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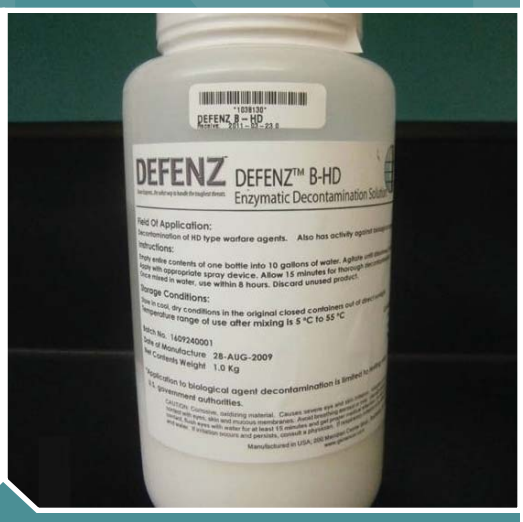
United States
Environmental Protection
Agency

Evaluation Report

Enzymatic Decontamination of Chemical Warfare Agents



Office of Research and Development
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Evaluation Report

Enzymatic Decontamination of Chemical Warfare Agents

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NORTH CAROLINA 27711

ERRATA Sheet

For the document: Enzymatic Decontamination of Chemical Warfare Agents (EPA/600/R-12/033) April 2012

Updated December 2012 with the following modifications:

- Section 2.6.2 (Page 11), the following text was added to clarify the procedure that was used:
  The test coupons were spiked with VX or TGD and allowed to weather for 30 minutes; then the DEFENZ VX-G enzyme was added for the specified contact time for the decontamination test. The positive control coupons were spiked with VX or TGD and allowed to weather for 30 minutes plus the specified contact time used for the test coupons.

- Section 2.6.3 (Page 13), the following text was added:
  The test coupons were spiked with HD and allowed to weather for 30 minutes; then the DEFENZ B-HD enzyme was added for the specified contact time for the decontamination test. The positive control coupons were spiked with HD and allowed to weather for 30 minutes plus the specified contact time used for the test coupons.

- A new Appendix A contains additional experimental data and discussion thereof that are directly related to the main body of the report. The data include the measurement of the enzymatic decontamination efficacy of the same enzyme containing decontamination products described in the main body of this report as derived from solution chemistry experiments without the presence of coupon surfaces. The additional data provides insights on the observed efficacies during bench scale coupon testing described in the main body of this report.

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EXECUTIVE SUMMARY

The U.S. Environmental Protection Agency (EPA) is the primary federal agency responsible for remediation in the aftermath of a terrorist release of chemical warfare agent (CWA). The imminent threat of release in a building or transportation hub resulted in EPA research on methods for effective neutralization/cleanup. As one of the potential tools/methods, EPA is systematically evaluating the effectiveness of enzyme-based decontamination technologies: DEFENZ™ VX-G (for decontamination of VX and G-type nerve agents) and DEFENZ™ B-HD (for decontamination of sulfur mustard [HD]). In addition, the extent to which the efficacy of the enzyme solutions changed after preparation and storage was evaluated. DEFENZ™ VX-G contains granulated organophosphorus acid anhydrolase (OPAA) and organophosphorus hydrolase (OPH) enzymes while DEFENZ™ B-HD contains an arylesterase enzyme that catalyzes a chemical reaction to produce peracetic acid.

Efficacy results, i.e., the difference in CWA recovered from positive controls and CWA recovered from decontaminated test coupons as a percentage of CWA recovered from positive control coupons, are summarized in Table ES-1. DEFENZ™ VX-G exhibited a statistically significant efficacy (Student's t-test $p < 0.05$) against VX on all materials tested except vinyl, with a 15-minute (min) contact time. Statistically significant efficacy means that the average measured amount of agent recovered from the test coupons after decontamination was statistically significantly lower than the average amount recovered from the positive control coupons (i.e., those without decontamination application). Tests on galvanized metal showed that efficacy against VX increased with increasing contact time and when higher concentrations of the enzymes were employed. DEFENZ™ VX-G exhibited a statistically significant efficacy against thickened soman (TGD) on carpet (but not against TGD on the other four test materials) with a 15-min contact time. DEFENZ™ VX-G enzymes applied to TGD on laminate showed that although no statistically significant efficacy was observed with a 15 min contact time, there was a higher efficacy with a 30-min contact time. After a 45-min contact time, less soman (GD) was recovered from laminate treated with enzyme than from positive controls, but the difference between the treated laminate and the positive controls was not statistically significant. Tests on galvanized metal showed that efficacy against TGD increased when higher concentrations of the enzymes were employed.

DEFENZ™ B-HD exhibited a statistically significant efficacy against HD on all five materials tested with 15-min contact time. Efficacy was increased by using a longer contact time (60 min, but not 30 min) for both vinyl and carpet.

No toxic byproducts were found to be produced by use of the DEFENZ™ VX-G or DEFENZ™ B-HD enzymes and no damage to the test material coupons was visually observed from the use of the enzymes.

Table ES- 1. Summary of Decontamination Efficacy Results

CWA	DEFENZ™ Enzyme	Material	Contact Time, min*	Concentration†	Mean Efficacy On Test Coupons
VX	VX-G	Laminate	15	1X	12% ($p < 0.01$)
VX	VX-G	Wood	15	1X	50% ($p = 0.05$)
VX	VX-G	Carpet	15	1X	19% ($p < 0.01$)
VX	VX-G	Vinyl	15	1X	19% ($p = 0.09$)
VX	VX-G	Galvanized metal	15	1X	11% ($p = 0.04$)
VX	VX-G	Galvanized metal	30	1X	23% ($p < 0.01$)
VX	VX-G	Galvanized metal	45	1X	26% ($p < 0.01$)
VX	VX-G	Galvanized metal	15	2X	29% ($p < 0.01$)
VX	VX-G	Galvanized metal	15	3X	39% ($p < 0.01$)
TGD	VX-G	Galvanized metal	15	1X	-5% ($p = 0.82$)
TGD	VX-G	Wood	15	1X	-9% ($p = 0.77$)
TGD	VX-G	Carpet	15	1X	42% ($p < 0.01$)
TGD	VX-G	Vinyl	15	1X	30% ($p = 0.19$)
TGD	VX-G	Laminate	15	1X	-37% ($p = 0.27$)
TGD	VX-G	Laminate	30	1X	48% ($p < 0.01$)
TGD	VX-G	Laminate	45	1X	24% ($p = 0.41$)
TGD	VX-G	Laminate	15	2X	68% ($p < 0.01$)
TGD	VX-G	Laminate	15	3X	51% ($p < 0.01$)
HD	B-HD	Galvanized metal	15	1X	24% ($p < 0.04$)
HD	B-HD	Laminate	15	1X	27% ($p < 0.01$)
HD	B-HD	Wood	15	1X	29% ($p < 0.01$)
HD	B-HD	Carpet	15	1X	16% ($p = < 0.01$)
HD	B-HD	Vinyl	15	1X	24% ($p = < 0.01$)
HD	B-HD	Vinyl	30	1X	7% ($p = 0.22$)
HD	B-HD	Vinyl	60	1X	35% ($p = 0.01$)
HD	B-HD	Carpet	30	1X	15% ($p = 0.09$)
HD	B-HD	Carpet	60	1X	30% ($p = < 0.01$)

* Manufacturer recommends 15-min contact time.
† 1X is enzyme diluted with deionized water per manufacturer's recommendation; 2X is diluted with half the recommended water; 3X is diluted with one-third of the recommended water.

While the DEFENZ™ VX-G and DEFENZ™ B-HD enzymes demonstrate efficacy, a substantial portion of the chemical agents (VX, TGD, and HD) can be extracted from the test

materials even after the longest contact times and using the highest enzyme concentrations evaluated. Longer contact times, or repeated applications, may be necessary to reduce the CWA to acceptable levels. Higher concentrations of DEFENZ™ VX-G than the manufacturer's recommendation may increase efficacy against VX and TGD. Likewise, use of longer contact times than the manufacturer's recommendation of 15 min for both DEFENZ™ VX-G and DEFENZ™ B-HD appears to increase efficacy. No loss of efficacy was observed for the DEFENZ™ VX-G and DEFENZ™ B-HD when prepared and stored according to manufacturer's recommendations.

Caution should be used in extrapolating from bench testing to field application of the enzymes. However, given the observed efficacies of the DEFENZ™ VX-G enzyme against VX and TGD and the DEFENZ™ B-HD enzyme against HD and the lack of visible damage to a range of indoor building materials, the enzymes appear to be technologies that might be considered for use against these CWA on indoor building materials after a terrorist release.

Activity of enzymes depends strongly on the manufacturer's production process. Hence, the results obtained for this report reflect solely on the commercially available DEFENZ™ enzyme decontamination products rather than the associated (OPAA and OPH) enzymes.

DISCLAIMER

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, funded and managed the research described here under EPA Contract Number EP-C-10-001, Work Assignment Number 2-04 to Battelle. This document has been subjected to the Agency's review and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency.

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FOREWORD

Following the events of September 11, 2001, EPA's mission was expanded to address critical needs related to homeland security. Presidential directives identify EPA as the primary federal agency responsible for the country's water supplies and for decontamination following a chemical, biological, and/or radiological (CBR) attack.

As part of this expanded mission, the National Homeland Security Research Center (NHSRC) was established to conduct research and deliver products that improve EPA's capability to carry out its homeland security responsibilities. One specific focus area of our research is on decontamination methods and technologies that can be used in the recovery efforts resulting from a CBR contamination incident. In recovering from an incident and decontaminating the area, it is critical to identify and implement appropriate decontamination technologies. The selection and optimal operation of an appropriate technology depends on many factors including the type of contaminant and associated building materials, temperature, relative humidity, decontaminant concentration, contact time, and others. This document provides information on how a commercially available enzyme containing decontamination product performed in treatment of chemical warfare agents (CWAs) deposited on interior industrial building materials at various operational conditions.

These results, coupled with additional information in separate NHSRC publications (available at www.epa.gov/nhsrc) can be used to determine whether a particular decontamination technology can be effective in a given scenario. With these factors in consideration, the best technology or combination of technologies can be chosen that meets the cleanup, cost and time goals for a particular decontamination scenario.

NHSRC has made this publication available to assist the response community to prepare for and recover from disasters involving chemical contamination. This research is intended to move EPA one step closer to achieving its homeland security goals and its overall mission of protecting human health and the environment, while providing sustainable solutions to environmental challenges.

Jonathan Herrmann, Director
National Homeland Security Research Center

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS/ACRONYMS

AMU	atomic mass unit
BBRC	Battelle Biomedical Research Center
°C	degrees Celsius
CCV	continuing calibration verification
CI	confidence interval
cm	centimeter(s)
CWA	chemical warfare agent
EPA	U.S. Environmental Protection Agency
FPD	flame photometric detection/detector
g	gram(s)
gal	gallon(s)
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
GD	soman
HD	sulfur mustard
HMRC	Hazardous Materials Research Center
HPLC	high performance liquid chromatography
IS	internal standard
kg	kilogram(s)
kHz	kilohertz
kPa	kilopascal(s)
L	liter(s)
LC/MS	liquid chromatography/mass spectrometry
m	meter(s)
MDL	method detection limit
MSD	mass selective detector
min	minute(s)
mL	milliliters(s)
mm	millimeter(s)

µm	micrometer(s)
µg	microgram(s)
µL	microliter(s)
ng	nanogram(s)
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
OPAA	organophosphorus acid anhydrolase
OPH	organophosphorus hydrolase
PAA	peracetic acid
PE	performance evaluation
psi	pounds per square inch
QA	quality assurance
QMP	quality management plan
QC	quality control
RDECOM	Research, Development and Engineering Command
RDS	research dilute solution(s)
RH	relative humidity
SD	standard deviation
SIM	selected ion monitoring
SRC	surrogate recovery compound
TBP	tributyl phosphate
TGD	thickened soman
TSA	technical systems audit

1.0 Introduction

1.1 Purpose

Protecting human health and the environment is the mission of the U.S. Environmental Protection Agency (EPA). The imminent threat of a chemical warfare agent (CWA) release in a building or transportation hub is driving the EPA to develop a research program that systematically evaluates potential decontaminants of CWAs. The EPA may be tasked to clean up these agents after a release. Most of the more efficacious decontamination technologies that have been identified are not compatible with all surface materials due to, e.g., their corrosive or bleaching characteristics, while some of the decontaminants may produce toxic by-products when they react with the CWA. A need therefore exists to identify decontamination methods that are nonreactive to building materials and that avoid toxic by-product formation. Enzymatic decontamination technologies are benign. However, effectiveness of available enzyme technologies against CWAs on many surfaces is unknown. In addition, the degree to which environmental conditions such as temperature and relative humidity (RH) affect decontamination is not known. The optimal decontaminant concentration and contact time have been determined primarily by vendors of decontaminants and are based predominantly on stirred reactor data. This report describes a systematic investigation to evaluate the efficacy of two enzyme-based technologies produced by Genencor® (a Danisco Division; Palo Alto, CA): DEFENZ™ VX-G (for decontamination of VX and G-type nerve agents) and DEFENZ™ B-HD (for decontamination of sulfur mustard [HD]). (In May 2011, DuPont acquired a majority stake in Danisco A/S and the Genencor® enzymes are now within DuPont Industrial Biosciences.) The effect of the decontaminant on the building material was assessed qualitatively.

Potential benefits for DEFENZ™ VX-G include lack of toxicity, high efficiency, high specificity and ease of use. DEFENZ™ B-HD provides an enzymatic method for on-site production of peracetic acid for HD decontamination, thereby avoiding safety issues associated with transportation and storage of this hazardous material.

1.2 Test Facility Description

All testing was performed at Battelle's Hazardous Materials Research Center (HMRC) or at the Battelle Biomedical Research Center (BBRC). Both facilities are located on the same Battelle site in West Jefferson, Ohio. Battelle is certified to work with chemical surety material at the HMRC through its Bailment Agreement W911SR-05-H-0001 with the U.S. Army Research, Development & Engineering Command (RDECOM). Battelle is certified to work with chemical surety material at the BBRC through its contract with the Defense Threat Reduction Agency (Contract Number: W81XWH-05-D-0001/DO 0001).

1.3 Project Objectives

The objective of this evaluation was to determine the decontamination efficacy of enzymatic decontamination technologies (DEFENZ™ VX-G against VX and thickened soman (TGD) and DEFENZ™ B-HD against HD) applied to coupons. The enzymes were initially prepared per manufacturer's label instructions and stored and used in accordance with the label

instructions. Efficacy of the enzymes when appropriately applied against VX, TGD, and HD was evaluated on each of five building materials (galvanized metal, decorative laminate, industrial carpet, wood flooring, and vinyl flooring) at one contact time (15 min as specified in the DEFENZ™ VX-G and DEFENZ™ B-HD instructions for use). Higher concentrations of DEFENZ™ VX-G and longer contact times were also evaluated. Specifically, a 2:1 and 3:1 mix of recommended enzyme to water was tested with a 15 min contact time of DEFENZ™ VX-G against VX and TGD. The recommended enzyme to water mix was also tested with a 30 and 45 min contact time for DEFENZ™ VX-G against VX and TGD while for DEFENZ™ B-HD, 30 and 60 min contact times were tested against HD.

