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United States
Environmental Protection
Agency

Preservation Study for Ultra-Dilute VX Standards

Office of Research and Development
National Homeland Security Research Center

Disclaimer

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Auspices Statement

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Acknowledgments

The authors wish to acknowledge the support of all those who helped plan and prepare this report. We also acknowledge Ro Leif of Lawrence Livermore National Laborotory for executing the ampoule sealing and for his helpful technical assistance and insights.

Abbreviations/Acronyms

CCV – continuing calibration verification

CWA – Chemical Warfare Agent

DCC – dicyclohexylcarbodiimide

DCM – dichloromethane

$(DES)_2$ – bis[2-(diisopropylamino)ethyl] disulfide, formula $C_{16}H_{36}N_2S_2$

DESH – 2-(N,N-diisopropylamino)ethanethiol, formula $C_8H_{19}NS$

DFTPP – decafluortriphenylphosphine, formula $C_{18}H_5F_{10}P$

DIC – diisopropylcarbodiimide

DOE – United States Department of Energy

EPA – United States Environmental Protection Agency

ERLN – Environmental Response Laboratory Network

EMPA – ethylmethylphosphonic acid

FPD – flame photometric detector

GC – gas chromatograph

GC-FPD – gas chromatography coupled with flame photometric detection

GC/MS – gas chromatography/mass spectrometry

IMPA – isopropyl methylphosphonic acid

LLNL – Lawrence Livermore National Laboratory

MS – mass spectrometer

NMR – nuclear magnetic resonance (spectroscopy)

PFTBA – perfluorotributylamine, formula $C_{12}F_{27}N$

Pyro A – O,O-diethyl dimethylpyrophosphonate, formula $C_6H_{16}O_5P_2$

Pyro B – O-ethyl, O-isopropyl dimethylpyrophosphonate, formula $C_7H_{18}O_5P_2$VX – O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothioate, formula $C_{11}H_{26}NO_2PS$

VOA – volatile organic analysis

VX – O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothioate

Executive Summary

Lawrence Livermore National Laboratory (LLNL) supplies ultra-dilute (10 µg/mL) chemical warfare agent (CWA) standards to the Environmental Response Laboratory Network (ERLN) laboratories to allow the use of authentic standards to assist in analyses required for a remediation event involving CWAs. These standards are synthesized by Lawrence Livermore National Laboratory specifically for the ERLN laboratories and are not commercially produced. For this reason, it is important to collect data regarding the shelf-lives of these standards. In a previous study (U.S. EPA, 2013), data collected by LLNL suggested that VX (O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothioate) was not stable over the long-term (e.g., greater than a month) when mixed with other CWAs, including sarin. Even stored as a single-analyte 10-µg/mL solution, the stability of VX in screw-capped vials was inconsistent. This instability has the potential to impact quality control in regional ERLN laboratories, resulting in data that are difficult to interpret. Thus, this study investigated the use of chemical stabilizers to increase the shelf-life of VX standards. VX standards with long shelf-lives are desirable, as long shelf-life would significantly reduce costs needed to ensure ERLN laboratory preparedness for VX is possible.

The goal of this work was to determine the efficacy of two stabilizers — diisopropylcarbodiimide (DIC) and dicyclohexylcarbodiimide (DCC) — for 10 µg/mL VX in dichloromethane (DCM) compared to a VX solution without any added stabilizer. We investigated the use of the stabilizers at concentrations of 1 µg/mL and 10 µg/mL. For these studies, the ultra-dilute VX standards were stored in both flame-sealed ampoules and 2-mL, Teflon®-lined, screw-capped vials. All standards were stored at 4 °C ± 2 °C for the twelve-month duration of the study. This temperature was selected to be representative of the storage conditions used by the ERLN laboratories. As the previous study had suggested that water in the solvent may be partly responsible for VX degradation, the water concentration of dried DCM was monitored when DCM was stored in 40-mL VOA vials and repeatedly opened and analyzed at each sampling point.

After the VX standards were prepared in DCM, 1-mL aliquots were transferred to amber glass ampoules and flame-sealed or transferred to screw-capped vials. Triplicate ampoules/vials were opened at predetermined times and VX, DIC, and DCC concentrations were measured by gas chromatography/mass spectrometry (GC/MS) and by gas chromatography coupled with flame photometric detection (GC-FPD). Concentrations were plotted as a function of time over the course of twelve months. Dunnett's test was used to determine if statistically significant decreases were observed in VX concentrations for the GC-FPD data collected during the course of the study. Water concentration in the DCM was measured by coulometric Karl Fischer titration.

Results of Dunnett's test (α=0.01) indicated that no continuing statistically significant decreases in VX concentrations occurred during the first 9 months of the study for any of the storage conditions tested; however, several random statistically significant decreases in VX concentrations were observed. By the twelfth month of the study, statistically significant degradation of VX (although still within -20% of the original concentration) was observed for seven of the ten samples. Based on these data, it is recommended that laboratories restock their VX standards every 6-9 months. It is also recommended that laboratories check (by analysis) the concentrations of VX standards prior to use to test for the possibility of receiving a standard that is not at its expected concentration. Data suggest that the use of a stabilizer was not warranted and no preference for storage of VX standards in sealed ampoules versus screw-capped vials exists. Based on the complexity and potential complications (soot formation, overpressure with purge gas, etc.) with flame sealing, it is recommended to store and ship standards in screw-capped vials. DCM was observed to absorb water from the environment suggesting that, when in use, vials of standards should be left open for as short a period as possible.

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Introduction

The Environmental Response Laboratory Network (ERLN) is EPA's national network of laboratories that can be accessed as needed to support large scale environmental responses. With the threat of a chemical, biological, and radiological attack to the United States becoming more complex, the need for accurate, timely environmental testing capabilities becomes even more crucial. Ultra-dilute (10 µg/mL) chemical warfare agent (CWA) standards are supplied by Lawrence Livermore National Laboratory (LLNL) to Environmental Response Laboratory Network (ERLN) laboratories to allow the use of authentic standards to assist in analyses required in remediation after an incident involving CWAs. As these standards are synthesized by LLNL specifically for the ERLN laboratories and are not commercially produced, knowledge regarding the shelf-lives of these standards is limited. Stability data collected by LLNL suggest that O-ethyl-S-(2-diisopropyl-aminoethyl) methylphosphonothioate (VX) is not stable over the long-term (e.g., more than a month) when mixed with sarin, soman, cyclosarin, or sulfur mustard (U.S. EPA, 2013). Previous investigations suggest that when stored as a single-analyte, 10 µg/mL solution in dichloromethane (DCM), VX in sealed vials was stable for only two months (20 % degradation of VX was observed by the third month of the study), with measurements at longer time-points yielding inconsistent results (e.g., VX in one vial had disappeared, while VX in a duplicate vial appeared to be stable, yielding an overall degradation of approximately 80 % after a year). VX standards in DCM that were stored in sealed ampoules appeared to be stable for a year; however, similarly-stored, sealed-ampoule, single-analyte solutions of VX in hexane showed ~20 % degradation after three months (U.S. EPA, 2013). The instability of VX has the potential to impact quality control in ERLN laboratories, yielding results that are difficult to interpret and possible misinterpretation of data. Thus, this study investigated the use of chemical stabilizers to increase the shelf-life of VX standards. VX standards with long shelf-lives are desirable, as long shelf lives for the standards that are produced would significantly reduce the costs associated with ERLN laboratory preparedness for VX.

