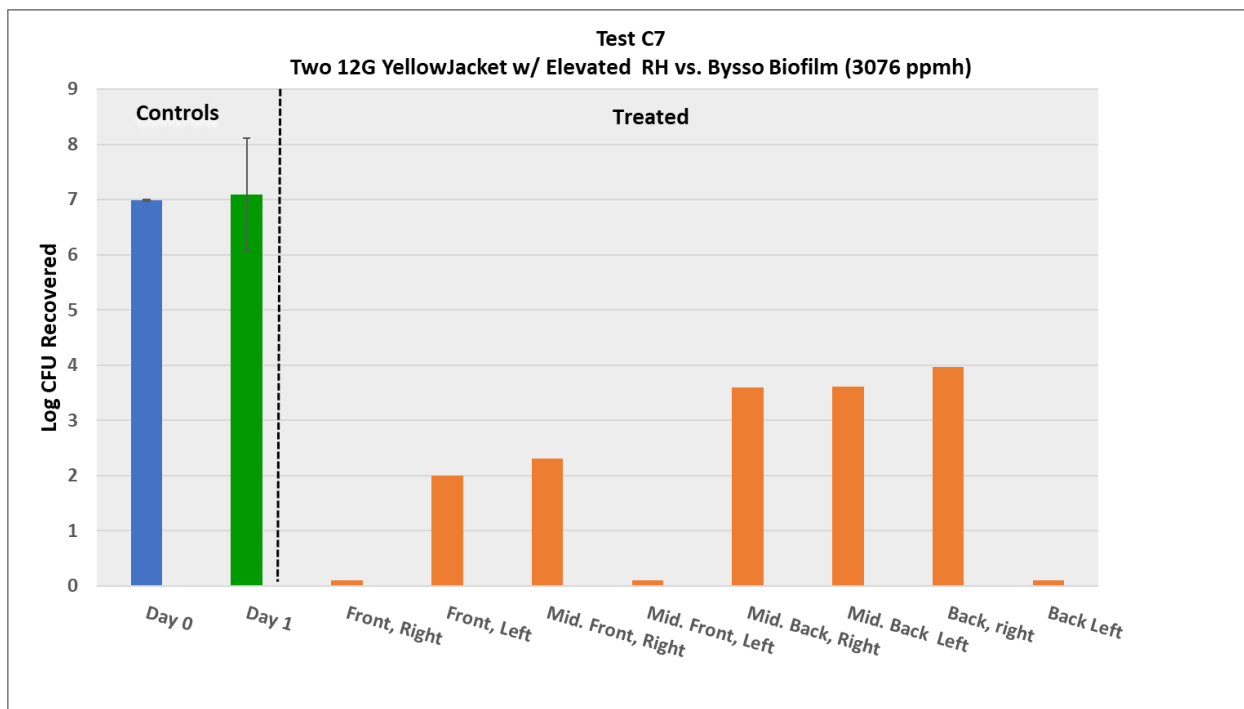


Figure B-7. Test C7 results

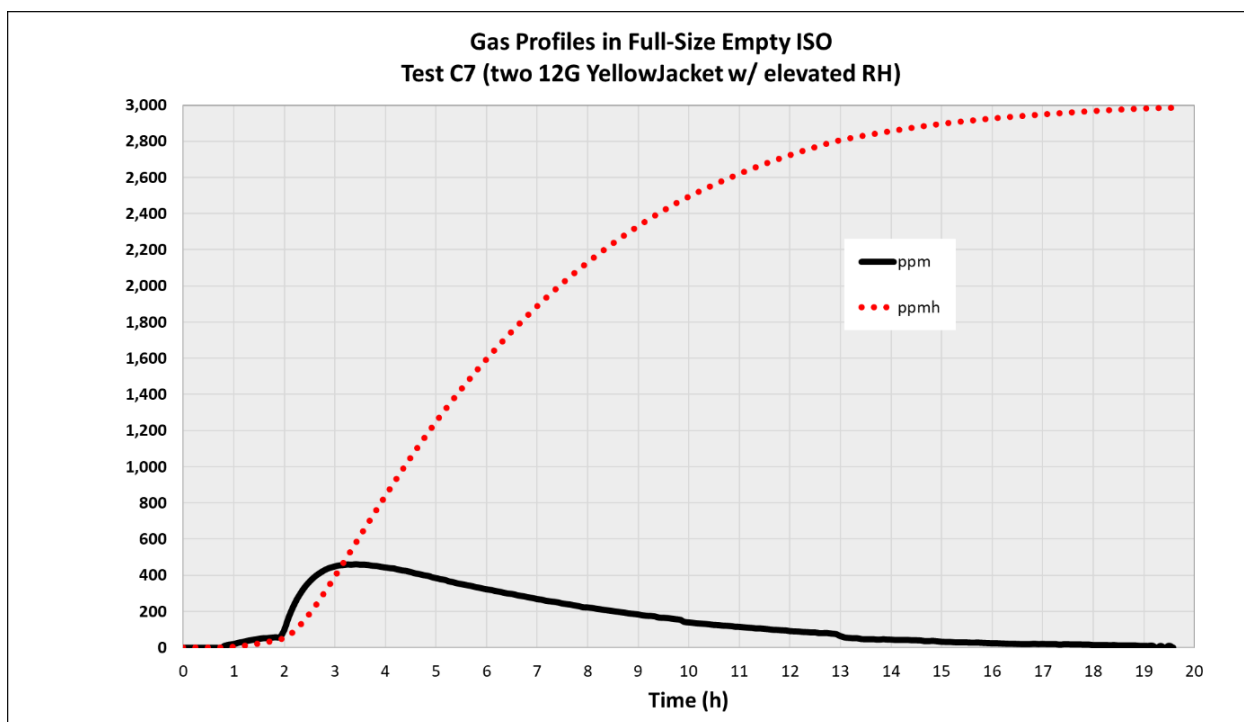


Figure B-8. Gas profile from Test C7

Test C8

This test was essentially a repeat of Test C7, although the air temperature in the test system was increased slightly through the aid of a household room heater. The heater only raised the mean temperature by 3 °C to 28 °C (data not shown). The RH ranged from 80-90% (data not shown). The treatment measured 2529 ppmvh, which was approximately 500 ppmvh less than Test C8. The level of kill was comparable to that of Test C7, and perhaps slightly lower (Figure B-9). The gas profile is shown in Figure B-10.

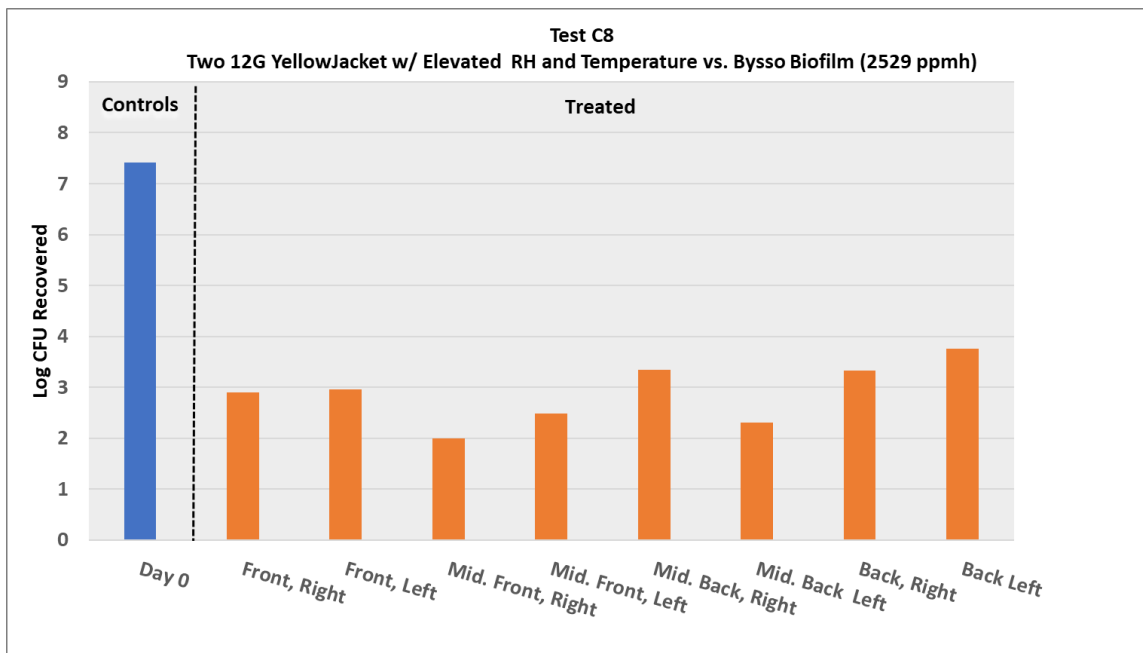


Figure B-9. Test C8 results

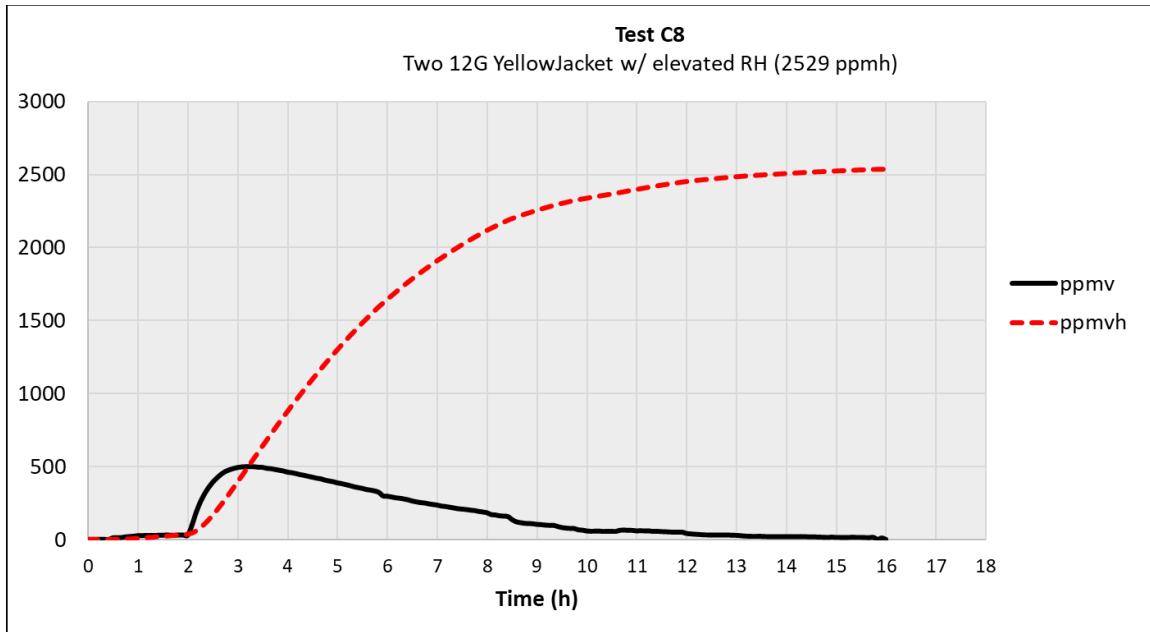
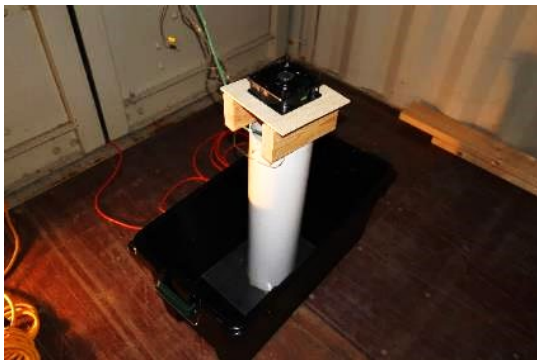


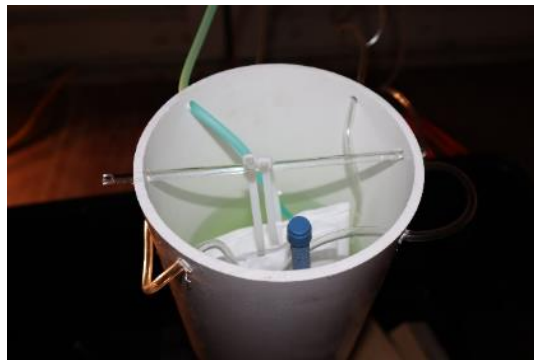
Figure B-10. Gas profile from Test C8

Test C9

METSS constructed a “column” gas dispersion system from 6-inch diameter PVC pipe to assess whether the rate of off-gassing can be increased using an alternative dispersion method. The column measured 24 inches in length and was retrofitted with bar across the opening from which up to three 12G micro-reactors could be suspended in 9 liters of water (Figure B-11). A 4-inch air diffusion disk was placed in the bottom of the column and connected to an aquarium manifold air pump. The intent was to allow the micro-reactors to generate ClO_2 for two hours inside the column after which the air pump connected to the diffusion disk would be turned on. The idea was that the small bubbles generated from disk diffusers would accelerate off-gassing. The efficacy results and gas profile are shown in Figure B-12 and Figure B-13, respectively. The treatment measured 2990 ppmvh.



(A) Column Gas Dispersion Unit



(B) Crossbar at top of column used to suspend micro-reactors



(C) Top down view inside column containing two 12G micro-reactors and water



(D) Air pump placed outside the shipping container and connected via silicon tubing to the air diffusers placed inside the column unit.