The stability of the efficacy of prepared solutions ("pot life") with proper storage was also evaluated. Because some decontaminants react with CWAs to produce toxic by-products, a qualitative assessment of decontamination by-products was performed. In addition to the chemical analyses, a qualitative visual assessment for obvious damage was made by comparing blank coupons exposed to enzyme solution to blank coupons not exposed to enzyme solution.

Testing was performed in accordance with *Test/Quality Assurance (QA) Plan for Enzymatic Decontamination of Chemical Warfare Agents, Version 2* (July 2010) [1].

2.0 Procedures

2.1 Technology Descriptions

DEFENZ™ VX-G and DEFENZ™ B-HD are enzyme-based technologies produced by Genencor® (a Danisco Division, Palo Alto, CA). Details of the technologies are proprietary. The instructions on how to create the default enzyme solutions are per vendor's directions. DEFENZ™ VX-G contains granulated organophosphorus acid anhydrolase (OPAA) and granulated organophosphorus hydrolase (OPH) enzymes which are present in a 1:10 mass ratio.

The DEFENZ™ VX-G product consists of a pouch containing two packets: (1) an enzyme packet (110 grams (g) of granulated powder and (2) a buffer packet (250 g of powder) containing predominantly sodium hydrogen carbonate ($NaHCO_3$). The enzyme packet contains two pre-mixed constituent powders: 10 g of "organophosphorous [sic] acid anhydrolase" enzyme (DEFENZ™ 120G) and 100 g of "organophosphorous [sic] hydrolase" enzyme (DEFENZ™ 130G). The enzyme and buffer dissolve in 10 liters (L) of water. According to the vendor, DEFENZ™ VX-G has a shelf life of 3 years when stored properly in an unopened and sealed container and a pot life (defined as time that the enzyme is active in aqueous solution) of 8 hours.

DEFENZ™ B-HD is a perhydrolase-based enzymatic system for generating peracetic acid (PAA) as the active ingredient in the presence of water, propylene glycol diacetate, and sodium percarbonate. It is delivered as one kilogram (kg) of slurry to which 37.85 L (10 gallons [gal]) of water is added to activate the technology. After mixing in water, the solution is allowed to sit for 20 min (for oxidant generation) before use. The solution must then be used within 8 hours of mixing with water.

2.2 Chemical Warfare Agents

The CWAs used to evaluate the efficacy of decontamination were VX, TGD, and HD (Table 1). The target purity of neat agent was at least 85% and was verified for the specific agent lot using gas chromatography (GC)-flame photometric detection (FPD) prior to beginning testing and monthly during testing. TGD was prepared by addition of approximately 5% acrylic polymer to neat GD at least one week prior to use in decontamination testing. This thickener was added to reduce the volatility of GD so that sufficient GD could be recovered from a positive control coupon.

Table 1. Chemical Warfare Agents Used

Agent	Manufacturer/Supplier Name	Preparation Applied to Coupons
VX	US Army from EPA stocks*	Neat agent (as supplied)
TGD	US Army from EPA stocks*	Neat agent (as supplied) with 5% acrylic polymer (weight: volume; Paraloid K125, Rohm and Haas Company, Philadelphia, PA)
HD	US Army from EPA stocks*	Neat agent (as supplied)

*EPA-owned stocks of CWAs are stored at Battelle's facilities in West Jefferson, OH.

2.3 Building Material Coupons

This bench-scale investigation utilized small coupons of interior building materials (presented in Table 2) contaminated with CWAs.

Table 2. Test Materials

Material	Description	Manufacturer/ Supplier Name	Coupon Surface Size L x W (cm)	Material Preparation
Galvanized metal ductwork	Industry heating, ventilation, and air conditioning standard; 24 gauge galvanized steel; thickness 0.7 mm (Adept Manufacturing)	Adept Products, Inc., West Jefferson, OH	3.5 x 1.5	Clean with acetone
Decorative laminate	Pionite® or Formica® laminate/white matte finish; grade 10; thickness ~1.2 mm	A' Jack Inc., Columbus, OH	3.5 x 1.5	None
Industrial grade carpet	Shaw Industries, Inc. EcoWorx thickness ~0.7 cm	Carpet Corporation of America, Rome, GA	3.5 x 1.5	None
Flooring material	Fir plywood (bare); thickness 0.9 cm	Lowe's, Columbus, OH	3.5 x 1.5	Clean with dry air to remove loose dust
Vinyl flooring material	Armstrong Excelon	Lowe's, Columbus, OH	3.5 x 1.5	None

2.4 Coupon Spiking

For each CWA, enzyme-based decontamination technology, contact time, and material combination:

- Five replicate test coupons were spiked with CWA with subsequent decontamination
- Five replicate positive controls were spiked with CWA without subsequent decontamination
- Two procedural blanks were not spiked with CWA but were decontaminated
- Two laboratory blanks were not spiked with CWA and were not decontaminated
- For DEFENZ™ VX-G 15-min contact times only, five replicate solution controls were spiked with CWA treated with the enzyme-free buffer solution to assess whether observed decontamination was due solely to enzymatic action or through an effect of the buffered solution without enzyme present
- For DEFENZ™ BH-D 15-min contact times only, five replicate solution controls were spiked with CWA treated with deionized water to assess whether observed decontamination was solely due to enzymatic action or through an effect of water.

Because this product is a premixed slurry, an enzyme-free product could not be evaluated for efficacy.

All test and positive control coupons were nominally spiked with 1 microliter (µL) of neat or thickened CWA. This spiking volume delivered approximately 0.9 milligram (mg) of VX, TGD, or HD. The contamination level was approximately 2 g/square meter (m^2) (0.9 mg/ [3.5 centimeters (cm) x 1.5 cm] = 0.17 mg/cm^2 = 1.7 g/m^2). VX and HD were dispensed using a Hamilton syringe (P/N 80565 [50 µL] equipped with a 22-gauge needle [P/N 91022] and repeating dispenser [P/N 83700], Hamilton Co., Reno, NV).

TGD was dispensed using a positive displacement pipette (P/N F148504 [5-10 µL] and C-10 [10 µL] tip, Rainin Instrument LLC, Oakland CA). The pipette was initially set to dispense 1.4 µL to account for losses along the pipette wall and tip, nominally yielding 1 µL applied to the coupon. Adjustments were made to the pipette setting based on ongoing experience to improve accuracy of the volume applied. For the initial lot of TGD, the volume was increased to 1.6 µL; for a new lot, the volume was decreased to 1.2 µL.

Polytetrafluoroethylene (Teflon®) spike control coupons (P/N 5Y43BYD, Thomas Scientific, Swedesboro, NJ) were evaluated, one at the beginning, one at the middle and one at end of each trial (total of three spike control coupons per trial). A day of decontamination and subsequent extraction and analysis is referred to as a "trial". Each spike control coupon was spiked with three droplets of neat or thickened CWA, using the same pipette and pipette settings as were used for spiking the test and positive control coupons, then immediately placed in 20 milliliters (mL) of extraction solution, shaken for 15 seconds and passively extracted for one hour. The first spike control coupon was prepared at the beginning of the evaluation. The second spike control coupon was prepared midway through application of agent to test coupons and positive controls. The final spike control coupon was prepared after the last test coupon was contaminated. The mass of CWA per spiked droplet applied to test and positive control coupons is assumed to be equal to the mean of the CWA per droplet recovered from the spike control coupons calculated as follows:

$$\alpha = \frac{\sum_1^3 \mathrm{CWA}_i}{9 \text{ droplets}} \quad (1)$$

where:

α = Mean mass of CWA per spiked droplet

CWA_i = Mass of CWA recovered from the i^{th} spike control coupon

2.5 Preparation of Enzyme-Based Decontamination Technologies

2.5.1 Preparation Procedure for DEFENZ™ VX-G

The DEFENZ™ VX-G enzyme pouch contained two types of enzymes appropriate for G-type agents (DEFENZ™ 120) and VX (DEFENZ™ 130). Because the enzymes were together in a single pouch but may not be thoroughly mixed, the following method was used to ensure homogeneity among enzyme solutions prepared using only a portion of the enzyme mixture to make less than 10 L of enzyme solution.

The enzyme packet and buffer packet were opened and the contents were separately weighed. The weight ratio between the enzyme and the buffer (110:250) was the ratio used to

create smaller quantities. Laboratory batches of the buffer (sufficient to produce 500 mL of enzyme solution) were prepared by dividing the contents of the buffer packet (nominally 250 g) into 20 equal portions (12.5 g ± 0.1 g each) in separate appropriately labeled scintillation vials (03-337-14/vial; 02-912-068/cap, Fisher Scientific, Pittsburgh, PA). The vials of buffer powder were stored at ambient temperature in a desiccator until needed.

Laboratory batches of enzymes (each sufficient to produce 500 mL of enzyme solution) were prepared, as shown in Figure 1, to ensure product uniformity as much as practical. The enzyme packet contents (DEFENZ™ VX-G enzyme; nominally 110 g) were divided into five equal portions (samples 22 g ± 0.1 g each) using an analytical balance (Model AX-205 ID # C21236, Mettler-Toledo, Toledo, OH). Each sample was retained in a weighing pan (08-732-103, Fisher Scientific, Pittsburgh, PA). Five mixed samples (22 g ± 0.1 g each) were then produced by transferring an equal amount (4.4 g ± 0.1 g) from each sample into each of five new weighing pans (08-732-103, Fisher Scientific, Pittsburgh, PA). Twenty batches (5.5 g ± 0.25 g each) were then produced by transferring an equal amount (1.1 g ± 0.05 g) from each mixed prepared sample into each of 20 scintillation vials (03-337-14/vial; 02-912-068/cap, Fisher Scientific, Pittsburgh, PA). Each vial, sufficient to prepare 500 mL of enzyme solution, was marked to indicate that the vial contained DEFENZ™ VX-G enzyme (5.5 g) and stored at ambient temperature in a desiccator until needed.

Enzyme solutions were prepared fresh each day of testing in accordance with manufacturer's instructions, but with smaller, proportionate amounts of enzyme (5.5 g) and buffer (12.5 g). The preparation of DEFENZ™ VX-G is shown in Table 3. Deionized water was used to prepare the solutions.

Table 3. Enzyme-Based Decontamination Technology Concentrations

Formulae for Preparing DEFENZ™ VX-G Solutions		
Enzyme (g)	**Buffer (g)**	**Water (mL)**
Weight (g) in packet/10,000 mL x 4,000 mL (nominally 44 g)	Weight (g) in packet/10,000 mL x 4,000 mL (nominally 100 g)	4000*
1 vial containing DEFENZ™ VX-G enzyme, 5.5 g ± 0.25 g	1 vial containing DEFENZ™ VX-G buffer (sodium hydrogen carbonate), 12.5 g ± 0.1 g	500†

* This solution was prepared and used in method development to establish the mass of DEFENZ™ VX-G that remains on coupons of various types when applied with a sprayer (see Section 2.6.1).
†These solutions were prepared for use in decontamination testing.

The pH of the enzyme solution was measured and documented prior to each day of testing using a pH meter (pH/Ion Analyzer Model 350, Corning, Lowell, MA). The enzyme solutions used were verified as being within a specified range of pH (8.3 ± 0.5).

The time that the enzyme solution was prepared was documented along with the time at which the enzyme solution was used (applied to test coupons); elapsed time from preparation to use was documented.

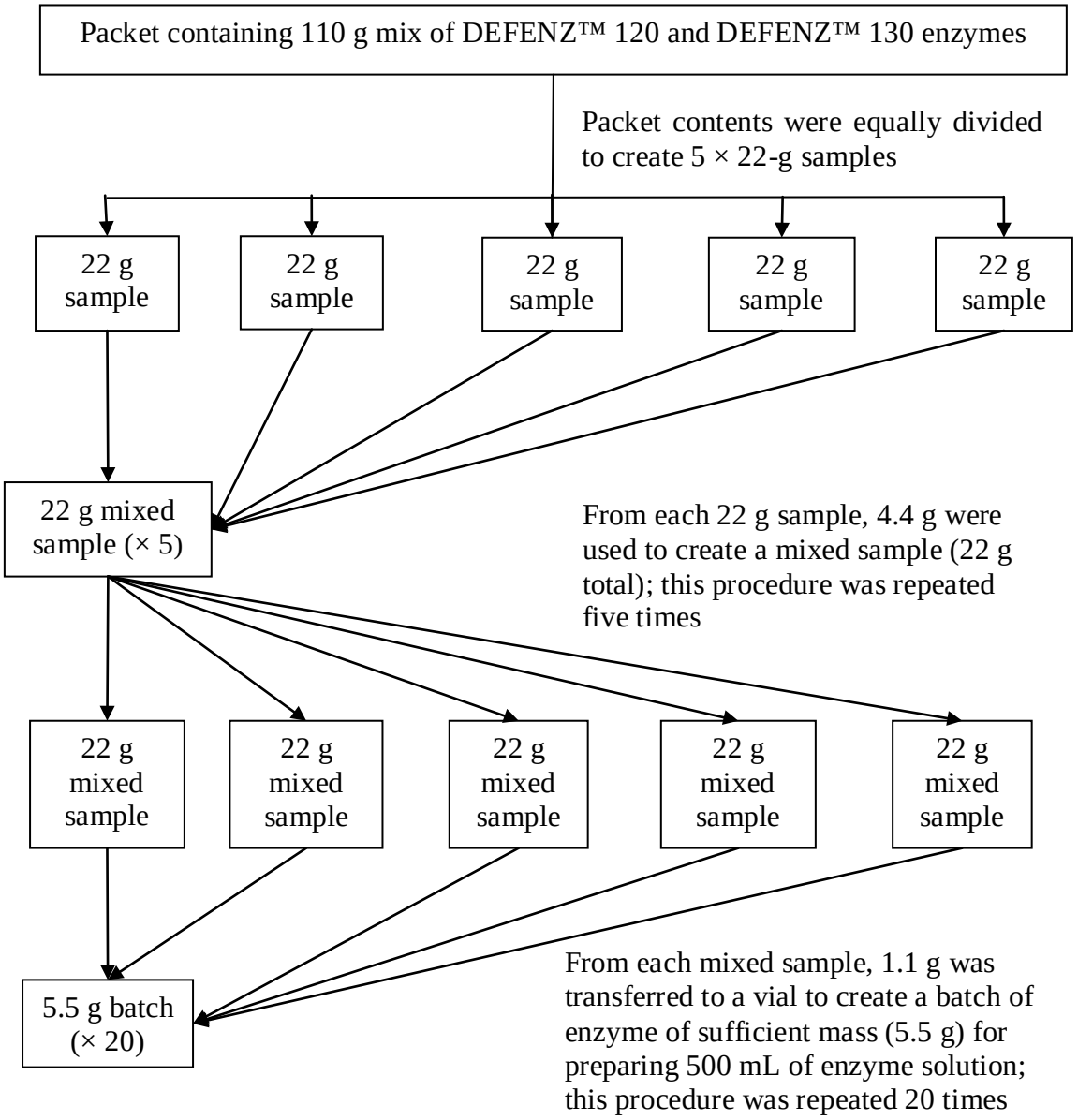

Figure 1. Approach used to ensure homogeneity of DEFENZ™ 120 and DEFENZ™ 130 enzymes in 500 mL enzyme test solutions.

2.5.2 Preparation Procedure for DEFENZ™ B-HD

Genencor® instructions for use of DEFENZ™ B-HD enzyme state: *Mix the entire contents (1.0 kg) into 10 gallons* [37.85 L] *of water. Agitate until dissolved and allow 20 min before use. Use within 8 hours.*

Because DEFENZ™ B-HD is a solution containing insoluble matter, a method was required to prepare user-ready solutions of less than 37.85 L. Genencor® recommended the use of good agitation followed by a fast transfer of the aliquots to make them as representative as possible. According to Genencor®, the key for successful operation of this enzyme is the

generated peracetic acid concentration. The amount of peracetic acid in each batch was measured before use.