The stability of VX can be reduced by reactions with water and other contaminants introduced in its manufacturing process and by the reaction of degradation products in the VX solution. Buckles et al. (1977), Pardasani et al. (2010), Black and Muir (2003), Munro et al. (1999), Yang (1999) and Yang et al. (1996) describe various processes of VX degradation that are based on its reactions with water, contaminants, and degradation products. Figures 1 through 3 show several degradation reactions for VX. U.S. EPA (2013) confirmed experimentally that O,O-diethyl dimethylpyrophosphonate (Pyro A) was produced by reaction of VX and ethyl methylphosphonic acid (EMPA) in a single-component standard; the presence of EMPA itself indicated that hydrolysis was a mechanism for VX degradation in the standards. That study also confirmed that the breakdown of VX in a multiple-component standard solution was initiated by the presence of isopropyl methylphosphonic acid (IMPA) where the initial attack on VX by IMPA initiates the autocatalytic breakdown cycle of VX, as seen in Figure 3.

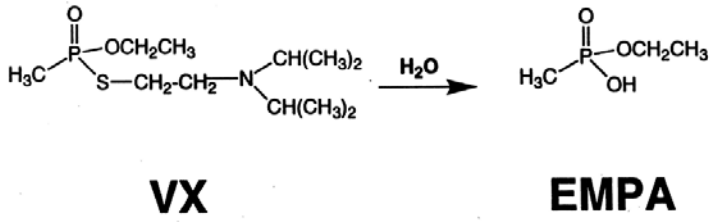


Figure 1. VX reaction with water to produce EMPA.

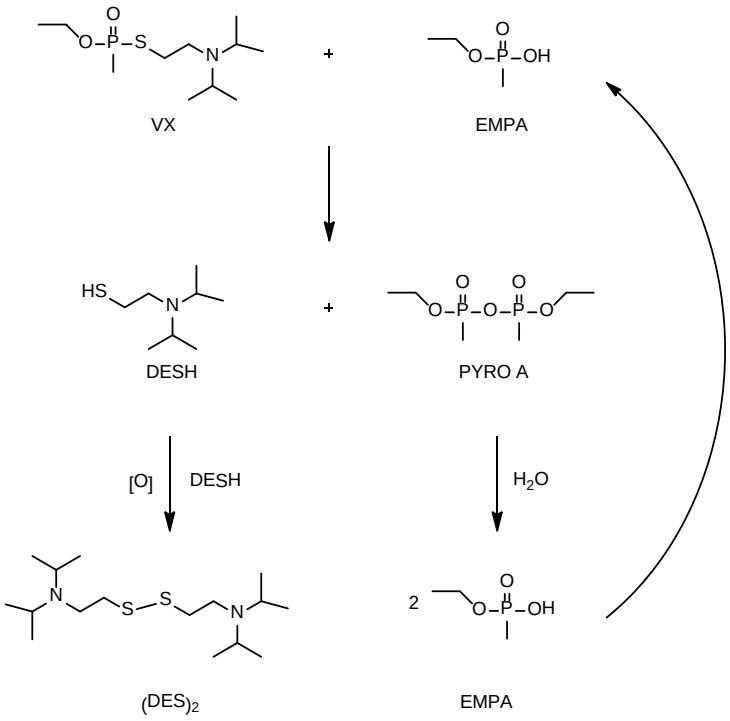
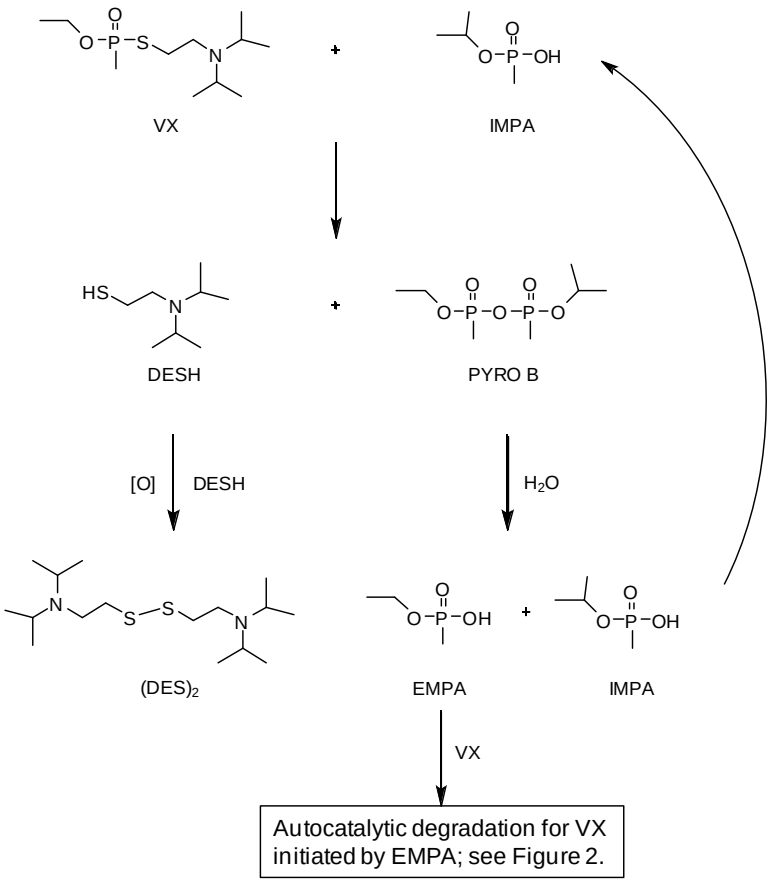


Figure 2. Autocatalytic degradation mechanism for VX initiated by EMPA.



EMPA – Ethyl methylphosphonic acid
IMPA – Isopropyl methylphosphonic acid
Pyro A – O,O-Diethyl dimethyl-pyrophosphonate
Pyro B – O-Ethyl, O-isopropyl-dimethylpyrophosphonate
DESH – 2-(N,N-diisopropylamino)-ethanethiol
$(DES)_2$ – *bis*[2-(diisopropylamino)-ethyl] disulfide

Figure 3. Autocatalytic degradation mechanism for VX initiated by IMPA.

Given the above degradation pathways, a desirable stabilizer would be one that could react with both water and the relevant contaminants or directly stabilize the VX. Buckles et al. (1977), Rosenblatt et al. (1996), and Rohrbaugh (1998) identified carbodiimide stabilizers as appropriate for VX. The rate of reaction of the stabilizers with residual water/contaminants was faster than the rate of reaction of the water/contaminants with VX. Because diisopropylcarbodiimide (DIC) and dicyclohexylcarbodiimide (DCC) were identified as stabilizers commonly used for VX stabilization, these stabilizers were selected for this study.

The goal of this work is to determine effective stabilization strategies for VX in ultra-dilute (10-µg/mL) standards. To achieve this goal, the study objectives are defined as:

- Investigate VX stability in standards of 10 µg/mL VX in dichloromethane (DCM), stored in flame sealed ampoules at 4 °C for 12 months. These standards were made using several different preparations, including:

  a) No stabilizer (control experiments)
  b) The stabilizer DIC at 1 µg/mL
  c) The stabilizer DIC at 10 µg/mL
  d) The stabilizer DCC at 1 µg/mL
  e) The stabilizer DCC at 10 µg/mL.

- Study the stability of standards of 10 µg/mL VX in DCM in 2-mL, Teflon®-lined, screw-capped vials at 4 ºC for 12 months. As above, these standards were prepared in varying ways, including:

  a) No stabilizer (control experiments)
  b) The stabilizer DIC at 1 µg/mL
  c) The stabilizer DIC at 10 µg/mL
  d) The stabilizer DCC at 1 µg/mL
  e) The stabilizer DCC at 10 µg/mL.

- Determine the water concentration in dried DCM when DCM is stored in 40-mL VOA vials and repeatedly opened and analyzed at various sampling points to understand the potential water exposure of VX standards under conditions of simulated laboratory use.