Figure B-11. Column gas dispersion unit

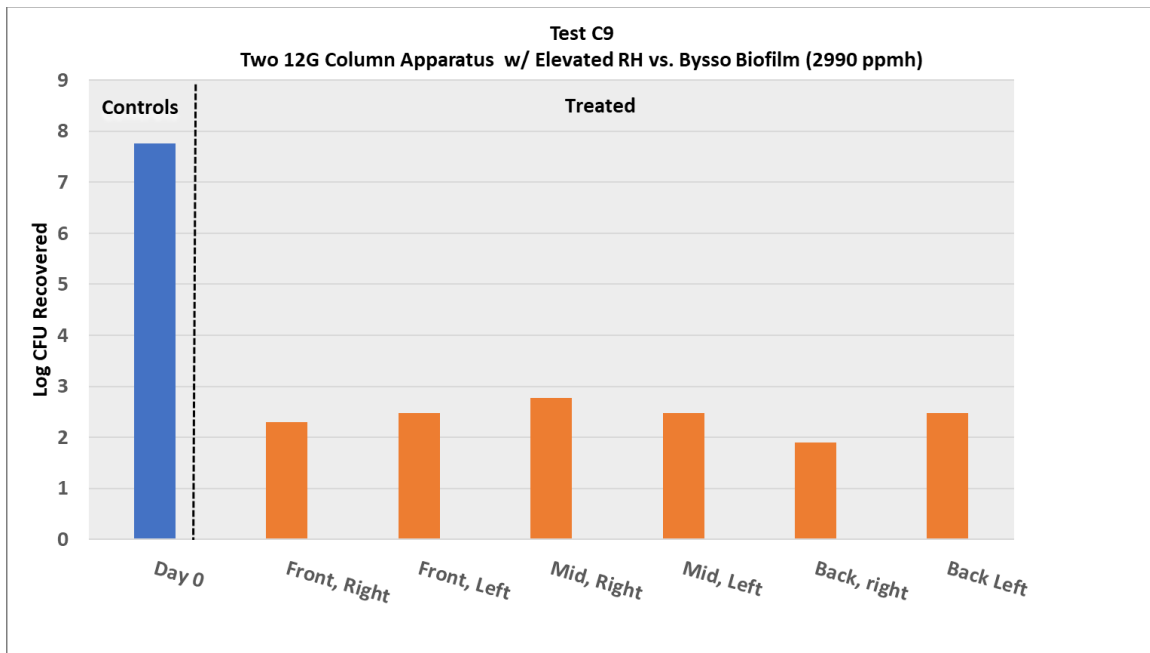


Figure B-12. Test C9 results

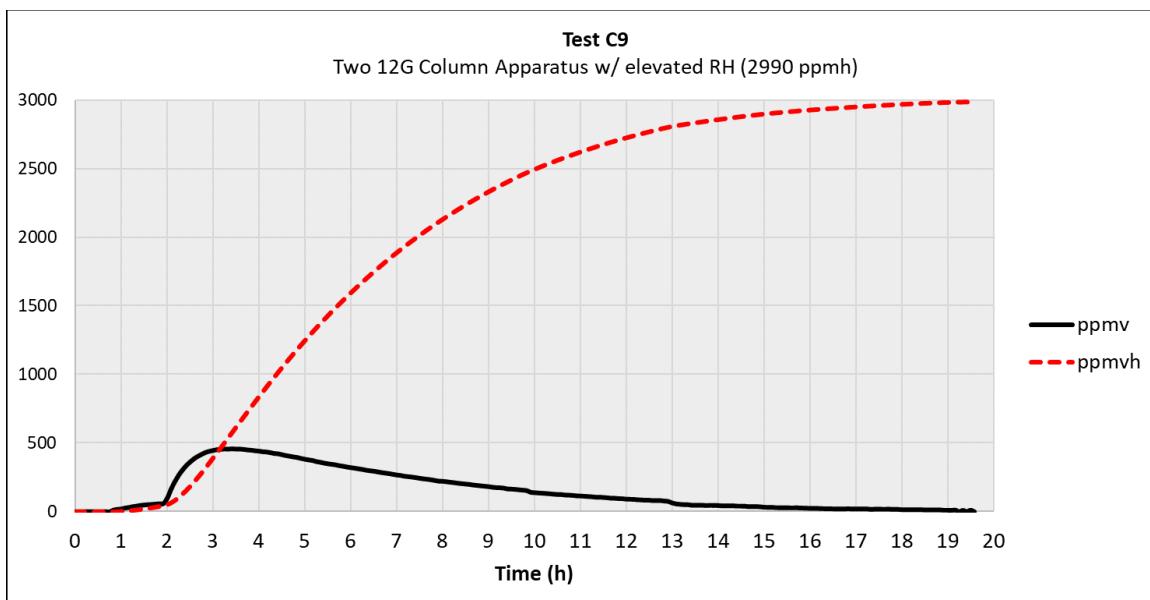


Figure B-13. Test C9 gas profile

Essentially the gas profiles using the column apparatus and Yellow Jacket were comparable and the rate of off-gassing was not enhanced. The efficacy levels between the two treatments (Tests C8 and C9) were comparable as well.

Test C10

This test was performed using only one 12G micro-reactor to assess both the level of gas treatment that would be achieved and the level of efficacy against *Byssochlamys* biofilm. The efficacy results and gas profile are shown in Figure B-14 and Figure B-15, respectively. As expected, the level of efficacy was less, at approximately 4-log reduction, and the treatment level was less, at 1547 ppmvh.

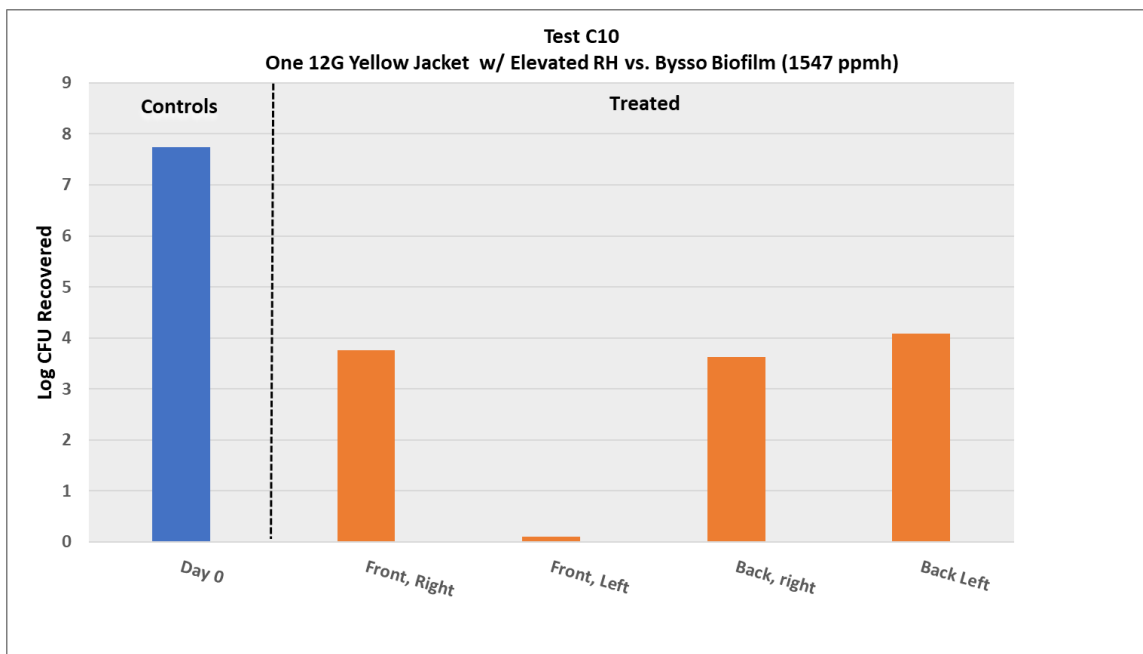


Figure B-14. Test C10 results

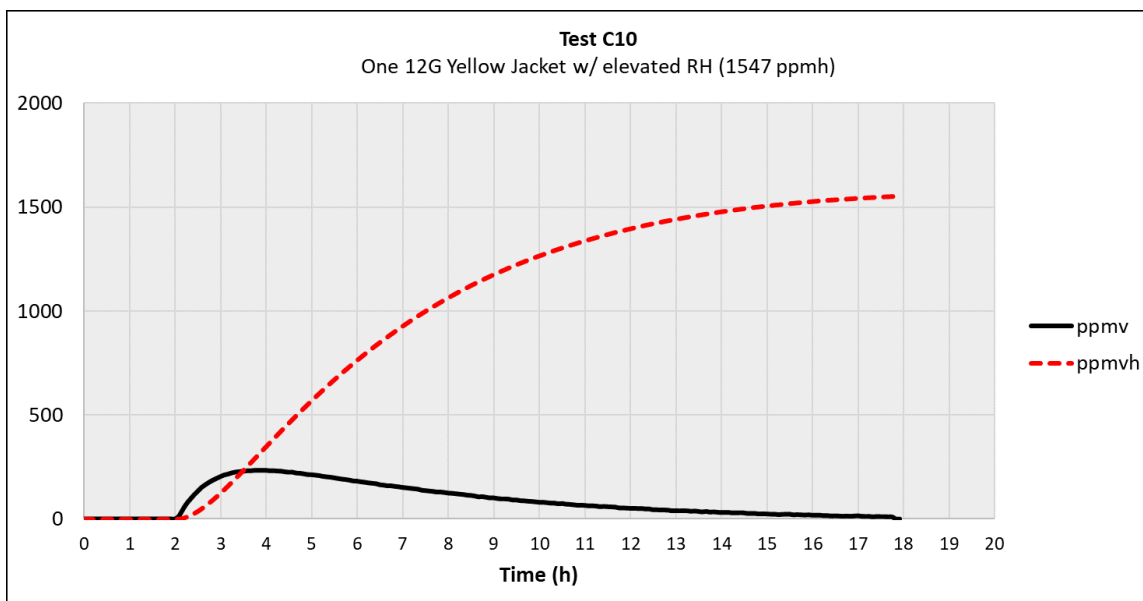


Figure B-15. Gas profile from Test C10

Test C11

Text C11 was performed to assess whether UV light could be used to quickly neutralize (i.e., inactivate) ClO_2 gas. ClO_2 gas is known to degrade rapidly upon exposure to UV light. Therefore, a test was performed to assess whether a UV lamp could be used to rapidly decrease the gas concentration at the end of the fumigation process, allowing the remediated tank to be returned to operation as quickly as possible. Three test runs were performed (denoted C11A, B, and C) in which the UV lamp was turned on at various stages of the treatment process. To assess the effects of UV light, a remote controlled, 15-inch dual UV lamp (BRIGHTINWD; Quartz Lamp; 110V 36W) was positioned on the floor in the middle of the shipping container and alongside the Yellow Jacket gas dispersion unit. During all three trials, the lamp was turned on three hours into the treatment cycle. In trial A, the Yellow Jacket remained on throughout the entire test. In trial B, the UV lamp was turned on and the Yellow Jacket was turned off. In trial C, the Yellow Jacket was turned off while the UV lamp remained off. The gas profile result along with a standard gas profile (from test C7) are shown in Figure B-16. Under these test conditions, using this particular UV lamp, the UV did not accelerate the gas decay rate, as indicated by the similarity in gas profiles from trials B and C (same configuration with and without the UV lamp turned on).

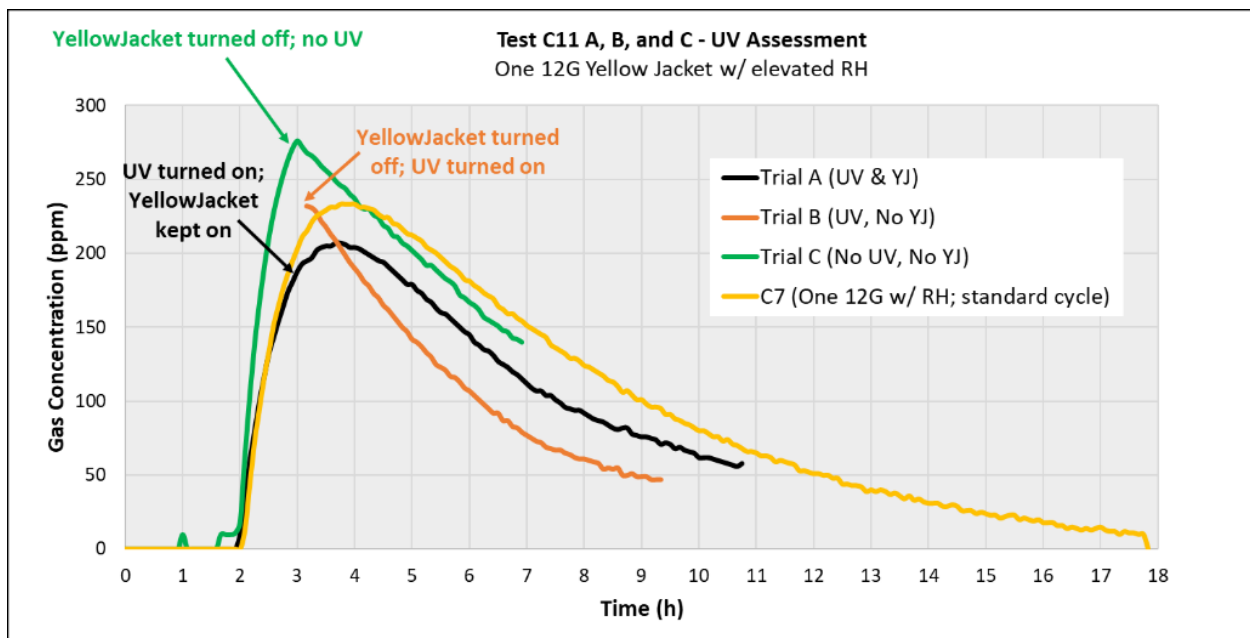


Figure B-16. Effect of UV Exposure on ClO_2 gas decay rate

Test C12

Following Test C11, a unique gas recirculation system was assembled, and its functionality was tested as described in Section 3.7.2.1. This system was designed to generate the ClO_2 gas and humidification outside of the tank within a contained, recirculating system that could be easily connected to the manway or gauge hatches. After the system was shown to operate as expected, a series of efficacy tests (C12 to C14) were performed.

