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RESEARCH, DEVELOPMENT & ENGINEERING CENTER
U.S. ARMY CHEMICAL AND BIOLOGICAL DEFENSE COMMAND

ERDEC-TR-378

TOXICITY OF HYDROLYZED CHEMICAL AGENTS TO AQUATIC ORGANISMS

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March 1997

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13. ABSTRACT (Maximum 200 words)

At the U.S. Army Edgewood Research, Development and Engineering Center (ERDEC), over 25,000 gal of solution containing agent breakdown products are disposed of each year through costly incineration and/or contractor disposal. Researchers investigated the toxicity of hydrolyzed/neutralized agents to determine if less restrictive disposal methods could be used. This research involved screening hydrolyzed agents in various media to determine their toxicity to selected aquatic organisms. Agents were hydrolyzed in 18% sodium hydroxide (GB and GD) and water (HD and VX), then neutralized for use in aquatic toxicity studies. The toxicity of the neutralized solutions were determined by conducting toxicity assays using brine shrimp, *Photobacterium phosphoreum* (MICROTOX), *Daphnia magna*, and sewage treatment plant biomass. Results showed that hydrolyzed HD was the least toxic to the test organisms, whereas hydrolyzed GB/GD and VX were the most toxic. Toxicity studies were also conducted on the individual break down products in an attempt to identify the toxic components of the hydrolysate. Due to the toxicity of GB, GD, and VX hydrolysate, it would not be feasible for disposal to the sanitary sewers at this time. However, HD may be the most likely candidate for less restrictive disposal methods.

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PREFACE

The work described in this report was authorized under Project Nos. 565WW4ED32 and 5605L6R401, Skunk Works and Alternative Technology Program, respectively. This work was started in November 1993 and completed in September 1995.

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TOXICITY OF HYDROLYZED CHEMICAL AGENTS TO AQUATIC ORGANISMS

1. INTRODUCTION

The U.S. Army Edgewood Research, Development and Engineering Center (ERDEC) currently generates over 25,000 gal of agent hydrolysis breakdown products annually.* Traditionally, an agent was hauled away by hazardous waste contractors for disposal once it had been decontaminated (hydrolyzed). A potential alternative disposal process not yet considered is disposal to the waste water treatment facility.[1,2] The local community is opposed to incineration at the installation. However, at local public information meetings, the community expressed interest in possible wastewater treatment facility disposal after hydrolysis and biodegradation of the agent. The Chesapeake Bay Foundation did not oppose this possibility as long as the discharge met the requirements set by the Environmental Protection Agency (EPA).

The Gunpowder Neck Waste Water Treatment Facility (GNWWTF) is located at ERDEC. The GNWWTF has a design flow of 2.7 million gal/day (mgd). The current average daily flow is approximately 0.8 mgd. The increased flow from subject waste will be minimal and will not require any modifications to the treatment facility. The handling cost for treatment facility discharge of solutions is negligible. If disposal to a wastewater treatment facility is approved, the ultimate resting place would be an aquatic ecosystem. Therefore, aquatic toxicity data is vital information needed to assess the effects of disposal.

The purpose of this research was to determine the aquatic toxicity of decontaminated (hydrolyzed) mustard (HD), Saran (GB), Soman (GD) and O-ethyl-S-(2-isopropylaminoethyl)methyl phosphonothiolate (VX). The toxicity of the hydrolysis breakdown products were assessed for the suitability of discharge to a wastewater treatment facility by using a variety of short term aquatic bioassays (MICROTOX, Daphnia, and Shrimp). Growth studies using biomass isolated from the water treatment facility were also conducted to investigate the effects of hydrolyzed agents on the biomass contained in the trickle filtration system.

2. METHODS

The HD samples used for hydrolysis reactions were taken from ton container storage devices. The GB, GD, and VX samples were taken from the Chemical Agent Standard Analytical Reference Materials (CASARM) Program.

