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Agricultural Pathogen Decontamination Technology – Reducing the Threat of Infectious Agent Spread

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Abstract

Outbreaks of infectious agricultural diseases, whether natural occurring or introduced intentionally, could have catastrophic impacts on the U.S. economy. Examples of such agricultural pathogens include foot and mouth disease (FMD), avian influenza (AI), citrus canker, wheat and soy rust, etc. Current approaches to mitigate the spread of agricultural pathogens include quarantine, development of vaccines for animal diseases, and development of pathogen resistant crop strains in the case of plant diseases. None of these approaches is rapid, and none address the potential persistence of the pathogen in the environment, which could lead to further spread of the agent and damage after quarantine is lifted.

Pathogen spread in agricultural environments commonly occurs via transfer on agricultural equipment (transportation trailers, tractors, trucks, combines, etc.), having components made from a broad range of materials (galvanized and painted steel, rubber tires, glass and Plexiglas shields, etc), and under conditions of heavy organic lead (mud, soil, feces, litter, etc). A key element of stemming the spread of an outbreak is to ensure complete inactivation of the pathogens in the agricultural environment and on the equipment used in those environments.

Through the combination of enhanced agricultural pathogen decontamination chemistry and a validated inactivation verification methodology, important technologies for incorporation as components of a robust response capability will be enabled. Because of the potentially devastating economic impact that could result from the spread of infectious agricultural diseases, the proposed capability components will promote critical infrastructure protection and greater border and food supply security.

We investigated and developed agricultural pathogen decontamination technologies to reduce the threat of infectious-agent spread, and thus enhance agricultural biosecurity. Specifically, enhanced detergency versions of the patented Sandia decontamination chemistry were developed and tested against a few surrogate pathogens under conditions of relatively heavy organic load. Tests were conducted on surfaces commonly found in agricultural environments. Wide spectrum decontamination efficacy, low corrosivity, and biodegradability issues were addressed in developing an enhanced detergency formulation. A method for rapid assessment of loss of pathogenic activity (inactivation) was also assessed. This enhanced technology will enable rapid assessment of contamination following an intentional event, and will also be extremely useful in routine assessment of agricultural environments.

The primary effort during the second year was progress towards a demonstration of both decontamination and viral inactivation technologies of Foot and Mouth virus (FMDv) using the modified SNL chemistry developed through this project. Lab studies using a surrogate virus (bovine enterovirus) were conducted using DF200, modified DF200 chemistry, and decontaminants currently recommended for use in heavily loaded organic, agricultural environments (VirkonS, 10% bleach, sodium hydroxide and citric acid). Tests using actual FMD virus will be performed at the Department of Homeland Security's Plum Island facilities in the fall of 2005. Success and the insight gained from this project will lead to enhanced response capability, which will benefit agencies such as USDA, DHS, DOD, and the agricultural industry.

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Nomenclature

AOAC	Association of Analytical Chemists
APHIS	Anima and Plant Health Inspection Service
ASTM	American Society for Testing and Materials
BCV	Bovine coronavirus
BEV-2	Bovine enterovirus
CBW	Chemical and Biological agent Weapon
CFR	Code of Federal Regulations
CMC	Critical micelle concentration
CPE	Cytopathic effect
DF200	Decontamination Foam, version 2
DHS	Department of Homeland Security
DOD	Department of Defense
EDTA	Ethylene diaminetetraacetic acid
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme-Linked Immunosorbent Assay
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FMDv	Foot and Mouth Disease virus
HRPO	Horse Radish Peroxidase
HRT	Human Rectal Tumor
IAHC	International Animal Health Code
MDBK	Madin Darby Bovine kidney cells
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SNL	Sandia National Laboratories
$TCID_{50}$	Tissue culture infective dose lethal to 50% of population
TMB	Tetramethylbenzidine, an ELISA reagent
USDA	United States Department of Agriculture
UV-VIS-IR	Ultraviolet-visible-infrared, regions of solar spectrum

1. Reducing the Threat of Infectious Agent Spread

Highly infectious agricultural diseases (e.g., foot and mouth disease virus) are a major concern to the economic well-being of the US whether they are maliciously introduced by a bioterrorist or result from a natural outbreak. These highly contagious diseases can be easily transmitted to uninfected areas. A small, local outbreak in the US or in a neighboring country could quickly spread to the entire country. Previous outbreaks (England 2001) have shown that movement of transportation vehicles can rapidly spread these diseases to uninfected areas. Currently, there are no acceptable methods for decontaminating vehicles exposed to infectious agents.

1.1. Decontamination Component

There are four problems with currently approved decontamination technologies: 1) they do not work against a wide spectrum of organisms, 2) they are corrosive, 3) they are inactivated by soil and other organic material, or 4) they are not environmentally friendly. Improved technologies are urgently needed to reduce the spread of infectious agents via transportation vehicles. We progressed in development of a novel decontamination formulation that will reduce the threat of infectious-agent spread to plants, animals, and humans via transport on exterior vehicle surfaces. The formulation is effective against a wide spectrum of organisms, has relatively low corrosivity properties, and demonstrates moderate improvement in detergency while maintaining decontamination efficacy.

The formulation could be used for both routine and emergency use. Availability of this validated decontamination technology is one element of a consequence management strategy enabling rapid restoration and recovery from either accidental or intentional introduction of agricultural pathogens. Because of the potentially devastating economic impact that could result from the spread of infectious diseases via transportation vehicles, the proposed technology will promote a more secure infrastructure and greater economic security.

This technology may benefit agencies such as USDA, DHS, and DOD and may be equally important to the agricultural industry.

Table 1 lists solutions recommended by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) for disinfecting contaminated equipment during a FMD outbreak.[i] (APHIS oversees the entry of cargo, personnel, equipment, personal property, and mail into the country.) Application is recommended to clean surfaces only. These disinfectants will not work if the surface is soiled because the organic load from soil, plant debris, milk, manure, etc, challenge the disinfectant by inactivating it or by protecting infectious agents from its active ingredients. These disinfectants also have poor surface wetting ability and detergency (detergency is the ability to remove particles from a surface). Additionally, hard water used to prepare the recommended disinfectants may reduce their activity. Using a disinfectant that is not fresh may provide a false sense of security.

Table 1. Decontaminants recommended for use by USDA

DISINFECTANT	NOTES
3% Sodium hypochlorite	Extremely corrosive to many surfaces, no detergency
4-5% Acetic Acid	Corrosive to many surfaces, limited effectiveness, no detergency
2% Sodium Hydroxide	Highly caustic, limited effectiveness, no detergency
4% Sodium Carbonate	Mildly caustic - may dull painted or varnished surfaces, limited effectiveness, no detergency

The United States is a member of the International Organization for Animal Health, which has promulgated the International Animal Health Code (IAHC).[ii] The IAHC recommends that the interior of aircraft destined for the carriage of animals be sprayed with a disinfectant that is suitable for the diseases that could be carried by the animals and does not cause problems with the aircraft. The IAHC suggests the use of 4% sodium carbonate and 0.1% sodium silicate or 0.2% citric acid. These disinfectants are not effective against a wide spectrum of infectious organisms and have no detergency properties. After use of the disinfectant, the aircraft is to be washed with clean water to avoid any damage to the aircraft structures.

Existing recommended commercial products are either ineffective against a wide variety of infectious agents; exhibit some other undesirable trait such as corrosivity or toxicity; or are easily inactivated by organic matter or hard water. Virkon S may be the best commercially available product. Virkon S contains peroxymonosulfate, a highly corrosive oxidizer with an acidic pH around 2.5. The manufacturers of Virkon S provide an in-depth biosecurity program for use in the agricultural community. However, their decontamination procedures call for the essential removal of all gross organic soil from the contaminated surfaces by an initial detergent application wash. Another commercially available product, Chlorhexidine, is a widely used disinfectant that is effective on skin surfaces, but is inactivated by organic debris and hard water. One Stoke Environ, Osyl, and Amphyl have good activity in hard water and in the presence of organic matter, but are not broad-spectrum; they are effective against tuberculosis and Johne's disease, but ineffective against FMD virus.[iii]

The disinfectants recommended by APHIS and IAHC are less effective, less broad-spectrum, more corrosive, or less environmentally friendly than the Sandia decontamination formulation. The DF-200 formulation was developed to decontaminate both chemical and biological agents, primarily in emergencies. As such, it is less than optimal for routine use to prevent the spread of infectious agricultural diseases. For example, the surfactants incorporated into DF-200 were not selected to maximize the detergency of the formulation but to catalyze reactions of the formulation with chemical warfare agents. Since reactions with chemical warfare agents are not a concern for this application, there are opportunities to select alternative surfactants with better detergency properties. Likewise, DF-200 is considered to be biodegradable to a level that is acceptable for an emergency-use product (it is approximately 30% biodegraded within 28 days). However, most consumer-type products that are used in routine applications are more quickly biodegraded (approximately 70-80% biodegraded within 28 days). We evaluated alternative surfactants (and other ingredients) that will enhance the biodegradability of the formulation.

We progressed in development of an effective biosecurity decontamination technology to overcome the inadequacies of the current recommended disinfectants and to improve upon our previous efforts. We utilized the basic chemistry of DF-200 that provides a mechanism for broad-spectrum kill of biological organisms and made moderate improvement upon other properties of the formulation (i.e., detergency, corrosivity, biodegradability, etc.) to develop a novel formulation that is ideal for biosecurity applications.

Of critical importance in decontaminating surfaces in an agricultural environment is the detergency effectiveness of the decontaminant. This is primarily imparted by the selection of appropriate surfactants and builders (builders are ingredients that are added to improve the performance of surfactants). Our work investigated alternative surfactants to the DF-200 technology for CBW agents. The surfactants in the current DF-200 formulation are of the cationic quaternary ammonium type. As noted above, these surfactants were chosen for their ability to catalyze the reaction rates of DF-200 against chemical warfare agents – not for maximum detergency. Above what is known as the critical micelle concentration (CMC), surfactants concentrate at interfaces and form aggregates known as micelles. At these concentrations, micelles can dissolve hydrophobic organic chemicals that are present in soils. Micelles also tend to reduce the interfacial tension between the hydrophobic organic chemicals and water. When the polluted soil is treated with surfactants, the hydrophobic organic chemicals are solubilized in surfactant micelles or partition to the sorbing surfactants. The process largely depends upon competition between micelle formation in the bulk liquid and the surfactant surface aggregates on soil for the uptake of hydrophobic organic chemicals.[iv] The type of soil, nature of exchangeable ions and the ionic strength of the aqueous solution have all been shown to be limiting factors for the sorption of cationic surfactants of the quaternary ammonium family.[v] Thus alternative surfactants may provide increased detergency characteristics and also be maximally biodegradable (quaternary ammonium surfactants are not highly biodegradable).

It is known that sub-CMC surfactant sorption onto soil is proportional to the organic carbon content of the soil, an indication that most of the sorbed surfactant is associated with soil organic matter. Additionally, surfactants vary in their sorption as well as solubilization enhancing properties. We investigated the use of various appropriate surfactants suitable to solubilize soil and contaminants for use in agricultural applications. In the soil/aqueous system, solubilization of contaminants occurs at surfactant concentrations greater than the surfactant CMC in clean water. The elevated surfactant concentration at which solubilization initiates in the presence of soil may be thought of as an effective CMC. The greater the soil/water weight-to-volume ratio, the greater the amount of surfactant required to decrease the surface tension. Surface tension measurements of the aqueous system and in the soil/aqueous system in the absence and presence of surfactant will provide information useful in identifying the CMC and amount of sorbed surfactant. Thus, we determined maximum sorption of surfactant onto soil to be used for the estimation of effective surfactant CMC in a soil/aqueous system. This provided valuable information which may be used for future development of realistic in-situ surfactant enhanced application methods of a biosecurity technology.