Coupons inoculated with *Byssochlamys* spores and *Byssochlamys* biofilms established in both fuel/water microcosms and in PDB were exposed to gas generated from two 12G micro-reactors under elevated RH conditions. The results (Figure B-17) indicated such a treatment (3173 ppmvh) was very efficacious (>3 log reduction) against all three types of contaminants. The gas profile is shown in Figure B-18.

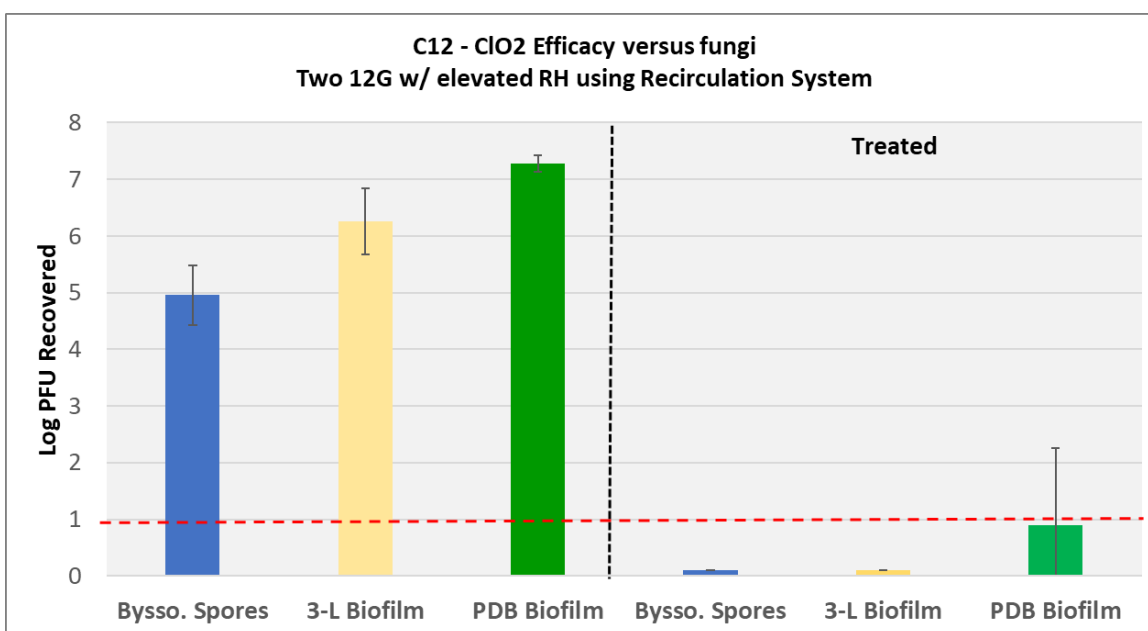


Figure B-17. Test C12 results

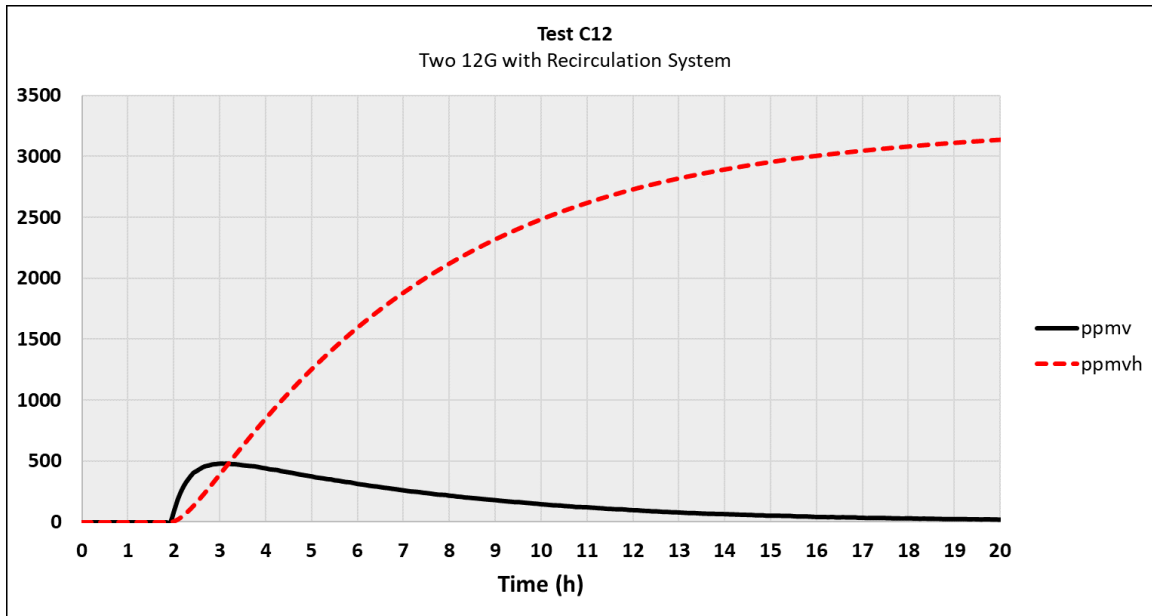


Figure B-18. Gas profile from Test C12

Test C13

This test was performed to show repeatability of the treatment process and was conducted in the same manner as Test C12 with the exception that efficacy testing was performed against *Geobacillus stearothermophilus* and *Bacillus atrophaeus* BIs that were placed throughout the test system. These BIs (sold commercially) are Tyvek coupons inoculated with >6 log spores and sealed within gas permeable membranes. Following treatment, the coupons are transferred aseptically to sterile growth medium and incubated for seven days. A color change in the medium occurs if viable spores grow in solution. Growth, therefore, indicates complete inactivation was not achieved. If no color change is observed, complete inactivation (or >6 log reduction) was achieved. CD Check Strips (ClorDiSys) that change from pink to light blue when exposed to at least 300 ppm gas were also placed throughout the test system. The results of the CD strips and BIs are shown in Figure B-19 and Figure B-20, respectively. The strips indicated the gas concentration did exceed 300 ppm throughout the shipping container, which is consistent with the gas profile shown in Figure B21. The results of the BIs indicated the treatment was very efficacious, as all Gs BIs tested negative for growth and five of six Bg BIs tested negative for growth.

Sample Type	Placement in Conex	Sample ID	Result (Pink or Blue)
Treated	Front Right Floor	1	Blue
	Front Left Floor	2	Blue
	Front Right elevated	3	Blue
	Front Left Elevated	4	Blue
	Mid Front Right Floor	5	Blue
	Mid Front Left Floor	6	Blue
	Mid Front Right elevated	7	Blue
	Mid Front Left Elevated	8	Blue
	Mid Back Right Floor	9	Blue
	Mid Back Left Floor	10	Blue
	Mid Back Right elevated	11	Blue
	Mid Back Left Elevated	12	Blue
	Back Right Floor	13	Blue
	Back Left Floor	14	Blue
Controls	Back Right elevated	15	Blue
	Back Left Elevated	16	Blue
	Outside front door	17	Pink
	Outside rear door	18	Pink
	Warehouse (at least 20 ft. from ISO)	19	Pink
	Laboratory bench-top	20	Pink
	Unexposed	21	Pink



Figure B-19. Test C13 CD Check strip results

Sample Type	Placement in Conex	Sample ID	BI Organism	Results (Pos. or Neg.)
Treated	Front bottom	1	Gs	Neg.
	Middle bottom	2	Gs	Neg.
	Rear Bottom	3	Gs	Neg.
	Front Top	4	Gs	Neg.
	Middle Top	5	Gs	Neg.
	Rear Top	6	Gs	Neg.
	Front bottom	7	Bg	Neg.
	Middle bottom	8	Bg	Neg.
	Rear Bottom	9	Bg	Neg.
	Front Top	10	Bg	Neg.
	Middle Top	11	Bg	Pos.
	Rear Top	12	Bg	Neg.
Neg. BI Controls	n/a	13	Gs	Pos.
	n/a	14	Bg	Pos.

Gs Biological Indicators



Bg Biological Indicators



Figure B-20. Test C13 BI efficacy results

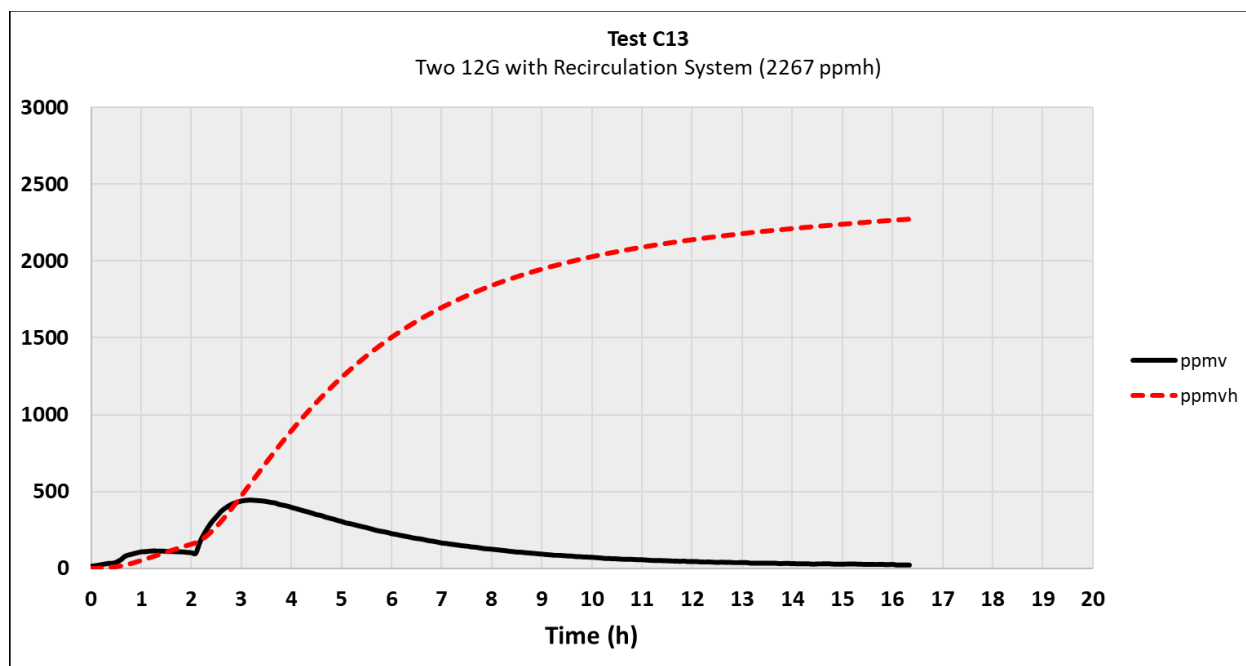


Figure B-21. Gas profile for Test C13

Test C14

Test C14 used the same test conditions as Test C13 to show repeatability of the ClO_2 decontamination system. Twelve Gs and Bg BIs were placed through the full-scale test system to further assess whether uniform gas dispersion was achieved. The treatment measured 2300 ppmvh (Figure B-22) and was comparable to that generated in Test C13. The results of the CD Check strips and BIs are shown in Table B-1. All Gs BIs tested negative, and ten of twelve Bg BIs tested negative. The two Bg BIs that tested positive were located on the floor at one end of shipping container.

This was the last efficacy test performed for this study and further demonstrated that using the recirculation system with two 12 micro-reactors with elevated RH (ranging 65-90%) would achieve the desired level of kill (>3 logs) to successfully remediate an empty biofilm-contaminated fuel storage tank.

At this stage of the study, the goal was to perform a field demonstration using the gas recirculation system on an active fuel tank system. At the time, no such system was available, so a mock-up field demonstration (referred to as the Dry Run) was conducted using the full-scale test system and performed at METSS as described in Section 3.7.2. When an active fuel tank system was available, a field demonstration was conducted as described in Section 3.8.

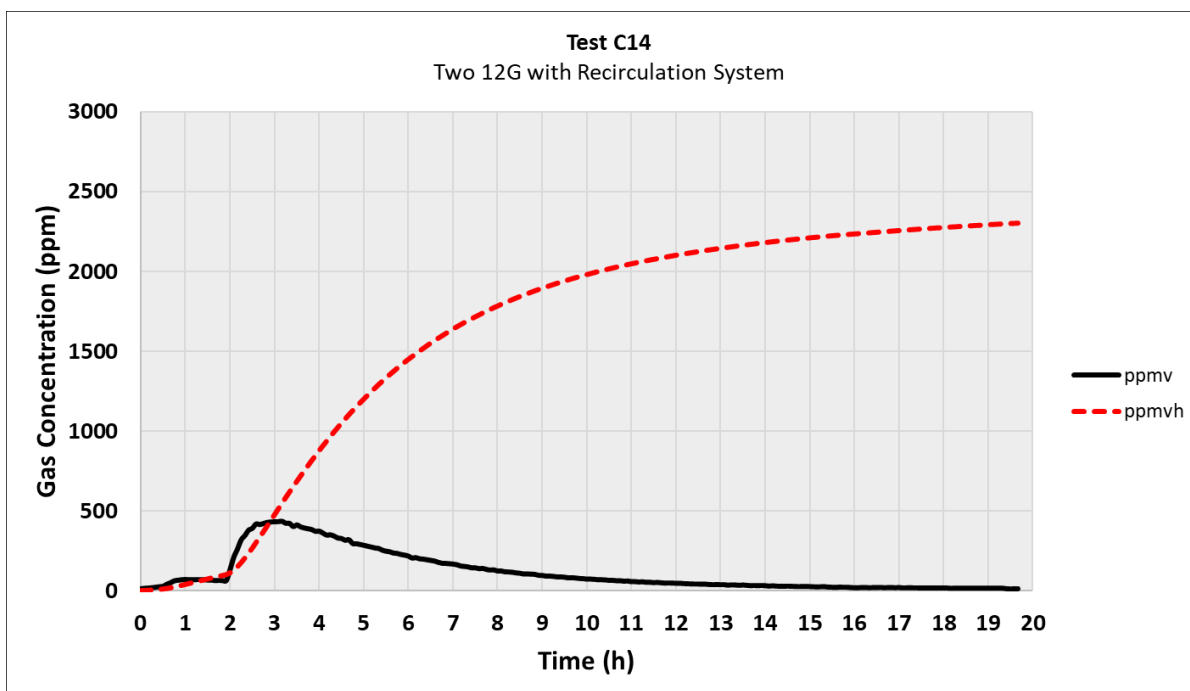


Figure B-22. Gas profile from Test C14

Table B-1. Results for Test C14

Sample Type	Placement in Conex	Sample ID	CD Check*	BI's (Pos. or Neg.)	
				Gs	Ba
Treated	Front Right Bottom Corner	1	Blue	Neg.	Pos.
	Front Right Top Corner	2	Blue	Neg.	Neg.
	Front Left Bottom Corner	3	Blue	Neg.	Pos.
	Front Left Top Corner	4	Blue	Neg.	Neg.
	Mid Right Bottom Corner	5	Blue	Neg.	Neg.
	Mid Right Top Corner	6	Blue	Neg.	Neg.
	Mid Left Bottom Corner	7	Blue	Neg.	Neg.
	Mid Left Top Corner	8	Blue	Neg.	Neg.
	Back Right Bottom Corner	9	Blue	Neg.	Neg.
	Back Right Top Corner	10	Blue	Neg.	Neg.
	Back Left Bottom Corner	11	Blue	Neg.	Neg.
	Back Left Top Corner	12	Blue	Neg.	Neg.
Controls	Neg. Control	13	Pink	Neg.	Neg.
	Pos.. Control	14	n/a	Pos.	Pos.