The following method was employed to prepare and ensure the quality of bench-scale batches of the enzyme:

1. The mass (1.001 kg) and volume (730 mL) of the parent product were measured.
2. The ratio of the solid phase (435 mL) to the total volume (730 mL) was determined to be 0.6.
3. Twelve aliquots of the stock enzyme, sufficient for about 650 mL of activated enzyme after the addition of water, were prepared. Solid material was transferred to graduated conical tubes to the 7.5 mL level (the level was measured after about 5 min of settling); the liquid phase was added to 12.5 mL. This procedure maintained a solid phase to liquid phase ratio of 0.6, the same as the parent stock solution. The stability of the ratio was verified after 1 hour; no increased settling of the solid phase was observed.
4. The net weight of each enzyme aliquot was calculated as the difference in pre-weight and post-weight of the conical tube. The mean and standard deviation of the samples are tabulated in Table 4.

Table 4. Weight of Enzyme Aliquots and Volume of Make-up Water Required

Aliquot #	Pre-Weight (g)	Post-Weight (g)	Net Weight of Enzyme Aliquot (g)	Make-up Water (mL)
1	12.79	29.15	16.36	619
2	12.93	30.40	17.47	662
3	12.80	29.83	17.03	644
4	12.86	29.99	17.13	649
5	12.94	30.76	17.82	674
6	12.84	29.70	16.86	638
7	12.69	30.69	18.00	681
8	12.74	30.38	17.64	668
9	12.77	30.31	17.54	664
10	12.81	29.94	17.13	648
11	12.72	30.27	17.55	664
12	12.66	29.92	17.26	653
		Average:	**17.32**	
		Standard Deviation (SD):	**0.45**	
		%Relative SD:	**2.60%**	

The following method was employed to activate and use the enzyme on each day of testing:

1. Deionized water ("make-up water") was added to an aliquot of the stock enzyme solution in a conical tube. The amount of water was based on the mass of that specific aliquot of enzyme so that the label instruction ratio of 1 kg/10 gal (1000 g/37,850 mL; 1 g/37.9 mL) was maintained.
2. The concentration of peracetic acid in the solution was determined 25 min after an aliquot of the DEFENZ™ B-HD was activated by diluting 1 mL of the prepared enzyme sample with 9 mL of deionized water. The pH was measured and adjusted to a range of two to five with acetic acid. A sample of the activated DEFENZ™ B-HD enzyme solution was diluted 1:10 (to get within the range of the test strips) and EM Quant® Peracetic Acid Test Strips (Number 100011, EMD Chemicals, Gibbstown, NJ) were then used to measure the peracetic acid concentration. The activated enzyme solution was considered acceptable for use if the peracetic acid concentration was >2500 ppm.
3. The pH of the enzyme solution was measured prior to each day of use using a pH meter (pH/Ion Analyzer Model 350, Corning, Lowell, MA) and documented.
4. Except for the delayed testing ("pot life test"), the enzyme solution was applied to the test coupons no sooner than 25 min after mixing the enzyme with water and no later than 60 min after mixing the enzyme with water.
5. The time that the enzyme solution was prepared each day was documented along with the time at which the enzyme solution was used (applied to test coupons); elapsed time from preparation to use was documented.

2.6 Test Matrices

2.6.1 Spray Application Demonstration to Select Enzyme Application Rate

In field application of the enzyme product, use of a sprayer would be likely. In laboratory tests, the enzyme-based decontamination technologies were delivered to coupon surfaces as measured amounts from syringes or pipettes in order to reduce variability in amounts applied when compared to a spray application. A demonstration was used to determine the mass of enzyme solution (DEFENZ™ VX-G) that would be applied to a surface in a typical spray application. These data provided material-specific target values for the amount of enzyme solution to be applied to coupons to evaluate decontamination efficacy. The amount of enzyme-based decontamination technology that carried over into neutralization or extraction was determined by: (1) weighing the coupon before application of the enzyme-based decontamination technology, (2) spraying the enzyme solution onto the coupons, (3) waiting for the shortest contact time, and (4) weighing the coupon. The residual enzyme solution was calculated as the difference in the mass of the coupon with residual enzyme-based decontamination technology after the contact time less the mass of the coupon before application of the enzyme-based decontamination technology.

DEFENZ™ VX-G solution (4,000 mL) was prepared as described in Section 2.5.1. The solution was held at ambient laboratory conditions for one hour before use in the sprayer.

Four 1.5 x 3.5 cm coupons of each test material (galvanized metal, decorative laminate, industrial carpet, wood flooring, and vinyl flooring) were weighed on a calibrated balance. The 20 coupons were placed on a horizontal surface and arranged side by side to form a row with the long sides next to each other and approximately 5 – 8 cm between the coupons.

The enzyme solution was applied to the coupons in controlled tests using a full-scale pressurized tank sprayer (Solo® Model 425 DLX, Solo, Newport News, VA). The sprayer was selected as representative of garden-type sprayers that would be commercially available to decontamination response teams in local stores across the nation.

The coupons were sprayed with a sweeping motion after establishing uniform flow of liquid decontaminant from the sprayer at 207 kPa (30 psi). The tip of the sprayer nozzle was held 0.5 – 0.6 meters above the coupons and at an angle of 90° to the substrate surface. Spraying was continued by sweeping side to side until a continuous film of liquid covered the surface of the material. The rate of the sweeping motion was approximately one linear foot per second.

After spraying was completed, the coupons were covered loosely with a Petri dish to hinder evaporation until the final weight of each coupon was determined. Each sprayed coupon was weighed on a calibrated balance (Mettler Toledo PG 5002-SDR, Zurich Switzerland) to obtain its final weight. The mass of the enzyme-based decontamination technology applied to the coupon was determined by subtracting the initial coupon weight from the coupon weight post-spraying. For each type of material, the average amount of each enzyme-based decontamination technology retained on a coupon was calculated.

The test was repeated with three additional sets of coupons in order to characterize average results for sprayer performance with DEFENZ™ VX-G enzyme-based decontamination technology. The density of the enzyme-based decontamination technology was used, along with the average mass found for spraying the liquid on each of the materials, to calculate the average volume of enzyme-based decontamination technology retained on the coupons. The results of the spray-and-weigh demonstration were the basis for selecting the amount of enzyme solution to be applied to the CWA on the coupons in subsequent bench-scale testing. The same amounts, by volume, of DEFENZ™ B-HD were used as DEFENZ™ VX-G; spray tests were not repeated.

All testing was conducted under ambient laboratory conditions (maximum and minimum range during testing were 17 degrees Celsius [°C] – 20 °C and RH 51% - 58%). The same amounts of enzyme solutions were applied to each spot of CWA contamination on the 3.5 cm by 1.5 cm coupons for both the DEFENZ™ VX-G and DEFENZ™ B-HD decontamination testing and are shown in Table 5. The amount of enzyme solution applied to each spot of CWA in this study was the same as the amount of cleaner applied to CWA in a prior study[2] for the following materials: galvanized metal – 0.06 mL, decorative laminate – 0.06 mL, wood flooring – 0.09 mL, and carpet – 0.12 mL. Vinyl flooring was not used in the prior study. Note that, in field use, mechanical removal of CWA by splash and runoff may occur in addition to the enzymatic degradation. In this investigation, an effort was made to differentiate the chemical degradation derived from the functioning of the enzyme solution from mechanical removal by gently applying the enzyme solution directly to the CWA spots. The amount of enzyme applied directly to the CWA spots was approximately equal to the mass of enzyme retained on the entire surface of the nonporous coupons. Both wood and carpet retain a high mass of enzyme solution. The higher mass retained on industrial carpet and wood flooring is assumed to have soaked into the coupon and may not reflect the mass of enzyme available to interact with the CWA.

(Note: The spray demonstration method used in this study to determine the amount of enzyme solution retained on coupons was the same method used in a prior study[2] to determine the amount of cleaning solutions that were retained on building materials. The mass of enzyme

solution retained on the coupons after a four-second spray [exclusive of splash and runoff] is shown in Table 5. The measured density of DEFENZ™ VX-G was 1.022 g/mL.)

Table 5. Enzyme Application Amounts for Bench-Scale Testing

Material	Measured Mass of Enzyme Solution, g (SD)	Enzyme Solution Application, mL
Galvanized metal	0.053 (0.018)	0.06
Decorative laminate	0.075 (0.020)	0.06
Wood flooring	0.220 (0.119)	0.09
Industrial carpet	0.166 (0.070)	0.12
Vinyl flooring	0.084 (0.022)	0.06

2.6.2 DEFENZ™ VX-G Test Matrices

The DEFENZ™ VX-G enzyme-based decontamination technology was evaluated against VX and TGD using a 15-min contact time and manufacturer-specified enzyme concentration for the material combinations shown in Table 6. The test coupons were spiked with VX or TGD and allowed to weather for 30 minutes; then the DEFENZ VX-G enzyme was added for the specified contact time for the decontamination test. The positive control coupons were spiked with VX or TGD and allowed to weather for 30 minutes plus the 15 minutes contact time used for the test coupons.

The only potentially toxic by-product from VX decontamination is EA 2192. EA 2192 cannot be determined using GC/MS. No potentially toxic by-products from TGD decontamination were expected. Therefore, no GC/MS analysis was performed to quantify toxic by-products from VX or TGD decontamination. Instead, LC/MS was used for qualitative analysis of EA 2192 in VX/decontaminant solutions as described in Section 2.11.

Table 6. Test Matrix for Decontamination of CWA with DEFENZ™ VX-G Prepared per Manufacturer's Recommendations and 15-Min Contact Time

Agent	Material	Test Coupons*	Positive Controls†	Solution Controls#	Procedural Blanks‡	Laboratory Blanks§
VX	Galvanized metal	5	5	5	2	2
VX	Decorative Laminate	5	5	5	2	2
VX	Industrial Carpet	5	5	5	2	2
VX	Wood Flooring	5	5	5	2	2
VX	Vinyl Flooring	5	5	5	2	2
TGD	Galvanized metal	5	5	5	2	2
TGD	Decorative Laminate	5	5	5	2	2
TGD	Industrial Carpet	5	5	5	2	2
TGD	Wood Flooring	5	5	5	2	2
TGD	Vinyl Flooring	5	5	5	2	2

* Test coupons are spiked with CWA and undergo decontamination.
† Positive controls are spiked with CWA but do not undergo decontamination.

[#] Solution controls were controls where the coupon with CWA applied to surface was able to interact with enzyme-free buffer solution.
[‡] Procedural blanks are not spiked with CWA but undergo decontamination; one additional procedural blank was held for 48 hours (or longer if over a weekend) and examined for visually-obvious changes.
[§] Laboratory blanks were not spiked with CWA and did not undergo decontamination.

An adaptive management approach was used in which testing results were used to modify subsequent testing. Because of low efficacies against VX on all types of materials tested here, no useful data would be generated by using shorter contact times as anticipated in the test/QA plan[1]. Therefore, DEFENZ™ VX-G efficacy was not evaluated at shorter contact times. Instead, efficacies at two higher enzyme concentrations (namely, 2X and 3X) were evaluated for VX on galvanized metal and TGD on laminate. Galvanized metal and laminate were selected because these materials exhibited the least efficacy observed with a 15-min contact time of DEFENZ™ VX-G against VX (shown in Section 4.2.3) and TGD (shown in Section 4.2.4), respectively. The question being answered was whether a higher enzyme concentration would increase efficacy for decontaminating materials on which the lower (vendor recommended) enzyme concentration had the least efficacy. The test matrix for higher concentration enzyme solutions is shown in Table 7.

Table 7. Test Matrix for Increased DEFENZ™ VX-G Enzyme Concentration

Agent	Material	Contact Time, min	Enzyme Concentration	Test Coupons	Positive Controls	Procedural Blanks	Laboratory Blanks
VX	Galvanized metal	15	2X	5	5	2	2
VX	Galvanized metal	15	3X	5	5	2	2
TGD	Decorative Laminate	15	2X	5	5	2	2
TGD	Decorative Laminate	15	3X	5	5	2	2

The manufacturer's instructions call for the contents of the enzyme packets to be mixed into 10 L of water. For the 2X concentration (i.e., two times the concentration recommended by the manufacturer), the contents would be mixed with 5 L of water and for the 3X concentration the enzyme would be mixed in 3.3 L of water. Actual mixtures were based on this proportion applied to the amounts of enzyme in the "batch packets" as follows:

- 2X mix: Add the contents intended for 500 mL to 250 mL of deionized water
- 3X mix: Add the contents intended for 500 mL to 167 mL of deionized water.

The granulated enzyme and buffer dissolve completely in deionized water at the 2X and 3X concentration levels. A 5X concentration was considered for testing; however, the enzyme or buffer products did not dissolve completely at this concentration and, therefore, a 5X concentration was not tested.

Efficacy as a function of pot life time, defined as the time between preparation of the enzyme solution and actual application onto the coupon surface, was evaluated using the test

matrix in Table 8. Enzyme solutions (3X DEFENZ™ VX-G) were prepared and placed in sealed containers in a refrigerator (approximately 4 °C) in accordance with the storage conditions for prepared enzyme that are recommended by Genencor®: "below 15 °C (59 °F) and out of sunlight." One hour before use (5, 14, and 23 hours, respectively), the 3X enzyme solutions were removed from the refrigerator and allowed to sit at room temperature. The 3X enzyme solution was then applied to decontaminate carpet spiked with VX or TGD. A 15-min contact time was used for the pot life test. Carpet was selected for the pot life test because carpet demonstrated high efficacy with a contact time of 15 min for DEFENZ™ VX-G against VX (shown in Section 4.2.3) and TGD (shown in Section 4.2.4), respectively. The extraction and analysis procedures were identical to the procedures used for the other efficacy testing.

Table 8. Test Matrix for Effect of Storage of Activated 3X DEFENZ™ VX-G Enzyme Solution on VX Decontamination Results

Agent	Material	Pot Life, hours	Test Coupons	Positive Controls	Procedural Blanks	Laboratory Blanks
VX	Carpet	6	5	5	2	2
VX	Carpet	15	5	5	2	2
VX	Carpet	24	5	5	2	2
TGD	Carpet	6	5	5	2	2
TGD	Carpet	15	5	5	2	2
TGD	Carpet	24	5	5	2	2

2.6.3 DEFENZ™ B-HD Test Matrices

DEFENZ™ B-HD was evaluated against HD following the test matrix in Table 9. The amounts of DEFENZ™ B-HD applied for decontamination of each type of material was the same as for DEFENZ™ VX-G and is shown in Table 5. The test coupons were spiked with HD and allowed to weather for 30 minutes; then the DEFENZ B-HD enzyme was added for the specified contact time for the decontamination test. The positive control coupons were spiked with HD and allowed to weather for 30 minutes plus the specified contact time used for the test coupons.

Table 9. Test Matrix for Decontamination of CWA with DEFENZ™ B-HD

Agent	Material	Test Coupons	Positive Controls	Solution Controls	Procedural Blanks	Laboratory Blanks
HD	Galvanized Metal	5*	5*	5*	2*	2*
HD	Decorative Laminate	5	5	5	2	2
HD	Industrial Carpet	5*	5*	5*	2*	2*
HD	Wood Flooring	5	5	5	2	2
HD	Vinyl Flooring	5	5	5	2	2

*Qualitative assessment of decontamination by-products was performed using full scan GC/MS.