Under the conditions described above, we could determine whether the presence of a stabilizer increased the amount of time that VX remained stable in a 10 µg/mL solution, relative to a VX solution with no added stabilizer. This study considers two sets of storage conditions — untouched, in flame-sealed glass ampoules (reflecting the manner in which the standards are shipped to the CWA laboratories, with the exception that standards are currently shipped at ambient temperature) and standards that are stored in screw-cap vials (which reflects the handling of standards that are "in use" by the laboratory). An analyte concentration of 10 µg/mL was selected for study because this concentration represents the maximum concentration of VX that can currently be handled in the EPA CWA laboratories. To determine VX stability in solution with a chemical stabilizer, large batches of 10 µg/mL solutions were made, and 1-mL aliquots were flame-sealed in multiple amber glass ampoules or stored in screw-capped vials. At predetermined intervals over the course of a year, ampoules or vials of the standards were opened and analyzed and the amounts of VX remaining in the standards were quantified to determine VX stability. This report covers the twelve months of observations.

Materials and Methods

Preparation of Standards

Standards of 10 µg/mL of VX in DCM were made containing either no stabilizer, a stabilizer concentration of 1 µg/mL, or a stabilizer concentration of 10 µg/mL. DCM used for making standards was obtained from Sigma-Aldrich (catalog number 34488, Fluka brand, which contains 25 mg/L amylene as a stabilizer). DCM was dried using a solvent distillation method with calcium hydride (Li et al., 2007) as a desiccant and refluxed over two days (Figure 4). While use of molecular sieves was considered for producing dry DCM, preliminary experiments suggested that the presence of molecular sieves may contribute to VX disappearance in a sample. To avoid the potential problem of VX disappearance as the result of molecular sieve use, solvent distillation was used to dry DCM. The water concentration remaining in the DCM used for standard preparation was determined by titration with an Aquamax Coulometric Karl Fischer Titrator (GR Scientific Ltd., Bedfordshire, United Kingdom) with a detection limit for water as low as 1 µg/mL. After drying the stock DCM, the measured concentration for water in the dried DCM used for preparing the VX standards was 25 µg/mL (the concentration of water in DCM fresh from the bottle was measured as 91 µg/mL; the water content for a bottle of DCM that was left open in the laboratory for 3 hours was 380 µg/mL).

Water content of the dried stock DCM was monitored during the course of the study. DCM was stored in a total of eleven 40-mL VOA vials, one vial for each sampling time point. The VOA vials were sealed with Teflon-lined silicone septa, similar to the septa used to seal the autosampler vials used to store the standard solutions containing VX. Because of the fragile nature of the ceramic frit and platinum electrode on the Karl Fischer Titrator and because of potential issues with decontamination of the electrode after exposure to VX, the standard samples containing VX and the stabilizers were not analyzed for water content. Thus, we have assumed that any changes in water content over time exhibited by the DCM in VOA vials would be similar to changes in water content exhibited by the VX standard samples.

VX used for this study was synthesized by LLNL and determined to be 96.0 % pure[1] by nuclear magnetic resonance spectroscopy (NMR, 600 MHz, Advance III, Bruker, Billerica, MA) and gas chromatography/mass spectrometry (GC/MS, Model 5975, Agilent Technologies, Santa Clara, CA) analyses. Impurities were identified by direct GC/MS analysis (non-polar compounds) and also by using N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, product no. 33024, St. Louis, MO) to form trimethylsilyl derivatives of polar compounds, which were then identified by GC/MS. Dilute standards were prepared gravimetrically from neat materials. Concentrations were also verified by GC-FPD analysis (7890A GC interfaced with a single P-channel FPD, Agilent Technologies, Santa Clara, CA), which allowed the comparison of phosphorus signal responses against those of certified reference materials (e.g., malathion, Fluka, product no. 31558, available from Sigma-Aldrich, St. Louis, MO).

All standards were refrigerated (4 ºC ± 2 ºC) until analysis to mimic storage conditions expected to be used by the laboratories. The refrigerator was equipped with a thermometer (part number

[1] VX impurities identified by GC/MS include O,S-diethyl methylphosphonothioate, diisopropylethyl mercaptoamine (detected as trimethylsilyl derivative), O-ethyl methylphosphonothioate (also identified as trimethylsilyl derivative), diisopropylaminoethyl chloride, and bis(diisopropylaminoethyl)disulfide.

20700T, H-B Instrument Company, Collegeville, PA) and the measured temperature was checked and recorded periodically during the course of the study. Had the temperature of the refrigerator exceeded the range of 2 ºC to 6 ºC at any point during the study, the standards would have been moved to a different refrigerator; however, such temperature fluctuations were not observed.

Standards were placed in either amber glass ampoules (part number 176796, Wheaton Science Products, Millville, NJ) that were flame-sealed or amber glass vials, closed with Teflon-lined silicone septa screw caps (part number 5182-0556, Agilent Technologies, Santa Clara, CA).

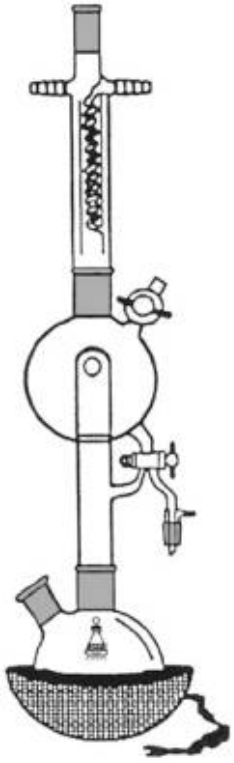


Figure 4. Solvent Distillation

Ampoule Sealing

One-milliliter aliquots of the working standards were transferred to 2-mL, pre-scored, amber, borosilicate ampoules (Wheaton Science Products, as previously specified) using a variable volume (0.5 – 5.0 mL) pipettor tipped with a long Pasteur pipet (Model 831, VWR, Radnor, PA). The ampoules were used as received from the vendor. The ampoules were loosely covered with a septum while their headspace was flushed with argon. The ampoules were then placed in liquid nitrogen (to freeze the solvent and prevent its evaporation) prior to flame sealing. Flame sealing was done using an Ampulmatic® automated ampoule sealing device (Bioscience, Inc., Allentown, PA), using a propane/oxygen flame. The procedures used to seal the ampoules were identical to those used by LLNL to seal ampoules of ultra-dilute standards prior to sending them to the ERLN laboratories.

Instrumentation

GC/MS analyses were performed with an Agilent 5975C MS coupled with an Agilent 7890A GC (both from Agilent Technologies, Santa Clara, CA). The GC/MS was tuned, as needed, with perfluoro-tributylamine (PFTBA), using the vendor's algorithms. Two nanograms decafluoro-triphenylphosphine (DFTPP) were injected into the GC/MS after instrument maintenance to establish, by monitoring ion current response and mass spectrum, that the GC/MS was functioning properly.

GC-flame photometric detector (FPD) analyses were performed with an Agilent 7890A GC interfaced with a single P-channel FPD (Agilent Technologies, Santa Clara, CA). Prior to analyses, the performance of the GC-FPD was tested with a standard containing malathion. This standard has been used for several years to test GC-FPD operation in our laboratory and has been observed to have a shelf-life greater than five years; a vendor of analytical standards has also noted stability of five years for a mixed pesticide standard containing 400 µg/mL malathion in 90/10 (v/v) hexane/acetone (Cerilliant 2011).

For both GC/MS and GC-FPD analyses, the GC was equipped with an HP-5ms column, 30 m x 0.25 mm i.d. x 0.25 µm film thickness (Agilent Technologies, Inc.). The GC oven was heated using the following program: isothermal for 1 min at 40 ºC, 15 ºC/min to 300 ºC, and held isothermal for 1 min, with the injector and detectors at 250 ºC, and helium at 3 mL/min as carrier gas.