*Blue= Positive; Pink= Negative

ClO₂ Decontamination Treatment Summary

The series of ClO₂ gas fumigation decontamination tests demonstrated the gas treatment effectively killed fungal biofilm and spores. The treatments were shown to be efficacious against fungal biofilm grown in fuel/water microcosms representing the type of growth in contaminated fuel storage tanks. ClO₂ was also effective against spores of fungal strains prevalent in contaminated tanks or the surrounding environment that may contribute to tank contamination. The test results indicated that a gas treatment of greater than 2000 ppmvh would reduce viability by approximately 6 logs (equivalent to 99.9999%). The tests also demonstrated that the gas could be easily and efficiently generated and dispersed into an enclosed container the size of large fuel storage tank by using a combination of easy-to-use Selectroicide gas-generating micro-reactors used in conjunction with a simple, low-cost gas dispersion. Bench-scale testing led to full-scale, proof-of-principle testing, which in turn, lead to the construction and testing of a gas recirculation system designed to decontaminate fuel tanks up to at least 8,000 gallons (a common size used at Air Force bases).

APPENDIX C – HAD MOCK-UP TRIALS

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HAD Mock-up Trial 1

The test set up used in Trial 1 is shown in Figure C-1 and used a 250K BTU GFE heater (Figure C-2). The objective was to assess the ability of a heater with this capacity to heat an empty container that was comparable in size and shape to a large fuel storage tank.

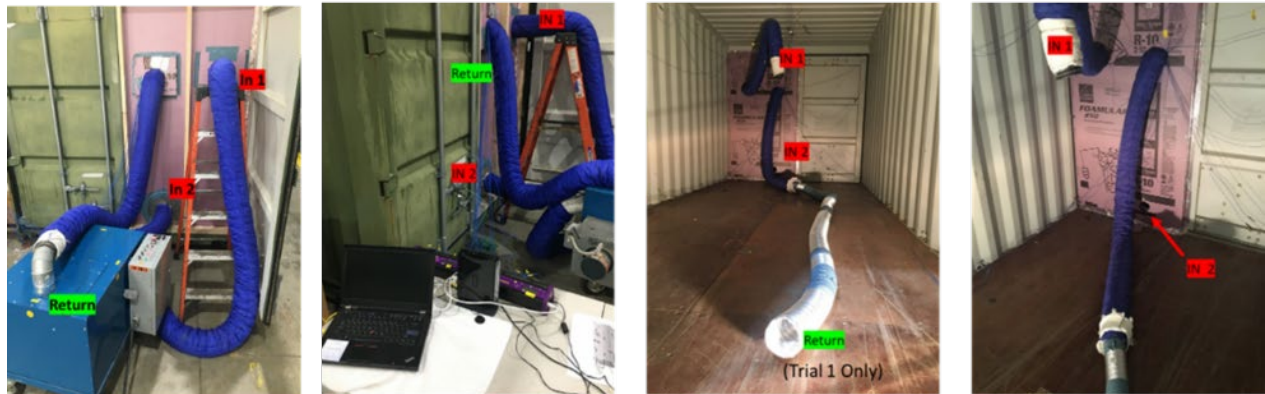


Figure C-1. Test system used for HAD Mock-up Trial 1

Humidification was not included in this trial. The heating system was set up as a closed loop in which hot air was blown into the shipping container and then returned via a second duct back to the heater which was positioned outside the shipping container. All exterior ducting was insulated to reduce heat loss. The heater was set to control the air temperature within the shipping container at 60 °C/140 °F for six hours.

Figure C-3 and Figure C-4 show the results of this trial which are expressed as thermal profiles generated from thermocouples placed on the interior surfaces and in the air, respectively. The surface temperatures never reached 60 °C and averaged approximately 45 °C. The air temperatures also never reached 60 °C and averaged approximately 53 °C.



Figure C-2. 250K BTU GFE heater used for Trials 1-3

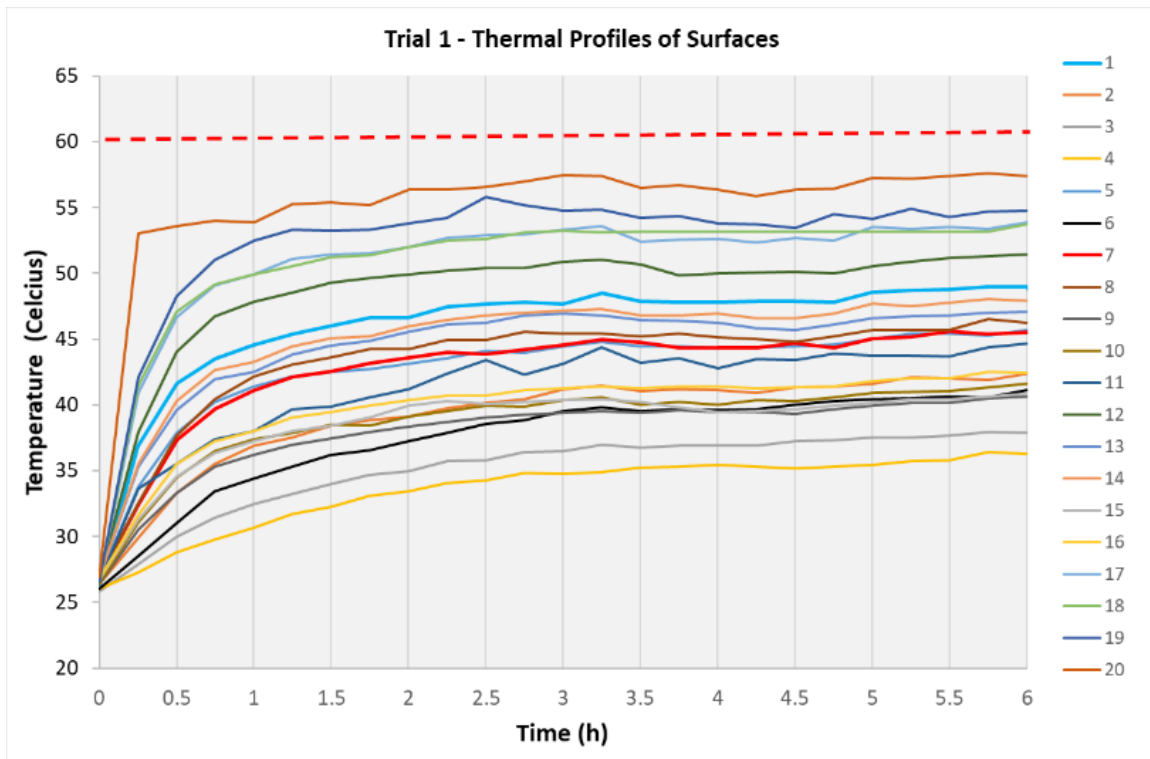


Figure C-3. HAD Mock-up Trial 1 thermal profiles on surfaces

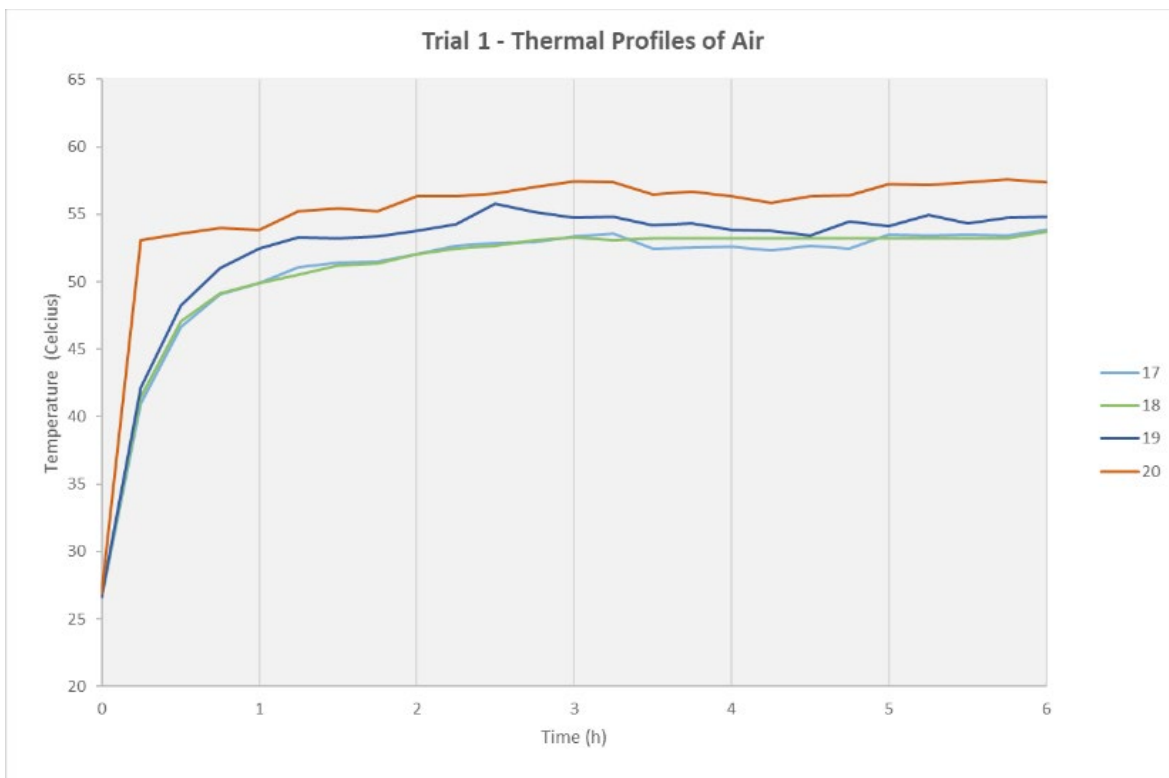


Figure C-4. HAD Mock-up Trial 1 thermal profiles in air

HAD Mock-up Trial 2

In this trial, the 250K BTU heater was used again; however, the air was not recirculated back to the heater. Figure C-5 and Figure C-6 show the results of this trial, which are expressed as thermal profiles generated from thermocouples placed on the interior surfaces and in the air, respectively. The heater was run only for two hours, as it quickly became evident that not returning the air to heater resulted in lower surface temperatures, averaging approximately 45 °C. The air temperatures also never reached 60 °C and averaged approximately 43 °C. Both surface and air temperatures were less than those achieved within two hours during Trial 1.