Because efficacies against HD were not high on any material after a 15-min contact time, DEFENZ™ B-HD efficacy was not evaluated at shorter contact times. Instead, efficacies at two higher contact times, 30 min and 60 min, were evaluated for HD on two materials (carpet and vinyl). Carpet and vinyl were selected for testing at the longer contact times because DEFENZ™ B-HD exhibited low efficacies against HD on these materials at a 15-min contact time (shown in Section 4.2.5). No solution controls were included and the test matrix is shown in Table 10.

Table 10. Test Matrix for Longer Contact Times with DEFENZ™ B-HD

Agent	Material	Contact Time, min	Test Coupons	Positive Controls	Solution Controls	Procedural Blanks	Laboratory Blanks
HD	Carpet	30*	5	5	0	2	2
HD	Carpet	60*	5	5	0	2	2
HD	Vinyl	30	5	5	0	2	2
HD	Vinyl	60	5	5	0	2	2

*Qualitative assessment of decontamination by-products was performed using full scan GC/MS.

Efficacy of DEFENZ™ B-HD solution as a function of pot life time was evaluated using the test matrix shown in Table 11. The DEFENZ™ B-HD solution was mixed as described in Section 2.5.2. The prepared enzyme solution was allowed to sit at room temperature for 2, 4, and 6 hours before use. The enzyme solution was then applied to decontaminate wood spiked with HD. The contact time was 15 min. Wood was selected for the pot life test because DEFENZ™ B-HD demonstrated high efficacy against HD with a contact time of 15 min (shown in Section 4.2.5).

Table 11. Test Matrix for Delayed Application of DEFENZ™ B-HD Enzyme Solution

Agent	Material	Pot Life, hours	Test Coupons	Positive Controls	Procedural Blanks	Laboratory Blanks
HD	Wood	2	5	5	2	2
HD	Wood	4	5	5	2	2
HD	Wood	6	5	5	2	2

2.7 Observation of Surface Damage

Representative digital photographs were taken of coupons before and after they were exposed to the enzyme solution decontamination for 15 min to document observed impacts or absence thereof. One of the decontaminated procedural blanks of each material type was rinsed with deionized water and allowed to dry. Additional photographs were taken of this procedural blank that was rinsed but not extracted two days after testing (or more if 'two days' fell on a weekend) to document any visually obvious changes that occurred to a procedural blank. They were visually inspected and compared to coupons not exposed to the decontamination treatment to look for obvious changes in appearance of the procedural blanks (for example, in the color, reflectivity, or apparent roughness of the coupon surfaces). Differences were recorded in the evaluation records. In addition, representative photographs were taken two days after testing to

document any visually obvious changes that may have occurred. Observations and photographs of pre- and post-decontaminant coupons are included in Section 4.4.

2.8 Extraction and Analysis

After the appropriate contact time the test, positive control, solution control, procedural blank, and laboratory blank coupons were transferred to individual extraction bottles (P/N 89044-462, VWR International, West Chester, PA) containing 10 mL of hexane with an internal standard (IS). The hexane extraction solvent contained naphthalene as an IS. The extraction bottles were sealed, shaken by hand for about 5-10 seconds, and placed into a sonicator. After all bottles containing coupons to be extracted for a given time point were placed in the sonicator, they were sonicated at 40 - 60 kilohertz (kHz) for 10 min. Within 30 min after the completion of sonication, a 1.0 mL aliquot was transferred to a GC vial (P/N 06-718-476 and 03-391-6, Fisher Scientific [Restek Corp], Hanover Park, IL 60133) and sealed. This process was repeated for all samples until each test, positive control, solution control, procedural blank, and laboratory blank coupon had been shaken, sonicated, and aliquoted for analysis.

All test, positive control, solution control, procedural blank, and laboratory blank coupons were individually extracted and the amount of CWA in the extraction solution was determined using a GC/mass spectrometer (MS, Model 6890, Agilent Technologies, Santa Clara, CA). Samples were analyzed using an Agilent (Palo Alto, CA) 6890N Series GC interfaced to a 5973 network quadrupole mass selective detector (MSD). Chromatographic separation of the analytes was conducted using an RTX-5 (cross-linked methyl silicone) fused silica capillary column, 30.0 m (or 29.5 m) length x 0.25 millimeter (mm) diameter x 0.5 micrometer (µm) coating thickness. The GC/MS parameters for VX, GD and HD analysis are shown in Table 12.

Table 12. Gas Chromatography/Mass Spectrometry Parameters for VX and GD Analysis

Parameters	Description	
	VX and GD	HD
Analysis Method:	GC/MS (Scan)	GC/MS (Scan)
Model & SN:	HP6890N GC (CN10331014) & 5973N MSD (US30985853)	HP6890N GC (US00042609) & 5973N MSD (US10460658)
Data System:	MSD ChemStation	MSD ChemStation
Liner Type:	4 mm Split/Splitless	4 mm Split/Splitless
Column:	RTX-5MS, 30.0 m or 29.5 m length, 0.25 mm diameter, 0.5 µm film coating thickness	RTX-5MS, 30.0 m length, 0.25 mm diameter, 0.25 µm film coating thickness
Mode:	Constant flow (1.5 mL/min)	Constant flow (1.3 mL/min)
Inlet (Injector) Temperature:	250 °C	250 °C
Detector Temperature:	230 °C	230 °C
Sample Size:	1 µL	1 µL

Oven Program for VX and GD Analysis:	50 °C (hold 2.0 min), increase at 20 °C/min to 250 °C (hold 0.0 min), increase at 35 °C/min to 300 °C (hold 0.5 min)	40 °C (hold 1.0 min), increase at 8 °C/min to 300 °C (hold 0.0 min), increase to 325 °C (hold 0.0 min)

The mass selective detector was operated in the full-scan mode for compounds ranging from 28 to 500 atomic mass units (AMUs). Where concentrations were too low to quantitate in the full-scan mode, the samples were scanned again in the selected ion monitoring (SIM) mode. The GC/MS measurements were used to compare and evaluate co-extractive sample components and CWA response. Table 13 outlines the ion masses which were used to quantitate the CWAs. CWA decontamination by-products were qualitatively assessed by examining the GC chromatogram in full scan mode.

Table 13. Pertinent Parameters for Target Chemicals

Analyte	SIM Ions
VX	114, 127, 72
TGD	99, 126, 69, 82
HD	158, 109, 160

2.9 Method Demonstration

2.9.1 Recovery of CWA from Test Coupons

Method demonstration was conducted and consistent with previous testing, to establish that extraction efficiencies (recoveries) from test coupons were sufficiently high and to establish method detection limits (MDL[s]) for CWAs and material combinations for which such information had not previously been demonstrated in the laboratory. The extraction efficiency was determined as a percent of the agent recovered from the spiked coupon relative to the amount spiked. The extraction method was acceptable if the extraction efficiency was 40% - 120% with a coefficient of variance between samples not exceeding 30%.

Hexane was selected to extract VX, TGD, and HD from the aqueous phase based on prior experience.[2, 3] Vinyl was the only material for which no recovery had been demonstrated. Therefore, eight vinyl coupons were each spiked with 10 µL of 875 micrograms (µg)/mL VX or 1,000 µg/mL GD research dilute solutions (RDS; CWA diluted with hexane [GC Resolv grade, Fisher Scientific, Pittsburg, PA]). The surrogate recovery compound (SRC) was also applied to the coupon surface. Sufficient hexane to cover the coupons (10 mL) was used for each extraction. The coupons were transferred into hexane within 0.5 min of spiking with dilute CWA solution. Immediately after transfer, the vial was capped and shaken by hand for 5-10 seconds and placed into a sonicator. After all vials containing coupons to be extracted were placed into the sonicator, the samples were sonicated at 50-60 kHz for approximately 10 min. Within 30 min after the completion of sonication, an aliquot of extract was transferred to a GC vial (P/N HP-5181-880, VWR [Agilent Technologies], West Chester, PA) and sealed. The amount of spiked CWA was confirmed using control samples where the same dilute solution was spiked directly into hexane and analyzed.

Because GD RDS recoveries were low, extraction efficiency demonstration was subsequently repeated by spiking each of two vinyl coupons with 1 µL of neat GD and 1 µL of neat SRC using a Hamilton syringe (P/N 80565 [50 µL]). The coupons were placed in 10 mL of hexane within 0.5 min of spiking with neat agent and SRC. The process and analysis, described in the preceding paragraph, was repeated for the extracts of vinyl spiked with neat GD.

HD recovery from vinyl was determined by spiking each of three vinyl coupons with 10 µg HD (10 µL of a 1 mg/mL RDS solution).

The aliquots of hexane extracts of coupons spiked with CWA (1 µL) and aliquots of hexane containing the same spike amount as applied to the coupons were analyzed for CWAs as described in Section 2.8.

Extraction efficiency was calculated using a series of equations. The CWA concentration in a coupon extract or spiked hexane sample was determined by Equation 2:

$$\frac{A_s}{A_{is}} = M\frac{C_s}{C_{is}} + W \tag{2}$$

where:

A_s = Area of the target analyte peak in the sample
A_{is} = Area of the internal standard peak
C_s = Concentration of the target analyte in the sample (µg/mL)
C_{is} = Concentration of the internal standard (µg/mL)
M = Slope of the GC calibration line
W = Y intercept of the GC calibration line

GC concentration results (µg/mL) were converted to total mass by multiplying by extract volume:

$$M_m = C \times E_v \tag{3}$$

where:

M_m = Measured mass of CWA (µg)
C = GC concentration (µg/mL)
E_v = Volume of extract (mL)

Extraction efficiency was then defined as:

$$Extraction\ Efficiency = \left(\frac{M_m\ of\ CWA\ on\ Test\ Coupon}{M_m\ of\ CWA\ in\ Hexane}\right) \times 100\% \tag{4}$$

2.9.2 MDL for VX, GD, and HD Extracted from Vinyl

The MDLs for VX, GD, and HD extracted from vinyl were determined following the EPA procedure.[4] The MDLs were calculated as follows:

$$MDL = t(n\text{-}1,1\text{-}\alpha = 0.99) \times SD \tag{5}$$

where:

$t(n-1,1-\alpha = 0.99)$ = the Student's t-value for a 99% confidence level and standard deviation estimate with n-1 degrees of freedom.
SD = standard deviation of the replicate analyses

2.9.3 Quench of Decontamination Reaction

Hexane extraction was expected to remove the CWA (reactant) from the aqueous phase in which the enzyme is active thereby halting (quenching) the decontamination reaction. Enzymes are not expected to be functional in the nonpolar phase so other additives are not expected to be needed to quench the reaction. The neutralization method was assumed not to be impacted by the coupon material. Quench methods were therefore evaluated using solution tests.

The use of hexane extraction as a quench method was assessed as follows:

1. Fifty µL of enzyme was added (using a positive displacement pipette (P/N M-250 [250 µL] and D-200 [2-200 µL] tip, Gilson Inc., Middleton, WI) to a vial containing 10 mL of hexane and IS (naphthalene-d_8) and 1 µL of CWA, shaken for 15 seconds, and allowed to stand for 10 min.
2. Distilled water, equivalent to the amount of enzyme solution in Step 1, was added (using a positive displacement pipette) to a vial containing 10 mL of hexane and IS (naphthalene-d_8) and 1 µL of CWA, shaken for 15 seconds, and allowed to stand for 10 min.
3. The extracts from Steps 1 and 2 were analyzed using GC/MS. Extraction alone, without additional neutralization, was acceptable for GC/MS analysis if the amount of CWA recovered in Step 1 (enzyme present) was at least 70% of the amount of CWA recovered in Step 2 (no enzyme present). All Agent recoveries with hexane exceeded the required 70% (Table 14).

Table 14. Recovery of Agent Using Hexane Extraction as Quench

Agent	Recovery with Water, µg/mL (SD) n = 3	Recovery with Enzyme, µg/mL (SD) n = 3	Mean % Hexane Recovery "with Quenched Enzyme" Compared to "with Water"
VX	20.1 (0.20)	18.1 (0.24)	90
GD	21.8 (0.18)	22.3 (0.33)	102
HD	21.0. (0.35)	19.0 (1.53)	90

2.9.4 EA 2192 LC/MS Analysis

Liquid chromatography/mass spectrometry (LC/MS) analysis for the EA 2192 by-product in the aqueous phase required that VX degradation by the enzyme be quenched. Hexane would be considered effective as a quench if (1) VX recovery from hexane was not reduced by enzyme in the aqueous phase (VX recovery >70%) and (2) the amount of EA 2192 recovered from the aqueous phase was >70% of an EA 2192 spike. This recovery was demonstrated by spiking 1 µL of neat VX containing 648 µg of VX (900 µg/µL for pure VX × 72% purity), using a Hamilton syringe (P/N 80565 [50 µL], into each of six 60-mL vials (GLC-04869, Qorpak, Bridgeville, PA)

containing 5 mL hexane with 5 μg/mL naphthalene-d8 (IS) and capping the vial. A solution containing deionized water (2 mL) and decontaminant (50 μL) was then added to each of three vials of VX in hexane with IS. Similarly, 2.05 mL of deionized water was added to each of the three remaining vials containing VX in hexane with IS. All vials were closed and sonicated for 10 min (50-60 kHz). The mixtures were allowed to sit for 15 min to enable the polar and nonpolar phases to separate. The hexane phase from each vial was analyzed for VX using GC/MS. The aqueous phase was frozen at -20 °C and subsequently analyzed for EA 2192 using LC/MS as a positive control (containing EA 2192 as a naturally-occurring by-product) to compare to EA 2192 present after VX was decontaminated with DEFENZ™ VX-G.

The recovery of EA 2192 in water or aqueous enzyme (when sonicated with hexane) was evaluated. Ten microliters of an EA 2192 solution (1.4 μg EA 2192/mL of deionized water) was spiked into each of six 60-mL vials (GLC-04869, Qorpak, Bridgeville, PA) containing 5 mL hexane IS. A solution of deionized water (2 mL) and decontaminant (50 μL) was added to three vials containing hexane and IS. Deionized water (2.050 mL) was added to each of the remaining three vials containing hexane and IS. The vials were closed and sonicated for 10 min (50-60 kHz), then allowed to sit for 15 min to enable the polar and nonpolar phases to separate. The aqueous phase was analyzed for EA 2192 using LC/MS.

The results of the hexane extraction of VX to quench the enzyme reaction are shown in Table 15. High percentages of VX were recovered from the hexane layer in the presence of water (90% recovered) or the aqueous solution of DEFENZ™ VX-G enzymes (103%). Further, there was no significant difference between the amount of VX recovered from hexane with water present or with enzyme present ($p = 0.35$). Because the recoveries of VX were >70%, hexane extraction of the VX reaction was considered sufficient to quench the enzyme reaction in subsequent decontamination efficacy testing.

EA 2192 is a degradation by-product that was present in the VX spike as observed in the aqueous phase of both the water/hexane (with VX) extract and the DEFENZ™ VX-G/hexane (with VX) extract. There was significantly less EA 2192 extracted from the neat VX (in hexane) with water present than from the neat VX (in hexane) with enzyme present ($p = 0.005$). However, these samples were transported between laboratories and stored at less than -20 °C for several months between the time of the VX quench test and the analysis of the EA 2192 quench test. Transportation and the passage of time may account for this anomaly. As noted below, when analyzed immediately, no significant differences were noted in EA 2192 in water or in DEFENZ™ VX-G solution.