An Aquamax Coulometric Karl Fischer Titrator was used to measure water content in the stock DCM solutions, dried DCM prior to standard preparation, and dried DCM stored in 40-mL VOA vials over the course of the study. The Aquamax Coulometric Karl Fischer Titrator is unique because of its excellent detection limits for water; this titrator can measure 1µg water in a 1-mL sample. A single, previously unopened, 40-mL VOA was opened and analyzed at each sampling time point. Additionally, all 40-mL VOA vials that had previously been opened and sampled at prior time points were also re-opened and analyzed at each sampling time point.

Analytical Procedure

<u>VX Analysis</u>

Prior to analysis of the VX standards, at each sampling interval, the standards stored in flame-sealed ampoules were transferred to screw cap vials. For both standards stored in flame-sealed vials (now in screw cap vials) and originally stored in screw cap vials, a 50-µL aliquot of the standard was transferred to a 250-µL vial insert and a 50-µL aliquot of 10 µg/mL malathion was added as an internal standard.

Individual standards were analyzed at the start of the experiment and subsequently as reported below.

- t=0 analysis completed on 6 June 2012
- t=2 weeks analysis completed on 21 June 2012
- t=4 weeks analysis completed on 2 July 2012
- t=6 weeks analysis completed on 18 July 2012
- t=2 months analysis completed on 7 August 2012
- t=3 months analysis completed on 4 September 2012
- t=4 months analysis completed on 2 October 2012
- t=5 months analysis completed on 6 November 2012
- t=6 months analysis completed on 4 December 2012
- t=9 months analysis completed on 7 March 2013
- t=12 months analysis completed on 4 June 2013

For the analyses of the standard samples containing VX only, VX with 1 µg/mL DIC, VX with 10 µg/mL DIC, VX with 1 µg/mL DCC, and VX with 10 µg/mL DCC, triplicate ampoules/vials were analyzed on each sampling day. Each analysis that was performed over the duration of the study for standards in both sealed ampoules and screw cap vials was accomplished using a new, freshly-opened ampoule or vial. Considering 11 time-points for sampling, that 3 replicate ampoules/vials were analyzed at each timepoint, and that 5 different conditions were studied, 165 ampoules (plus a few extra) were prepared and stored. Because the starting conditions at t=0 for the ampoules and vials were the same, only 150 vials (plus a few extra) were prepared and stored. Note that the sampling of the ampoules and vials was conducted in a manner that would provide data on the stability of the standards prior to their opening and prior to their continued use in a laboratory.

Water Analysis

Water concentrations were analyzed at the start of the experiment and subsequently as reported below.

- t=0 analysis completed on 11 June 2012
- t=2 weeks analysis completed on 22 June 2012
- t=4 weeks analysis completed on 6 July 2012
- t=7 weeks analysis completed on 31 July 2012
- t=2 months analysis completed on 13 August 2012
- t=3 months analysis completed on 12 September 2012
- t=4 months analysis completed on 9 October 2012
- t= 5 months analysis completed on 20 November 2012
- t= 6 months analysis completed on 13 December 2012
- t=9 months analysis completed on 28 March 2013
- t=12 months analysis completed on 28 June 2013

For the DCM, water analysis was performed using both a new, freshly-opened vial and aliquots that had been repeatedly opened and closed during the course of the study; this practice was followed to simulate how standards would be stored in vials and used by the CWA laboratories (i.e., to understand if repeated opening and closing of the vials would affect the water content of the DCM). Water analysis was performed in triplicate.

Quantitation of Target Analytes

Each batch of samples was analyzed with a corresponding solvent blank, which consisted of dried DCM from the same lot that was used to make the VX standards. A calibration curve using VX and malathion was run prior to analysis to assess GC/MS and GC-FPD performance. In addition, at least every 9th sample analyzed and evaluated was a continuing calibration verification (CCV) standard near the midpoint of the calibration range. The CCV response was required to be within 20 % of the response of the initial calibration for the data collected between CCV checks to be considered valid. Quantitation was performed by the external standard method and with consideration of the practices suggested in EPA Method 8000C (U.S. EPA, 2003). A minimum of five calibration levels were used for quantitation, covering a range from 0.5 µg/mL to 10 µg/mL so that the analyte concentrations would fall within the calibration range. VX standards and associated stabilizers to be measured were analyzed at 5 µg/mL (assuming 100 % recovery of their known t=0 concentration). VX and the stabilizers were quantified using quadratic regression calibration curves. R-Squared values for all calibration curves were >0.99 and continuing calibration standards showed that the standard responses remained within ±20 % of their expected values during analyses.

The water content of the DCM samples was analyzed per the vendor instructions provided with the Karl Fischer Titrator.

Results and Discussion

The concentrations of VX and the stabilizers were measured at various times to determine the efficacy of the two stabilizers for maintaining the concentration of the 10-µg/mL VX in DCM during the course of the study. DCM was selected as the solvent of interest because ultra-dilute CWA standards are currently prepared and shipped to the ERLN laboratories in DCM. In contrast to the previous study (U.S. EPA, 2013), attempts were made to ensure that dry DCM was used in this study to minimize the potential for VX hydrolysis. While the concentration of water in the DCM used in the previous study was unknown (vendor specifications suggested an upper limit of ~200 µg/mL, straight from the bottle), in this study, the DCM was dried by distillation before use and the initial water concentration in DCM was kept as low as practical (25 µg/mL). Standards were stored in both sealed amber glass ampoules and in amber glass vials with Teflon-lined silicone septa screw caps. The sealed glass ampoules represent how the ultra-dilute CWA standards are currently shipped to the ERLN laboratories and how the standards would be stored prior to their use. Screw-capped vials represent the storage conditions of working solutions in use by laboratories.

The initial concentration of VX in each standard was 10 µg/mL. The initial concentrations of the two stabilizers were 1 µg/mL and 10 µg/mL. The standards were diluted by a factor of two prior to analysis to ensure that the linear ranges of the GC/MS and GC-FPD were not exceeded. The results of the analyses are presented in Figures 5–9 as graphs of concentration versus time for each component. The data presented in Figures 5–7 show no marked changes in VX concentrations as a function of time under any of the conditions studied. The corresponding data for DIC and DCC in Figures 8 and 9 likewise show no marked changes in stabilizer concentrations as a function of time.