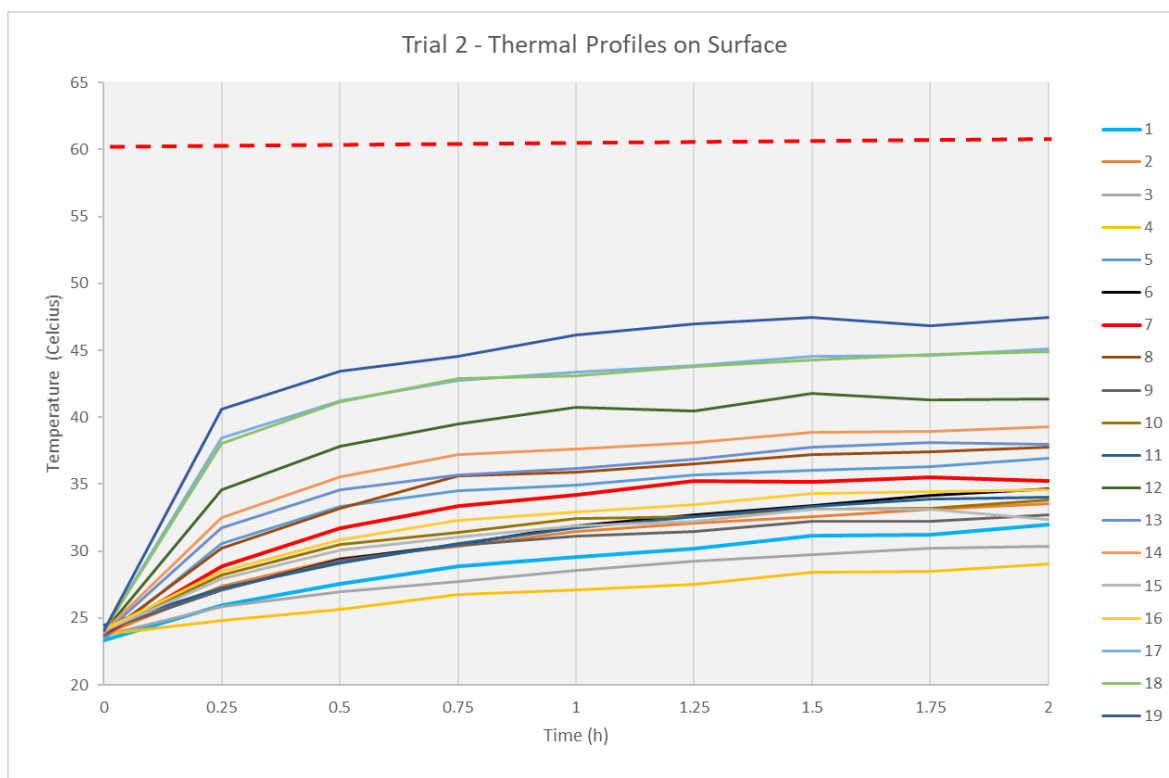


Figure C-5. HAD Mock Trial 2 thermal profiles on surfaces

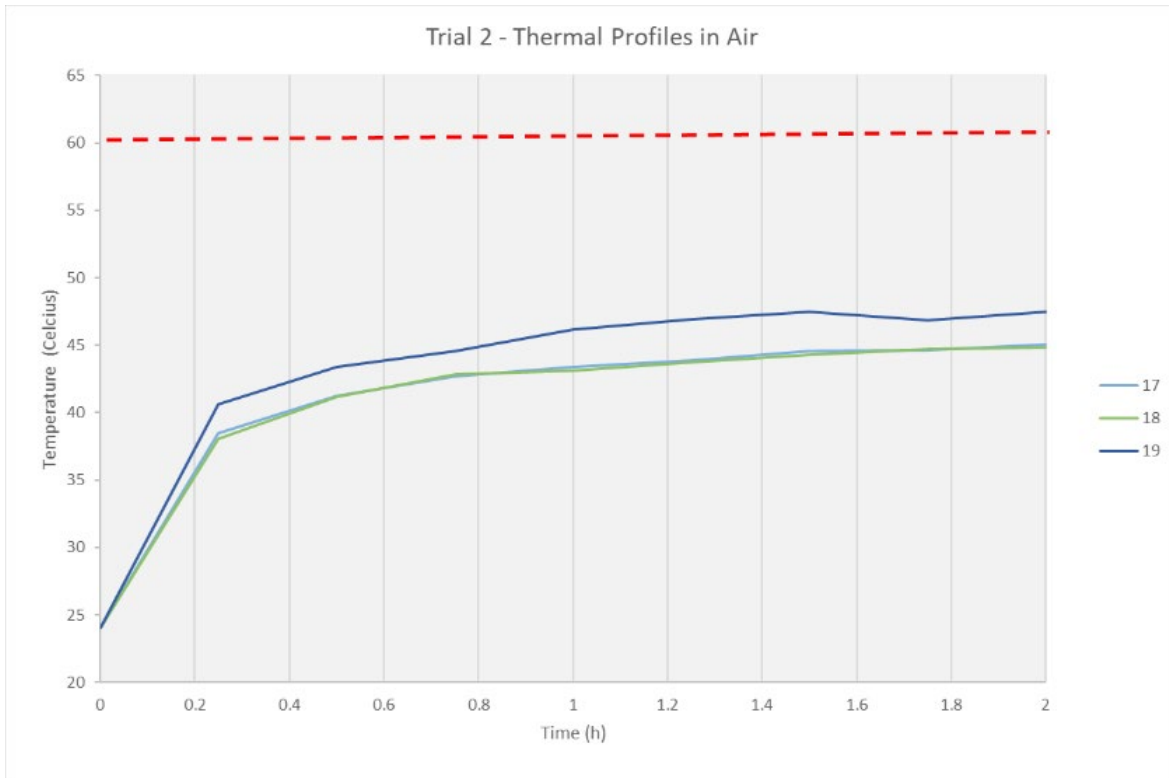


Figure C-6. HAD Mock-up Trial 2 thermal profiles in air

HAD Mock-up Trial 3

In this trial, the 250K BTU heater was used in conjunction with a Caren steam generator (Figure C-7) to assess whether increased humidity could be reached and potentially aid in increasing temperatures more uniformly throughout the container volume. The generator was placed just outside the shipping container and plumbed to a steam diffuser positioned within the shipping container. At 1.5 hours into the trial the generator, set at 20% capacity, was turned on. Then at four hours, the generator was set to run at 100% capacity.

Figure C-8 and Figure C-9 show the thermal profiles on the surfaces and in the air. Once again, the surface temperatures did not reach 60 °C and averaged approximately 45 °C at six hours. The air temperatures averaged approximately 55 °C after six hours. As shown in Figure C-10, the RH reached approximately 30% when the generator was set to 20% capacity, but did increase to 65%, on average, when set to 100% capacity.



Figure C-7. 250K heater (left) and steam generator (right) used for Trial 3

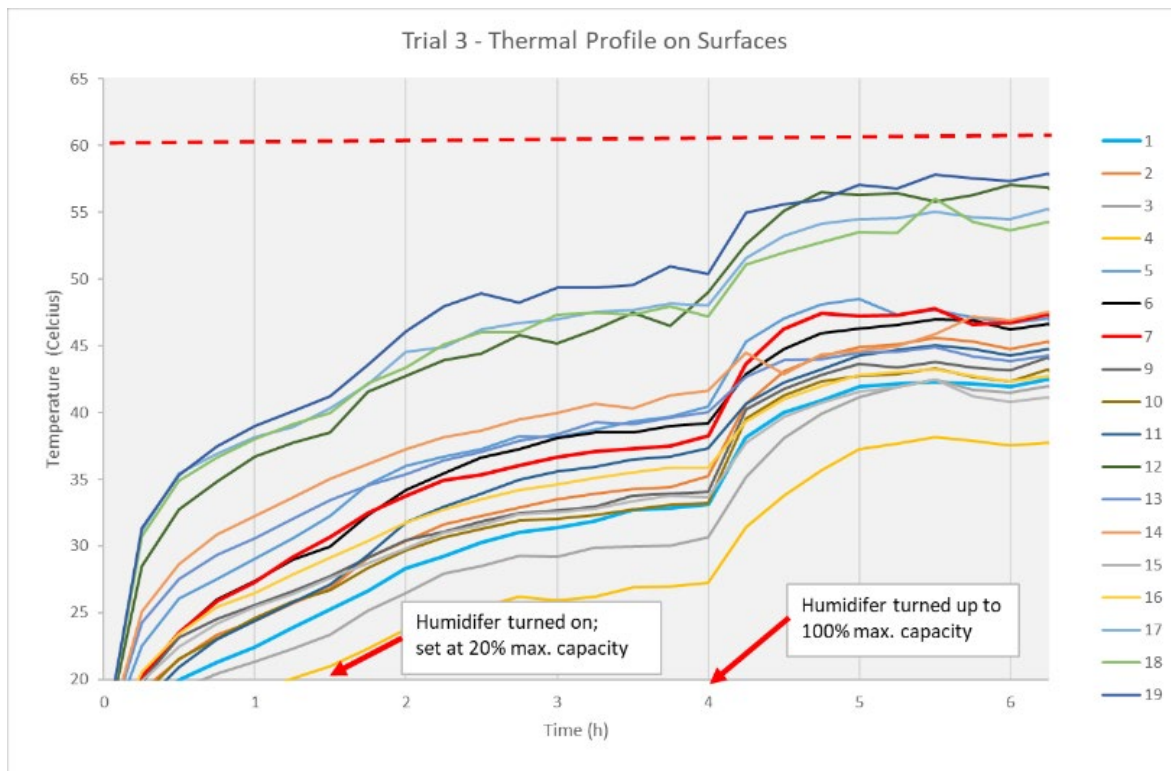


Figure C-8. HAD Mock-up Trial 3 thermal profiles on surfaces

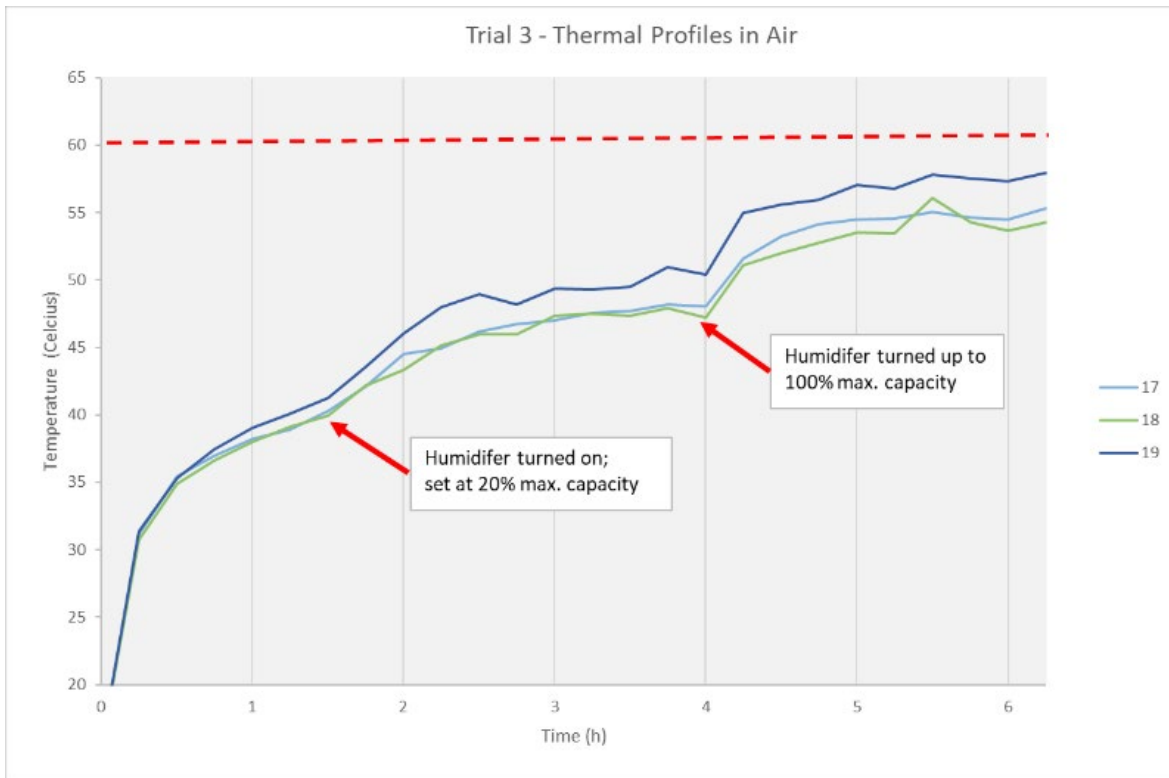


Figure C-9. HAD Mock-up Trial 3 thermal profiles in air

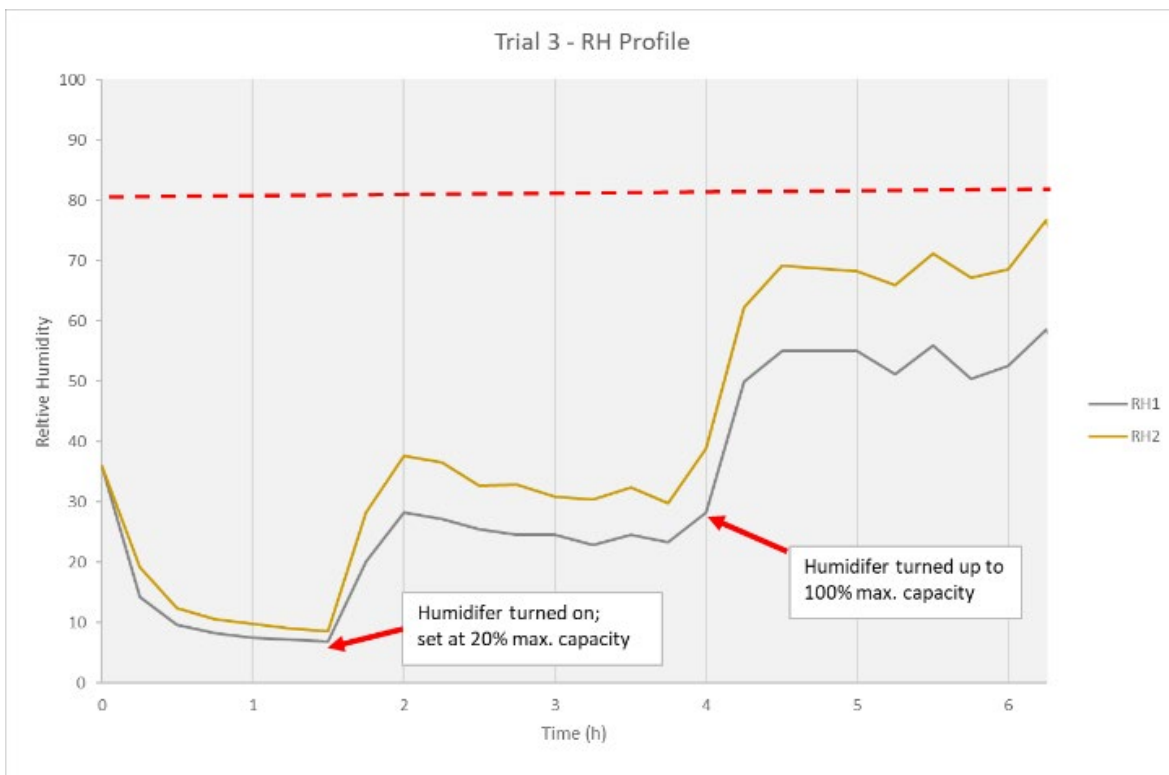


Figure C-10. HAD Mock-up Trial 3 RH profile

HAD Mock-up Trial 4

Since the 250K BTU heater used in Trials 1-3 was incapable of heating the shipping container rapidly (within hours) to 60 °C, a higher capacity (325K BTU; Flagro Model FVO-400) heater (Figure C-11) was rented and used to conduct Trials 4-7. The heater was diesel-powered and produced an air circulation rate of 2500 cfm from two 18-inch outlet ports. The heater was designed for air recirculation. Both heater outputs were connected to the retrofitted door panel of the shipping container. Since the heater generated a very high air output rate which could not be controlled, air was vented from the shipping container through a 6-inch port placed in the door panel to avoid over pressurizing the container. Trial 4 was conducted for 1.5 hours.