The results of the EA 2192 stability demonstration are shown in Table 16. From water without enzyme mean recovery was 99%. From water with enzyme mean recovery was 79%. The recoveries of the EA 2192 spike from the aqueous phase of the DEFENZ™ VX-G/hexane extraction met the test/QA criterion of exceeding 70% of the amount of EA 2192 recovered from the aqueous phase of the water/hexane extract receiving comparable treatment, so hexane extraction of VX was considered sufficient to quench the enzyme reaction. There was no significant difference in the amount of EA 2192 extracted by hexane with water present or with enzyme present ($p = 0.15$).

To test for potential ion suppression by the enzyme solution that could result in artificially low EA 2192 values, a known mass of EA 2192 (2.55 ng) was added to both decontamination samples and positive control samples and quantified. A mean of 95% (SD 35%)

of the added EA 2192 was recovered from the positive control samples and a mean of 82% (SD 7%) of the added EA 2192 was recovered from the test samples. Based on the results from this test, there was no ion suppression.

Table 15. Demonstration of Hexane as Quench for Enzyme Reaction Prior to LC/MS Analysis for EA 2192

Sample Type	VX Spike, µg	VX Recovered, µg	Mean VX Recovery, µg (SD)	% Recovery	Mean VX % Recovery	EA 2192 Recovered, µg	Mean EA 2192 Recovery µg (SD)
Water in Hexane	648	572.5	584.8 (60.0)	88%	90%	0.763	0.864 (0.191)
Water in Hexane	648	650		100%		0.744	
Water in Hexane	648	532		82%		1.084	
DEFENZ™ VX-G in Hexane	648	589	665.2 (112.6)	91%	103%	1.365	1.735 (0.382)
DEFENZ™ VX-G in Hexane	648	794.5		123%		2.128	
DEFENZ™ VX-G in Hexane	648	612		94%		1.712	

Table 16. Recovery of EA 2192 from Aqueous Phase after Hexane Extraction

Sample Type	EA 2192 Spike, ng	EA 2192 Recovered, ng	EA 2192 Recovery	Mean EA 2192 Recovery, ng (SD)	Mean EA 2192 % Recovery
EA 2192 (Water/ Hexane)	14	11.77	84%	6.74 (1.07)	99%
EA 2192 (Water/ Hexane)	14	16.11	115%		
EA 2192 (Water/ Hexane)	14	13.55	97%		
EA 2192 DEFENZ™ VX-G in Hexane	14	12.44	89%	5.41 (0.66)	79%
EA 2192 DEFENZ™ VX-G in Hexane	14	9.76	70%		
EA 2192 DEFENZ™ VX-G in Hexane	14	11.05	79%		

2.10 Efficacy Determination

Decontamination efficacy was determined by measuring the amount of residual CWA on test coupons and comparing them with positive controls (spiked with CWA, not decontaminated and analyzed after the same "contact time" as the test coupons). Aliquots of extracts from blanks, positive controls, and decontaminated coupons were analyzed for CWAs according to methods described in Section 2.8. Decontamination efficacy was calculated as follows:

1) Calculation of CWA (or SRC) concentration in a coupon extract sample is determined by Equation 5:

$$\frac{A_s}{A_{is}} = M\frac{C_s}{C_{is}} + W \tag{5}$$

where:

A_s = Area of target analyte peak in sample
A_{is} = Area of internal standard peak
C_s = Concentration of target analyte in sample (µg/mL)
C_{is} = Concentration of the internal standard (µg/mL)
M = slope of the GC calibration line
W = Y intercept of GC calibration line.

2) GC concentration results (µg/mL) are converted to total mass by multiplying by the extract volume:

$$M_m = C_s \times E_v \tag{6}$$

where:

M_m = Measured mass of target analyte (CWA or SRC) (µg)
C_s = GC concentration (µg/mL) of target analyte
E_v = Volume of extract (mL).

3) Decontamination efficacy (percent removal achieved during decontamination) is then defined as:

$$E = \left(1 - \frac{M_m \ of \ CWA \ on \ Test \ Coupon}{M_{\bar{m}} \ of \ CWA \ on \ Positive \ Control \ Coupon}\right) \times 100\% \qquad (7)$$

where:

M_m = Measured mass of CWA (μg) on an individual test coupon (or solution control coupon)

$M_{\bar{m}}$ = Mean measured mass of CWA (μg) from five positive control coupons.

The mean efficacy is the average efficacy from five test coupons included in a given decontamination test (i.e., enzyme type, enzyme concentration, and contact time). A statistically significant efficacy is defined when the average measured amount of agent recovered from the test coupons after decontamination is statistically significantly (Student's t-test $p < 0.05$) lower than the average amount recovered from the positive control coupons (i.e., those without decontamination application).

2.11 Analysis of By-Products

Based on previous non-enzymatic decontamination testing,[1] a variety of by-products may be produced during decontamination of HD, such as: o-mustard; 2-thiophene acetonitrile; 3-chloro-2-methylthiopropene; thiocyanic acid, 2-(2-butoxyethoxy) ethyl ester; 1,3-bis(ethylthio) propane; divinyl sulfone; bis(beta-chloroethyl) sulfone (mustard sulfone); and bis(beta-chloroethyl) sulfoxide (mustard sulfoxide). The GC/MS instrumentation was operated in the full scan mode to detect such toxic CWA by-products in coupon extracts. A National Institute of Standards and Technology (NIST) 2002 mass spectral library was used to tentatively identify compounds in the mass spectra. Reports were generated using ChemStation software (Version D.01.02.16 [15 June 2004], Agilent, Santa Clara, CA).

LC/MS was used for qualitative analysis for EA 2192 in VX/decontaminant solutions. EA 2192 is a highly toxic VX degradation by-product. LC/MS was used because EA 2192 cannot be resolved using GC analytical methods. The test matrix for the LC/MS by-product analysis is shown in Table 17.

Table 17. Test Matrix for LC/MS EA 2192

Agent	Sample Type	# Replicates
VX	DEFENZ™ VX-G concentrated (3X) enzyme solution with CWA (test solutions)	3
VX	Water (rather than enzyme) with CWA (positive control)	3
None	Neutralized enzyme solution (solution blank)	3

For each "test solution" sample, 50 μL of the liquid decontaminant was added using a Hamilton syringe (P/N 80565 [50 μL]) to a 20-mL vial (66022-060, VWR) containing 1 μL of neat VX and mixed. The contact time between the concentrated (3X) enzyme and the VX solution was 15 min. The "3X" decontaminant is the enzyme solution prepared with only one-third the label-specified dilution with water. As positive control solutions, deionized water (50 μL) was added to each of three 20-mL vials containing 1 μL of neat VX.

All vials containing test and positive control solution were closed and sonicated for 1 min (50-60 kHz) after decontaminant or deionized water, respectively, was added. The mixture was allowed to sit for 15 min. The reaction was halted at the end of the contact time by extraction with hexane to remove the VX substrate from the aqueous solution containing the active enzyme. Specifically, 50 µL of deionized water and 5 mL hexane were added to each of the test and positive control solutions. After the hexane was added to the vials, they were closed and sonicated for 10 min (50-60 kHz), then allowed to sit for 15 min to enable the polar and nonpolar phases to separate. The aqueous phase of each positive control and test solution was serially diluted with deionized water (1:10 of 1:25 of 1:50).

The EA 2192 was analyzed using an LC/MS system that consisted of a Shimadzu 20_{XR} solvent delivery system, a Prodigy ODS-3, 2.1 x 150 mm, 5 µm analytical column (Phenomenex, Torrance, CA) for chromatographic separation, and an Applied Biosystems 5500 mass spectrometer operated using positive electrospray. LC/MS parameters are shown in Table 18. Data were acquired for ion transitions 240>139 and 240>128. Samples that were not analyzed the same day were stored at -20 °C or lower.

During method demonstration, a standard curve of EA 2192 in water was analyzed at 0.2, 0.5, 1.0, 2.5, 5.0, 9.8, and 25.2 ng/mL. The response to EA 2192 was found to be quadratic over this range of concentrations. Quench efficacy using hexane to remove VX (thereby halting decontamination by the DEFENZ™ VX-G solution) was verified in method demonstration. Effectively recovery of EA 2192 from the aqueous phase after extraction with hexane was also verified.

Because the analysis was qualitative, calibration with EA 2192 standards was not included in the analysis of EA 2192 as a by-product of enzymatic decomposition. Rather, the relative proportion of EA 2192 extracted from a VX solution, with or without enzymatic decontamination (DEFENZ™ VX-G “3X” solution), was determined.

Table 18. Liquid Chromatography/Mass Spectrometry Parameters for EA 2192 Analysis

Equipment and Parameters	Description		
High Performance Liquid Chromatography (HPLC)	Shimadzu 20_{XR} Series		
Mass Spectrometer	AB SCIEX Triple Quad™ 5500		
Mass Spectrometer Source	TurboIonSpray® probe (Electrospray), positive ion mode		
Mass Spectrometer Software	Analyst 1.5.1		
HPLC Column	Phenomenex Prodigy ODS-3, 2.1 x 150 mm, 5 µm		
HPLC Column Temperature	Ambient		
Mobile Phase Components	A = water: acetonitrile, 98:2 (v:v)		
	B = 0.2% formic acid in acetonitrile: isopropanol 80:20 (v:v)		
Gradient Profile	Time, min	%B	Flow rate, mL/min
	0	0	0.2
	1	0	0.2
	8	25	0.2
	8.5	25	0.2
	8.6	0	0.3
	20	0	0.3
Injection Volume	10 µL		
Run Time	20 min		

3.0 Quality Assurance/Quality Control

3.1 Control of Monitoring and Measuring Devices

QC requirements and results are shown in Table 19.

Table 19. Data Quality Objectives and Results for Test Measurements

Parameter	Measurement Method	QC Requirement	Results
Time	Timer/data logger	Two seconds/hour; check once before beginning testing	Passed requirement
Mass	Balance with daily calibration check using standard weights	Balance precision at least 0.1x lowest measured value	Daily balance calibration check passed QC requirement
pH	pH meter	Calibrate with two standard buffer solutions spanning range of interest	Daily 2-point calibration was performed
Background Contaminants	Analyze blank solvent using GC- or LC/MS	<MDL for analyte; include with each batch of samples	No background contamination detected
Mass of CWA (in extraction solvent)	Extract in solvent and analyze using GC- or LC/MS	>70% of CWA spike is recovered; determine once during method demonstration	All CWA recoveries met the QC requirement
Mass of CWA (in neutralized enzyme solution)	Extract in solvent and analyze using GC- or LC/MS	>70% of CWA, spike is recovered; determine once during method demonstration	All CWA recoveries met the QC requirement
Mass of SRC (test and positive control coupons and laboratory and procedural blanks)	Extract in solvent and analyze using GC/MS	>70% recovery of SRC (which provides a check for matrix effects)	All CWA recoveries met the QC requirement
Mass of CWA (on positive controls)	Extraction/ chromatographic quantitation	Results were considered an outlier if the recovery value for analyte from a coupon fell outside three standard deviations of the mean. Criterion applies only if concentration of analyte is ≥5 times the MDL	Outliers are noted where applicable
Mass of CWA (on spike controls)	Extraction/ chromatographic quantitation	≥85% of CWA spike target	The spike control recoveries were sometimes lower than 85% of the spike target, documented in the deviations
Mass of CWA (on laboratory blank)	Extraction/ chromatographic quantitation	<MDL for CWA	No CWA was detected on any laboratory blank coupon

3.2 Chemical Analysis Equipment Calibrations

A six-point calibration for CWA and tributyl phosphate (TBP) as an SRC was generally used with a lower calibration level of 0.5 µg/mL and an upper range of approximately 50 µg/mL. Due to saturation, some analytes had only a five-point curve with an upper range of 25 µg/mL. Naphthalene-d_8 was used as an IS for quantitation of CWAs and TBP. An average response (relative standard deviation <15%) or linear regression (or, in a few cases, quadratic regression) curve fit was applied to the calibration data. Samples exceeding the upper calibration limit were diluted to a concentration within the calibration range and reanalyzed.

Continuing calibration verification (CCV) standards were included prior to sample analysis, following every fifth sample and at the end of each batch of samples. Two or more CCV concentrations were used, one of which was equal to the low calibration standard (0.5 µg/mL and 10.0 µg/mL for VX and TGD; 0.5 µg/mL and 12.0 µg/mL for HD) and CCV response within 25% of nominal concentration was acceptable. Samples analyzed prior to or following CCVs that were outside acceptance limits were re-analyzed.

For GC/MS, the neat CWA was diluted with hexane to prepare standard solutions that were analyzed to construct a standard curve within an appropriate range. The standard solutions were included each day that an analysis was performed. The GC/MS or LC/MS calibration curves met the following performance requirements:

- r^2 greater than 0.98
- % bias for the lowest standard less than 25%
- % bias for the remaining standards less than 15%
- % bias for the lowest calibration check standard less than 35%
- % bias for the remaining calibration check standard less than 20%
- Difference between replicate samples less than 20%.

3.3 Technical Systems Audit (TSA)

The QA Manager performed a TSA during the performance of the decontamination testing. The purpose of the TSA was to ensure that testing was performed in accordance with the test/QA plan[1]. In the audit, a QA Officer reviewed the sampling and analysis methods used, compared actual test procedures to those specified in the test/QA plan[1], and reviewed data acquisition and handling procedures. The QA Manager prepared a report, the findings of which were addressed either by modifications to the test procedures or by documentation in the test records.

The TSA report noted that efforts to compensate for TGD losses caused by adhesion to pipette wall and tip were documented as a deviation from the test/QA plan. [1] The impact of the inconsistent TGD applications to coupons was to have high variability that made it less likely that significant efficacy would be detected.

The TSA report also noted that the samples not used on the same day were stored at -20° C rather than 4° C specified in the test/QA plan. [1] The lower temperature was a more stringent condition than that specified in the test/QA plan[1] and was not expected to have any adverse impacts on the results.

The TSA report also noted that the sonication at 50-60 kHz corresponds to the factory specification and was not verified. The test/QA plan[1] did not require verification of the frequency of sonication and precise sonication frequency was not considered to be a critical parameter.

3.4 Performance Evaluation Audits

A performance evaluation (PE) audit was conducted for each performance parameter shown in Table 20 to assess the quality of the measurements made during testing. The audits for temperature, RH, concentration, and time were performed once during testing by analyzing a standard that is independent of standards used during the testing. Table 20 summarizes the acceptance criteria and results for the PE audit.

Table 20. Performance Parameters to be Audited

Parameter	Audit Procedure	Expected Tolerance	PE Audit Results
Time	Compare time to independent clock or watch value	±2 seconds/hour	Both timers used during testing were compared and found to be within 2-second requirement
Chemical Mass	Use GC/MS to measure SRC from secondary source and compare to primary source	±10%	Primary and secondary sources were found to be within +/-10% tolerance requirement
	Determine mass of agent delivered to Teflon® spike control coupons and compare to target application level	≥85% of spike target	For determining mass of agent delivered to Teflon, see Section 3.6 below
Mass	Use balance to determine the mass of a reference weight	±0.1 g	Balance used was within annual calibration and calibration checks performed regularly to ±0.1 g criterion

3.5 Data Quality Audit

The Battelle QA Manager audited at least 10% of the investigation data and traced the data from initial acquisition through reduction and statistical comparisons to final reporting. All data analysis calculations were checked.