The chemical agents, GB and GD (2% by volume), were reacted with 18% NaOH and neutralized with 10% HCL (Figure 1). The HD (1% by volume) was reacted with water and pH adjusted using NaOH (Figure 2). The VX (92% by volume) was also reacted with water (Figure 3). The final solutions were diluted and subjected to toxicity studies that

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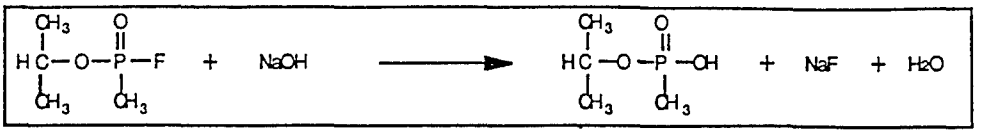
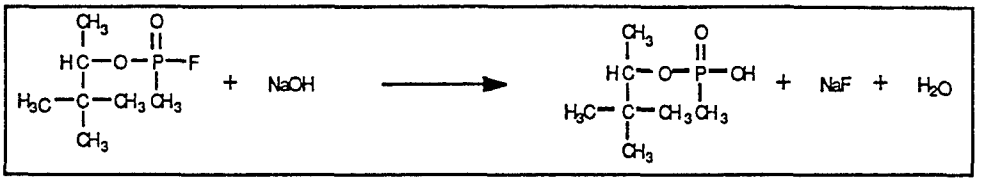
*Personal communication, J.A. Ware, Operations Directorate, ERDEC, November 1996.

are described in the following text. Several of the breakdown products were subjected to toxicity studies in an attempt to determine which component may be causing the majority of the toxicity.



Isopropyl methylfluorophosphonate (GB - Sarin)

Isopropyl methylphosphonic Acid (IMPA)



Pinacolyl methylfluorophosphonate (GD - Soman)

Pinacoyl methylphosphonic Acid (PMPA)

Figure 1. Hydrolysis of GB and GD[2]

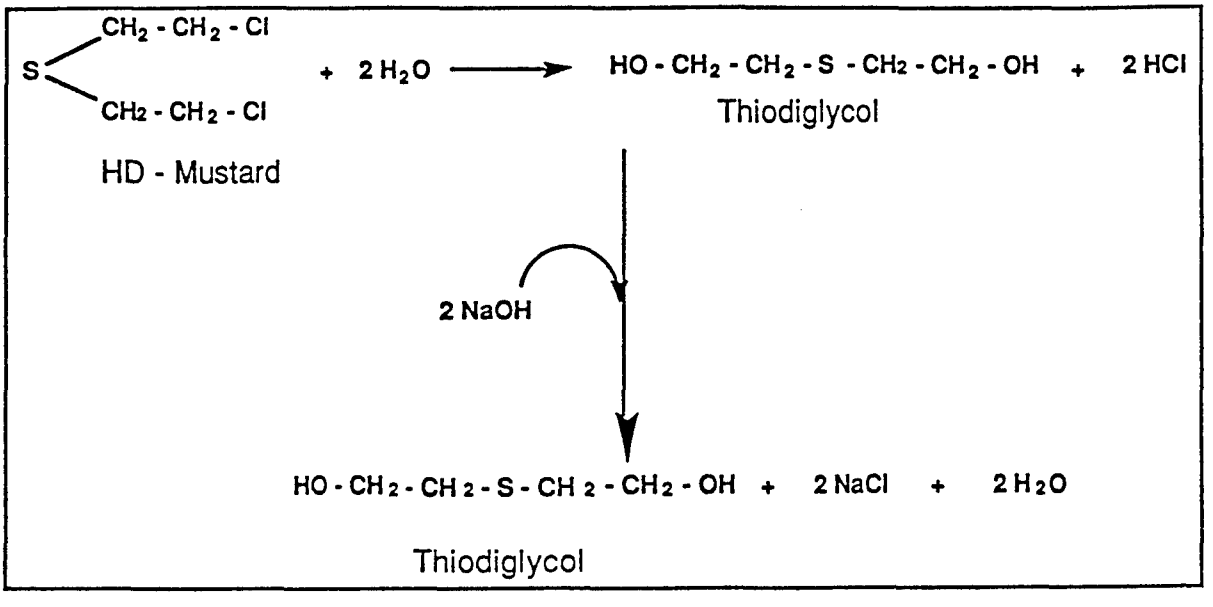


Figure 2. Hydrolysis of HD[3]