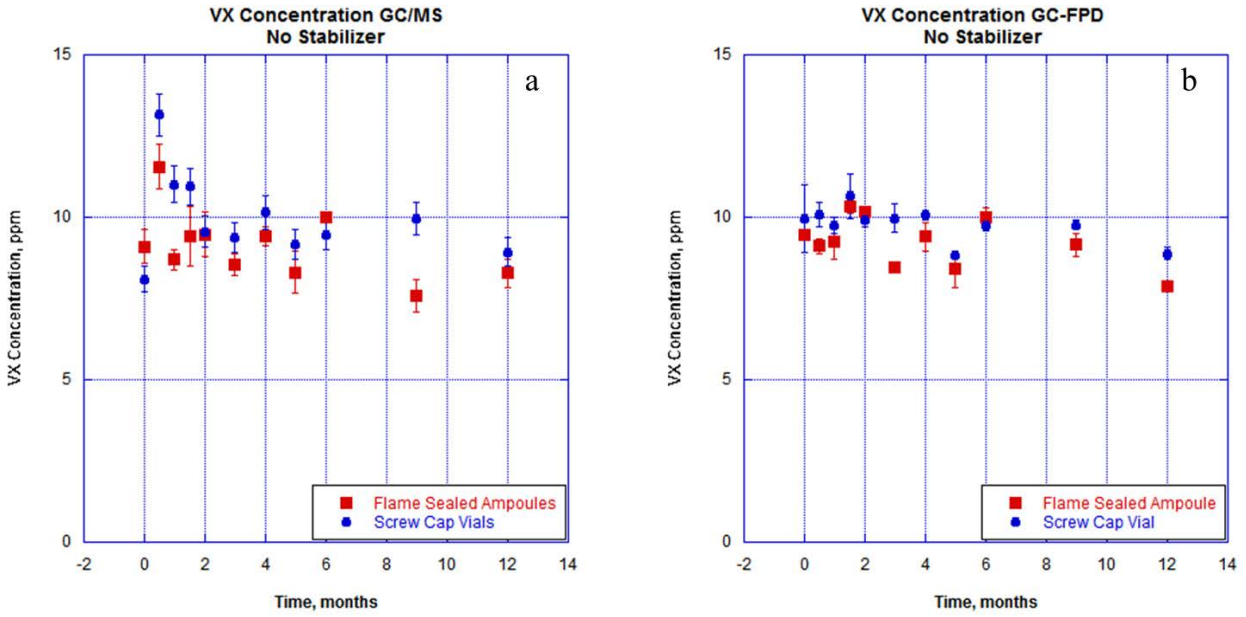


Figure 5. VX concentration in standards without stabilizer analyzed by (a) GC/MS and (b) GC-FPD.

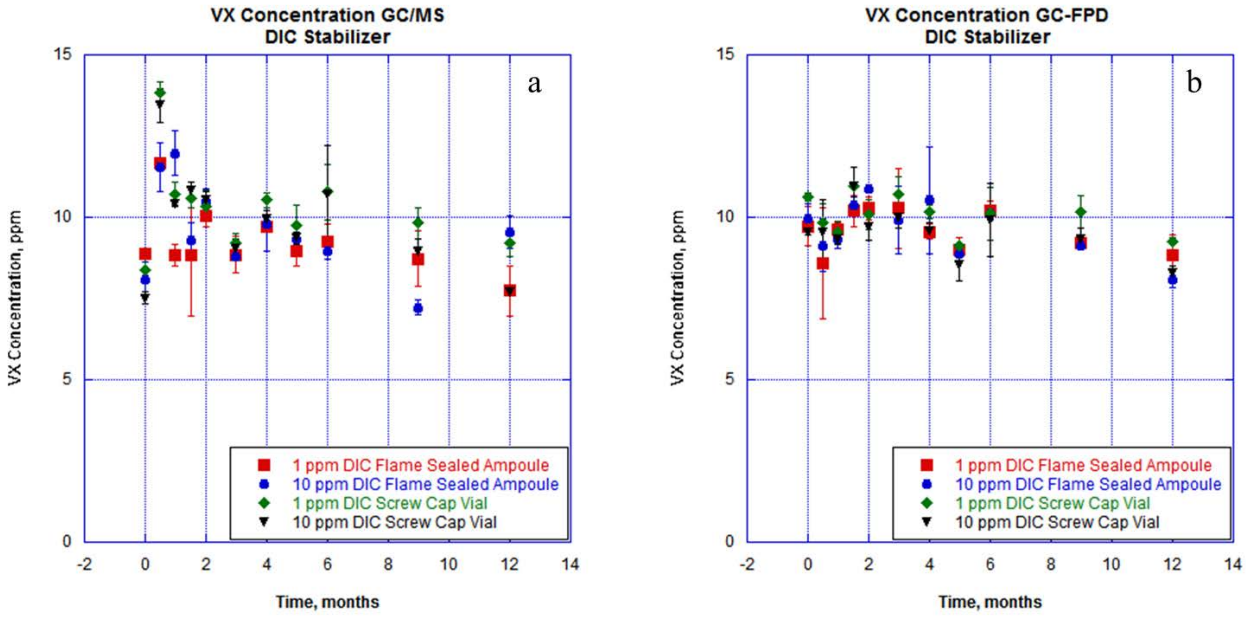


Figure 6. VX concentration in standards with DIC stabilizer analyzed by (a) GC/MS and (b) GC-FPD.

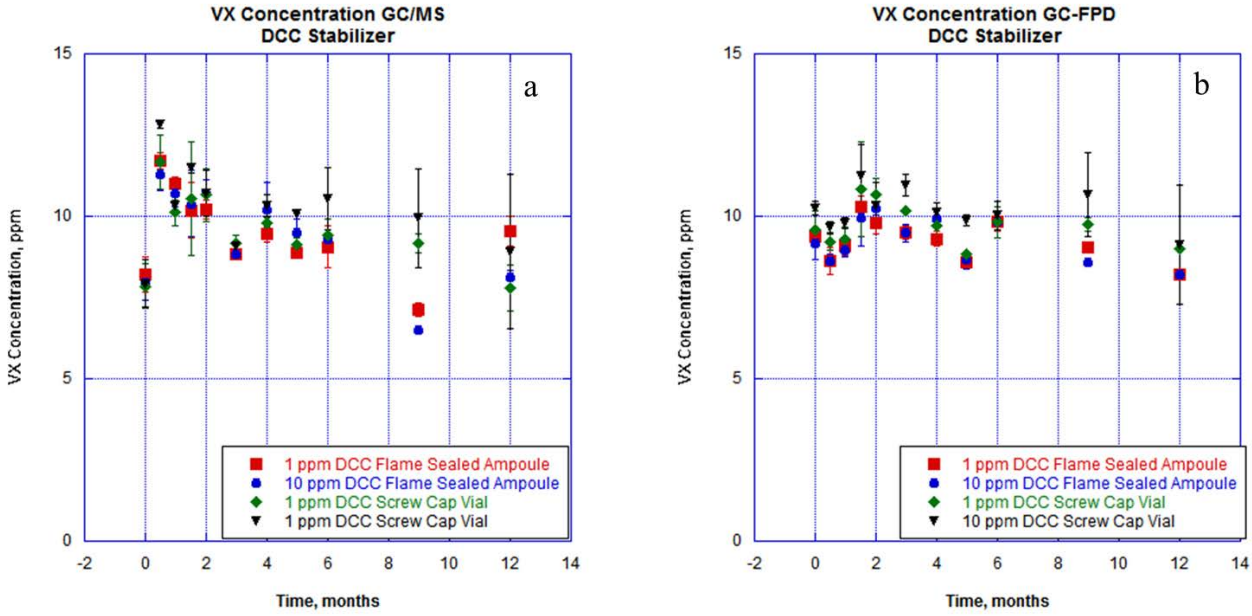


Figure 7. VX concentration in standards with DCC stabilizer analyzed by (a) GC/MS and (b) GC-FPD.