We investigated the effectiveness of alternative cationic surfactants, as well as anionic surfactants, anionic/non-ionic surfactant combinations (i.e., mixed micelle systems which can form highly stable micelles), or cationic/non-ionic combinations. Modified biosecurity formulations will ideally contain relatively low concentrations of surfactant, oxidizer, oxidizer activator and carbonate. They are also expected to be efficacious in decontamination against the broad spectrum of infectious agents and to be readily biodegradable. To address the ultimate materials compatibility issue, the formulations were evaluated by standard electrical impedance spectroscopy (EIS) methodology.

The greatest technical challenges of this work were in overpowering the organic load competition for the oxidizer and surfactant properties necessary for effective kill of infectious agents. The solution to these challenges was to develop an effective decontaminant with components demonstrated to improve detergency while maintaining efficacy.

1.2. Virology Component

Foot and Mouth disease virus (FMDV) is a global disease occurring in domestic and cloven-hoofed animals. FMDV is considered the most infectious disease known to exist due to rapid and logarithmic spread (3). Once an outbreak occurs, FMDV is extremely difficult to halt. Culling and slaughter practices were implicated in the FMDV outbreak in the UK in 2001, along with disinfection of affected premises.

Although mortality rates in adult animals are low, FMDV severely impacts production and imparts critical international trade restrictions on animals and livestock products. Due to the non-endemic status of FMDV, the US agriculture sector remains vulnerable to the intentional or unintentional introduction of this disease. Rapid containment and eradication of infected herds and disinfection of contaminated livestock holding facilities is essential in order to minimize industry losses. Case studies have revealed that the estimated economic loss to the UK from the 2001 FMDV outbreak was about $13 billion US dollars (2, 3, 9).

Chemical disinfectants are important in containing and further preventing the spread of infectious disease. Proper sanitation, disinfection, and virus inactivation are important to effectively control viral diseases. Highly effective disinfection procedures have been identified as one way to help aid in the containment of an FMDV outbreak; and such procedures are best conducted in combination with other efforts including herd culling and vaccination for a systems approach to more efficiently halt an epidemic. While there is considerable published information and disinfection efficacy data regarding bacteria and fungi, the efficacy of chemical disinfectants against viruses has not been as well studied. In general, virucidal activity and mechanism of action of various disinfectants is largely understudied. Standards for virucidal testing are being developed, however none are mandated at this time for verifying virus inactivation. It is largely accepted and recommended to follow Association of Official Analytical Chemists (AOAC) germicidal methods for virucidal efficacy testing (1). The major structural targets of disinfectants against viruses include the viral envelope (predominantly lipid), the capsid (predominantly protein), and the genome (nucleic acid material) (6).

In this research, three studies were conducted. In the first study, bovine coronavirus (BCV) was used preliminary as a respiratory model surrogate virus to study virus inactivation. The BCV model was primarily used to study a rapid verification for loss of viral infectivity based on degradation of RNA following disinfectant treatment. In the second study, experiments were conducted to study the recovery of virus samples from environmental matrices including soil and fecal material. The third study was the use of bovine enterovirus 2 (BEV-2) as a surrogate to Foot and Mouth Disease virus (FMDV) to study viral inactivation. BEV-2 virus inoculum was treated with an equal amount of test decontamination formulation for various exposure durations (1, 10, 20 minutes). Additionally, the decontamination formulation was challenged with 0, 10, 25, or 50% bovine fecal challenge (10% bovine feces weight/volume). Samples were washed via ultracentrifugation (100K X G, 1 hour, 4^{o}C) to remove disinfectant and pellets were reconstituted in appropriate infectivity media. Samples were then evaluated using tissue culture infective dose 50 ($TCID_{50}$) or with reverse transcriptase polymerase chain reaction (RT-PCR) to evaluate post-treatment infectivity and intact RNA, respectively. Three replications were conducted for each sample treatment.

2. Year 1 Results and Accomplishments

2.1. Decontamination Component

2.1.1. Task 1- Formulation Modification

We progressed in development of a biosecurity decontamination technology with demonstrated increased detergency, capable of infectious agent decontamination in the presence of heavy organic loads. The increase in detergency was accomplished by use of alternative surfactants and detergency builders. Initial tests indicated a treatment-dependent increase in surface detergency, as measured by changes in surface reflectance of selected surfaces (polypropylene, anodized steel and butyl rubber) following soil application and exposure to various formulations. Decontamination efficacy data are based on treatment exposure times of 15 and 60 minutes

A variety of surfactant chemistries were assessed in standard spore kill efficacy tests. The effectiveness of alternative cationic surfactants, as well as anionic surfactants, anionic/non-ionic surfactant combinations (i.e., mixed micelle systems which can form highly stable micelles), or cationic/non-ionic combinations was explored. Progress was made in development of a preliminary biosecurity decontaminant by the selection of a surfactant that demonstrated increased activity against biological pathogen simulants and characteristic soil microorganisms. The surfactant, Barquat 4280Z, is a cationic surfactant mix of ethyl and benzyl quaternary alkyl ammonium chlorides. The surfactant demonstrates an established synergistic effectiveness against a broad spectrum of biological pathogens including algae, fungi, viruses and bacteria. This characteristic makes this surfactant applicable for use in an agricultural environment, where broad-spectrum microorganisms are often prevalent. The use of this specific cationic surfactant, low levels (<4%) of oxidizer and oxidizer activator, carbonate, solvents and added detergent builders combine to provide increased detergency. Additionally, the Food and Drug Administration permits this surfactant for use in no-rinse food contact sanitizers at no more than 200 ppm, per 21 CFR part 178.1010. **This fact may be leveraged in further development of Sandia decontamination technologies as applicable to the food processing industry.**

Tests in our laboratory indicated a formulation with active Barquat 4280Z surfactant concentration of 0.05% achieved 7-8 log reductions of *B. atrophaeus* within 15 minutes. In contrast, at recommended use concentrations of 1%, Virkon S (a commercial product for decontaminant use in agricultural environments) was ineffective at killing *B. atrophaeus* spores present in a sterile deionized water matrix, with an observed kill of less than 1 LOG reduction. It is important to note that the active ingredient of Virkon S is peroxymonosulfate, a strong oxidizing agent. This product does not contain the additional components present in the Sandia decon formulation, notably key surfactant and solvents required to provide infectious agent solubility. In particular, the surfactants form micelles that dissolve hydrophobic organic materials that are present in soils. The relatively poor performance of Virkon S is indicative of this observation. This observation may be used in future efforts to understand inactivation mechanisms and methodology.

Additionally, standard spore kill tests were also completed in the presence of a 10% organic challenge. Select formulations, DF200 and modified “Ag”, were also tested in the presence of 25% and 50% organic challenge. The source of the organic challenge was soil obtained from the Kansas Prairie research area in Northeast Kansas. Note: A general microbiology assessment was conducted on the soil samples: APC (total aerobic plate count),

ECC (*E. coli* and coliform count), Entero (*Enterobacteriaceae*), and Y&M (yeast and mold count). All organic challenges (10%, 25% and 50%) were inoculated with 10^7 *B. atrophaeus* spores/ml. The combination of the Konza soil and spore inoculum provided a rigorous organic challenge to the decontamination technology, as would be expected in a real-world agricultural environment. Table 2 below summarizes a small portion of the efficacy testing. Results represent triplicate analyses. Neutralizer tests were performed on all modified formulations to confirm inactivation of alternate surfactant and peroxide activity. Note also that all modified formulations included the use of the Barquat 4280Z surfactant in place of Variquat 80MC.

Table 2. Summary of Spore Kill Efficacy following exposure to various decontaminant formulations

Formulation Description	Test Matrix	Log CFU/ml Total Aerobic Count, 15-min.	Log CFU/ml Total Aerobic Count, 60-min.	Log CFU/ml Control Total Aerobic Count	Comments
Virkon S, 1%	Sterile DI H2O	7.90	7.43	8.00	No surfactant
DF-200	10% organic load	5.34	4.69	7.97	
DF-200D	Sterile DI H2O	7.91	0	8.03	
DF-200D	10% organic load	3.96	4.09	7.94	
Formula A	Sterile DI H2O	0	0	7.99	Very low level surfactant
Formula A	10% organic load	7.41	7.35	8.02	Very low level surfactant
Modified "Ag"	10% organic load	3.70	3.66	7.99	Increased surfactant
Modified "Ag/EDTA"	10% organic load	4.10	3.68	7.78	
Modified "Ag/Q2"	10% organic load	4.51	3.68	8.05	
DF200/Barquat	10% organic load	4.29	0	7.91	Barquat used as cationic surfactant in place of Variquat
5830/Barquat	10% organic load	3.55	3.63	7.37	Added wetting agent
5840/Barquat	10% organic load	3.66	3.90	7.25	Added wetting agent
DDTAB	10% organic load	3.88	3.56	7.86	Alternative Gemini surfactant
DTAB	10% organic load	3.07	3.51	7.87	Alternate cationic surfactant
DF200	50% organic load	7.09	6.81	7.88	
DF200	50% organic load	7.23	7.21	7.78	
Modified "Ag"	50% organic load	7.43	7.41	7.95	
Modified "Ag"	50% organic load	6.48	6.05	7.84	

DF200	**25% organic load**	**7.32**	**7.42**	**7.83**	
DF200	**25% organic load**	**4.98**	**4.82**	**7.53**	
Modified "Ag"	**25% organic load**	**7.09**	**6.88**	**7.85**	
Modified "Ag"	**25% organic load**	**6.64**	**6.29**	**7.85**	

Among tests completed at the 10% organic challenge level, the DF200/Barquat formulation demonstrated the best kill efficacy. Many of the modified formulations performed slightly better than DF200. Insignificant decontamination efficacy was demonstrated at the 50% organic challenge level; minimal efficacy was demonstrated in the 25% organic challenge.

In the soil/aqueous system, contaminants are made soluble at surfactant concentrations greater than the surfactant CMC in clean water. The elevated surfactant concentration at which solubilization initiates in the presence of soil may be thought of as an effective CMC. The greater the soil/water weight-to-volume ratio, the greater the amount of surfactant required to decrease the surface tension. This is demonstrated in Table 2, as greater concentrations of surfactant were required for more effective spore kill efficacy in highly soiled environments.

Additionally, the use of wetting agents could be desirable for an effective biosecurity formulation. Wetting agents promote substrate wetting by effectively lowering both equilibrium (static) and dynamic surface tension in water systems. A wetting agent will rapidly migrate to the formulation interface providing coverage over low-energy hydrophobic or contaminated surfaces. As such, a wetting agent will aid in the rapid dispersal of biosecurity decontamination formulations over difficult to wet surfaces and, together with formulation stability, will provide a means of prolonging the contact of formulation with contaminated surfaces. Although wetting agents are typically designed for use in high-performance applications, it is reasonable to predict that their use would be advantageous in the biosecurity formulation for decontamination and reduction of spread of infectious agents.

Use of chelating agents may be beneficial in two different ways: The use of chelating agents to bind metals from soils, thus assisting in the removal of soils from surfaces. Additionally, the chelating agents may bind metals (calcium and/or magnesium) from bacteria. In some cases, lipopolysaccharide may also be removed by the use of chelating agents.[vii]

2.1.2. Task 2 – Assessment of Surface Detergency

Detergency was assessed based on changes in test substrate surface reflectance following soil application and subsequent contact in various decontaminants. The measurement of surface reflectance prior to and following decontaminant contact is specified per ASTM D4488-95, a standard method commonly used in the detergency industry. Thus, an efficient and standardized method was used to assess detergency of modified decontamination formulations.