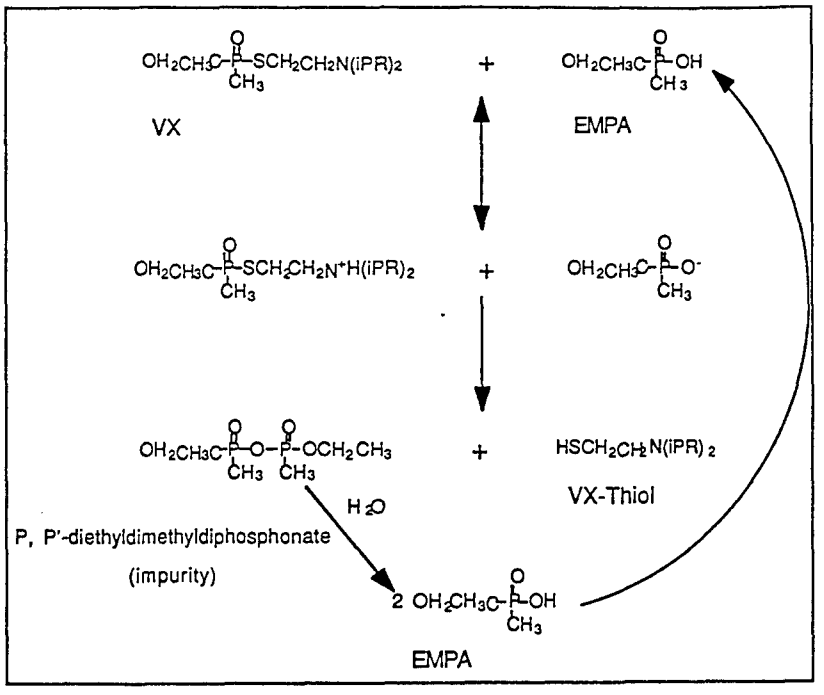


Figure 3. Hydrolysis of VX*

2.1 Water Source.

A 375-ft deep well located next to building E3224 at the Edgewood Area, Aberdeen Proving Ground, was the water source for daphnia testing. The water was passed through an in-line air injector system, pH buffer tank, iron removal system, activated charcoal filters, particulate filters, and an ultraviolet (UV) sterilizer. Well water was analyzed semiannually by Watercheck National Testing Laboratories, Incorporated (Ypsilanti, MI) for 96 different ground water contaminants ranging from heavy metals to pesticides.[3] This was done to insure that contaminants from shallow aquifers were not leaching to the deeper aquifer.

2.2 MICROTOX Assay.

The MICROTOX bioassay exposes a bioluminescent marine bacterium to a toxicant and measures the change in light output as the means of determining the effects on the organisms.[4] The bacterium, *Vibrio fischeri*, is a standardized test organism, cultured and lyophilized by Microbics Corporation (Carlsbad, CA). The bacterium (stored frozen) is reconstituted immediately before testing. Each bioassay uses <3 mL of toxicant and is performed in a temperature controlled photometer. Light output readings are measured after 5 and 15 min into the exposure. The change in light output is compared to the light output of the control at the same intervals, and the EC_{50} is calculated using MICROTOX statistical software.

*Yang, Y.-C., "Neutralization Reactions for Chemical Warfare Agents," In Workshop Proceedings on Advances in Alternative DemilitarizationTechnologies, U.S. Army Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD, September 1995, UNCLASSIFIED Report, unpublished data.

The MICROTOX 100% Test Protocol is a 1:2 serial dilution assay. The assay is performed in glass cuvettes that are incubated in the temperature controlled wells of the MICROTOX photometer. Thirty test wells are available, so several samples or replicates can be performed simultaneously. The assay must have a minimum of four dilutions and exhibit a dose-response for optimum accuracy in predicting an EC_{50}.