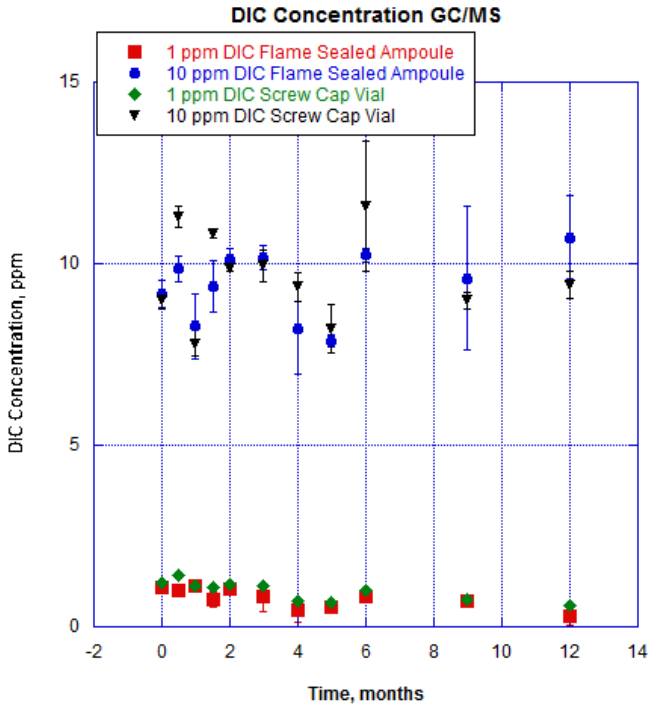


Figure 8. DIC Concentration

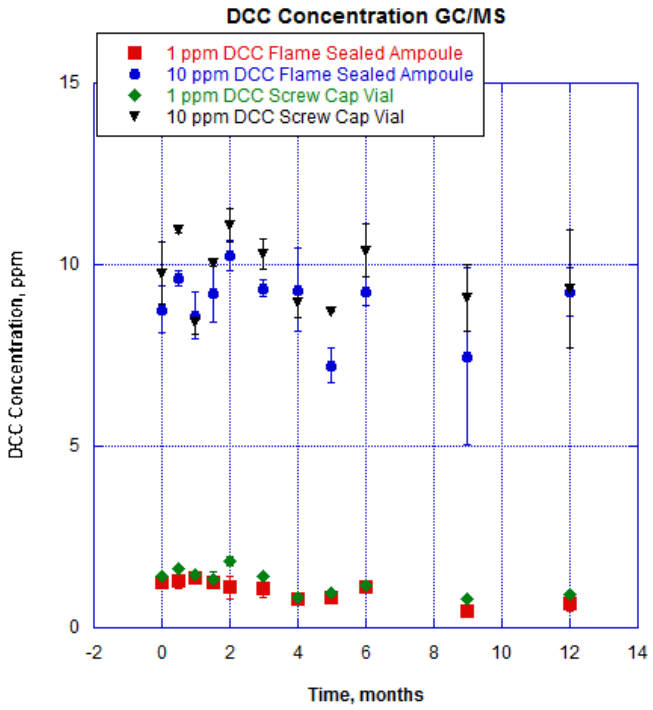


Figure 9. DCC Concentration

A program called "R" (R Core Team, 2012) was used to determine whether differences in VX concentrations as a function of time could be discerned using statistical analyses. Statistical analyses were performed using the data collected by FPD. While data were collected by both GC/MS and GC-FPD, statistical analyses were performed using only the FPD data because GC/MS measurements collected after the initial time point of the experiment appeared to be greater in concentration than the initial measurements, suggesting issues with the GC/MS data collected at the first time point only. As it is not possible to form VX under the conditions of the experiment, it is possible that GC/MS column (e.g., active sites) and source conditions might have resulted in a low initial measurement. Because it has been our experience that GC-FPD is a simpler and more stable detector, data collected with GC-FPD were selected for statistical analyses.

Dunnett's test (Hsu, 1996) was performed separately for each set of experimental conditions (each combination of stabilizer and container) to compare the VX concentrations measured at each time point (i.e., $t > 0$) with the initial measured VX concentrations ($t = 0$). The null hypothesis was that the average VX concentrations at the later times are greater than or equal to the initial VX concentration. The alternative hypothesis was that one or more average VX concentrations at a later time was less than the initial VX concentration (a one-sided test). Results of these comparisons are shown in Table 1.

Although each set of experimental conditions was evaluated repeatedly over time, the vials or ampoules from which samples were extracted were different at each time. That is, the solution analyzed at any given time point was taken from a different vial or ampoule than every other time point. Therefore, the measurements at each time point are statistically independent of those at other time points (this would not have been the case if solution from each vial or ampoule had been extracted at multiple time points).

At a significance level (α) of 0.01 [a conservative value of $\alpha = 0.01$ was chosen over the commonly used value of $\alpha = 0.05$ to compensate for the increased rate of statistical false positives resulting from multiple applications of Dunnett's test], statistically significant lower VX concentrations were observed for VX stored in ampoules with no stabilizer, at three, five, and twelve months of storage, and for VX stored in ampoules with 1 µg/mL DCC (Table 1, "DCC-lo") at two weeks and five and twelve months, and for VX stored in vials with 10 µg/mL of DIC ("DIC-hi") at five and twelve months. At month twelve of the study, seven of the ten different conditions tested showed statistically significant decreases in VX concentration; only VX in an ampoule with 10 µg/mL DIC ("DIC-hi"), VX in a vial with 1 µg/mL DCC ("DCC-lo"), and VX in a vial with 10 µg/mL DCC ("DCC-hi") did not show statistically significant decreases in concentration.

Table 1. Significance levels (p values) for comparison of VX concentrations measured by GC/FPD at time *t* (months) and time 0; p<0.01 indicates statistically significant concentration decrease.

Type	Stabilizer	t_0	$t_{0.5}$-t_0	t_1-t_0	$t_{1.5}$-t_0	t_2-t_0	t_3-t_0	t_4-t_0	t_5-t_0	t_6-t_0	t_9-t_0	t_{12}-t_0
Ampoule	NOST	0.0	0.35	0.62	1.0	1.0	<0.01	0.84	<0.01	1.0	0.41	<0.01
Ampoule	DIC-lo	0.0	0.27	0.43	0.99	1.0	0.88	1.0	0.13	0.95	0.26	<0.01
Ampoule	DIC-hi	0.0	0.44	0.88	0.98	0.98	1.0	0.89	0.68	0.96	0.74	0.45
Ampoule	DCC-lo	0.0	<0.01	0.28	1.0	1.0	0.98	0.80	<0.01	1.0	0.26	<0.01
Ampoule	DCC-hi	0.0	0.20	0.70	1.0	1.0	1.0	1.0	0.23	1.0	0.15	<0.01
Vial	NOST	0.0	0.96	0.72	1.0	0.88	0.91	0.96	0.01	0.73	0.74	<0.01
Vial	DIC-lo	0.0	0.90	0.67	1.0	0.99	1.0	0.95	0.08	1.0	0.88	<0.01
Vial	DIC-hi	0.0	0.09	0.02	0.99	0.30	0.95	0.40	<0.01	0.31	0.38	<0.01
Vial	DCC-lo	0.0	0.58	0.66	1.0	1.0	1.0	0.93	0.21	0.92	0.98	0.25
Vial	DCC-hi	0.0	0.67	0.73	1.0	0.93	0.99	0.87	0.79	0.84	0.98	0.92

Notes: "NOST"= no stabilizer, "DIC-lo" = 1 µg/mL of dicyclohexylcarbodiimide as stabilizer, "DIC-hi" = 10 µg/mL of dicyclohexylcarbodiimide as stabilizer, "DCC-lo"= 1 µg/mL dicyclohexylcarbodiimide as stabilizer, and "DCC-hi" = 10 µg/mL dicyclohexylcarbodiimide as stabilizer.