The formulations most efficacious in successful spore kill were consequently subjected to an assessment of detergency on soiled substrate surfaces. All experiments were performed in triplicate. The following substrates were tested: butyl rubber (Compound No, 60-T-3, Specification BMS 1-33, Grade 60, 1/16" thickness), polypropylene (White polypropylene, manufactured by Poly Hi Solidur Worldwide, 1/8" thickness), and anodized steel (USS Posco, ASTM A653-02 CS Type A, 22 gauge galvanized tin). Surface areas were chosen to accommodate the port size of reflectance instruments; the use of reflectance methodology dictated the use of smooth test surfaces.

Reflectance Methodology

Baseline reflectance (265-2500 nm) measurements were taken of representative substrates using Cary 5E UV-VIS-NIR Spectrophotometer and the Solar Spectrum Reflectometer, model SSR. Substrates were coated with soil slurry using soil obtained from the Kansas site. Samples were horizontally placed in an oven heated to 70°C and allowed to dry overnight (~18 hours). The next day, substrate weights and reflectance measurements were obtained. Using the Solar Spectrum Reflectometer, model SSR, pre-decon reflectance measurements were taken of the contaminated substrates. Three readings were taken of each sample, each reading being an average across a specified wavelength range. The wavelength ranges average as follows (approximate): L1, IR range between 0.8 – 2.5 microns; L2, Red wavelength range between 0.5-1.1 microns; and L3, Blue wavelength range between 0.375-0.85 microns. To obtain percent soil removal data based on weight difference, substrates were weighed prior to, and following soil slurry application, and again following decontamination. Substrates were then exposed to decon formulations at an application rate of 0.01 ml/mm^2 for 5, 15 or 30 seconds. This ratio is ~ 20X that of the application rate recommended for the commercial product, DF200. This application rate was chosen for initial testing to ensure adequate solution contact with the contaminated substrates; geometry of plastic beaker bottom precluded using less solution volume. Following decontaminant exposure, each sample substrate was rinsed by briefly dipping substrate (5 seconds each) in two sequential deionized water baths. The decontamination test matrix was sampled and the reaction was neutralized, then serially diluted and plated onto PetriFilm^{TM} to assess total aerobic count. Substrates were allowed to dry 1 hour in flow hood at ambient temperature. Post-decon weights and reflectance measurements of each substrate were obtained. Using the Solar Spectrum Reflectometer, model SSR, post-decon reflectance measurements were taken as described above.

Detergency effectiveness was determined by evaluating the difference in post-test reflectance vs. the pre-test reflectance, as suggested in the methodology of ASTM D4488-95, i.e., by comparing the reflectance on the unsoiled, pre-test substrates vs. the post-test, soiled substrates following the decon/detergency treatment. Differences of post-test vs. pre-test (unsoiled) surface reflectance of <0.05 are within standard error for the method. These tests may also be completed using soil samples inoculated with *B. atrophaeus* to assess both detergency and spore decontamination in a challenging organic environment.

Effort continued to further demonstrate increased detergency by additional formulation modification. It should be noted that within the different surfaces, the magnitude of reflectance change varied among the wavelength ranges, i.e., changes in reflectance varied more within some wavelength ranges than others for any given material. For instance, the stainless steel and polypropylene exhibited the most significant changes in reflectance in the L3 (blue portion) wavelength range. Preliminary data indicate modified formulations provided increased detergency based on changes in surface reflectance measurements. The change in L3 reflectance of stainless steel exposed to a modified biosecurity formula was 68% less than that of stainless steel exposed to DF200. Refer to Table 3 for representative reflectance data. Post-reflectance surfaces are depicted in Figure 1. Surface detergency analyses of select modified formulations continued into the second year of the project.

Table 3: Surface Detergency Testing

FY'04 Progress and Accomplishments
Surface Detergency Testing

- Results indicated various levels of treatment-dependent detergency ranging from nearly 100% effective (insignificant change in surface reflectance following treatment) to delta reflectance of ~70%

Reflectance changes following 5-minute treatment

Formulation	Delta L1	Delta L2	Delta L3
Virkon S	-0.023	-0.007	-0.045
DF200D	-0.092	-0.084	-0.132
"A"	-0.020	-0.012	-0.059
"B"	-0.056	-0.058	-0.078
"C"	-0.056	-0.054	-0.087

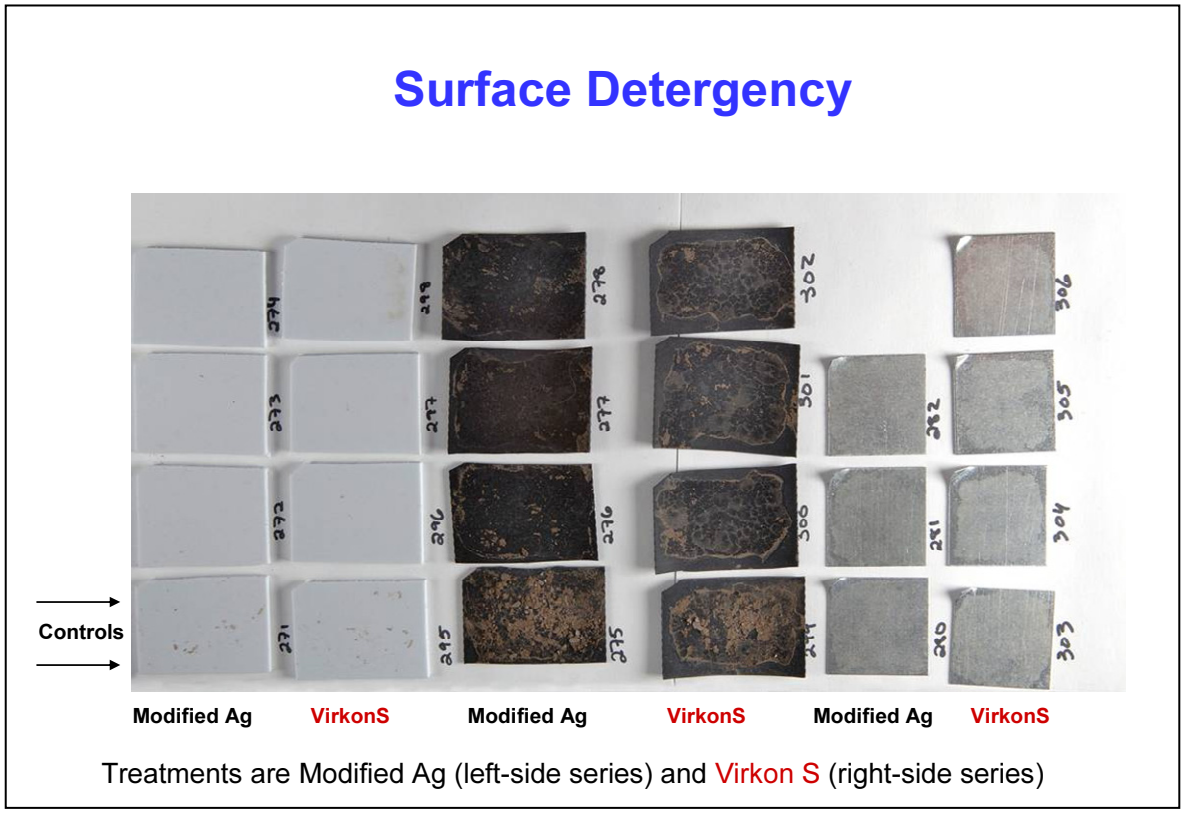


Figure 1: Surface Detergency

2.2 Virology Component – Materials and Methods

2.2.1. Methods and Materials, Study 1

Study 1 with Bovine Coronavirus

Virus and cells. Bovine coronavirus isolate Wisconsin 1sk was propagated in Human Rectal Tumor-18 (HRT) cells as previously described (5). Culture media was made by adding Minimal Essential Medium with Earle's salts and L-glutamine (Invitrogen Corporation, Carlsbad, CA) and 2.2 g/L sodium bicarbonate (Fisher Scientific, Hampton, NH) collectively referred to as MEM with the addition of trypsin (Fisher Scientific) (5 µg/ml) and pancreatin (Fisher Scientific) (5 µg/ml) (4). Cells were maintained in MEM containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) supplemented with antibiotics [2.5 mg/L amphotericin B; 0.67 g/L streptomycin; and 0.3 g/L penicillin G (all from Fisher Scientific)].

Virus Inactivation Protocol to Assess Viral RNA Integrity. 200 µl BCV was added to sterile microcentrifuge tubes. Then, 200 µl of the test formulation (Sandia DF200d, bleach, ethanol, Virkon® S) was added to each tube using the following dilutions made with sterile deionized water of the recommended concentration: Sandia DF200d (50%, 25%, 12.5%), bleach (1%, 10%), ethanol (70%), and Virkon® S (1%, 0.5%). Samples were mixed thoroughly and exposed for 1. Similar studies have also been conducted on BCV inoculated onto metal (anodized steel), butyl rubber (tire rubber), and polypropylene following similar studies for viral inactivation on surfaces using 50% DF200d and 10% bleach. After exposure duration of 1 minute, the viral RNA was extracted from the samples using the QIAGEN Mini Viral RNA extraction protocol.

Qiagen OneStep RT-PCR. Reverse Transcription PCR was performed on the samples with a forward primer (5'- GCC GAT CAG TCC GAC CAA TC -3') and a reverse primer (5'- AGA ATG TCA GCC GGG GTA T -3'). The RT-PCR product was detected by electrophoresis on an agarose gel stained with ethidium bromide and digital image capture under UV excitation.

2.2.1.1. Results, Study 1

Study 1 with Bovine Coronavirus

In preliminary studies using BCV, we were able to demonstrate visible degradation of viral RNA following treatment with various concentrations of the DF-200d decontamination formulation. Using the mechanism of viral inactivation based on RNA degradation for rapid verification of loss of viral infectivity was submitted as a technical advance as a result of these experiments. Visualization of degraded RNA is shown in the figures below.

Lane 1: DNA ladder
Lane 2: Positive BCV
Lane 3: Negative BCV
Lane 4: BCV treated with 0.1M PBS
Lane 5: BCV + 12.5% DF-200D
Lane 6: BCV + 25% DF-200D
Lane 7: BCV + 50% DF-200D

Figure 2: BCV RNA following RT-PCR post treatment with DF-200d.

2.2.2. Methods and Materials, Study 2

Study 2 Recovery of Virus from Soil and Feces

Sample collection. A small farm (500 acres, 204 head) in eastern central Kansas has been experiencing chronic diarrhea among its adult cattle. Fecal samples (n = 6) collected over a two month period tested positive for BCV using bovine coronavirus ELISA. To investigate the source of BCV on the farm, various soil samples were collected surveying the land of the farm by geographical positioning system map supplied by USDA.

General Micro Analysis on Konza Soil Samples. Soil samples were obtained from the Konza Prairie research area in Northeast Kansas. A general microbiology assessment was conducted on the soil samples by diluting the soil samples ten fold in 0.1% peptone buffer and then making serial 1:10 dilutions using 9 ml 0.1% peptone blanks. Samples from each dilution including the original 10% wt/volume sample were plated onto the following Petrifilm™ for general microbiological analysis: APC (total aerobic plate count), ECC (*E. coli* and coliform count), Entero (*Enterobacteriaceae*), and Y&M (yeast and mold count).