Due to the test organism being marine, parameters of the samples were measured and adjusted prior to performing the assay. Cloudy solutions and those with precipitates were centrifuged and the supernatant decanted to provide a clean sample. Samples with salinity <10 parts per thousand (ppt) were adjusted at the beginning of the assay with 22% NaCl solution.

The assay was set up with a predetermined number of dilutions and one control. All but the 100% cuvette received 1 mL of MICROTOX diluent. The 100% cuvette received 2 mL of sample, then two-fold serial dilutions were made by removing a 1-mL sample from the 100% cuvette and adding it to the adjacent 50% cuvette. The 50% dilution was mixed, and 1 mL was removed and added to the 25% cuvette. This procedure was continued for each dilution until the control was reached. The 1-mL sample from the final dilution was discarded.

Bacteria were transferred to the cuvettes in 10 mL aliquots and swirled. Addition of bacteria is referred to as time zero. Five minutes after time zero, the control cuvette was used to calibrate the photometer to 100% light output. The control and test cuvettes were returned to the incubator and remeasured at 15 min. Data was analyzed with the MICROTOX 100% Test Protocol software to determine the EC_{50} for both time intervals.

2.3 Brine Shrimp Assays.

The process of decontaminating GB and GD involves the use of 18% NaOH. After being neutralized with 10% HCL, the NaOH concentration was approximately 4.2%. Salt concentrations this high are not tolerated by daphnia. Therefore, brine shrimp were selected as a screening tool for estimating the toxicity.[5]

Brine shrimp eggs are commercially available and easily stored in the laboratory for extended periods of time. This assay uses small quantities of sample and is concluded in 24 hr.

Growth media was prepared by dissolving Forty Fathoms salt mix in distilled/deionized water and mixing thoroughly to produce a salt concentration of 32 ppt. The media was aerated over night to provide maximum oxygen saturation at test conditions. Undissolved particulate was removed by passing the solution through a 0.45 μm filter. Shrimp eggs (Brazilian strain) were added to a 1-L separatory funnel (hatching chamber) and filled with 800 mL of media. The eggs were incubated at 25 °C for 18-24 hr under continuous lighting and aeration. To ensure a homogenous second and third instar shrimp population, shrimp were removed from the hatching chamber and placed into graduated cylinders filled with fresh media and incubated for an additional 18-24 hr.

The test was performed in 100 mm x 20 mm borosilicate glass petri dishes. Shrimp were transferred from the graduated cylinder to the petri dishes with Pasteur capillary pipettes. Each petri dish contained 20 shrimp in 30 mL of solution. Because brine shrimp demonstrate a positive photoactic response, the test was performed in total darkness to prevent overcrowding in one area of the petri dish. After 24 hr, the number of dead were recorded, and mortality data was evaluated using the probit analysis that was developed by Tidepool Scientific Software (McKinleyville, CA).[6]

2.4 *Daphnia Magna* Assay.

The fresh water organism, *Daphnia magna,* was obtained from Dr. Freida Taub at the University of Washington in Seattle[7] and reared using methods described by Goulden, et al.[8] Daphnia stock cultures were fed a mixture of vitamin enriched *Ankistrodesmus falcatus, Selenastrum capricornutum,* and *Chlamydomonas reinhardi.*

Test protocols were based on guidelines from the EPA and the American Society for Testing and Materials (ASTM).[9,10] Test beakers (250 mL) were placed into a temperature controlled room at 20 °C having a light/dark cycle of 16/8 hr set at 315 ft candles. Two replicates per concentration containing 10 daphnia <24 hr old were used. The total volume of solution in the test vessels was 100 mL. The pH and oxygen measurements were taken at the start of each test. At 24 and 48 hr, the daphnia were checked for immobility. If the daphnia could not actively swim for 15 s, it was considered immobilized. The EC_{50} (the effective concentration at which 50% of the organisms were immobilized) values were computed using the probit analysis and tabulated graphically using a least square regression analysis to verify all probit results.

2.5 Biomass Growth Study.

Biomass from the GNWWTF was sampled from the head of the biofiltration system and from the discharge directly after the filtration system. The samples were placed on a table top shaker and agitated overnight to suspend bacteria from the filtration bed matrix. Liquid samples (1 mL) were placed in nutrient-rich broth (3 g beef extract and 5 g peptone/L) and allowed to grow for 24 hr to reach log phase growth.