3.6 Spike Control Data

HD recoveries from spike control coupons are shown in Table 21 for each day of testing. Examination of the mass spectra revealed that no HD was detected on any laboratory blank or procedural blank coupons (except one procedural blank that was inadvertently spiked).

Table 21. HD Recovery from Spike Control Coupons

Date	Recovery, µg/mL (SD, n = 3)	Percent of Expected Concentration
8/2/2011	33	92
8/18/2011	45	125

Because of deviations from the test/QA plan, [1] the spike control data for VX and TGD are shown in Section 3.8.

3.7 Amendments

Seven amendments were incorporated into the test/QA plan. [1] A brief summary of the amendments follows:

- Amendment 1: Additional details were added to decrease losses during the spray-and-weigh demonstration; language was added detailing the LC/MS evaluation of EA 2192.
- Amendment 2: A required deliverable of the work assignment, the amendment described test/QA changes to perform additional efficacy testing at shorter or longer times for high and low efficacy materials, respectively, and evaluate loss of efficacy as a function of time after the enzyme solution is prepared.
- Amendment 3: Added language to perform neat GD extractions to determine extraction efficiency; added language to describe method demonstration for the use of hexane to quench the aqueous solution for LC/MS analysis.
- Amendment 4: Provided details for preparing uniform aliquots of DEFENZ™ VX-G from the bulk materials received.
- Amendment 5: Provided flexibility to modify tests specified for "high efficacy" materials when high efficacy was not observed for any materials.
- Amendment 6: A required deliverable of Amendment 1 to contract EP-C-10-001 Work Assignment 1-04, provided test/QA details necessary to apply the plan to testing of DEFENZ™ B-HD.
- Amendment 7: A revised LC/MS analysis to compare EA 2192 extracted from VX with and without enzyme present.

3.8 Deviations

Deviation 1: On March 4, after testing had begun on March 2, the monthly purity tests showed that actual delivery of neat agent was 80.7% VX (Table 22) rather than the 85% required by Section B1.1 of the test/QA plan. [1] VX is known to become unstable and degrade unpredictably and rapidly during storage. Because of this phenomenon, U.S. Department of Defense research typically requires 70% purity as the acceptance criterion. Monthly quantitative analysis of VX stock solutions is performed to identify stocks that are degrading. Normal, but unpredictable, degradation of VX occurred between the monthly test of purity and the use of the VX in testing.

Table 22. Monthly VX Purity Data Showing Gradual Degradation

Date	Agent	Purity %
4-Mar-11	VX	80.7
3-Feb-11	VX	89.3
7-Jan-11	VX	91.3

The deviation is not believed to have impacted test results. The testing results indicated little or no decontamination efficacy at the lower contamination level generated by the less pure (80.7%) VX challenge. If the decontamination is ineffective at 80.7%, ineffectiveness at the full (85%) challenge is expected. Therefore, all samples were included in the analysis.

Deviation 2: The spike controls from the TGD trials showed actual delivery of CWA was sometimes outside the range required by the test/QA plan. [1] Thickened agent was particularly difficult to deliver with accuracy and precision. Microliter levels of application of thickened agent are imprecise due to the high viscosity of the material and drag on the pipette tip. Excess TGD from the reservoir sticks to the outside of the pipette tip. Wiping off this excess TGD from the tip can pull the TGD aliquot out of the pipette. As specified in the test/QA plan, [1] based on prior experience the pipette was set to dispense 1.4 µL of TGD in order to deliver 1 µL (expected measurement of 100 µg/mL). However, the TGD recovered from controls corresponded to a volume higher than target being delivered to the coupons. Specifically, rather than observing recoveries equivalent to 100 µg/mL, deliveries to the coupon ranged from a mean of 134 µg/mL for galvanized metal to 288 µg/mL for carpet.

Based on the accuracy of the first tests, professional judgment was used to adjust the pipette setting in subsequent testing to get closer to the test/QA plan[1] targets. The TGD was also replaced with fresh material before subsequent testing.

Deviation 3: Teflon® spike control coupons are used by Battelle as a standard to detect unexpected problems, e.g., low VX purity. VX recoveries from spike control coupons are shown in Table 23 for each day of testing. VX recoveries from spike control coupons were 72% or greater. However, recoveries were generally lower than the 85% recovery specified in the test/QA plan. [1]

Because the testing was repeated with longer decontamination times, the deviation was not believed to impact conclusions drawn from the testing.

Table 23. VX Recovery from Spike Control Coupons

Mean Recovery, µg/mL (SD) n = 3	% of Target
107.7 (6.4)	72
128.4 (0.9)	86
126.9 (3.8)	85
114.8 (1.6)	77
118.2 (1.6)	79
115.8 (2.1)	77

4.0 Results/Discussion

4.1 Method Demonstration Results

The extraction methods accepted for use met the acceptance criterion (see Section 2.9.1) of being in the range of 40% - 120% recovery with a coefficient of variance between samples not exceeding 30%. Use of RDS of VX, GD, and HD for the extraction efficiency demonstration was a deviation from the test/QA plan.[1] VX and HD recoveries were above 70% and therefore acceptable (Table 24). GD RDS recoveries were below 70% and therefore repeated with neat GD. Extraction efficiencies with neat GD were above 70%.

Table 24. Extraction Efficiencies for CWAs from Vinyl Coupons

CWA	Mean Extraction Efficiency	Coefficient of Variance
VX (RDS)	114%	7.0%
GD (Neat)	107%	11%
HD (RDS)	94%	3.0%

The MDLs for VX, GD, and HD extracted from vinyl using 10 mL of hexane are shown in Table 25.

Table 25. MDL Values for VX, GD, and HD Extracted from Vinyl Using Hexane

	MDL, µg (10 mL Extract)		
Material	**VX**	**GD**	**HD**
Vinyl	1.81	0.96	1.52

4.2 Decontamination Results

4.2.1 Measurement of pH of Enzyme Solution on Coupons

The pH of the enzyme solutions on the various coupons was measured using broad range (0-14) pH indicator strips (#9590, EMD, Gibbstown, NJ). The DEFENZ™ VX-G solution, ready for use, had a pH between 8 and 9 as measured with the pH indicator strips. The pH indicator strips were selected because a pH meter could not measure small (100 μL) droplets on the various material surfaces. A broad range pH indicator was selected because the potential pH range from the various surfaces was not known. The DEFENZ™ VX-G on the surface of laminate, wood, carpet, and vinyl, measured after a 15 min contact time, exhibited a pH of 9, except for galvanized metal which exhibited a pH of 10. The DEFENZ™ B-HD enzyme preparation exhibited a pH of about 8. No change in pH (pH remained about 8) was observed for the enzyme after 15 min contact time with the surface of the galvanized metal, laminate, wood, carpet, and vinyl coupons.

4.2.2 Measurement of Peracetic Acid in DEFENZ™ B-HD Enzyme Solution

The results of the measurement of the peracetic acid concentration in the activated DEFENZ™ B-HD enzyme solution are shown in Table 26. Each activated enzyme preparation was greater than the minimum recommended 2500 ppm level. The strips measure in 100 mg/mL increments and the test solution was diluted 1:10 before the test strips were used. A reading of 100 mg/mL measured in the dilute solution was therefore reported as 1000 mg/mL in the activated solutions.

Table 26. Peracetic Acid Measurements for Activated DEFENZ™ B-HD Enzyme

Test Date	Use	Time Prepared	Time Peracetic Acid Tested	Peracetic Acid, mg/mL
7/22/11	Method development (quench)	1018	1056	4000
7/22/11	Method development (quench)	0926	0959	4000-5000
8/2/11	15 min contact time	0950	1014	4000
8/2/11	15 min contact time	1053	1116	4000-5000*
8/2/11	15 min contact time	1148	1215	4000
8/18/11	6 hr pot life test	0710	0752	4000
			1315	3000-4000*
8/18/11	4 hr pot life test	0717	0801	4000
			1117	3000-4000*
8/18/11	2 hr pot life test	0930	0814	4000
			0930	4000
8/18/11	30 and 60 min contact time	0838	0907	4000
			1143	3000-4000*

* Test strip key is in 100 mg/mL increments and was used to test a 1:10 dilution of the enzyme. A 400 mg/mL measurement in the dilute solution was therefore reported as 4000 mg/mL in the full strength enzyme solution. The readings that were intermediate between color values were reported as a range, e.g., 4000-5000 indicating a measurement above 4000, but below 5000.

4.2.3 VX Decontamination

No VX was found on any laboratory blank coupon. No VX was found on any procedural blank coupon.

Decontamination efficacy results (mean and SD) using DEFENZ™ VX-G enzymes prepared in accordance with manufacturer's instructions are shown in Table 27. The p-value is the result of comparison of the mean of the positive control coupon and the test coupon recoveries using the two-tailed Student's t-test. Differences were considered statistically significant if $p \leq 0.05$. This means that the average measured amount of agent recovered from

the test coupons after decontamination was statistically significantly lower than the average amount recovered from the positive control coupons (i.e., those without decontamination application).

A statistically significant efficacy was observed for DEFENZ™ VX-G enzymes with a 15 min contact time for VX on all materials except vinyl. Efficacy was observed for VX on vinyl, but, because of variability in the results, the efficacy was not statistically significant. Results from the solution controls indicate some efficacy against VX on wood or carpet that may be attributable to ingredients in the DEFENZ™ VX-G product other than the enzyme.

The standard DEFENZ™ VX-G enzyme preparation was tested at longer contact times (30 and 45 min) to evaluate whether efficacy would increase. Galvanized metal was used because of the low efficacy observed for VX on galvanized metal at 15 min. Results for decontamination of VX using longer contact times are shown in Table 27. Efficacy was observed to increase with increasing contact time.

Table 27. VX Decontamination Results Using DEFENZ™ VX-G

Material	Contact Time, min	Mean Positive Controls, µg (SD; 95% Confidence Interval [CI])	Mean Solution Control Coupons, µg (SD; 95% CI)	Mean Test Coupons, µg (SD; 95% CI)	Mean Solution Control Efficacy	Mean Test Coupons Efficacy
Laminate	15	700 (21; 681-719)	683 (53; 637-730)	618 (34; 589-648)	2%	12% p < 0.01
Wood	15	410 (134; 293-527)	299 (115; 199-400)	207 (143; 82-332)	27%	50% p = 0.05
Carpet	15	718 (46; 678-759)	458 (108; 363-553)	580 (57; 530-630)	36%	19% p < 0.01
Vinyl	15	646 (74; 581-712)	703 (34; 673-733)	524 (118; 421-627)	-9%	19% p = 0.09
Galvanized metal	15	716 (34; 686-747)	747 (43; 709-785)	635 (62; 582-689)	-4%	11% p = 0.04
Galvanized metal	30	918 (54; 871-965)	891 (9; 883-899)	711 (29; 686-736)	3%	23% p < 0.01
Galvanized metal	45	874 (30; 848-900)	859 (17; 844-874)	645 (42; 608-681)	2%	26% p < 0.01

Higher concentrations of DEFENZ™ VX-G enzymes were tested with a 15 min contact time to evaluate whether efficacy would increase. Results for decontamination of VX using higher concentrations than the manufacturer's recommendation (2X and 3X) DEFENZ™ VX-G solutions with a 15 min contact time are shown in Table 28. Efficacy was observed to increase with increasing enzyme concentration. A 5X concentration resulted in incomplete dissolution of the enzyme or buffer products and was not tested.

Table 28. VX Decontamination Results with Concentrated Enzyme Solutions

Material	Enzyme Concentration	Contact Time, min	Mean Positive Controls, µg (SD; 95% CI)	Mean Test Coupons, µg (SD; 95% CI)	Mean Test Coupons Efficacy
Galvanized metal	2X	15	854 (23; 834-875)	608 (33; 579-637)	29% $p < 0.01$
Galvanized metal	3X	15	841* (79; 772-910)	509* (45; 458-561)	39%* $p < 0.01$

*Only three values used due to sample processing error.

Results for decontamination of VX using concentrated (3X) DEFENZ™ VX-G solutions stored at 4 °C after preparation for use are shown in Table 29. The prepared enzyme solution was stored in a refrigerator for the time shown in Table 29 minus one hour, and then exposed to ambient conditions for one hour. Contact time was 15 min. The p-value resulting from comparison of the mean of the 6-hour delay test coupons to the 24-hour delay coupons ($p = 0.35$) indicates that no significant difference in efficacy was observed after the longer delay period. The p-value resulting from comparison of the mean of the 15-hour delay test coupons to the 24-hour delay coupons ($p = 0.37$) indicates that no significant difference in efficacy was observed after the longer delay period. No VX was found on any laboratory blank or procedural blank coupons. Refrigerated storage for up to 24 hours appears to maintain the activity of the DEFENZ™ VX-G enzyme solution when used for decontamination of VX.

Table 29. Effect of Storage of Activated Enzyme Solution on VX Decontamination Results

Material	Enzyme Concentration	Pot Life, hours	Contact Time, min	Mean Positive Controls, µg (SD; 95% CI)	Mean Test Coupons, µg (SD; 95% CI)	Mean Test Coupons Efficacy
Carpet	3X	6	15	810* (44; 788-833)	528 (130; 415-642)	35% $p < 0.01$
Carpet	3X	15	15		520 (104; 429-611)	36% $p < 0.01$
Carpet	3X	24	15		429 (183; 269-588)	47% $p < 0.01$

*Mean of 15 positive control coupons.

4.2.4 TGD Decontamination

Application of small, precise amounts of thickened agent was difficult. Pipette settings were manipulated based on historical applications and trial-to-trial observations. As shown in Table 30, within trial and between trial variability in the mass recovered from spike control coupons was high.

Table 30. TGD Spike Recovery from Spike Control Coupons

Pipette Setting, µL	Recovery, µg/mL (SD, n = 3)	% of Target	CWA Lot
1.4	420 (169)	280	1
1.2	163 (33)	108	1
1.2	187 (54)	125	1
1.2	61 (7)	41	2
1.2	71 (4)	47	2
1.6	202 (8)	135	2

Results for decontamination of TGD using DEFENZ™ VX-G are shown in Table 31. The p-value is the result of comparison of the mean of the positive control coupon and the test coupon recoveries using Student's t-test. No GD was found on any laboratory blank or procedural blank coupon.

Statistically significant efficacy was observed for DEFENZ™ VX-G against GD (applied as TGD) on carpet after a 15 min contact time. No efficacy was observed for DEFENZ™ VX-G against TGD on galvanized metal, wood, vinyl, or laminate after a 15 min contact time. With longer contact times (30 min), a statistically significant efficacy was observed for DEFENZ™ VX-G against TGD on laminate. At 45 min contact time, efficacy was observed, but, due to variability, was not significantly different from the positive controls.

Data shown in Table 31 suggest that the solution controls containing the DEFENZ™ VX-G ingredients without the enzymes have consistent higher recoveries than positive controls. This may be due to the reduced evaporation of TGD in the presence of a buffered water droplet covering TGD. Analysis of p-values from the comparison of the mean of the positive control coupon and solution control recoveries using Student's t-test shows, however, that the differences are not statistically significant (p value ranges from 0.08 to 0.67 across the five materials).

Based on the high variability in the use of small volumes of thickened agents, future testing should apply larger volumes of thickened agent (to correspondingly larger coupons). Further, longer contact times may be appropriate to increase the likelihood of observing significant differences.