Quantitative ELISA. Nineteen samples were collected and analyzed using a quantitative BCV antigen ELISA capture using the Z3A5 neutralizing antibody of the spike subunit of BCV as previously described. Z3A5 was diluted to 250 ng/ml (1:4000) using 0.05M carbonate coating buffer (pH 9.6) and then 50 µl was added to each well of an Immulon 1 (Dynex Technologies, Chantilly, VA) flat-bottom microtiter plate and incubate overnight at 4°C. After overnight incubation, wash thoroughly five times with PBS containing 0.05% Tween 20 (Sigma), desiccate, and then store at 4°C until use. 100 µl of a 0.5% protein block (0.2 g powdered glycine in 40 ml 0.01M PBS) was added to each well and plates were incubated at 37°C for 30 minutes before thorough washing with PBS-Tween. Then 50 µl of each sample was added in triplicate to each well (to achieve an average optical density for each sample) including a known positive and a known negative sample and plates were incubated at 37°C for 30 minutes before thorough washing with PBS-Tween. Primary polyclonal anti-BCV reagent was prepared at a 1:500 dilution in the blocking solution (0.5% glycine in 0.01M PBS) and plates were incubated at 37°C for 30 minutes before thorough washing with PBS-Tween. Then 50 µl of the anti-pig HRPO-conjugated antibody was diluted 1:16,000 in 0.01M PBS was added to each well and plates were incubated at 37°C for 30 minutes before thorough washing with PBS-Tween. Finally 50 µl TMB substrate was added to each well and plates were incubated at 37°C for 30 minutes. After the 30 minute TMB substrate, stop the reaction by adding 50 µl of 1N H_2SO_4. Optical density following the assay is determined by reading the absorbance at 450 nm on a plate reader. The average OD reading of the negative samples was doubled to determine the cutoff OD. Anything higher than the cutoff was considered positive.

RNA extraction and RT-PCR. Samples testing positive for BCV by the ELISA were subjected to RNA extraction by using 10% weight/volume in 0.01M phosphate buffered saline (PBS) and vortexing for 1 minute. The samples were then centrifuged at 2000 rpm for 15 minutes. The supernatant was used for the RNA extraction process using the QIAGEN Mini Viral RNA extraction kit. A fecal sample positive with BCV MN-1988 was used as a positive control. Following RNA extraction, RT-PCR was performed on the samples with a forward primer (5'- GCC GAT CAG TCC GAC CAA TC -3') and a reverse primer (5'- AGA ATG TCA GCC GGG GTA T -3'). Analysis of RT-PCR product amplification was conducted by agarose gel electrophoresis stained with ethidium bromide.

Virus Isolation. Samples were clarified, filtered and inoculated into human rectal tumor 18 cells to assess the presence of viable BCV based on typical cytopathic effect (CPE) and hemagglutination with rodent erythrocytes.

2.2.2.1. Results, Study 2

Study 2 Recovery of Virus from Soil and Feces

General Micro Analysis of Konza Soil Following 24-48 hours of incubation at 35°C, colony forming units were counted on the various types of petrifilm used to determine the microbial load from the Konza soil samples. Three replications were conducted and averages were determined.

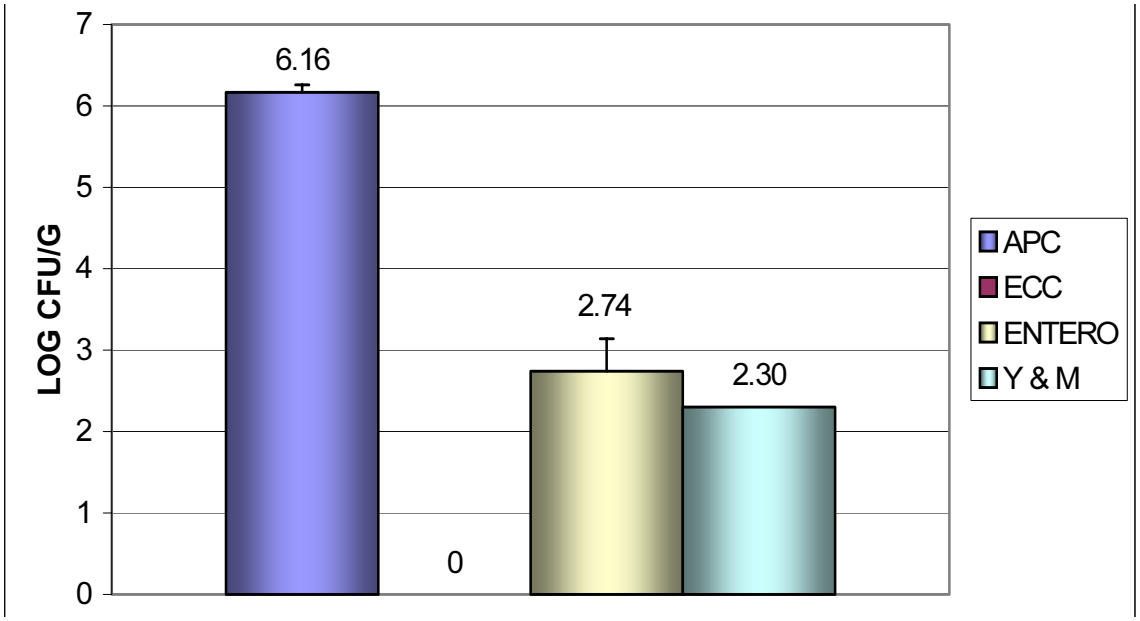


Figure 3: Aerobic Plate Count (APC), E. coli and coliform count (ECC), enterobacteria (Entero), and yeast and mold (Y & M) counts of soil used in study 2.

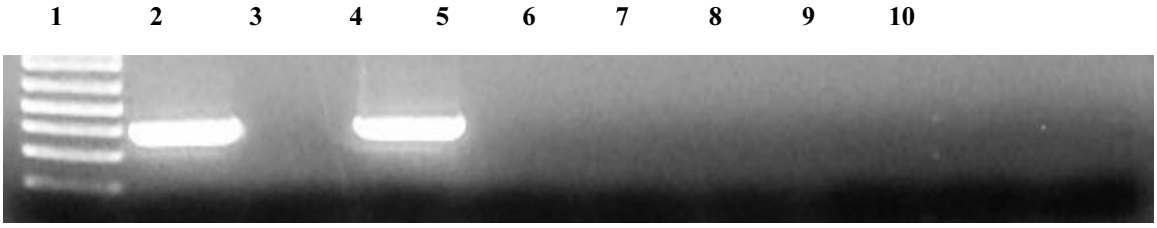
Quantitative ELISA. Six of the 19 samples tested positive for BCV by the ELISA. The sample taken near the common watering area (location J, corral) resulted in the highest level of BCV based on the OD reading. This location was heavily soiled with visible manure. Additionally, animals are more likely to be in close proximity to one another near the water and feed troughs, thus increasing the risk of infection in these locations.

Table 4: Areas in which BCV was recovered from soil and or manure.

Sample	Average OD	Result
6. South pasture, near trees	**0.225 (1+)**	**Low positive**
8. Southeast creek	**0.239 (1+)**	**Low positive**
10. Northeast creek	**0.171 (1+)**	**Low positive**
12. North pasture (with cattle)	**0.168 (1+)**	**Low positive**
16. Northwest of coral	**0.194 (1+)**	**Low positive**
18.Corral (near water/feed)	**0.468 (2+)**	**Low positive**

***OD cutoff was determined to be >0.164 = 1+, >0.328 = 2+, >0.492 =3+, >0.656 = 4+ (high positive)**

RNA extraction and RT-PCR. These results indicate that the fecal sample from the experimentally infected calf was positive for BCV by this RT PCR extraction protocol. The soil samples however, were not positive by this method, but were previously positive based on ELISA. Further efforts will be conducted in trying different RNA extraction protocols from soil as the silica constituent of soil may be binding the RNA and interrupting the extraction. Additionally, PCR inhibitors could have been present in the various soil samples and further analysis is ongoing. RT-PCR Detection of Soil samples obtained from test farm location**.**



Lane 1: DNA ladder
Lane 2: BCV positive control
Lane 3: BCV negative control
Lane 4: Feces with BCV MN 1988
Lane 5: Soil sample #6 (location E)
Lane 6: Soil sample #8 (location F)
Lane 7: Soil sample #10 (location G)
Lane 8: Soil sample #12 (location H)
Lane 9: Soil sample #16 (location J)
Lane 10: Soil sample #18 (corral, J)

Figure 4. RT-PCR of Soil Samples

Virus Isolation. HRT 18 monolayers were negative for the presence of viable BCV based on typical cytopathic effect (CPE) and hemagglutination with rodent erythrocytes. Previous studies have also shown that HRT 18 cells allowed replication of BCV (10^5-10^6 PFU/ml at passage 4, but did not allow replication of field isolates. Based on the physiochemical properties, we concluded that BCV doesn't survive in soil on this farm. Because adult cattle can be chronically infected with BCV and shed virus intermittently, an all-in-all-out system of management was recommended. These results indicate that BCV will survive poorly in soil from this farm.

3. Year 2 Results and Accomplishments

3.1 Decontamination Component

Effort continued throughout the second year to produce modified decontaminant formulations with increased detergency, while maintaining efficacy in highly organic environments. It is important to note that most modified formulations included the Barquat surfactant in place of the Variquat surfactant. Various factors such as wettability, corrosivity and detergency were assessed throughout the year. Additionally, a soil surfactant sorption study was undertaken to assess potential sorption characteristics of the Variquat and Barquat surfactants. Results of these studies are presented in the following sections.

3.1.1 Wettability (measured by contact angle)

A cross-comparison was performed to assess wettability performance of all formulations on the various surfaces. Contact angle measurements were made to assess the wettability of various decontaminants on steel, butyl rubber and polypropylene. Nine different aqueous decontaminants were evaluated. The decontaminants used in this study were identified as follows:

- Formulation #1, DF200 (EasyDecon, commercial product of Envirofoam Technologies, Inc.)
- Formulation #2, "Barquat/5830"
- Formulation #3, "Barquat/5840"
- Formulation #4, DF200D/Barquat
- Formulation #5, DF200D
- Formulation #6, "Ag"
- Formulation #7, "Ag/EDTA"
- Formulation #8, "Ag/Q2"
- Formulation #9, 1% VirkonS (manufacturer's recommended concentration)

Contact Angle Method

Contact angle was measured in the following manner. The goniometer (contact angle apparatus) was leveled, both at the base and at the platform area which is located near the center of the apparatus. A horizontal surface was verified by viewing the surface through a scope and ensuring the horizontal line indicator lined up from end to end with the edge of the test surface. The tangent angle indicator line was ensured to be at 0° and resting directly on top of the horizontal indicator line. Using a plastic transfer pipette, a drop of test solution was placed on the edge of a test surface (steel, butyl rubber or polypropylene). Consistency in drop placement (height and force of drop placement) was attempted with each replication. By viewing the solution droplet through a scope, the contact angle was determined by rotating the tangent line indicator properly to correspond to the line tangent to the solution droplet curvature at the horizontal surface interface. Note, the scope produced an inverted, backward image of the test solution, and thus appeared as a hanging droplet. Contact angle measurements were made as quickly as possible, minimizing the spread effect of the droplet on the test surfaces. Triplicate measurements were made of three different drops immediately following placement of each test formulation drop on the test surface.

Contact Angle Assessment Conclusions

In general, all measurements of Sandia formulations with hydrogen peroxide were of lower contact angle than formulations without peroxide. The addition of the peroxide increased the wettability of the test formulation. Average measurements were 25.22° for the polypropylene; 20.06° for the butyl rubber; and 16.38° for the anodized steel, indicative of the relative hydrophobic nature of the surfaces.