Toxicant stocks were prepared by dissolving powdered nutrient in hydrolyzed agent and autoclaving for 15 min at 240 °C. The toxicant was added to growth tubes and diluted with sterilized nutrient broth (growth tubes consisted of sterilized 150 mm x 20 mm glass test tubes with snap on caps). Samples of log phase growth bacteria (0.5 mL) were added to the growth tubes to yield a total volume of 15 mL. Within 10 min of adding the bacteria, the growth tubes were placed in a spectrophotometer to measure absorbency at a wave length of 590 nm. Absorbency was measured every hour thereafter for 9 hr. In between measurements, the growth tubes were placed in an oscillating incubator set at 30 °C, oscillating at 200 rpm.

Absorbency readings were plotted against time to determine the area of the growth curve. Growth curves from the treatment groups were compared to the control groups to determine the effects (inhibition/stimulation). From growth curve data, an EC_{50} was determined by conducting a regression analysis.

3. RESULTS/DISCUSSION

Hydrolyzed VX was extremely toxic to all the test organisms. Hydrolyzed GB, GD, and HD were the least toxic, respectively. Table 1 lists the toxicity of the various hydrolyzed agents. The units associated with the EC_{50} (the concentration that produces an effect on 50% of the organisms) values presented in Table 1 represent percent volume/ volume dilution of hydrolyzed agents. Table 1 lists the salt concentrations (as determined by using a density refractometer) in the various hydrolyzed agents after pH adjustments were completed. The daphnia and MICROTOX assays were the most sensitive to the hydrolyzed agents with the shrimp being the least sensitive.

Table 1. Toxicity of Hydrolyzed Chemical Agents
(Data represent EC_{50} values in vol/vol %.)

Assay	GB-XXX	GD-XXX	VX-XXX	HD-XXX
MICROTOX	1.3 %	1.1 %	1.4×10^{-2} %	1.4 %
Shrimp	2.1 %	1.4 %	8.5×10^{-2} %	N.T.
Daphnia	---	---	3×10^{-4} %	32.3 %
Bio-Mass	1.8 %	1.9 %	5×10^{-2} %	N.T.
Salt Concentration	4.8 %	4.7 %	0 %	1.5 %

N.T. - not toxic @ 100 %.

In an attempt to determine which components of the hydrolysates were the major contributors to toxicity, the most abundant breakdown products were subjected to toxicity screening. Solutions of neutralized NaOH were also screened for toxicity to determine the effects of high salt concentrations.

The most abundant breakdown product of HD hydrolysis is thiodiglycol with sodium hydroxide being used to neutralize the acid formed during the hydrolysis process. Therefore, the toxicity of thiodiglycol in water and in neutralized sodium hydroxide was determined. Data show that the salt increased the toxicity to daphnia and remained approximately the same for MICROTOX (Table 2). Because the bacteria used in the MICROTOX assays are marine, it was expected that the salt would not affect the toxicity. Daphnia, however, are fresh water organisms and cannot tolerate salt. Therefore, an increase in toxicity due to salt addition was expected. The toxicity of hydrolyzed HD to MICROTOX (1.4%) was not expected, because the toxicity to daphnia (traditionally a more sensitive organism) was 32.3%. The MICROTOX bacteria happen to be more sensitive to hydrolyzed HD than daphnia.

Table 2. Toxicity of Several Chemical Agent Hydrolysis By-Products

By-Product	MICROTOX EC_{50}	Daphnia EC_{50}	Toxicity Ranking* (Based on Daphnia Results)
NaOH	15,580 ppm	3,000 ppm	4
IMPA	2,029 ppm	0.3 ppm	9
PMPA	1,668 ppm	13 ppm	6
Na-flouride	>2,100 ppm	733 ppm	2
Thiodiglycol (in NaOH)	6,031 ppm	2,075 ppm	0
Thiodiglycol (in H2O)	5,445 ppm	8,913 ppm	0

* Scale 0-9, 9 being the most toxic.[11]