Figure C-12 and Figure C-13 show the thermal profiles of the surfaces and in the air, respectively. The surface temperatures appeared to reach a steady state within the first 20 minutes, averaging approximately 40 °C. One location peaked over 60 °C and another came close to 60 °C, but these thermocouples were close to the heat inlets. The air temperatures did increase rapidly within the first 15 minutes and averaged around 60 °C.



Figure C-11. 325K rented heater used for Trials 4-7

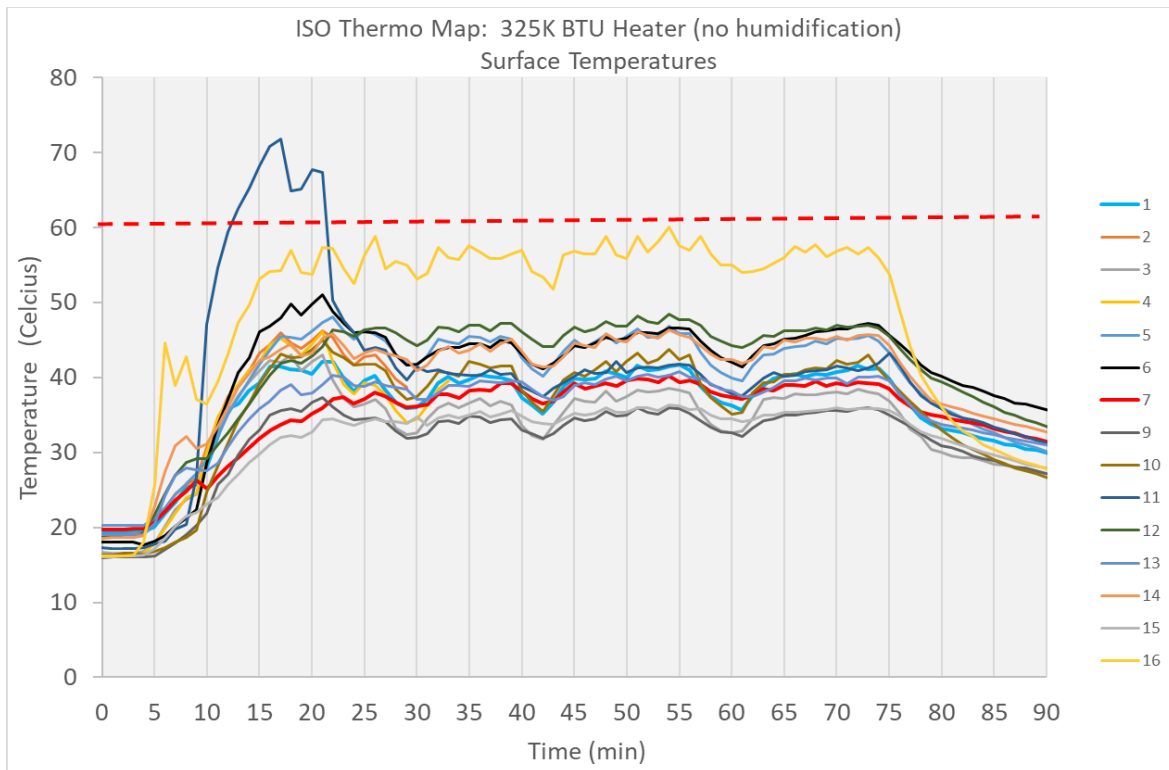


Figure C-12. HAD Mock-up Trial 4 thermal profiles on surfaces

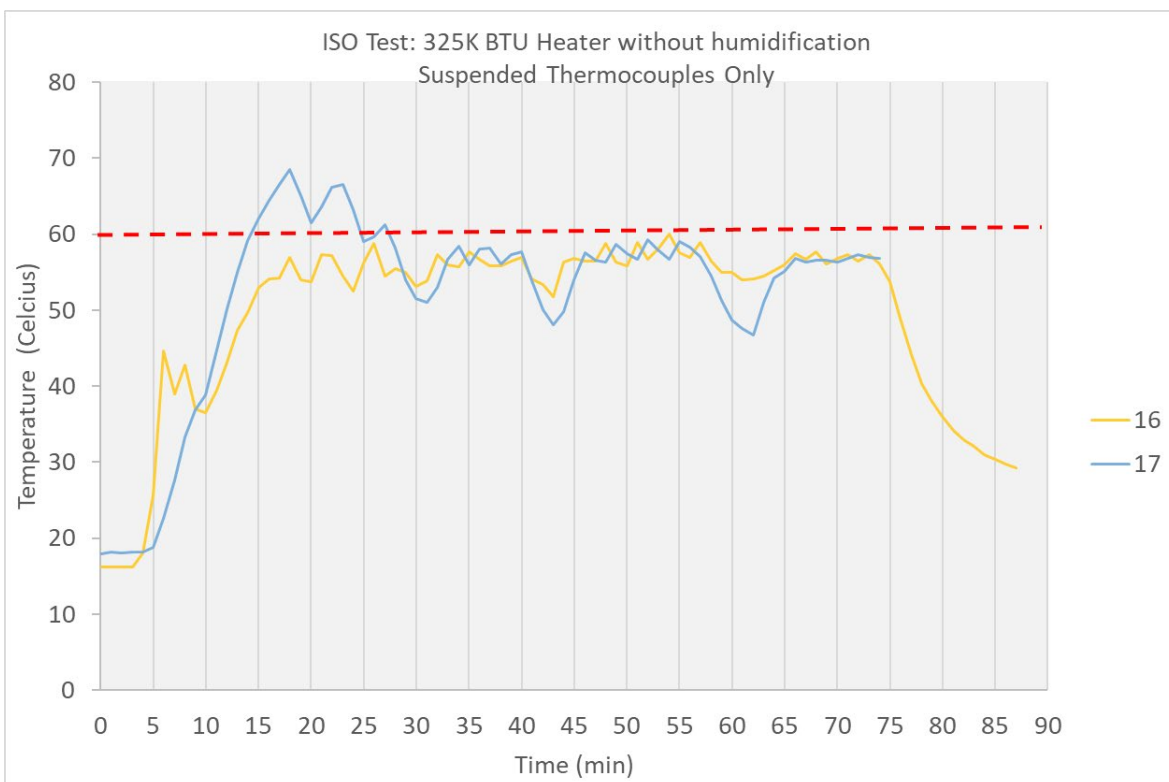


Figure C-13. HAD Mock-up Trial 4 thermal profiles in air

HAD Mock-up Trial 5

In this trial, the 325K BTU heater was run for seven hours; the steam generator was used after three hours to assess the ability of this test set up to elevate RH. Figure C-14 and Figure C-15 show the thermal profiles on the surfaces and in the air, respectively. The surface temperatures appeared to reach a steady state at about 1.5 hours, averaging approximately 50 °C with one location reaching 60 °C (note: this location was close to the heat inlets). The air temperatures increased rapidly within the first 15 minutes to 60 °C or greater. Two thermocouples held steady around 60 °C while the two other couples held fairly steady between 80-90 °C. Due to the high air capacity of the heater, it was observed around six hours that air pressure within the shipping container started dislodging the retrofitted door. To prevent this from worsening, the door at the opposite end of the shipping container was cracked open to allow for more efficient venting. By doing so, the surface and air temperatures actually increased. However, the RH profile (Figure C-16) illustrates the steam generator did not have the capacity to elevate humidity over 20%, as the air flow through the shipping container was simply too high.