A cross-comparison was performed to assess wettability performance of all formulations on the various surfaces. A point system was developed in which formulations were evaluated based on their average contact angle measurement on each test surface. Formulations were ranked by assigning points based on relative contact angle measurement within surface treatment, i.e., 10 points was assigned for the formulation with lowest contact angle reading for steel, 9 points for the formulation with the next highest contact angle

reading for steel, etc. Points were totaled for each formulation and the formulations were then ranked based on the total number of points. Results indicate wettability of the solutions as ranked in the following order: Ag Q2> DF200D and Barquat/5830 (tie) >Ag >DF200 > DF200D/Barquat >Barquat/5840 > Ag/EDTA > Virkon S.

3.1.2 Detergency (measured by change in surface reflectance; based on ASTM methodology)

Surface detergency analyses of select modified formulations continued in the second year of the project. In general, detergency performance was both surface and solution dependent. No formulation performed optimally on all three surfaces.

In conclusion, all Sandia formulations performed better than Virkon S on polypropylene and rubber, with significant improved performance of Sandia solutions on rubber (as compared to Virkon S). As demonstrated by least change in pre and post-organic load contamination change in surface reflectance, AgQ2 demonstrated optimal detergency performance on polypropylene; Ag, Ag/EDTA, Ag5830 and DF200D/Barquat performed comparably and demonstrated optimal detergency performance on butyl rubber; DF200D demonstrated optimal detergency performance on stainless steel.

3.1.3 Surfactant Soil Sorption

The basis for this study was the inherent capacity of surfactant sorption onto soils. Given a soil saturated with a known surfactant concentration, a portion of the surfactant will sorb onto the soil and a portion of the surfactant will exist in the bulk solution. Surface tension measurements were taken of various surfactant concentrations in soil and surface tension was plotted against the surfactant concentration. At the point of critical micelle concentration, cmc, a break occurs in the plotted curve, indicating the approximate concentration at which the bulk solution is saturated with surfactant. It is predicted that at this point where the curve breaks, further increases in surfactant concentration will not result in significant surface tension change, i.e., the slope of the curve will flatten out. This is due to the surfactant being saturated in the bulk solution and surfactant transfer between the aqueous phase and soil phase is at or very near equilibrium. Thus, when comparing the control trend line to the soil sorption trend line, we will gain insight into the surfactant concentration that is sorbed onto the soil and unavailable for microbial kill. We can thus predict additional surfactant concentration required to attain sufficient microbial kill.

This information is useful in theoretically determining the surfactant concentration unavailable for killing microorganisms and thus provides insight into additional surfactant concentration required for adequate microbial kill. Parameters that would affect results may be the nature of the surfactants, soil ionic strength and the total cations and/or cation exchange capacity of the soil. Cationic surfactants tend to sorb more strongly onto most soils than other surfactant species, thus there may be greater sorption and loss of effective surfactant than we might expect.

This study consisted of a comparison of two different quaternary ammonium compounds (quats): that used in DF200 (Variquat); and Barquat, a quaternary ammonium compound used in most of the modified formulations tested throughout the project. The fundamental difference between the two quats is that the Barquat series are blends of alkyl dimethyl benzyl ammonium chlorides and alkyl dimethyl ethylbenzyl ammonium chlorides. Variquat is comprised only of the alkyl dimethyl benzyl ammonium chloride. The Barquat series surfactants are broad spectrum biocides, FIFRA registered as an active ingredient in

hard surface disinfectants, sanitizers and some water treatment products. Lonza, manufacturer of the Barquat series claims the product is also effective in heavy organic soil loads.

As determined by plots of surface tension versus surfactant concentration, both in deionized water matrix and with 10% added soil (all analyses performed in triplicate), the critical micelle concentration of the Barquat solutions (refer to Figure 5) in deionized water is equivalent to the critical micelle concentration of the Variquat (Figure 6) solutions. However, the slope of the Barquat control line rises at a much slower rate than the slope of the Variquat control; thus, the difference between the average slope of the control versus soil sorption surface tension lines is significant when comparing the Barquat test series to the Variquat test series. This trend suggests that Barquat may have greater soil sorption capacity than the Variquat.

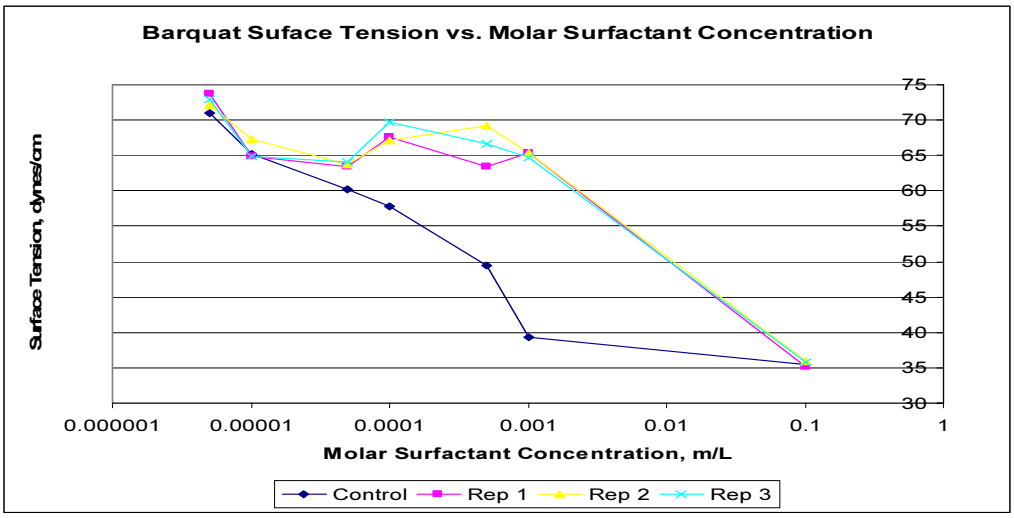


Figure 5. Barquat 4280Z Surfactant Soil Sorption

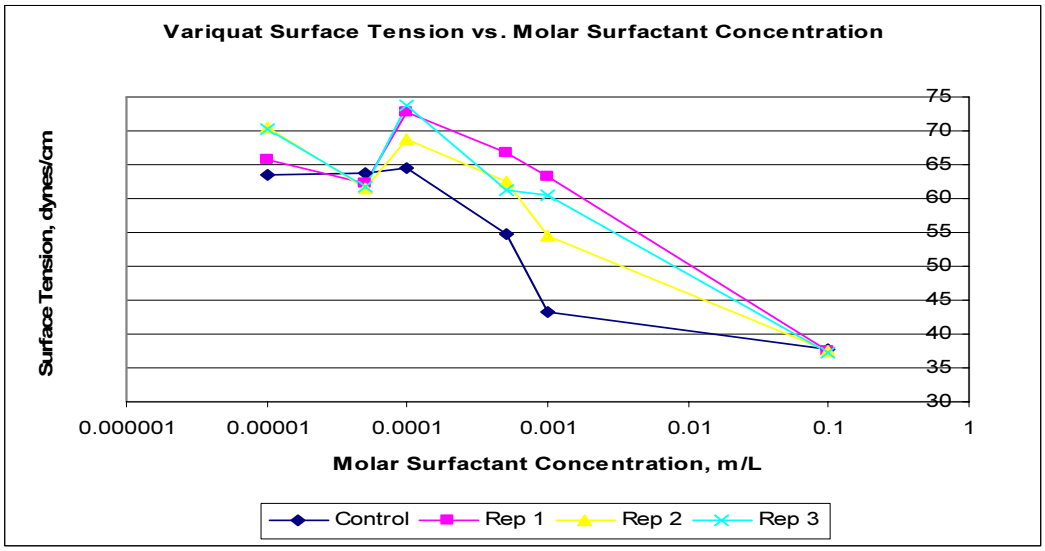


Figure 6. Variquat 80MC Surfactant Soil Sorption

3.1.4 Corrosion and Material Degradation

Material description

Test coupons (1.5”x1.5”) were cut from commercially available zinc-coated (galvanized) low carbon steel (CS Type A) sheet. Galvanizing was performed per ASTM A 653 specifications.

Electrochemical Impedance Spectroscopy (EIS)

EIS is an analysis technique that has gained wide acceptance for determining the electrochemical properties of metal-solution systems, including corrosion rates. It involves applying a low level (<25 mV) sinusoidal voltage signal to a metal-solution interface and monitoring the current response (magnitude and phase). The voltage is applied over a range of frequencies. The interface is modeled as an R-C circuit in order to extract the desired electrochemical parameters (e.g. solution resistance, coating capacitance and polarization resistance). The use of this technique has several advantages over traditional methods for determining corrosion rates (e.g. mass loss): It is non-destructive, highly sensitive and has short test times on the order of minutes to hours. The polarization resistance (R_p) of a corroding metal is indirectly proportional to the corrosion current (I_{corr}) as shown by Equation 1:

$$I_{corr} = B/ R_p \qquad \text{Equation 1}$$

where I_{corr} is the corrosion current (A)
B is a proportionality constant, usually 25 mV
and R_p is the polarization resistance (ohms)

The corrosion current is in turn directly related to the corrosion rate as shown in Equation 2, a variation of Faraday’s Law:

$$CR=0.129ai/(nD) \qquad \text{Equation 2}$$

where CR is the corrosion rate in mils/year
(1 mil = 0.001 in)
a is atomic weight (65.38 g for Zn)
i is the corrosion current density (A/cm^2)
n is the number of equivalents/mol (2 eq/mol for Zn)
and D is density (7.14 g/cm^3 for Zn)

EIS Results

The exposure of galvanized steel to various decontaminants results in large differences in corrosion rate dependent on the formulation chemistry. The corrosion rates (in mils/year) of galvanized steel exposed to DF200, Virkon S and 10vol% bleach as determined by EIS are presented in Figure 7. The instantaneous corrosion rate at exposure times of 5 minutes, 2 hours and 24 hours are shown. Virkon S was determined to be the most corrosive decontaminant in this study, resulting in a corrosion rate at least an order of magnitude higher than for any of the other decontaminants. Exposure to Virkon S also resulted in a high

corrosion rate (approximately 100 mpy) that was maintained throughout the 24 hour test period, unlike the behavior observed for other decontaminant solutions. The Sandia decontaminant technologies, including DF200, demonstrated much lower corrosivity. The corrosion rate due to exposure to DF200 is initially high (up to 9 mpy) at early exposure times (up to 3 hours), and eventually falls to levels that industry considers as zero (less than 1 mpy). The temporarily elevated corrosion rate can be attributed to the peroxide chemistry which is characteristic of all the Sandia decontaminants.

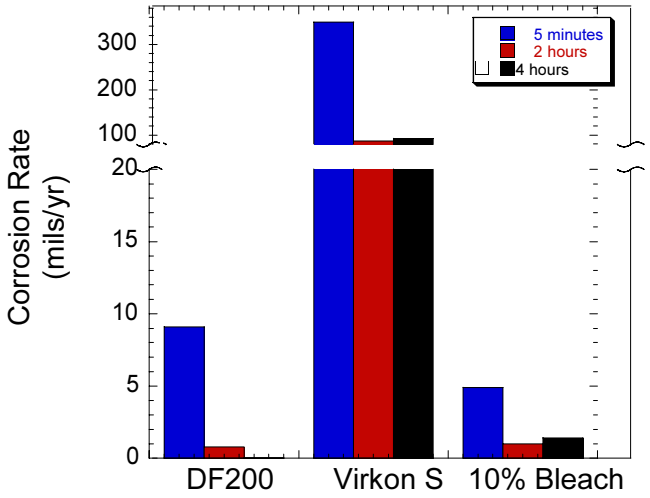


Figure 7: Corrosion rates of galvanized steel during exposure to DF200, Virkon S and 10vol % bleach determined by Electrochemical Impedance Spectroscopy (EIS).