The major breakdown products of GB and GD hydrolyses are isopropyl methylphosphonic acid (IMPA), pinacoyl methylphosphonic acid (PMPA), and sodium fluoride (Figure 1). Because salt was one of the more toxic components of HD hydrolysate, it was assumed that the salt in the GB and GD hydrolysates would also be the most toxic component. This was not the case. The sodium fluoride component was not toxic to MICROTOX and only slightly toxic to daphnia. The GB and GD acids were toxic to daphnia and only slightly toxic to MICROTOX (Table 2). Results indicate that the NaOH is less toxic to daphnia than IMPA and PMPA. On the other hand, the MICROTOX assays were more sensitive to NaOH than to the acid breakdown products (Table 3).

Table 3. Toxicity of Neutralized NaOH (4.7%) Decon Solution to Aquatic Organisms (Data represent EC_{50} values in vol/vol %.)

MICROTOX	Shrimp	Daphnia	Biomass
36.6 %	56.7 %	7.4 %	24.6 %

Hydrolyzed VX was the most toxic to the test organisms by several orders of magnitude. The VX was hydrolyzed using water; therefore, the toxic effects of salt in solution did not influence the toxicity. At the time these studies were conducted, there were no isolated VX breakdown products (Figure 3) available for testing. Therefore, no assumptions can be made at this time as to which by-product may be contributing the major portion of the toxicity. This issue will have to be addressed at a later date.

Standards for the discharge of pollutants to water treatment facilities are governed by Section 403 of the Code of Federal Regulations, entitled General Pretreatment Regulations. The purpose of these regulations is to control pollutants that pass through or interfere with water treatment facilities and to protect the sewer system, treatment plant, and water quality of the receiving stream. An issue of concern when discharging to a water treatment facility is "pass through." Pass through is a legal term for discharge that exits the water treatment facility into waters of the United States in concentrations that, alone or in conjunction with other discharge, will cause harmful effects to the environment. This can occur in the following two ways:

- The biofiltration system can be disrupted causing the biotreatment to shut down.

- The biotreatment may not be effective against the new discharge.

Wastewater treatment facility disposal of hydrolysate could potentially create pass through and interference. If hydrolyzed agents disrupt (kill) the biomass in the filter beds, the waste feeding into the water treatment facility may pass through without receiving any biological treatment. To test the effects of discharging hydrolyzed agent on the biomass, growth studies were conducted. Biomass (bacteria/sludge) from the top of the filter beds, as well as from the effluent passing out of the beds, was sampled and used in growth studies. Hydrolyzed HD stimulated bacterial growth by 6.5% (Figure 4). Increased growth indicates the biomass is using components of the hydrolysate. However, the differences in the mean growth values among treatment groups were not enough to exclude the possibility that the differences were due to random sampling variability. Never the less, the discharge of hydrolyzed HD should not disrupt the biological treatment process at the sewage treatment facility. The hydrolysates of GB, GD, and VX caused the biomass growth to shut down and scored EC_{50}s below 2% (Table 1). These hydrolysates (GB, GD, and VX) would most likely cause the wastewater treatment facility biofilter to be disrupted and cause pass through and interference. After an extended exposure period (24 hr) in 100% GB and GD hydrolysates, the bacteria growth recovered (cell density similar to control). The biomass has such diversity that over time natural selection allowed the re-population of the biomass with a less sensitive strain. However, the diversity of the entire bacteria population and the overall efficiency of the biomass to treat the entire waste stream may have decreased. The GB, GD, and VX hydrolysates disrupt the biomass growth rates to the point that straight discharge into the sanitary sewer would not be feasible.