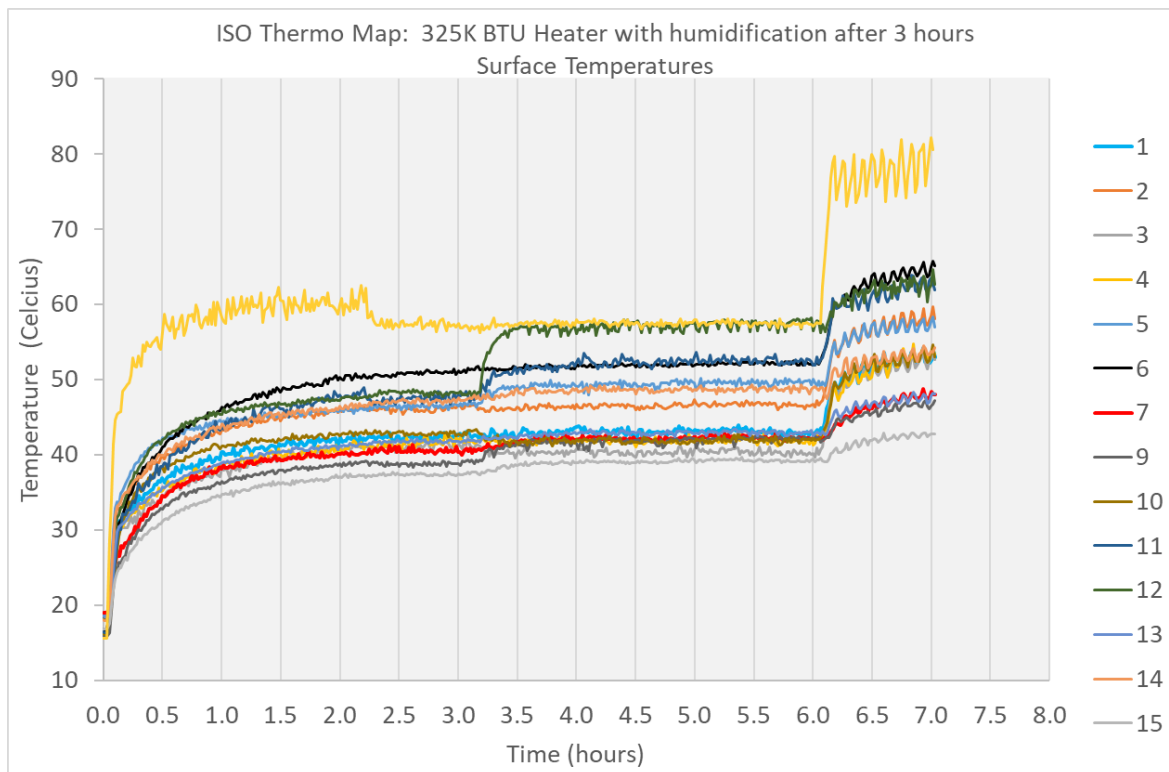


Figure C-14. HAD Mock-up Trial 5 thermal profiles on surfaces

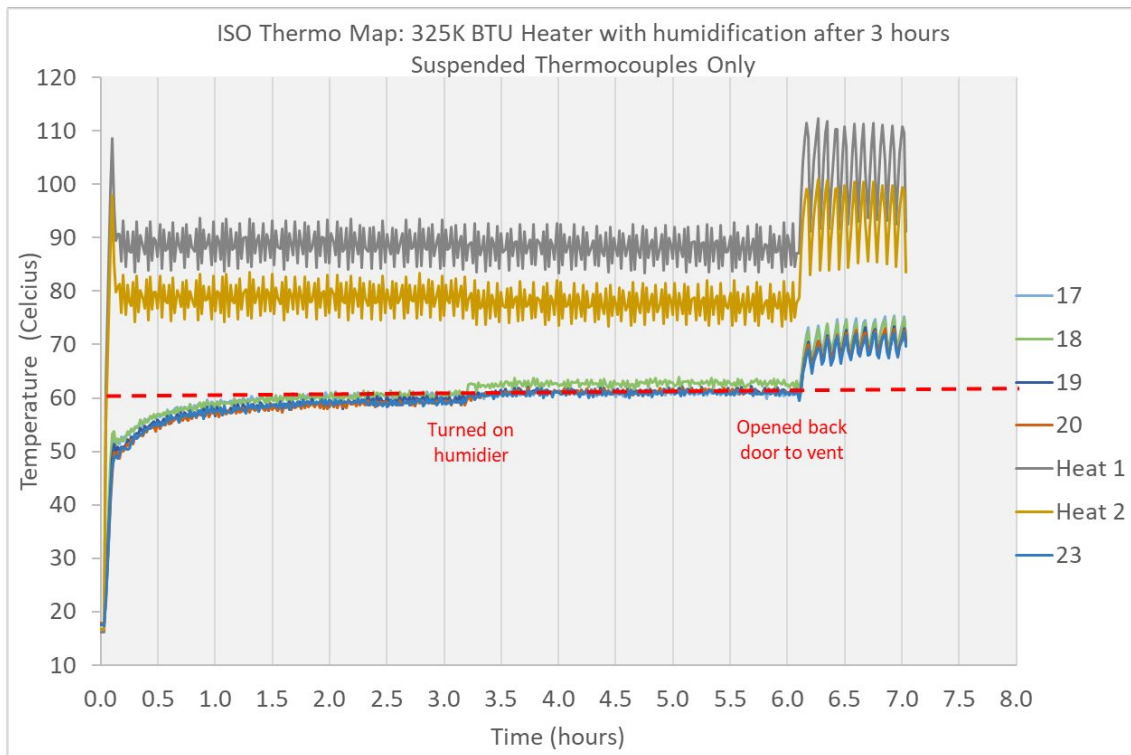


Figure C-15. HAD Mock-up Trial 5 thermal profiles in air

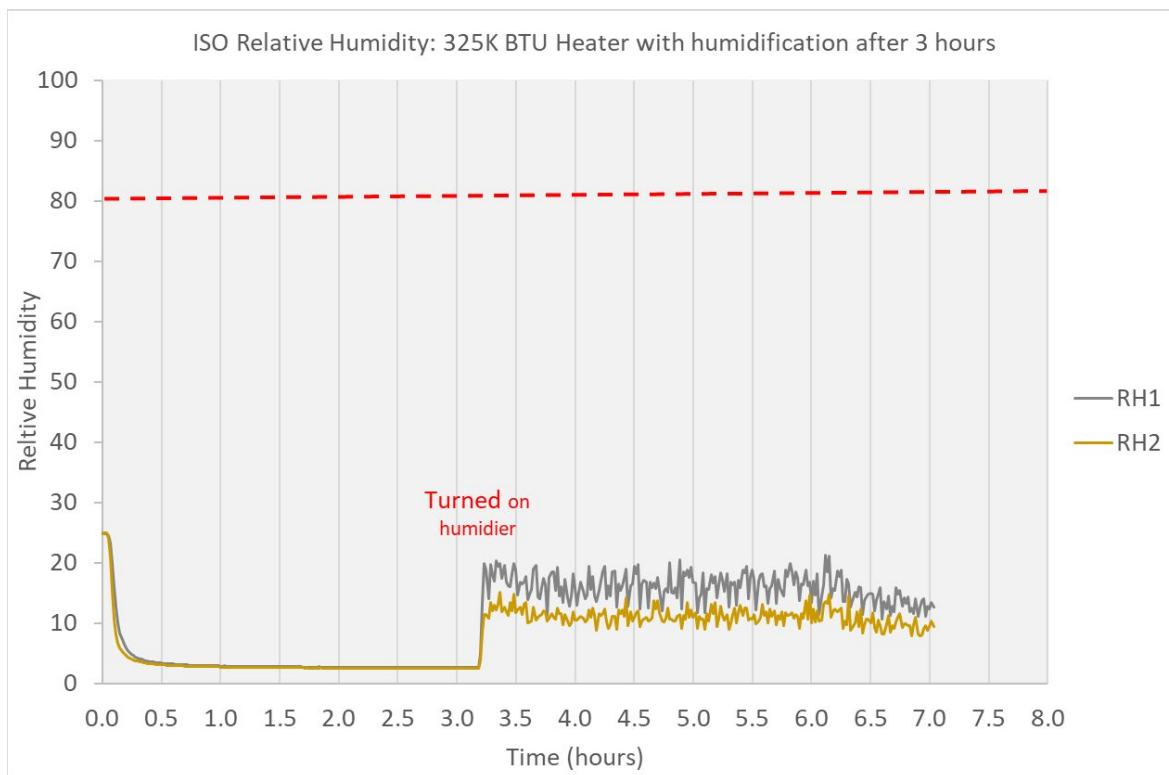


Figure C-16. HAD Mock-up Trial 5 RH profiles

HAD Mock-up Trial 6

Trial 6 was essentially a repeat of Trial 5 with the exceptions that the rear door of the shipping container remained cracked open throughout the entire test and the steam generator was turned on earlier (at one hour versus three hours). Figure C-17 and Figure C-18 show the thermal profiles on the surfaces and in the air, respectively. The surface temperatures did reach higher values than all previous tests with a mean nearly 60 °C, however, approximately half of the locations ranged from 40-55 °C. The air temperatures all exceeded 60 °C, ranging from approximately 65 °C to 110 °C. As shown in Figure C-19, the RH never exceeded 15%.

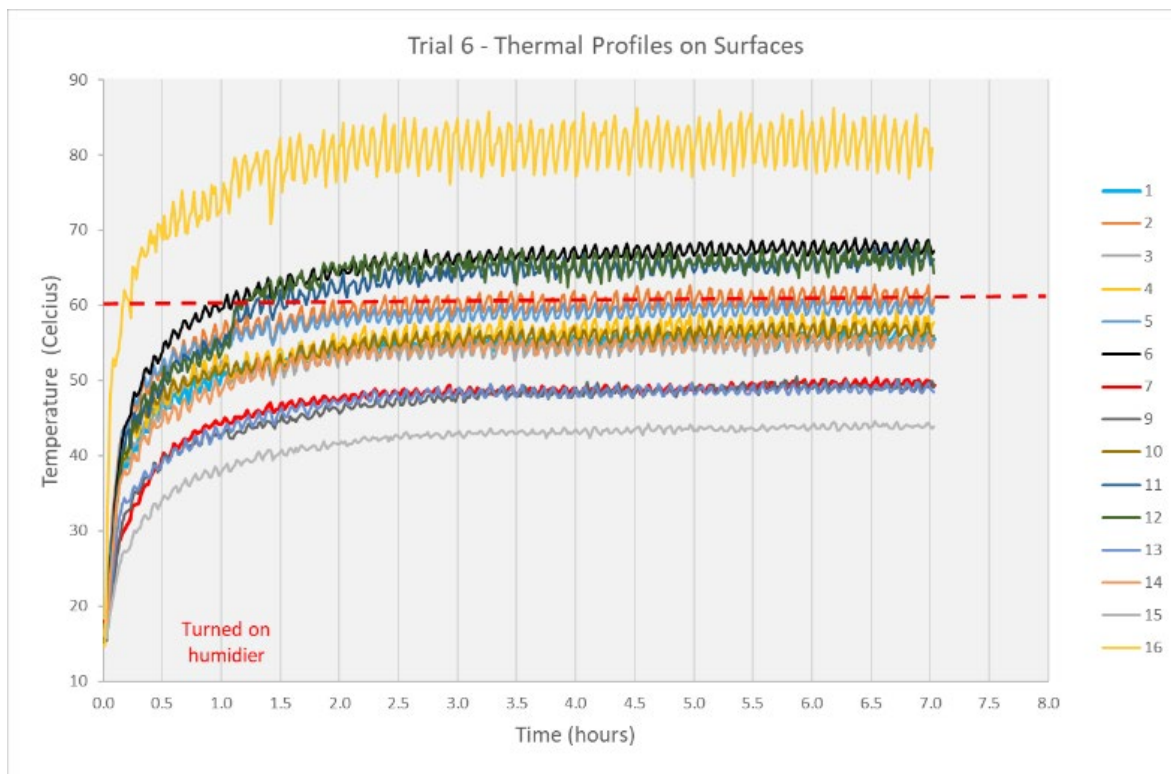


Figure C-17. HAD Mock-up Trial 6 thermal profiles on surfaces

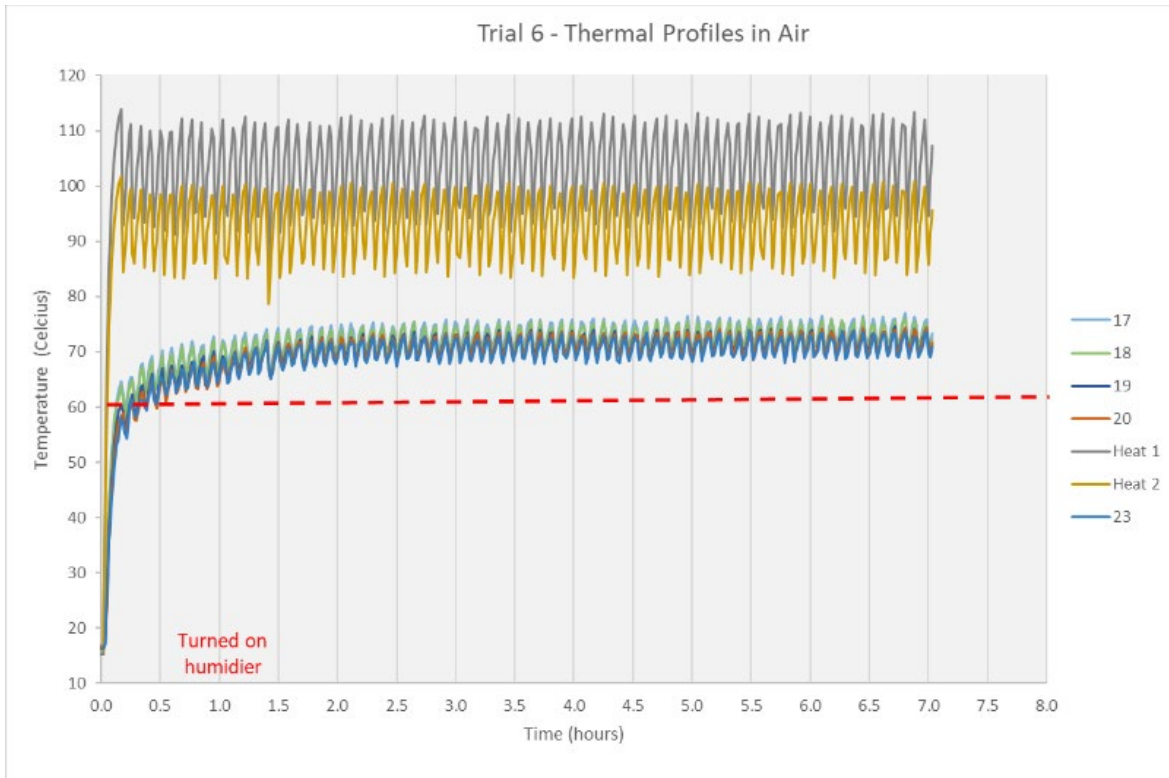


Figure C-18. HAD Mock-up Trial 6 thermal profiles in air

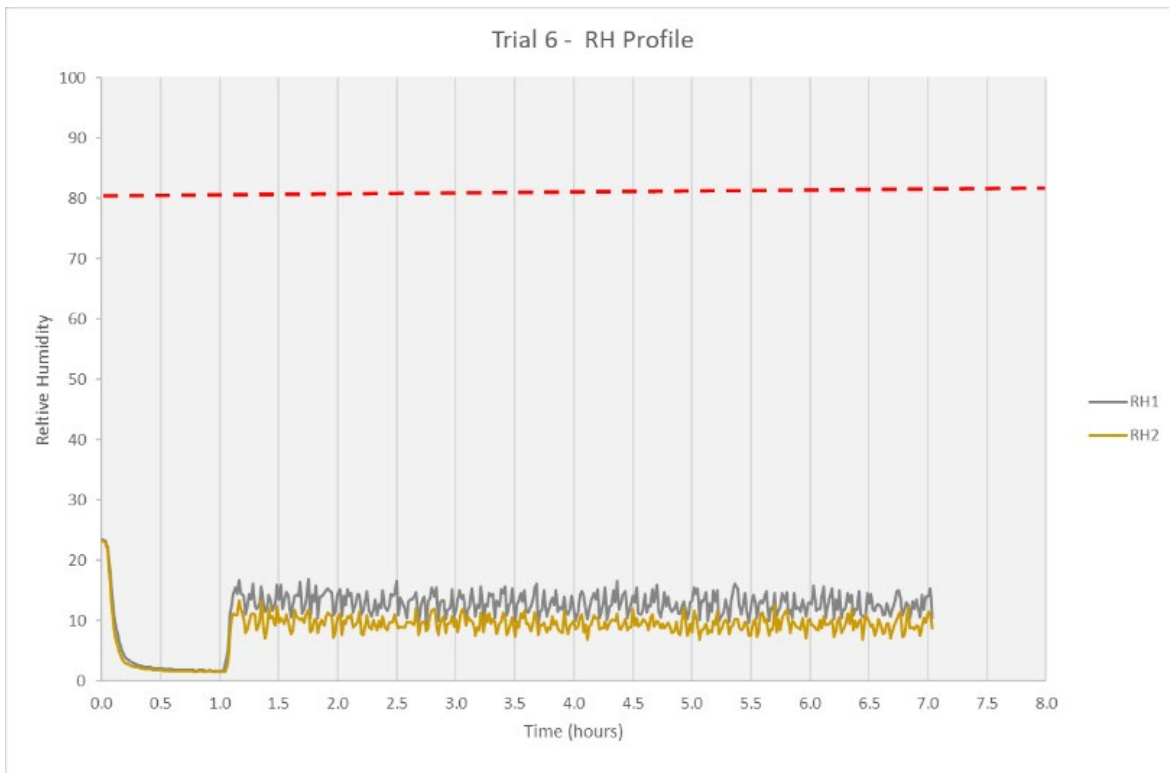


Figure C-19. HAD Mock-up Trial 6 RH profile

HAD Mock-up Trial 7

In this test, a combination of heating and humidification scenarios were assessed in which the heater and steam generator were turned on and off intermittently. In addition, the heater was initially operated using both outputs; after approximately one hour and 20 minutes, only one output was used. The sequence of events is depicted in Figure C-20, Figure C-21, and Figure C-23, showing surface temperature, air temperature, and RH profiles, respectively.

The temperature and RH profiles tracked as expected. Higher temperatures were reached with both heater outputs were used and the RH increased slightly to approximately 30% when only one heater output was used. When the heat was turned off, the RH quickly approached 100%.