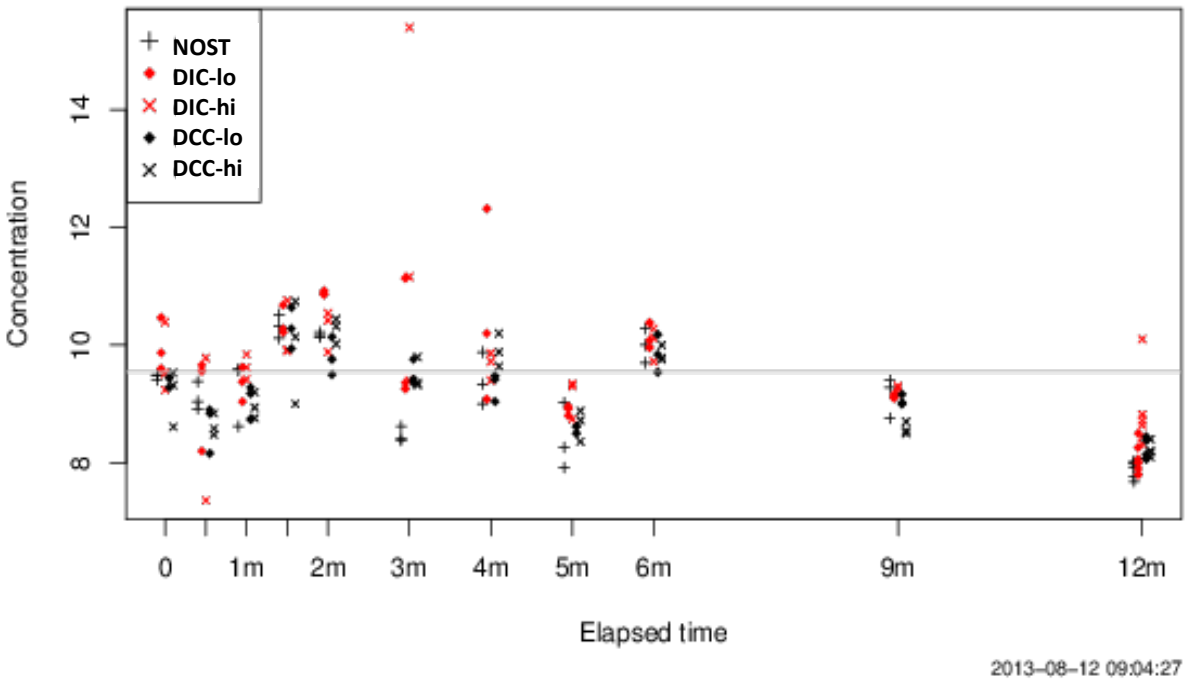
Figures 10 and 11 plot VX concentrations as measured with GC-FPD versus time for all conditions of VX standards stored in sealed ampoules and vials, respectively. While all data were collected on the same day, the values representing each experimental condition are slightly offset to facilitate comparison of VX concentrations.

Ultimately, it is desired to use the above data to inform decisions regarding recommendations about how long ERLN laboratories can store VX standards and how LLNL should best prepare such standards for these laboratories. When interpreting the above data, several assumptions were made. First, statistical tests assume equality of all VX concentrations of specific conditions, until otherwise indicated. It has also been assumed that all aliquots of VX of the same conditions, or treatment (e.g. storage container type, stabilizer type, or no stabilizer), will behave similarly and, once begin to degrade, will degrade, continuously, at a constant rate. It is expected that once an indication of degradation is detected at a time point (as determined by a p-value supporting the rejection of the null hypothesis), continued degradation at subsequent time points should be observed. While several instances of statistically significant decreases in VX concentrations were observed during the first six months of the study (t=0.5 months for ampoule with DCC-lo; t=3 months for ampoule with no stabilizer; t=5 months for ampoules with no stabilizer and DCC-lo, and vials with no stabilizer and DIC-hi), subsequent analyses at 6 months and 9 months showed no statistically significant decreases in VX concentrations (i.e. no trends in continued decreases in VX concentrations were observed). Consequently, the standards were considered stable for the first nine months of the study. Further testing is needed to confirm VX stability at nine months and investigate the random instances of decreased VX concentration noted above.

Because several time points prior to 9 months showed statistically significant decreases in VX concentrations, it is possible that “bad ampoules/vials” may sometimes be encountered; however,the reason for this is unclear. Possiblities include reaction of VX with water contamination in the vials (e.g. water introduced during any procedure where the VX ampoules were being handled), reaction of VX with impurities from either the stabilizers or from VX synthesis by products, or some other process not completely understood. Although several samples exhibited statistically significant decreases in VX concentration, these concentrations were still within -20% of their initial concentration. By month 12, because the majority of conditions (7 out of 10) showed statistically significant decreases in concentration, it cannot be concluded with certainty that the standards are stable for 12 months. Further testing is necessary, including repeating the holding time study, using the same conditions, to determine the stability for VX under all tested conditions, but was outside the scope of this study. There were no data to suggest that DIC or DCC stabilizers prevented VX degradation under the conditions of this study.

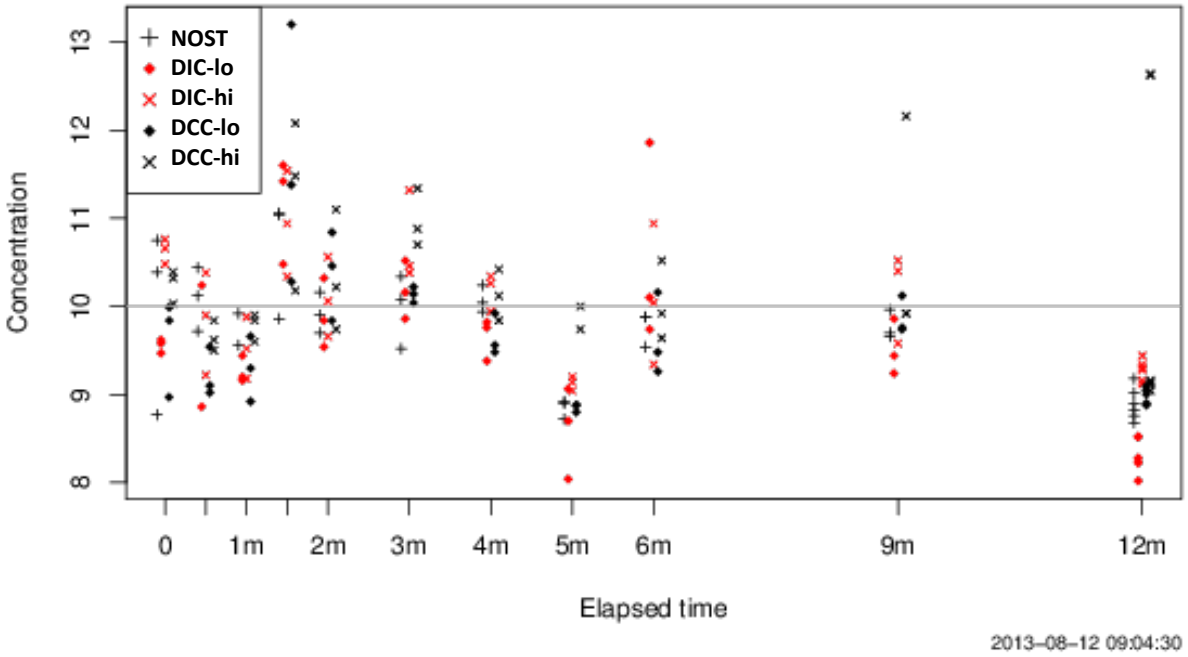
During this study, the amount of atmospheric water that a standard might adsorb during use was considered. Figure 12 shows a plot of water concentration in DCM versus the number of times a vial was opened. One previously-unopened 40-mL vial was opened at each time point, along with other vials that had been opened at preceding time points. Only a single vial was opened 10 times during the study, and the error bars associated with this data point represent the standard deviation of three independent, water measurements that were made for the DCM in this vial. Ten vials were opened once, with the corresponding data point in Figure 12 reflecting the deviations of triplicate measurements for water concentrations in each of the 10 vials. Figure 12 indicates that, as vials are opened and closed, the solvent DCM absorbs water from the environment. It is important to note that the vials were opened and closed multiple times over the course of the study.