The GNWWTF (Edgewood Area, APG) discharges into the fresh waters of the Bush River, which flow to the Chesapeake Bay. Salt discharge into the sanitary sewer may create concern of possible impact to the ecosystem. The Maryland Department of the Environment has a water monitoring station located in the Bush River (East of Gum Point,

39° 26′ 06"). Data from this station is presented in Figure 5. From 1990 to 1993, the salinity ranged from 0 to 3.6 ppt.[12] The salt concentration of hydrolyzed HD is 1.6% (16 ppt). However, when added to the sanitary sewer system, the dilution factor would reduce the salt concentration considerably. The dilution scenario depicted in Figure 6 uses an average flow rate of 800,000 gal/day. Because most of the activity at ERDEC accrues during a 16-hr period, the scenario was calculated using 50,000 gal/hr. If discharged into the sewage system, the dilution factor would be approximately 1:223. The final salt concentration would be approximately 0.07 ppt (0.007%), which is less than 65% of the recorded salinity values from 1990 to 1993 and should not impact the ecosystem.

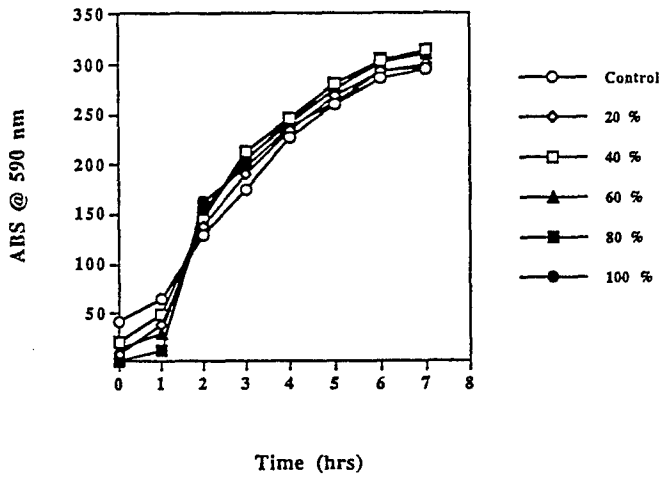


Figure 4. Biomass Growth on Hydrolyzed HD

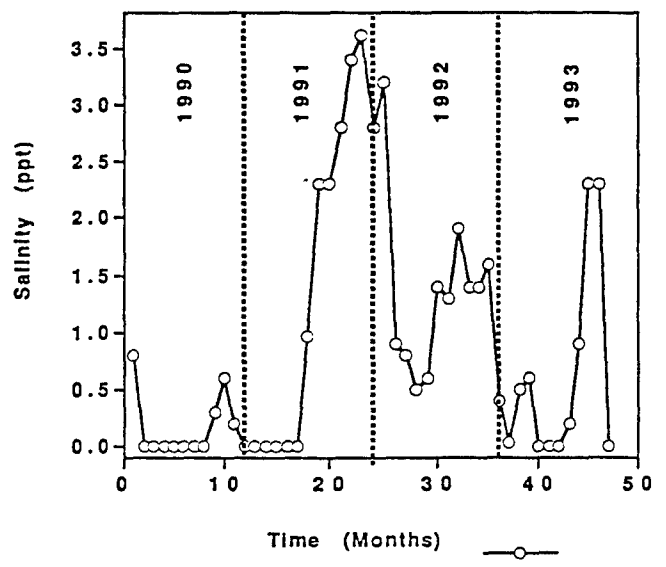


Figure 5. Surface Salinity Reading Taken by the Department of the Environment (Bush River Station MWT1.1)

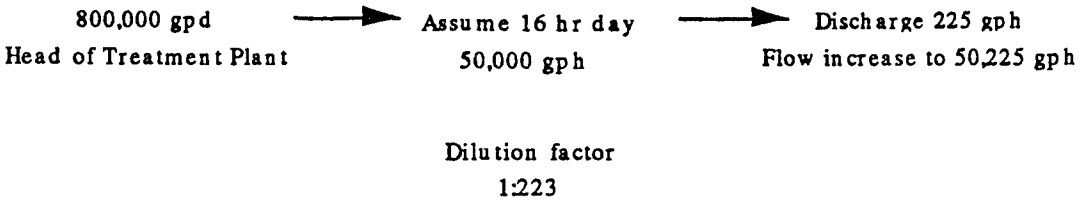


Figure 6. Discharge Scenario for Hydrolyzed HD

The Clean Water Act and the Code of Federal Regulations 40 CFR 122.4[13] prohibit the discharge of any radiological, chemical, or biological warfare agent. This prohibition pertains to the discharge of subject waste to the "waters of the United States." Some issues that must be addressed as this prohibition applies to waste generated by the hydrolysis of chemical agents are as follows.