The corrosion rates determined by EIS are consistent with visual observations and mass loss measurements of the samples after exposure to the decontaminant solutions. The zinc surfaces are clearly etched after exposure to Virkon S and 10vol% bleach indicating uniform corrosion (see Figure 8). The etching of the surface is noticeable after 2 hours and becomes more pronounced after 24 hours. By comparison, samples exposed to DF200 show no etching after 2 hours exposure and only slight etching after 24 hours of exposure. The mass loss measurements also indicate a greater thickness loss associated with exposure to Virkon S and the bleach solutions compared to DF200, on the order of 0.1 mil compared to 0.01 mil after 24 hours.

In conclusion, our findings indicate that the corrosion rates due to DF200, DF200D and DF200D/Barquat exposures are highly comparable and are the lowest overall corrosion rates measured in this study. Virkon S resulted in the highest overall corrosion rate of all the solutions tested.

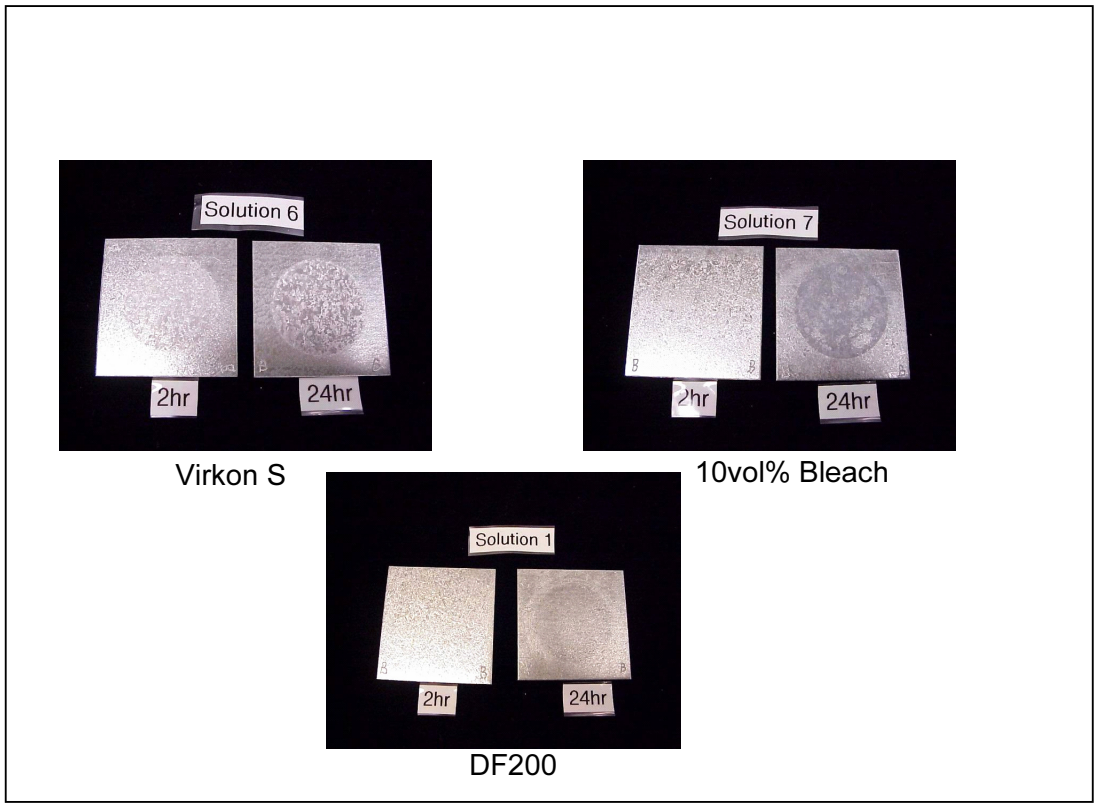


Figure 8: Photo comparison of galvanized steel surfaces after 2 hour and 24 hour exposures to Virkon S, 10vol% bleach and DF200.

Preliminary modified Ag formulation results indicate potential agreement with modified formulation results mentioned above; however, with repeated analyses, the other modified formulations did not perform as well as the DF200/Barquat series. Ag/EDTA results are understandable of higher corrosivity due to presence of acidic salt. Bleach and DF100 demonstrate long-term, intermediate corrosion rates; however, the initial corrosion rate of both bleach and DF100 is lower than all other formulations, with the exception of Virkon S.

Additionally, the butyl rubber and polypropylene polymer surfaces were analyzed by attenuated total internal reflection (ATR) infrared spectroscopy or photoacoustic infrared spectroscopy.

Butyl Rubber

Black butyl rubber analyses by ATR and photoacoustic infrared spectroscopy failed to produce meaningful results, primarily due to the high loading of carbon in the butyl rubber material. In the first case, the black polymer attenuated the infrared radiation, yielding a very low infrared signal, resulting in spectra that contained rather broad spectra features that could

not be used to determine if chemical degradation of the decontaminant exposed material had occurred. Results of the photoacoustic spectroscopy indicated carbon black loadings of >50%, at least 10-15% greater than the upper limit of this methodology.

Polypropylene

Analysis of the polypropylene surfaces to the various decontaminants showed no effects to surfaces following exposure. The samples were all exposed to the solutions overnight, rinsed with DI water and dried over kimwipes before analysis. Spectra were collected and are shown in Figure 9 below. Solutions are defined as follows:

- Solution 1, DF200
- Solution 2, DF200D
- Solution 3, DF200D-Barquat
- Solution 4, Ag
- Solution 5, Ag EDTA
- Solution 6, Virkon S
- Solution 7, 10% bleach

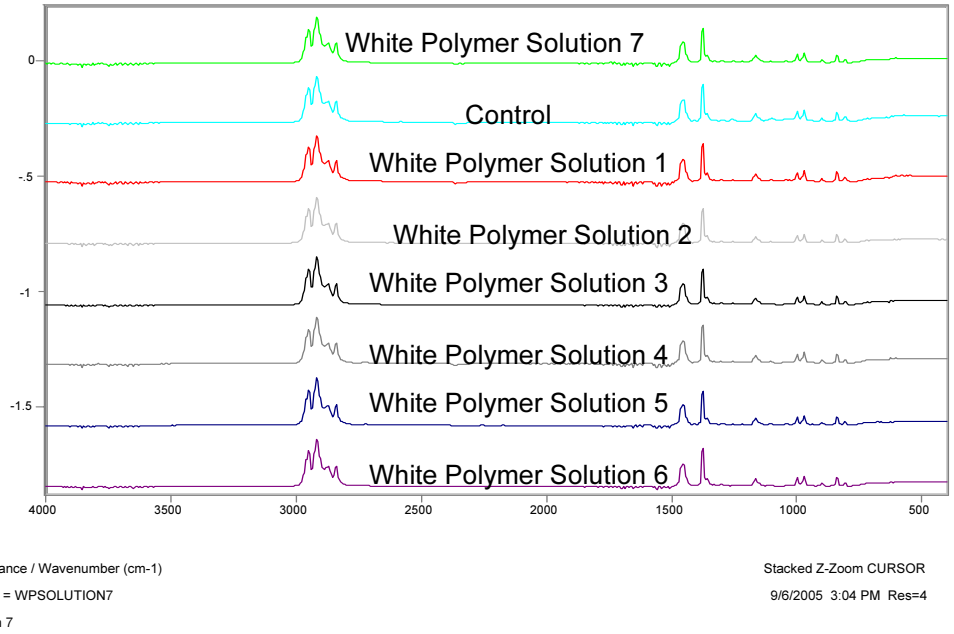


Figure 9: Spectra of white polymer material following exposure to various decontaminants.

3.2. Virology Component

3.2.1. Methods and Materials, Study 3

Study 3 with Bovine Enterovirus-2

Virus and cells. BEV-2 (obtained from the National Veterinary Services Laboratory, Ames, IA) was propagated in Madin Darby Bovine Kidney (MDBK) cells. Culture media was made by adding Dulbecco's Modification of Eagle's Medium (Cellgro®, Herndon, VA) with 4.5 g/L glucose and L-glutamine without sodium pyruvate collectively referred to as DMEM. Cells were maintained in DMEM containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) supplemented with antibiotics [2.5 mg/L amphotericin B; 0.67 g/L streptomycin; and 0.3 g/L penicillin G (all from Fisher Scientific)].

Test Disinfectants. Test disinfectants included various modifications of the Sandia National Laboratories Decontamination Formulations (DF-200) shown in Table 5 below.

Ag DF-200	**Ag DF-200/EDTA**	**Ag DF-200/Variquat**	**Ag DF-200/EDTA/Variquat**
3.76 g Part A (with barquat)	3.76 g Part A (with barquat)	3.76 g Part A (with variquat)	3.76 g Part A (with variquat)
0.3 g potassium bicarbonate	0.4 g potassium bicarbonate	0.3 g potassium bicarbonate	0.4 g potassium bicarbonate
0.2 g potassium carbonate	0.05 g EDTA	0.2 g potassium carbonate	0.05 g EDTA
4.4 g 8% hyrdrogen peroxide	4.4 g 8% hyrdrogen peroxide	4.4 g 8% hyrdrogen peroxide	4.4 g 8% hyrdrogen peroxide
0.2 g diacetin	0.2 g diacetin	0.2 g diacetin	0.2 g diacetin
Ag/Q2 formulation	**DF-200d**	**DF-200d/Barquat**	
3.66 g Part A (Ag/Q2)	7.3 g Part A (with variquat)	7.3 g Part A (with barquat)	
0.3 g potassium bicarbonate	2.5 g 8% hyrdrogen peroxide	2.5 g 8% hyrdrogen peroxide	
0.2 g potassium carbonate	0.2 g diacetin	0.2 g diacetin	
4.4 g 8% hyrdrogen peroxide			
0.2 g diacetin			

Table 5: Modified decontamination formulations used in this study. The "Part A" or surfactant portion, is comprised of different components in each formulation.

Organic Matrices. The organic matrices used in this study included a 10% weight/volume (wt/vol) solution of bovine feces. The bovine feces was prepared by collecting fecal material rectally from healthy cattle, weighing out 10 g of the feces, and adding 90 ml 0.01 M PBS and mixing thoroughly. This preparation was used in all replications.

Virus Inactivation. Approximately 10 ml of BEV-2 cell culture prepared inoculum was added to 10 ml test disinfectant and were incubated (1, 10, 20 minutes, room temperature), with 0% fecal challenge. For 10% organic challenge, 10 ml of test virus was added to 9 ml of test disinfectants containing 1 ml of a 10% bovine fecal slurry and were incubated (1, 10, 20 minutes, room temperature). For 25% organic challenge, 10 ml of test virus was added to 7.5 ml of test disinfectants containing 2.5 ml of a 10% bovine fecal slurry and were incubated (1, 10, 20 minutes, room temperature). For 50% organic challenge, 10 ml of test virus was added to 5 ml of test disinfectants containing 5 ml of a 10% bovine fecal slurry and were incubated (1, 10, 20 minutes, room temperature). Samples were washed by ultracentrifugation (100,000xG, 1 hour, 4^{o}C). Pellets were reconstituted with 1 ml of DMEM. Samples were stored at -80^{o}C until further processing.