In contrast, the VX standards were opened only once at the appropriate analytical time point (i.e., at any given time point, the analyzed solution was taken from a different vial or ampoule than the vial or ampoule at every other time point) and were not exposed to water from the environment. At the beginning of the study, the water content of the DCM was approximately 27 µg/mL; by the end of the study, the water content of the DCM that had been opened ten times was 94 µg/mL. Vendor analyses claim that the water content of DCM, fresh from the bottle, is approximately 200 µg/mL. Note that the water content of the DCM observed at the end of the study remained below 200 µg/mL. In a previous study, we observed that some VX standards made using DCM fresh from the bottle were unstable under



Notes: Grey line indicates average VX concentration at t=0; "NOST"= no stabilizer, "DIC-lo" = 1 µg/mL of dicyclohexylcarbodiimide as stabilizer, "DIC-hi" = 10 µg/mL of dicyclohexylcarbodiimide as stabilizer, "DCC-lo"= 1 µg/mL dicyclohexylcarbodiimide as stabilizer, and "DCC-hi" = 10 µg/mL dicyclohexylcarbodiimide as stabilizer.

Figure 10. VX concentrations in sealed ampoules *vs.* time.



Notes: Grey line indicates average VX concentration at t=0; "NOST"= no stabilizer, "DIC-lo" = 1 µg/mL of dicyclohexylcarbodiimide as stabilizer, "DIC-hi" = 10 µg/mL of dicyclohexylcarbodiimide as stabilizer, "DCC-lo"= 1 µg/mL dicyclohexylcarbodiimide as stabilizer, and "DCC-hi" = 1 µg/mL dicyclohexylcarbodiimide as stabilizer.

Figure 11. VX concentrations in vials *vs.* time.

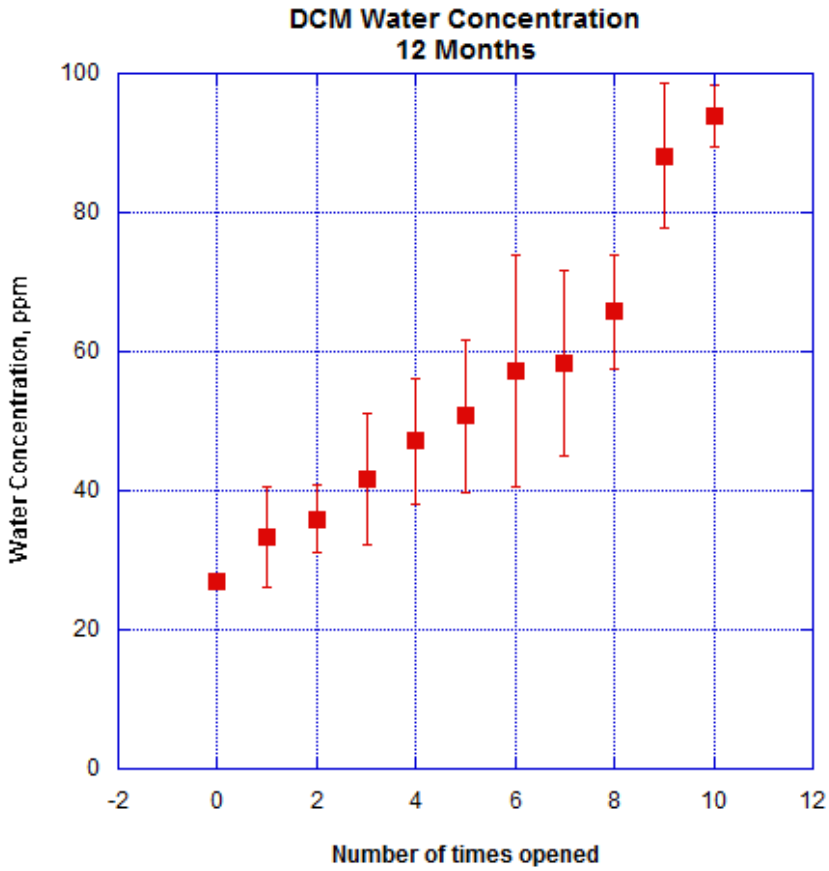


Figure 12. Water concentration in DCM stored in screw capped vials as compared to the number of times the vials were opened over the 12 month study period.

certain conditions, and we now speculate that this instability might have been caused by hydrolysis of VX initiated by the introduction of water during preparation or use of the standards. However, we can only speculate that water content above the 200 µg/mL threshold may cause VX hydrolysis; note that some support for this idea is suggested by the fact that we observed that VX in sealed ampoules containing DCM was stable for 12 months, while VX in hexane, which could have contained as much as 500 µg/mL water, stored in sealed ampoules, was stable for only 3 months (U.S. EPA, 2013). The question of at what water concentration in DCM adversely affects VX concentration was beyond the scope of this study. However, the data suggest that, when in use, vials of standards should be left open for as short a period as possible.

Conclusions

VX concentrations were measured at various time points over the course of a year towards the goal of determining the efficacy of two stabilizers — DIC and DCC — for 10 µg/mL VX in DCM compared to a VX solution without any added stabilizer. Using the data obtained, some guidance may be given about supplying the ERLN with VX standards.

With regards to preparing standards, the data did not suggest that the use of either DIC or DCC as a stabilizer was warranted; thus, the current practice of supplying 10-µg/mL VX standards in DCM, without stabilizer, to the ERLN laboratories should be continued. The distillation step used to dry the DCM prior to making the VX standards may help preserve the VX (i.e., prevent its hydrolysis, the only plausible mechanism for VX degradation in DCM). In the previous study, the water content of the DCM and hexane used to make VX standards was not controlled, and the same VX standard vial was opened at each sampling interval, repeatedly exposing the VX standard to the environment (note that in the previous study, only the vials, and not the ampoules, were repeatedly opened). In this study, a new ampoule or vial was opened at each sampling interval preventing exposure to the environment over the course of the study. While we cannot conclude that drying of DCM by distillation (residual water concentration of ~25 µg/mL) is better than using DCM directly from the bottle (residual water concentration of ~200 µg/mL), as a best practice, we recommend that the driest solvents possible should be used when preparing VX standards. Conducting laboratory manipulations with conscious thought about minimizing the potential for the analyte to be exposed to water appears to be a prudent practice.

With regards to packaging standards, statistical analysis did not show a clear preference between the use of sealed ampoules and screw-capped vials. However, the only statistically significant degradation observed before the five-month time point (t=0.5 months with DCC-lo and t=3 months with no stabilizer) was in a sealed ampoule. Because liquid nitrogen is used to cool the DCM during the flame-sealing process, it may be possible to unintentionally, and randomly, introduce water into the standard during ampoulation, which might contribute to VX degradation. Based on the complexity and potential complications (soot formation, overpressure with purge gas, etc.) with flame sealing, we recommend storing and shipping standards in screw-capped vials.

Based on the statistical anaylsis of the holding time data points, no continuous trend, with respect toVX degradation, was observed at Month 9 of the study, under any of the storage conditions (i.e., consecutive data points evaluated at different holding times exhibiting a statistically significant difference). Therefore, VX standards of 10 µg/mL in DCM may not degrade at a significant

levels for at least 9 months. However, some statistically significant decreases in VX were observed at random time points prior to 9 months making it is possible for a lab to occasionally receive a standard that will not be at its expected concentration. Thus, it is strongly recommended to check (by analysis) the concentrations of such standards prior to use. If an ERLN laboratory receives a vial/ampoule of standard that appears to provide a lower than expected response for VX, that laboratory may choose to open and use another vial of standard.

Further testing is necessary, including multiple holding time studies using the same conditions, to determine a more accurate depection of the stability for VX under all tested conditions, including a longer holding time. Multiple evaluations of VX under the exact storage condtions provided in this document will provide a more detailed description of what may be occurring, with respect to degradation. Given the variability of some of the data, a follow-up study using a different analytical technique, such as liquid chromatography/mass spectrometry (Love, 2004), which would be able to detect both VX and its hydrolysis product, may be desirable.

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