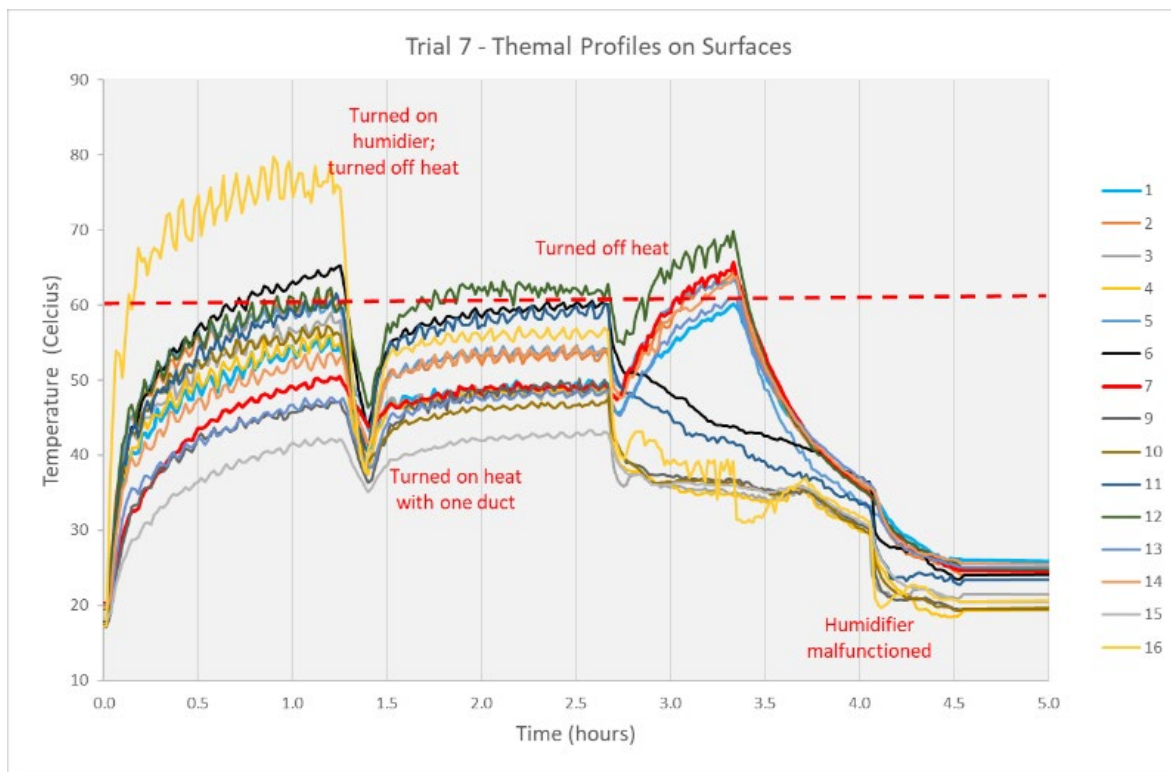


Figure C-20. HAD Mock-up Trial 7 thermal profiles on surfaces

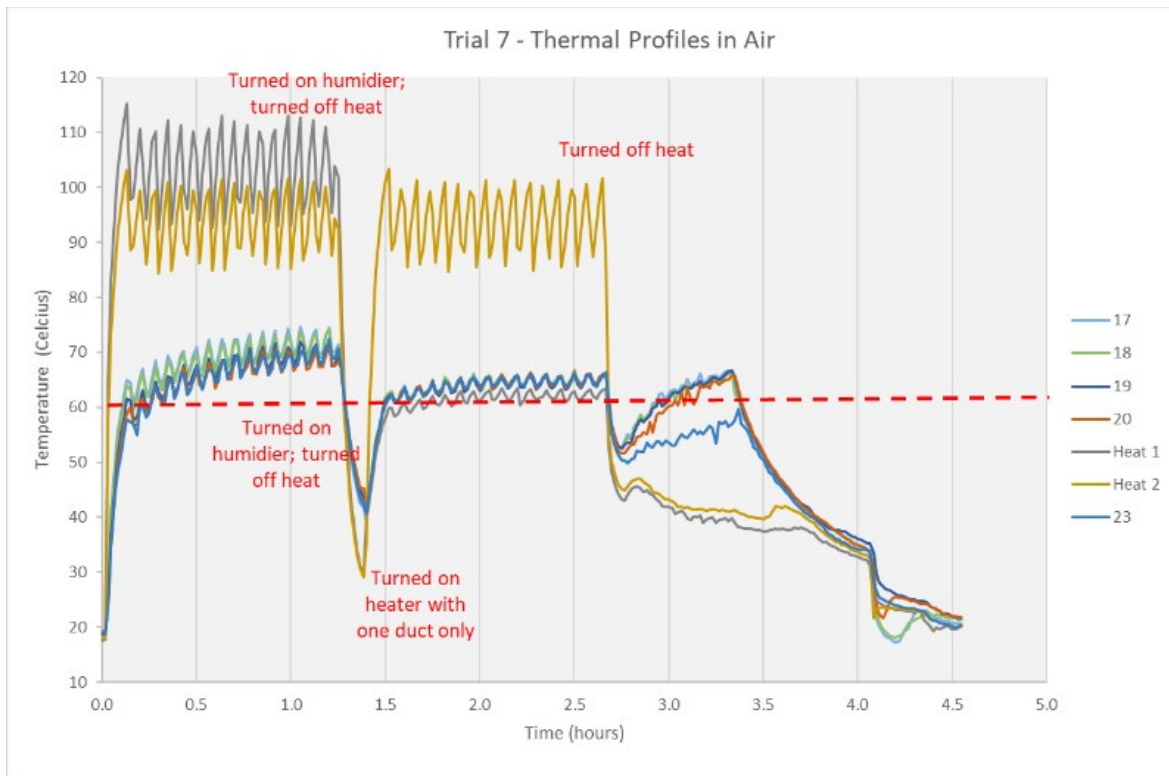


Figure C-21. HAD Mock-up Trial 7 thermal profiles in air

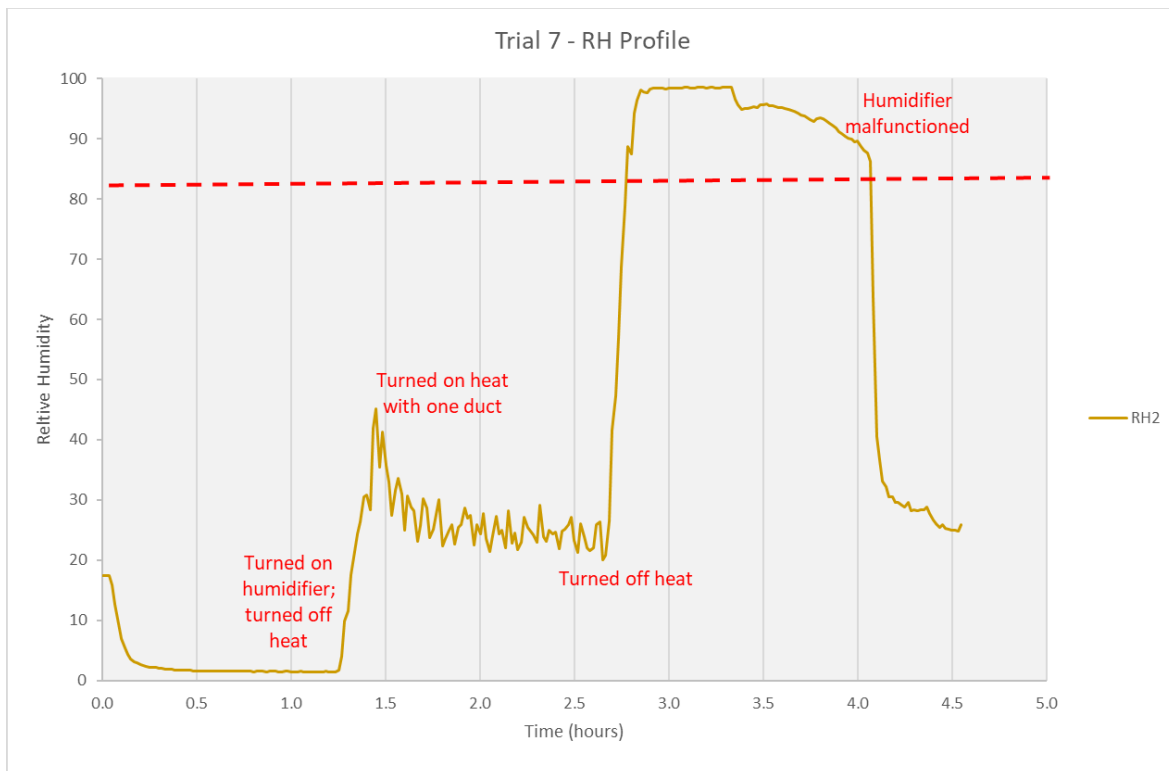


Figure C-22. HAD Mock-up Trial 7 RH profile

HAD Mock-up Trial 8

This final HAD trial assessed the ability of two household room heaters and a household large room-scale humidifier to heat and humidify the shipping container. Both heaters were set to maximum heating capacity and the humidifier was set to control RH at 75% (the max setting of the unit). The test was run for just over seven hours. Figure C-24 and Figure C-25 show the thermal profiles on the surfaces and in the air, respectively. The temperatures slowly and gradually increased to, but not more than, 32 °C. The RH did, however, reach 80% after approximately three hours (Figure C-25).

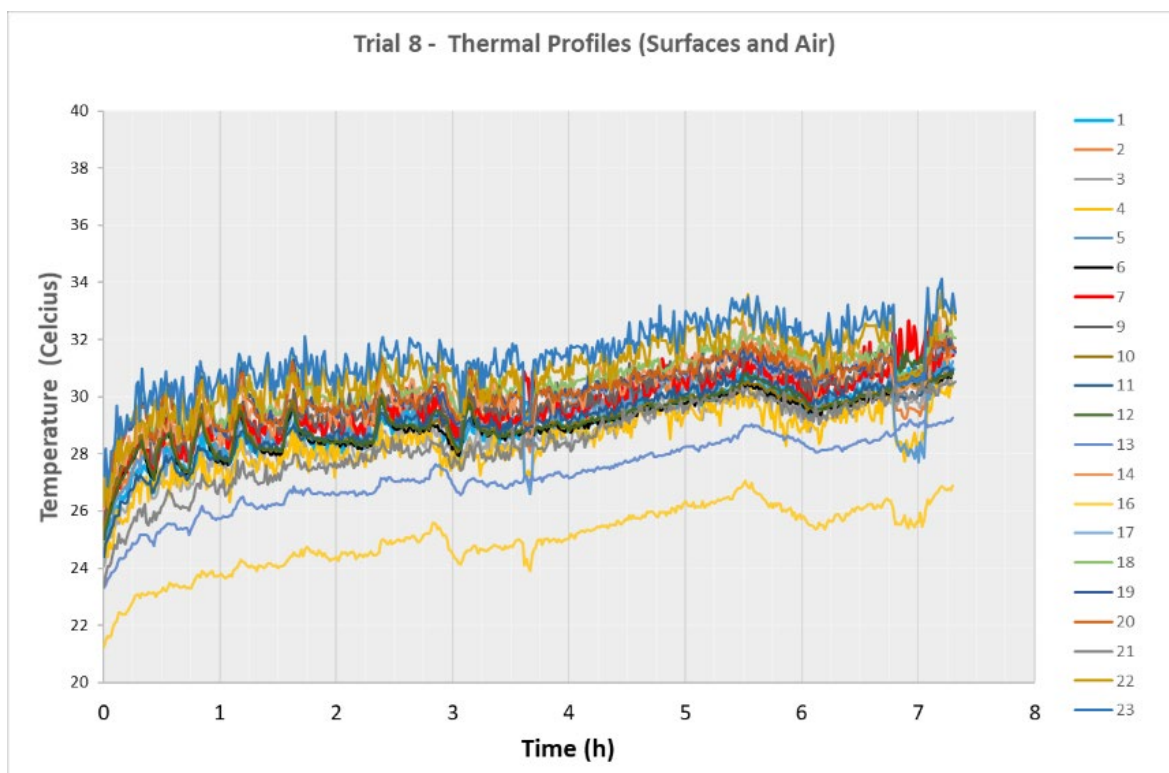


Figure C-23. HAD Mock-up Trial 8 thermal profiles on surfaces and in air

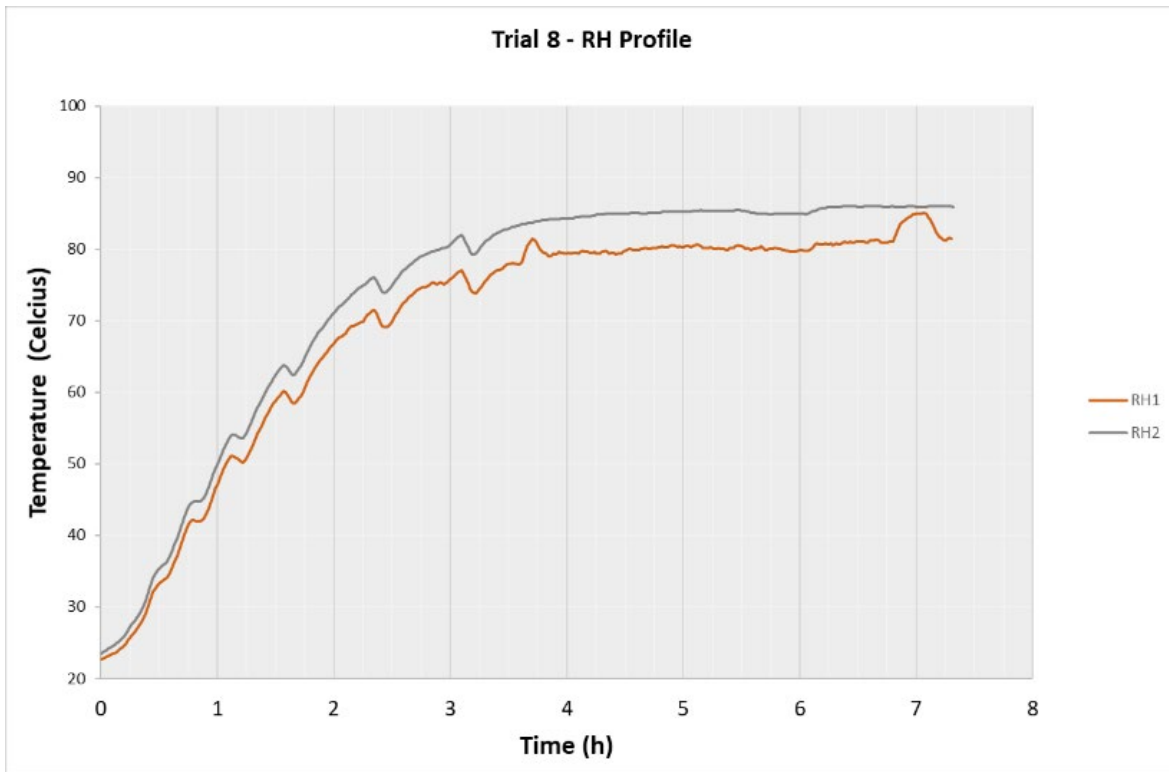


Figure C-24. HAD Mock-up Trial 8 RH profile