Tissue Culture Infective Dose 50 ($TCID_{50}$). To determine infectious viral titer, BEV-2 was quantified using an endpoint method tissue culture infective dose 50 ($TCID_{50}$). The Reed-Muench method (8) was calculated in a 96-well format to determine the dilution of virus resulting in infection of fewer than 50% replicate wells. Nunc 96-well cell culture microtiter plates (Fisher Scientific) were seeded with MDBK cells and incubated (approximately 24 hrs, at 37^{o}C with 5% CO_2). Once cells were monolayer, the media was removed and plates were washed once with 0.01 M phosphate buffered saline (PBS, pH 7.2). Treated and washed disinfectant samples were then serially diluted (ten fold) in culture media to a 10^{-9} dilution. The microtiter plates were inoculated by adding 25 µl of each virus dilution to each well. Negative controls were inoculated with 25 µl of uninfected culture media. An additional 75 µl culture media was added to each well and the plate was incubated (48 hrs, 37^{o}C with 5% CO_2). Infectivity was determined by cytopathic effect (CPE) as indicated by the presence of rolling up and sloughing of infected cells. $TCID_{50}$ was calculated using the Reed-Muench method for estimating 50% endpoints of infectivity (8).

Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR). RT-PCR was used to determine the presence of BEV-2 specific nucleic acid sequences and was visualized by EtBr-stained gels. Samples were extracted using the Qiagen Mini Viral RNA extraction kit (Qiagen, Valencia California) and RT-PCR was conducted using commercial kits (Qiagen OneStep RT-PCR kit). A Smartcycler thermocycler (Cepheid, Sunnyvale CA) was used for all RT-PCR reactions. Viral RNA was extracted from the test samples following 1, 10, or 20 minute exposure to the test disinfectants using the Qiagen Mini Viral RNA extraction kit. Following RNA extraction, RT-PCR was performed on the samples using a forward primer (5'- GCC GTG AAT GCT GCT AA -3') (7) and a redesigned reverse primer (5'- AGC AAT GTT CAA TGG CAA GGT CGC -3') to amplify a larger amplicon. For real time detection, a fluorescently labeled TaqMan probe (5' AAC CTC CGA GCG TGT GCG CA 3') labeled with 6-FAM™ at the 5'-end and Iowa Black Fluorescent Quencher at the 3'-end, (Integrated DNA Technologies) was used.

For each 25 µl reaction, 2.5 µl of the RNA sample was added to 22.5 µl of master mix was that contained 5 µl OneStep buffer, 1 µl dNTP, 1 µl enzyme (all from Qiagen's OneStep RT-PCR kit), and 0.5 µl each of the forward and reverse primers (1 µM concentration), and 14.5 µl RNAse-free water. The SmartCycler thermocycler was set with the following settings: Stage 1 hold (42°C, 1800 seconds without optics), Stage 2 hold (95°C, 900 seconds without optics), Stage 3 was repeated 35 times using a 3-temperature cycle (94°C, 15 s without optics; 50°C, 15 seconds without optics; and 72°C, 30 seconds with optics on). Analysis of RT-PCR amplification products were completed by agarose gel electrophoresis. Briefly, aliquots from RT-PCR were loaded onto 1% agarose gels and electrophoresed (90 V, 45 min), and subsequently stained with ethidium bromide. A successful BEV-2 RT-PCR would result in the visualization of a 190 base pair amplicon.

3.2.2. Results, Study 3

Study 3 with Bovine Enterovirus-2

Tissue Culture Infective Dose 50 ($TCID_{50}$). Following various treatment parameters with the various test disinfectants, BEV-2 infectious titer was quantified using $TCID_{50}$ in MDBK cells. The data generated is presented in three separate figures (figures 10-12) representing the 1, 10, and 20 minute exposures. Within each figure, the 0, 10, 25, and 50% organic fecal challenged is presented.

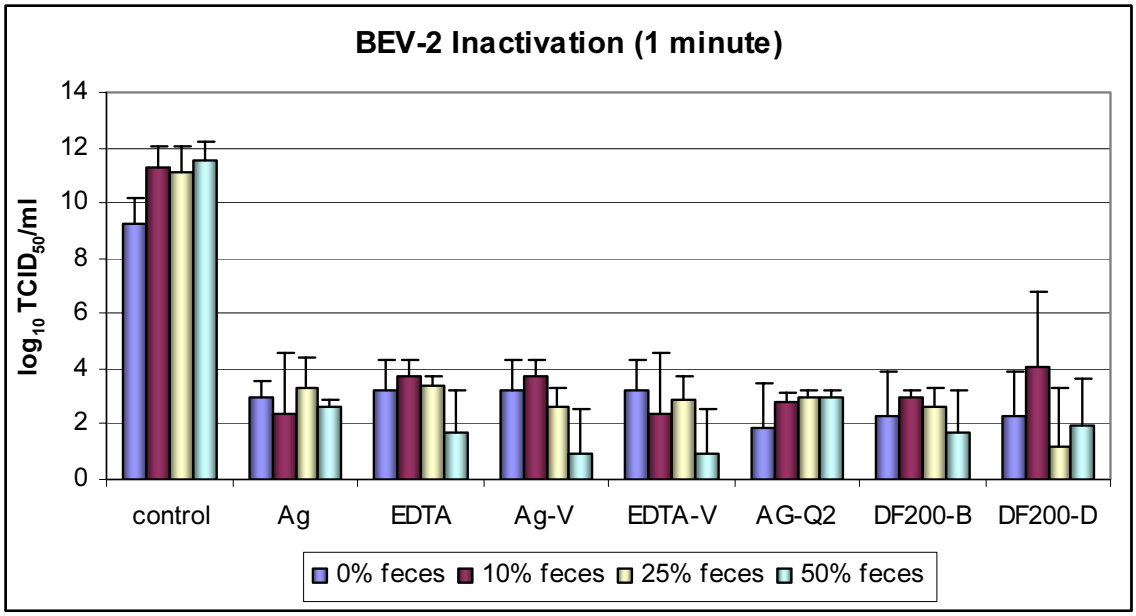


Figure 10: BEV-2 inactivation following 1 minute exposure with 0, 10, 25, and 50% fecal challenges. Standard deviation is depicted with error bars. (Control, no treatment; Ag, Ag DF-200, EDTA, Ag DF-200/EDTA; Ag-V, Ag DF-200/Variquat; EDTA-V, Ag DF-200/EDTA/Variquat; AG-Q2, Aq-Q2 formulation; DF200-B, DF-200/Barquat; DF200-D, DF-200d)

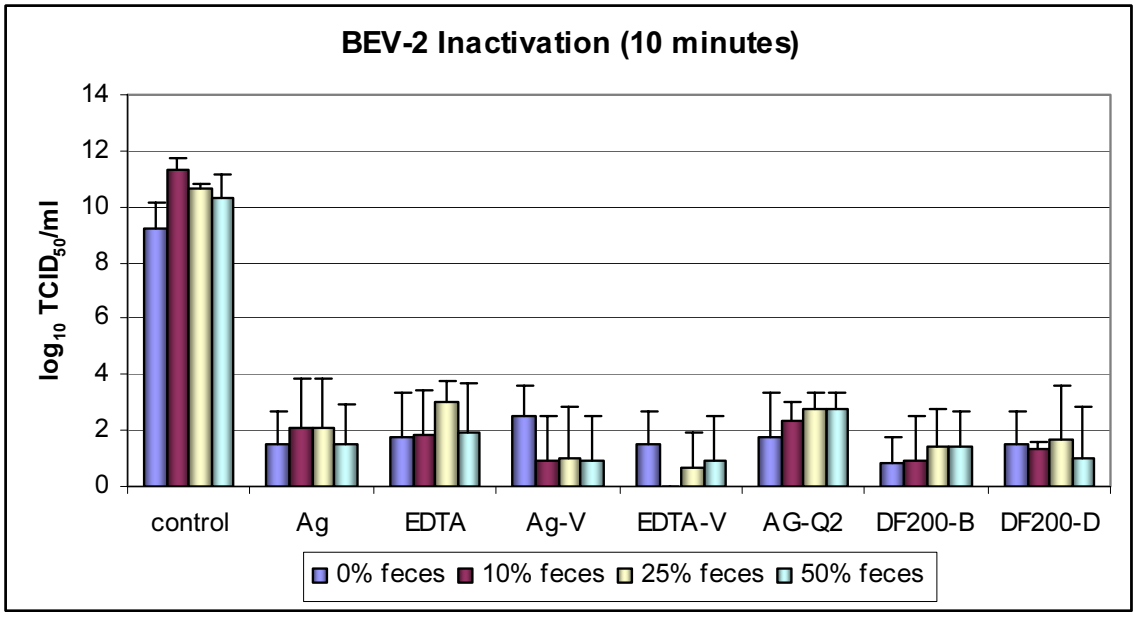


Figure 11 BEV-2 inactivation following 10 minute exposure with 0, 10, 25, and 50% fecal challenges. Standard deviation is depicted with error bars. (Control, no treatment; Ag, Ag DF-200, EDTA, Ag DF-200/EDTA; Ag-V, Ag DF-200/Variquat; EDTA-V, Ag DF-200/EDTA/Variquat; AG-Q2, Aq-Q2 formulation; DF200-B, DF-200/Barquat; DF200-D, DF-200d)

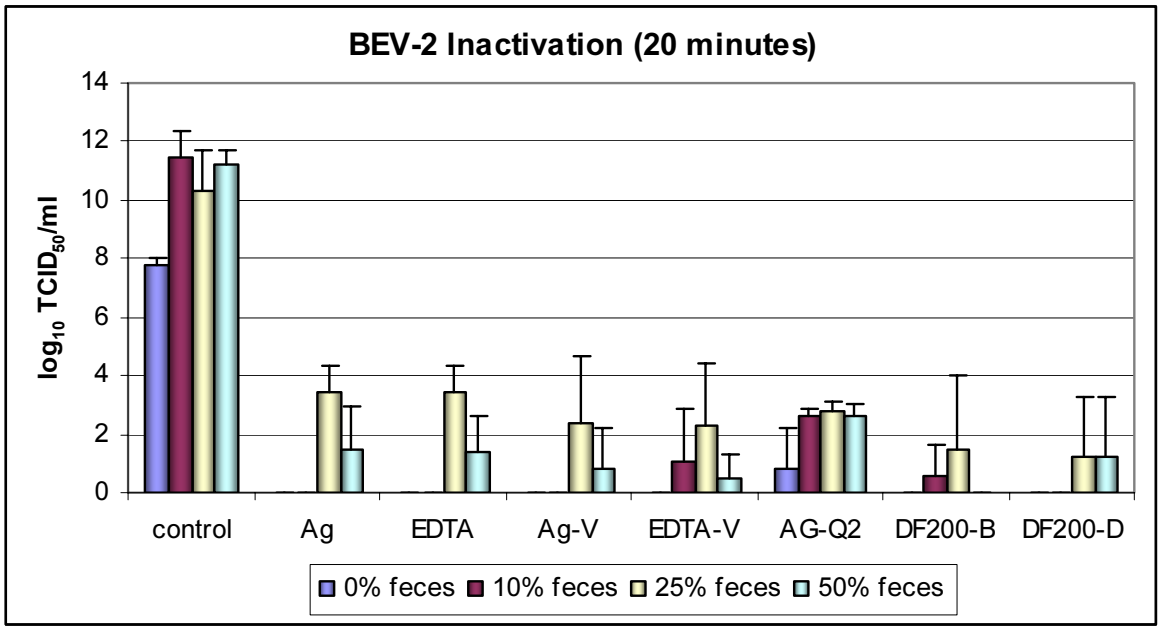


Figure 12: BEV-2 inactivation following 20 minute exposure with 0, 10, 25, and 50% fecal challenges. Standard deviation is depicted with error bars. (Control, no treatment; Ag, Ag DF-200, EDTA, Ag DF-200/EDTA; Ag-V, Ag DF-200/Variquat; EDTA-V, Ag DF-200/EDTA/Variquat; AG-Q2, Aq-Q2 formulation; DF200-B, DF-200/Barquat; DF200-D, DF-200d)

The 20 minute exposure time was the most effective for all treatments in reducing the level of BEV-2 infectious titer. Overall, there were not significant differences in the level of virus reduced following treatment with the various modified decontamination formulations (Ag DF-200, Ag DF-200/EDTA, Ag DF-200/Variquat, Ag DF-200/EDTA/Variquat, DF-200d, DF-200/Barquat, Aq-Q2 formulation). In the presence of higher organic challenge (25, 50% feces), Ag DF-200/EDTA/Variquat, DF-200/Barquat, and DF-200d remained the most efficacious.

Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR). Following various treatment parameters with the various test disinfectants, BEV-2 RNA was quantified using an RT-PCR based detection assay. The data generated is presented in three separate figures (figures 13-15) representing the 1, 10, and 20 minute exposures. Within each figure, the 0, 10, 25, and 50% organic fecal challenged is presented.

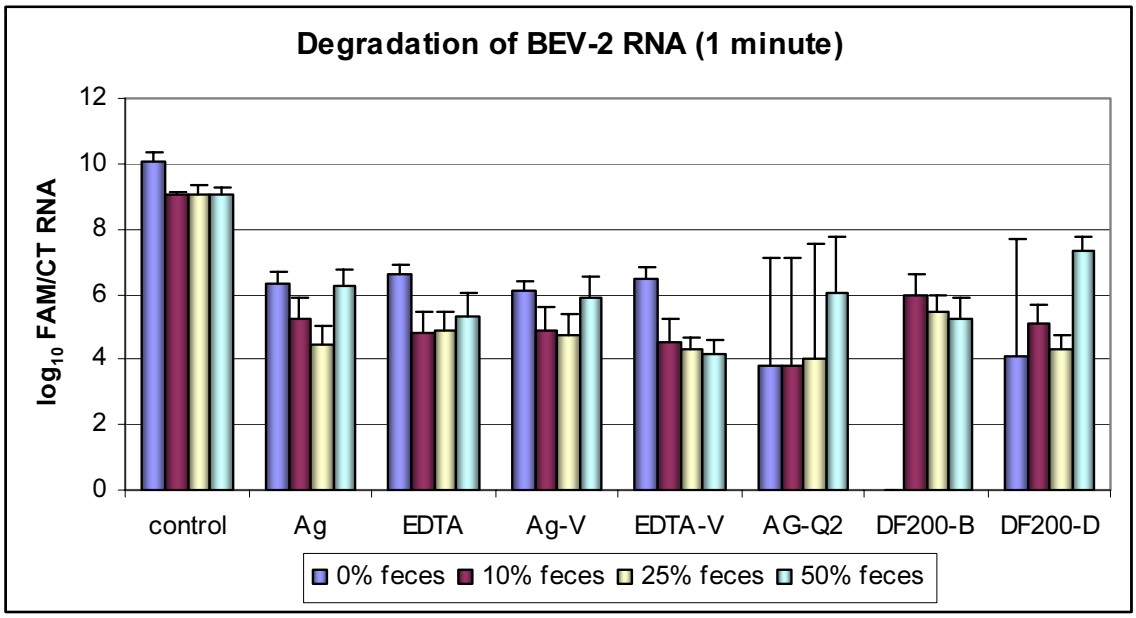


Figure 13: Degraded BEV-2 RNA following 1 minute exposure with 0, 10, 25, and 50% fecal challenges. Standard deviation is depicted with error bars. (Control, no treatment; Ag, Ag DF-200, EDTA, Ag DF-200/EDTA; Ag-V, Ag DF-200/Variquat; EDTA-V, Ag DF-200/EDTA/Variquat; AG-Q2, AG-Q2 formulation; DF200-B, DF-200/Barquat; DF200-D, DF-200d)

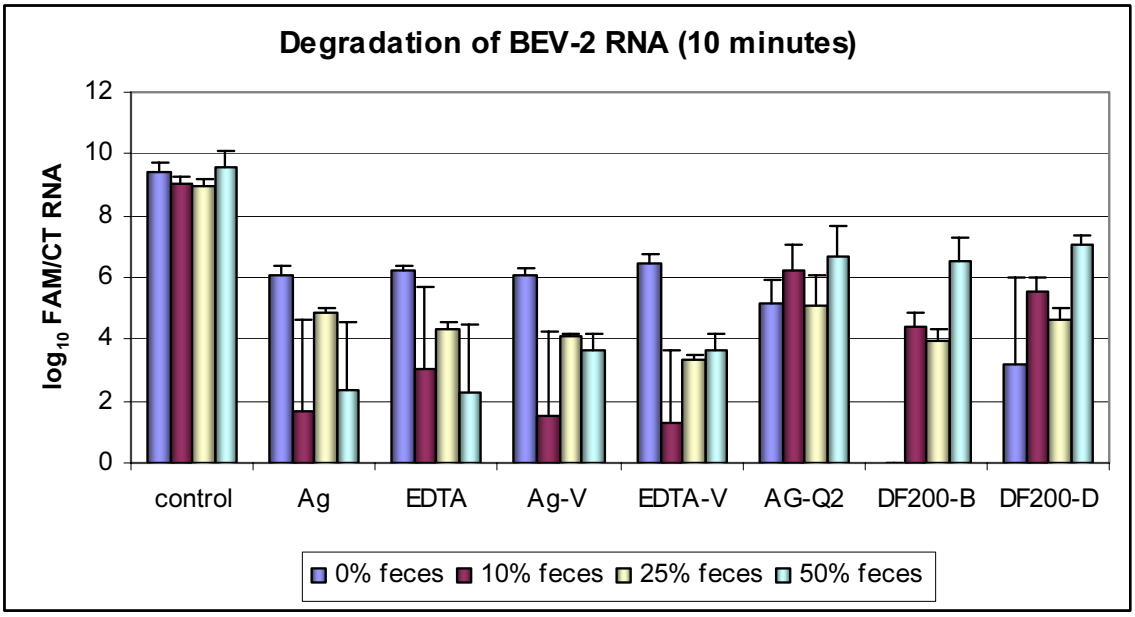


Figure 14: Degraded BEV-2 RNA following 10 minute exposure with 0, 10, 25, and 50% fecal challenges. Standard deviation is depicted with error bars. (Control, no treatment; Ag, Ag DF-200, EDTA, Ag DF-200/EDTA; Ag-V, Ag DF-200/Variquat; EDTA-V, Ag DF-200/EDTA/Variquat; AG-Q2, Aq-Q2 formulation; DF200-B, DF-200/Barquat; DF200-D, DF-200d)

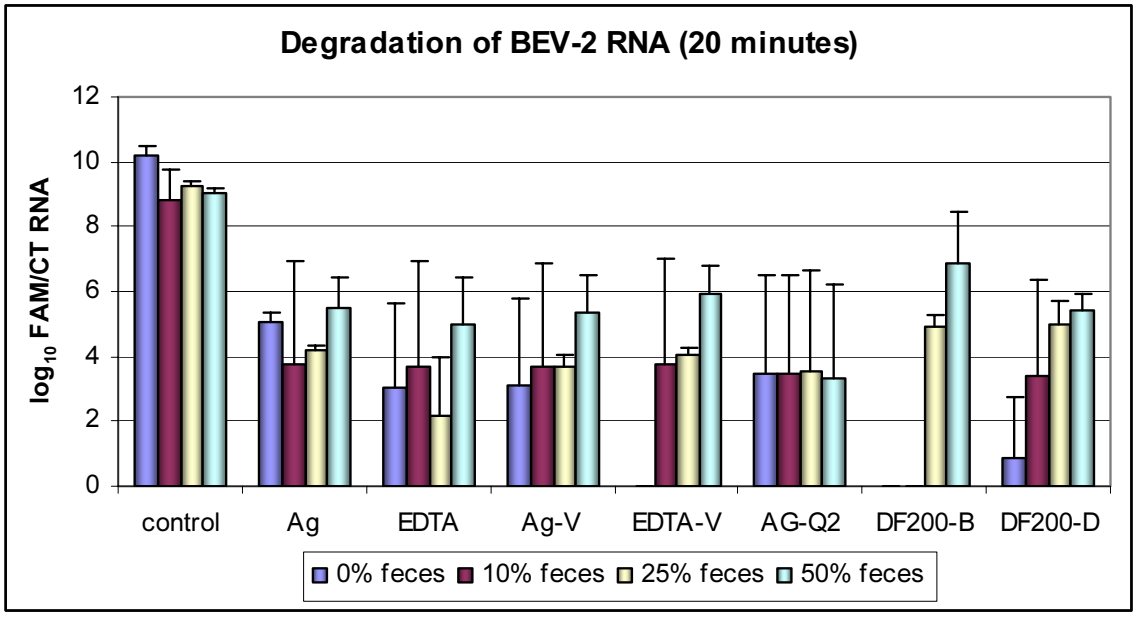


Figure 15: Degraded BEV-2 RNA following 20 minute exposure with 0, 10, 25, and 50% fecal challenges. Standard deviation is depicted with error bars. (Control, no treatment; Ag, Ag DF-200, EDTA, Ag DF-200/EDTA; Ag-V, Ag DF-200/Variquat; EDTA-V, Ag DF-200/EDTA/Variquat; AG-Q2, Aq-Q2 formulation; DF200-B, DF-200/Barquat; DF200-D, DF-200d)

Preliminary tests were conducted to ensure that the extracted samples containing the modified decontamination formulations did not have an inhibitory effect on the RT-PCR assay. None of the modified decontamination formulations demonstrated any such effect on inhibiting this assay (data not shown). In most cases, treatment with DF-200/Barquat was the most effective for degrading BEV-2 RNA. There did not appear to be significant differences in the amount of BEV-2 RNA that was degraded with the remaining modified decontamination formulations (Ag DF-200, Ag DF-200/EDTA, Ag DF-200/Variquat, Ag DF-200/EDTA/Variquat, DF-200d, Ag-Q2 formulation).

4. Conclusions

Modified formulations, overall conclusion: Based on favorable results of corrosion and spore kill tests, potential enhanced soil sorption of Barquat surfactant, and comparable performance on wettability, DF200/Barquat was the modified formulation selected for testing (along with DF200, DF200D, 10% bleach, 1%VirkonS, citric acid and sodium hydroxide) with live infectious agent, FMDv, at Plum Island. This modified formulation demonstrated increased detergency on butyl rubber surfaces, as compared to other test disinfectants.

To summarize the project, a modified decontaminant formulation with demonstrated moderate increase in detergency effectiveness on butyl rubber was selected for testing with live FMDv infectious agent at Plum Island. The modified formulation selected for live agent testing, as well as other modified formulations, maintained or slightly improved spore kill efficacy in the presence of 10% organic loads.

In relation to the project objective of corrosivity assessment, an industry standard test was performed on numerous disinfectants. The Sandia formulations were demonstrated as relatively non-corrosive on galvanized steel coupons as compared to 1% VirkonS, the disinfectant currently recommended by the USDA for use in agricultural environments.

Finally, Sandia formulations will be among those tested for efficacy against FMDv at Plum Island, beginning in the fall of 2005.

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