- Can chemical warfare agents be successfully treated and the resultant waste be discharged to a waste water treatment facility?

- Neither the Clean Water Act nor the Federal Regulations define "any" agent. If it is assumed "any" means zero, what is the appropriate method to establish an enforceable zero discharge number?

A general counsel opinion from the EPA, dated 21 April 1977, clarified that the application of high temperature incineration of Herbicide Orange will not violate the federal regulations for discharge, because the products of the incineration are not closely related to the original technical ingredients. The nature of the waste changes dramatically; therefore, the EPA does not consider the Herbicide Orange to be a chemical or biological warfare agent after the incineration. The preliminary waste characterization of hydrolyzed HD shows a similar situation with the HD hydrolysis products being dramatically different from the original HD. The Alternative Technology Program has taken the treatability of HD one step further by biodegrading hydrolyzed HD using sequencing batch reactors before suggesting final disposal. The toxicity results of this work will be addressed in later publications.

The minimum detection level is defined in the Code of Federal Regulations as the concentration at which the entire analytical system must give a recognizable signal at an acceptable calibration point. This concentration is based on the "Method Detection Point," which is the minimum concentration of analyte that can be measured and reported with 99% confidence that the analyte concentration is >0. The Water Quality Based Effluent Limits (WQBEL) are used when technology-based effluent guidelines are deemed not stringent enough to protect the quality of the receiving stream. In the case of discharging hydrolyzed HD, there are no effluent guidelines. The WQBELs are a system to control the release of toxic pollutants and state that no toxic pollutants in toxic amounts are to be discharged. Guidance is given to express water quality permits as calculated based on health effects. When these limits are set below quantitative levels, the minimum detection level is used as the quantitative level and is included in the permit as a footnote to the WQBEL. According to EPA guidance,[14] analytical results that fall below the minimum

detection level should be reported as "0." This approach is being used as a legally defensible compliance tool by EPA Region VI for dioxin limits. Therefore, a regulatory zero mark is attainable. There is also a ground water clean up project being conducted at the OLD O-Field site (Edgewood Area, APG), where ground water is contaminated with agent by-products. The water is extracted from the ground, treated with chemical precipitation, UV oxidation, monitored using fish exposures, and then discharged into the Bay. Target clean up and acute toxicity levels have been set for this particular situation. The remedial methods are meeting target levels, and the discharge of material that contained agent by-product was approved. The research presented in this paper faces similar situations. If the materials to be discharged are no longer agents and the discharge meets toxicological and analytical requirements, can it be discharged into a water treatment facility?

4. CONCLUSIONS

Water hydrolyzed mustard (HD) was the overall least toxic hydrolyzed agent tested. The solution was not detrimental to the Gunpowder Neck Waste Water Treatment Facility (GNWWTF) biomass. Data indicate that the major portion of the toxicity to daphnia is associated to the salt concentration.

Hydrolysis breakdown products of Saran (GB) and Soman (GD) were toxic to all the organisms tested. The GNWWTF biomass was completely shut down. However, after 24 hr, the biomass growth did recover. Hydrolysis by-products isopropyl methyl-phosphonic acid (IMPA) and pinacoyl methylphosphonic acid (PMPA) were the most toxic components of the hydrolysates.

Hydrolyzed O-ethyl-S-(2-isopropylaminoethyl)methyl phosphonothiolate (VX) was the most toxic of all the hydrolyzed agents. The individual break down products were not screened for toxicity due to availability of the materials.

Hydrolyzed HD is the best candidate for possible discharge into a waste water treatment facility. Treatability studies (biomass growth) have shown no significant difference in cell growth between the control and the 100% treatment groups. Also, the dilution factor of 1:223 would reduce the concentration of hydrolyzed HD to $<0.5\%$, and therefore, substantially reduce possible interference to the biomass of the water treatment facility. Possible disposal into a wastewater treatment facility should be investigated further.

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