Table 31. TGD Decontamination Results Using DEFENZ™ VX-G

Material	Contact Time, min	Mean Positive Controls, µg (SD; 95% CI)	Mean Solution Control Coupons, µg (SD; 95% CI)	Mean Test Coupons, µg (SD; 95% CI)	Mean Solution Control Efficacy	Mean Test Coupons Efficacy
Galvanized metal	15	1347 (257; 1121-1572)	1867 (617; 1326-2408)	1410 (545; 932-1888)	-39%	-5% p = 0.82
Wood	15	1525 (169; 1377-1673)	1694 (489; 1266-2123)	1665 (883; 800-2530)	-11%	-9% p = 0.77
Carpet	15	2877 (172; 2727-3027)	3290 (780; 2606-3973)	1659 (366; 1338-1979)	-14%	42% p < 0.01
Vinyl	15	2177 (679; 1582-2773)	2362 (648; 1794-2930)	1524 (749; 868-2180)	-8%	30% p = 0.19
Laminate	15	1381 (162; 1239-1523)	2016 (610; 1481-2550)	1887 (890; 1107-2668)	-46%	-37% p = 0.27
Laminate	30	460 (92; 379-540)	624 (110; 528-720)	238 (61; 185-292)	-36%	48% p < 0.01
Laminate	45	634 (180; 476-792)	820 (501; 381-1260)	481 (337; 186-777)	-29%	24% p = 0.41

Higher concentrations of DEFENZ™ VX-G enzymes were tested with a 15-min contact time to evaluate whether efficacy would increase. Results for decontamination of TGD using more concentrated DEFENZ™ VX-G solutions are shown in Table 32. The p-value is the result of comparison of the mean of the positive control coupon and the test coupon recoveries using Student's t-test. No GD was found on any laboratory blank coupon or procedural blank coupon.

Statistically significant efficacy at 15 min was observed with both the 2X and 3X enzyme concentrations against TGD on laminate. This contrasts with no statistically significant efficacy measured with the standard enzyme solution (following manufacturer's recommendation) at 15 min.

Table 32. TGD Decontamination Results with Concentrated Enzyme Solutions

Material	Enzyme Concentration	Contact Time, min	Mean Positive Controls, µg (SD; 95% CI)	Mean Test Coupons, µg (SD; 95% CI)	Mean Test Coupons Efficacy
Laminate	2X	15	226 (35; 195-257)	72 (23; 52-92)	68% $p < 0.01$
Laminate	3X	15	225 (31; 198-252)	110 (53; 63-156)	51% $p < 0.01$

Results for decontamination of TGD using concentrated (3X) DEFENZ™ VX-G solutions stored at 4 °C after preparation for use are shown in Table 33. The prepared enzyme solution was stored in a refrigerator for the time shown in Table 33 minus one hour and then exposed to ambient conditions for one hour. Contact time was 15 min. The p-value is the result of comparison of the mean of the positive control coupon and the test coupon recoveries using Student's t-test. The p-value resulting from comparison of the mean of the 6-hour delay test coupons to the 24-hour delay coupons ($p = 0.67$) indicates that no significant difference in efficacy is observed after the longer delay period. The p-value resulting from comparison of the mean of the 15-hour delay test coupons to the 24-hour delay coupons ($p = 0.43$) indicates that no significant difference in efficacy is observed after the longer delay period.

No GD was found on any laboratory blank coupon. Small amounts of GD were recovered from carpet procedural blanks in the 6 hour pot test (10.0 µg), 15 hour pot test (9.9 µg), and 24 hour pot test (12.4 µg).

Refrigerated storage for up to 24 hours appears to maintain the efficacy of the DEFENZ™ VX-G enzyme solution when used for decontamination of GD.

Table 33. Effect of Storage of Activated DEFENZ™ VX-G Enzyme Solution on TGD Decontamination Efficacy

Material	Enzyme Concentration	Pot Life, hours	Contact Time, min	Mean Positive Controls, µg (SD; 95% CI)	Mean Test Coupons, µg (SD; 95% CI)	Mean Test Coupons Efficacy
Carpet	3X	6	15	1597* (399; 1395-1799)	1074 (797; 376-1773)	33% (p = 0.22)
Carpet	3X	15	15		1038 (296; 779-1298)	35% (p < 0.01)
Carpet	3X	24	15		907 (186; 744-1070)	43% p < 0.001

*Mean of 15 positive control coupons.

4.2.5 HD Decontamination

Results for decontamination of HD using DEFENZ™ B-HD solution prepared per label instructions are shown in Table 34. Contact time was 15, 30, or 60 min. The p-value is the result of comparison of the mean of the positive control coupon and the test coupon recoveries using Student's t-test. The p-values resulting from comparison of the mean of the test and positive control coupons indicates that statistically significant efficacy ($p \leq 0.05$) of DEFENZ™ B-HD against HD is observed for galvanized metal, laminate, wood, carpet, and vinyl coupons with a 15-min contact time. The standard DEFENZ™ B-HD enzyme preparation was tested at longer contact times (30 and 60 min) to evaluate whether efficacy would increase. Carpet and vinyl were used because of the low efficacy observed for HD on carpet and vinyl at 15 min. Efficacy of DEFENZ™ B-HD against HD on vinyl and carpet was higher after a 60 min contact time than after a 15- or 30-min contact time.

Table 34. HD Decontamination Results Using DEFENZ™ B-HD

Material	Contact Time, min	Mean Positive Controls, µg (SD; 95% CI)	Mean Solution Control Coupons, µg (SD; 95% CI)	Mean Test Coupons, µg (SD; 95% CI)	Mean Solution Control Efficacy	Mean Test Coupons Efficacy
Galvanized metal	15	1111 (105; 1019-1203)	1038 (25; 1016-1060)	843 (125; 702-984)*	7%	24% p <0.04
Laminate	15	1222 (128; 1096-1348)†	1092 (21; 1073-1110)	894 (89; 816-972)	11%	27% p < 0.01
Wood	15	849 (94; 767-932)	754 (26; 731-777)	601 (64; 545-657)‡	11%	29% p < 0.01
Carpet	15	1171 (71; 1109-1233)	1146 (72; 1083-1209)	983 (67; 924-1041)	3%	16% p = <0.01
Vinyl	15	1094 (43; 1056-1132)	1062 (60; 1009-1114)	826 (73; 762-890)	3%	24% p = < 0.01
Vinyl	30	816 (64; 760-872)	Not attempted	761 (65; 705-818)	Not attempted	7% p = 0.22
Vinyl	60	947 (188; 782-1112)	Not attempted	613 (53; 566-660)	Not attempted	35% p = 0.01
Carpet	30	1063 (146; 935-1191)	Not attempted	905 (105; 812-997)	Not attempted	15% p = 0.09
Carpet	60	990 (64; 934-146)	Not attempted	690 (56; 641-739)	Not attempted	30% p = <0.01

*Results only for three coupons because two coupons were tipped or dropped during transfer.

†Results for only four coupons because one coupon was tipped or dropped during transfer.

‡One outlier excluded from analysis.

Results are shown for decontamination of HD using DEFENZ™ B-HD solution prepared per label instructions and stored at ambient laboratory temperature (approximately 21° C) after preparation for use. The prepared enzyme solution was stored for the time shown in Table 35 before use. Contact time was 15 min. The p-value is the result of comparison of the mean of the positive control coupon and the test coupon recoveries using the two-tailed Student's t-test. The p-value resulting from comparison of the mean of the 2-hour delay test coupons to the 6-hour delay coupons ($p = 0.24$) indicates that no significant difference in efficacy is observed after the longer delay period. Storage for up to 6 hours at approximately 21° C appears to maintain the efficacy of the DEFENZ™ B-HD enzyme solution.

Table 35. Effect of Storage of Activated DEFENZ™ B-HD Enzyme Solution on HD Decontamination Efficacy

Material	Pot Life, Hours	Contact Time, min	Mean Positive Controls, µg (SD)	Mean Test Coupons, µg (SD)	Mean Test Coupons Efficacy
Wood	2	15	662 (32)	611 (129)	8% p = 0.44
Wood	4	15	755 (109)	728* (160)	4% p = 0.79
Wood	6	15	930 (167)	718 (138)	23% p = 0.06

*Results for only four coupons; outlier excluded, possible dilution error.

4.3 By-Product Analysis

Bis (beta-chloroethyl) sulfone was the only HD by-product of interest detected in coupon extracts or positive control samples using full scan GC/MS. Bis (beta-chloroethyl) sulfone was detected only in the hexane extract for the 60-min decontaminated carpet samples. The peak was very small (just above baseline).

The peak areas for EA 2192 visible in the LC/MS method with and without application of DEFENZ™ VX-G to VX are shown in Table 36. Note that these are raw peak areas for positive control and test solutions diluted with deionized water as follows: 1:10, 1:25, 1:50. Qualitative analysis showed EA 2192 to be present with and without decontamination. This observation may be expected because EA 2192 is a by-product from natural degradation of VX. Further, significantly less EA 2192 was observed ($p < 0.005$) after application of the enzyme than in the controls (without enzyme). Assuming that the calibration standards were stable since preparation, EA 2192 in positive controls averaged 78.5 µg/mL and EA 2192 in test solutions averaged 27.5 µg/mL. (Because the work was semi-quantitative, the stability of calibration standards was not verified. However, the quadratic fit for the seven point calibration curves had an $r^2 = 0.993$.)

DEFENZ™ VX-G in contact with VX (containing natural EA 2192 by-product) does not appear to produce EA 2192 but rather to reduce EA 2192 that may be naturally present as a VX contaminant.

Table 36. Results from Analysis for EA 2192 (Toxic VX By-product)

Sample ID	Peak Area	Average Peak Area	% Remaining Compared to Positive Control	% Less Than Positive Control
Positive Control Sample 1	5549039	5289832	NA	NA
Positive Control Sample 2	4803737			
Positive Control Sample 3	5516719			
Test Solution Sample 1	2773475	1964603	37.1	62.9
Test Solution Sample 2	1452236			
Test Solution Sample 3	1668098			

4.4 Observations of Damage to Coupons

The DEFENZ™ VX-G treatment resulted in no obvious change to any coupons. The acrylic polymer used to thicken GD was visible on coupons after decontamination. Typical photographs before, during, and after treatment are shown in Figures 2 and 3. The DEFENZ™ VX-G treatment resulted in no obvious visible damage to any of the coupons either immediately after decontamination or two days after the decontamination. Typical photographs taken before, during, and after treatment are shown in Figure 4.

No detailed examination or testing for structural damage was included in this evaluation. Damage, if any occurred, that is not readily visible would not be likely to be detected in this evaluation.

Figure 2. Coupons before application of CWA (top), during DEFENZ™ VX-G decontamination (center) and after enzymatic decontamination (bottom) with residual plastic thickener from TGD visible, e.g., as shown by arrow.

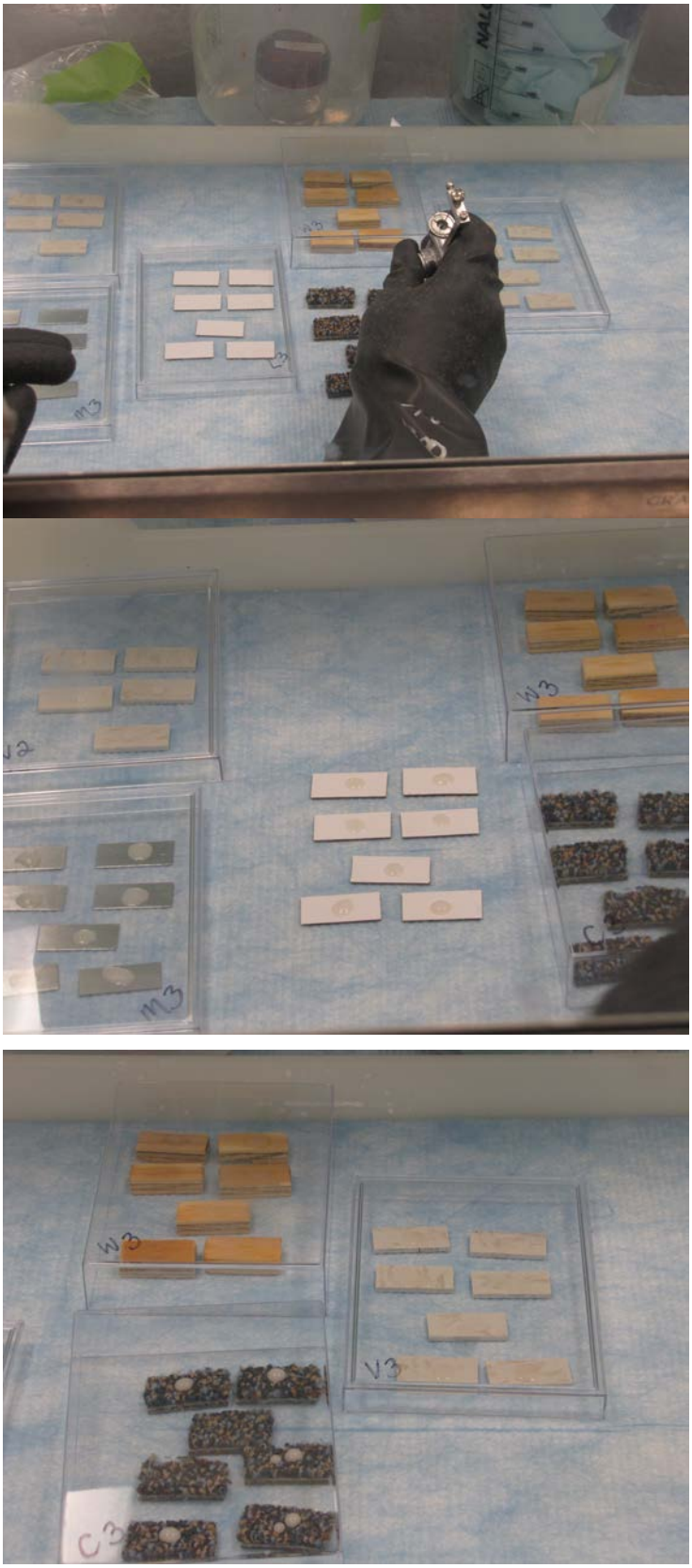

Figure 3. Coupons during application of VX (top) and during DEFENZ™ VX-G decontamination (center, bottom).

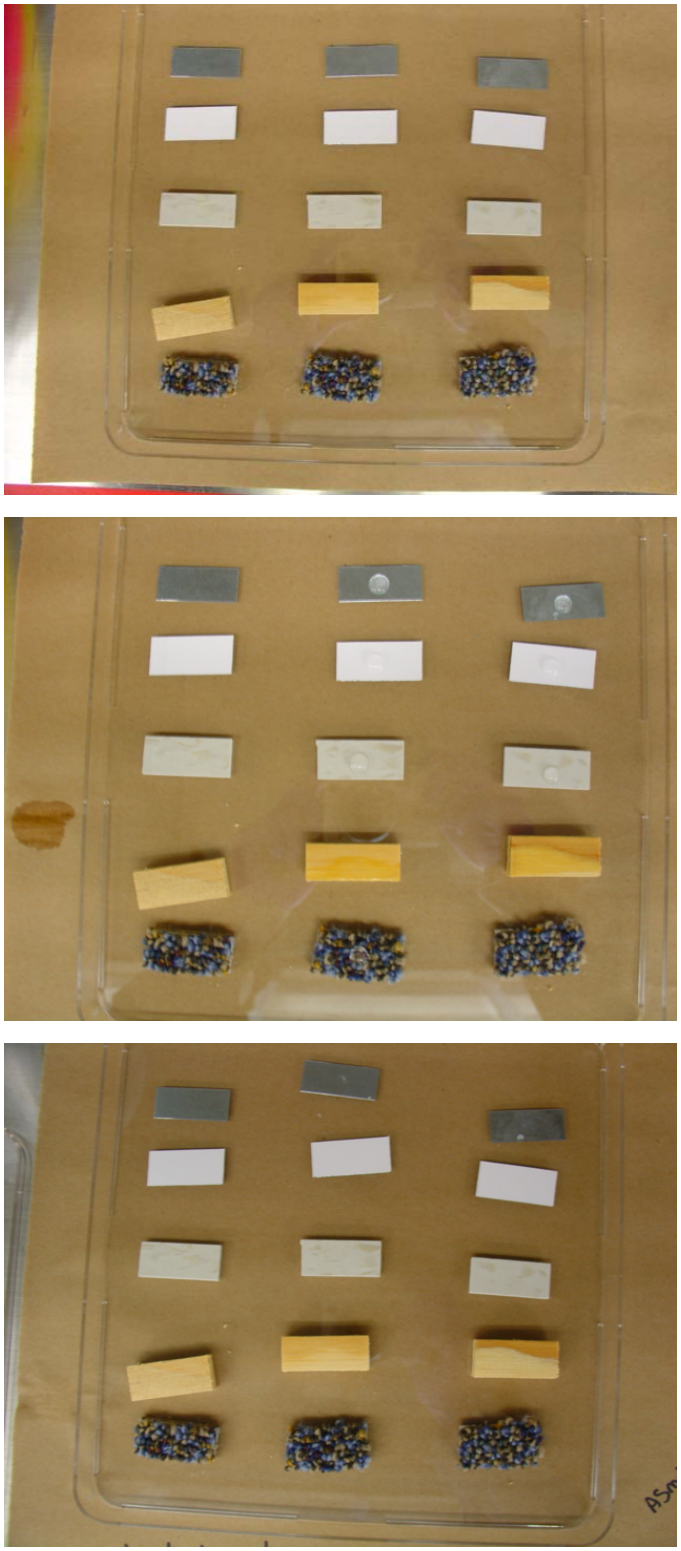

Figure 4. Coupons before application of HD (top), during DEFENZ™ B-HD decontamination (center) and 48 hours after decontamination (bottom).

5.0 Conclusions

DEFENZ™ VX-G exhibited statistically significant efficacy ($p < 0.05$) against VX on laminate, wood, carpet, and galvanized metal) with a 15 min contact time; DEFENZ™ VX-G did not exhibit statistically significant efficacy against VX on vinyl with a 15 min contact time ($p = 0.09$). Tests on galvanized metal showed that efficacy increased with increasing contact time (30 and 45 min) and when higher concentrations of the enzymes (2X and 3X manufacturer's recommendations) were employed.

DEFENZ™ VX-G exhibited statistically significant efficacy ($p < 0.05$) against TGD on carpet, but not against TGD on galvanized metal ($p = 0.82$), wood ($p = 0.77$), vinyl ($p = 0.19$) or laminate ($p = 0.27$) with a 15-min contact time. DEFENZ™ VX-G enzymes applied to TGD on laminate showed that although no statistically significant efficacy was observed with a 15-min contact time, there was statistically significant efficacy with a 30-min contact time. After a 45-min contact time, less GD was recovered from laminate treated with enzyme than from positive controls, but the difference was not significant. High variability in recoveries of GD and substantial loss of GD from coupons due to natural attenuation challenge efforts to determine efficacy. While the results are valid, future testing involving thickened agents should use larger application volumes to reduce variability.

DEFENZ™ B-HD exhibited statistically significant efficacy against HD on all five materials tested (laminate, wood, carpet, vinyl, and galvanized metal).

No toxic by-products were found to be produced by use of the DEFENZ™ VX-G or DEFENZ™ B-HD enzymes and no damage to the test material coupons was visually observed from the use of the enzymes.

The observed modest efficacies of the DEFENZ™ VX-G or DEFENZ™ B-HD enzymes are similar to those obtained with e.g., diluted bleach products[2] under similar test conditions. Combined with the observation that toxic by-products are not produced and the lack of visible damage to a range of indoor building materials, the enzymes appear to be technologies that might be considered for use against VX, G-agents, or HD on indoor building materials after a terrorist release.

Caution should be used in extrapolating from the bench testing to field application of the enzymes. A full-scale test using spray equipment and larger surfaces is warranted to ensure that the laboratory results are scalable. With the preceding caveat, based on the results of this evaluation, the following application guidance is suggested:

1. Use a higher concentration of DEFENZ™ VX-G (dilute with only one third the amount of water recommended by the manufacturer) and at least a 45-min contact time to increase efficacy against VX when a variety of surface types may be contaminated.
2. Natural attenuation of GD (even when thickened) will likely be high from many surfaces, but the use of DEFENZ™ VX-G may increase the rate of removal of the agent. Use a higher concentration of DEFENZ™ VX-G (use only one third the amount of water recommended by the manufacturer) and at least a 45 min contact time for surfaces contaminated with TGD.

3. Use a 60-min contact time for DEFENZ™ B-HD enzymes, prepared according to manufacturer's instructions, for decontaminating HD.
4. While the DEFENZ™ VX-G and DEFENZ™ B-HD enzymes demonstrate modest efficacy, a substantial portion of the chemical agents (VX, TGD, and HD) can be extracted from the test materials even after the longest contact times and using the highest enzyme concentrations evaluated. Use of longer contact times or repeated applications may further reduce the chemical agent to acceptable levels.

The effectiveness of using longer contact times or repeated applications as approaches to reduce chemical agents to acceptable levels is an important knowledge gap; further investigation is recommended. Temperature effects were not tested in this evaluation and testing was performed under ambient laboratory conditions. Temperature typically impacts enzyme performance (rule of thumb, reaction rates increase by 50% to 100% with a 10° C rise in temperature). Efficacy would be expected to decrease at lower temperatures and increase with higher temperatures within an (unknown) optimal range. Higher temperatures will denature enzymes. Further investigation is recommended to understand the impact of temperature on efficacy.

Activity of enzymes depends strongly on the manufacturer's production process. Hence, the results obtained in this report reflect solely on the commercially available DEFENZ™ enzyme products rather than the associated enzymes.

6.0 References

1. Test/Quality Assurance (QA) Plan for Enzymatic Decontamination of Chemical Warfare Agents, Version 2 (July 2010). Available upon request from EPA
2. U.S. EPA. 2011. Evaluation of Household or Industrial Cleaning Products for Remediation of Chemical Agents. U.S. Environmental Protection Agency. EPA/600/R-11/055.
3. Rogers, J., T. Hayes, D. Kenny, I. MacGregor, K. Tracy, R. Krile, M. Nishioka, M. Taylor, K. Riggs, H. Stone, and S. P. Ryan. 2008. Decontamination of Toxic Industrial Chemicals and Chemical Warfare Agents on Building Materials Using Chlorine Dioxide Fumigation and Liquid Oxidant Technologies. U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-08/125.
4. Code of Federal Regulations Title 40: Protection of Environment Part 136 – Guidelines establishing test procedures for the analysis of pollutants. Appendix B - Definition and Procedure for the Determination of the Method Detection Limit—Revision 1.11

APPENDIX A

SIMULATED ENZYME REACTOR TESTS

SUMMARY:

Simulated enzyme reactor testing was performed to determine the decontamination efficacy of enzyme decontamination technologies (DEFENZ™ VX-G against GD and VX and DEFENZ™ B-HD against HD) without potential confounds arising from application to and extraction from material coupons. This simulated reactor test is defined here as a test where neat CWA interacts with the enzyme solution in a vial (no coupon surface present) and includes sonication during a contact time of 15 min as a simulation of the stirring process. The results of the simulated reactor tests are shown in Table AS-1. DEFENZ VX-G exhibited efficacy against GD and VX. Ninety-nine percent or more of both G agents was decomposed within the 15 min contact time. Lower efficacy was observed against VX (23%). DEFENZ B-HD exhibited a 44% efficacy in 15 min against HD.

Table A-1. Simulated Reactor Results for Enzymes and CWA

CWA	Enzymes Used	Blank Solution, µg	Mean Positive Control Total Mass, µg (SD)	Mean Test Total Mass, µg (SD)	Mean Efficacy
GD	DEFENZ™ VX-G	ND*	430 (18)	ND*	>99%
VX	DEFENZ™ VX-G	ND*	480 (11)	370 (10)	23%
HD	DEFENZ™ B-HD	ND*	1110 (5)	620 (270)	44%

*ND indicates no CWA was detected

APPROACH:

Procedures were followed as described in the main text. Deviations from the coupon testing procedures are described here.

Chemical Warfare Agents

The purity of neat CWA (Table A-2) used was greater than 85%, except for VX which had purity of 71%. The neat VX purity was accepted for the single simulated reactor test. This change to the test/QA plan was documented in an amendment. VX is known to degrade once an ampoule is opened. Thus, 85% purity is a difficult criterion to achieve except for the initial aliquot removed from an ampoule. The U.S. Army accepts VX with a purity of 70% ± 10% for testing. Therefore, the decision was made not to open a new ampoule of VX, but to use the VX from the opened ampoule and to take the purity into account when making analytic standards.

Table A-2. Purity of Chemical Warfare Agents Used in Testing

Agent	Manufacturer/Supplier Name	Neat CWA Purity
VX	US Army from EPA stocks*	71%
GD	US Army from EPA stocks*	91%
HD	US Army from EPA stocks*	99%

*EPA-owned stocks of CWA are stored at Battelle's facilities in West Jefferson, OH

Test Matrix

A simulated reactor test was performed for GD and VX utilizing DEFENZ™ VX-G and for HD utilizing DEFENZ™ B-HD. The simulated reactor test is defined here as a test where a neat CWA interacts with the enzyme solution in a vial (no coupon surface present). The test involved sonication of the vial at 50-60 kHz during a contact time of 15 min as a simulation of the stirring process. The test matrix is shown in Table A-3. The simulated reactor test was not performed for thickened GD (TGD) to avoid difficulties in dispensing TGD as described in Section 4.2.4.

One microliter (1 µL) of neat agent was pipetted using a calibrated Hamilton syringe (P/N CAL80975 [50 µL] equipped with a 22-gauge needle [P/N 91022] and repeating dispenser [P/N 83700], Hamilton Co., Reno NV) into each vial designated as a test sample or positive control. Sixty microliters (60 µL) of the appropriate enzyme decontaminant was added to each test sample. This amount was selected because it is consistent with the application to nonporous surfaces in coupon testing. The CWA and enzyme solution were always in contact during sonication. Positive control samples were vials spiked with CWA to which 60 µL of DI water was added (i.e., no enzymatic decontamination). Blanks are defined as vials with only the 60 µL enzyme solution and no CWA.

CWA was extracted individually by transferring the solution from each test, positive control, and blank vial each into a separate 40 mL glass bottle (S236-0040, Fisher Scientific, Pittsburgh, PA) that contained 10 mL of hexane/IS, then sonicating at 50-60 kHz for 10 min. The CWA amount present in the vials was determined by the GC/MS analysis method in use for analysis of the coupon extracts. Samples that were not analyzed the same day were stored at -20 °C ± 3 °C or colder. GC/MS results were reviewed to identify by-products from CWA decontamination.

Table A-3. Test Matrix for Simulated Reactor Testing

Agent	Enzyme Product	Number of Test Samples	Number of Positive Controls	Number of Blanks
GD	DEFENZ™ VX-G	3	3	1
VX	DEFENZ™ VX-G	3	3	1
HD	DEFENZ™ B-HD	3	3	1

Extraction and Analysis

The GC/MS parameters where different from the main text during analysis of the simulated enzyme reactor testing for VX, GD, and HD analysis are shown in Table A-4.

Table A-4. Gas Chromatographic/Mass Spectrometry Parameters for VX, GD, and HD Analysis where different from Coupon Testing (See Table 12, main body of text)

Parameters	VX and GD	HD
Analysis Method	GC/MS (Scan)	GC/MS (Scan)
Mode	Constant Pressure	Constant Pressure
Oven Program for Analysis	40 ℃ (1.0 min), 100 ℃ (0.0 min) @ 30 ℃/min, 150 ℃ (0.0 min) @ 5 ℃/min, 275 ℃ (0.0 min) @ 15 ℃/min, 325 ℃ (1.0 min)	40 ℃ (2.0 min), 150 ℃ (0.0 min) @ 15 ℃/min, 280 ℃ (0.0 min) @ 30 ℃/min, 300 ℃ (0.0 min) @ 30 ℃/min, 325 ℃ (3.0 min)

Quality Assurance/Quality Control where Different from Coupon Testing

Amendments

Revised the purity criterion for accepting VX for use in testing from 85% to 71%; lower limit is consistent with the U.S. Army purity acceptance criterion (70% ±10%).

RESULTS/DISCUSSION OF SIMULATED ENZYME REACTOR TESTS

The results of the simulated reactor results are summarized in Table A-5. DEFENZ™ VX-G (mixed with water at the ratio recommended by the manufacturer) demonstrated efficacy against GD and VX. No GD was detected after the 15 min contact time; mean efficacy was >99%. Mean efficacy against VX was 23% after 15 min. DEFENZ™ B-HD (prepared per manufacturer's guidance for small volumes and diluted with water in the recommended ratio of water to enzyme solution) after a contact time of 15 min demonstrated 44% efficacy against HD compared to positive controls. The temperature profile for fifteen min sonication was demonstrated to result in a rise of about 7 °C from 17.4 °C when sonication begins to 24.2 °C at 15 min.

Table A-5. Simulated Reactor Results for Enzymes and CWA

CWA	Enzymes Used	Blank Solution, µg	Mean Positive Control Total Mass, µg (SD)	Mean Test Total Mass, µg (SD)	Mean Efficacy
GD	DEFENZ™ VX-G	ND*	430 (18)	ND*	>99%
VX	DEFENZ™ VX-G	ND*	480 (11)	370 (10)	23%
HD	DEFENZ™ B-HD	ND*	1110 (5)	620 (270)	44%

*ND indicates no CWA was detected

No CWA was detected in any blank solution in the simulated reactor testing.

The simulated reactor GC/MS data were examined for qualitative differences between control and test samples. The qualitative differences in peaks in the test sample compared to the control sample were as follows:

- GD – increased GD diester [Dipinacolyl methylphosphonate]
- VX – increased VX sulfide [Bis(2-diisopropylaminoethyl) sulfide] and butyric acid
- HD – methane sulfonamide, chloroacetic acid, and carbonic acid were found only in test samples.

Note that certain known by-products, particularly the EA 2192 from VX, may be present, but would not be detected using GC/MS.

CONCLUSIONS SIMULATED REACTOR TESTING

Simulated reactor testing demonstrated significant efficacy of DEFENZ VX-G against VX and GD with a 15 min contact time. GD amount was reduced by > 99%. Mean efficacy against VX was lower, 23%. Simulated reactor testing demonstrated significant efficacy of DEFENZ B-HD against HD with a 15 min contact time. Mean efficacy against HD was 44%. In most instances, simulated reactor testing efficacies were higher than observed efficacies during coupon testing, albeit in the same range. The largest difference was observed for DEFENZ VX-G product against GD as the presence of the thickener to create TGD as used during coupon decontamination may have resulted in a significantly reduced efficacy against TGD.

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