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AQUATIC TOXICITY OF THE DECONTAMINATING AGENT: MULTIPURPOSE (DAM) DECONTAMINATION SOLUTION

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13. ABSTRACT *(Maximum 200 words)*

A new formulation, Decontaminating Agent: Multipurpose (DAM) Decontamination Solution, is being considered as a replacement to the DS-2 decontaminating solution. The new formulation is composed of calcium hypochlorite and N-cyclohexyl-2-pyrrolidinone. Since this is a new formulation little environmental data exists. To estimate potential impact to an aquatic environment, *Daphnia magna* and *Photobacterium phosphoreum* (a luminescent marine bacterium) were exposed to the DAM solution and to the individual components (Calcium hypochlorite and N-cyclohexyl-2-pyrrolidinone). The toxicity of the DAM solution to *D. magna* and *P. phosphoreum* was 5.0×10^{-4} and 5.3×10^{-4}, respectively (highly toxic). The toxicity of calcium hypochlorite and N-cyclohexyl-2-pyrrolidinone to daphnia was 0.04 mg/L (highly toxic) and 107 mg/L (moderately toxic), respectively.

14. SUBJECT TERMS

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Daphnia magna	*Photobacterium phosphoreum*
Aquatic toxicity	N-cyclohexyl-2-pyrrolidinone

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PREFACE

The work described in this report was authorized under Sales Order No. 2FK4. This work was started in May 1992 and completed in December 1992.

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QUALITY ASSURANCE

This study, conducted as described by Protocol 22092000X044, was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792 (effective 17 Aug 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase inspected	Date	Date reported
Dosing	19 May 92	21 May 92
Data & Final Report	26 Oct 93	26 Oct 93

To the best of my knowledge, the methods described were the methods followed during the study. The report was determined to be an accurate reflection of the raw data obtained.

Dennis W. Johnson 26 Oct 93

DENNIS W. JOHNSON
QA Coordinator, Research & Technology

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AQUATIC TOXICITY OF THE DECONTAMINATING AGENT: MULTIPURPOSE (DAM) DECONTAMINATION SOLUTION

1. INTRODUCTION

The U.S. Army is working on replacing the currently used decontaminating solution DS-2. One of the components of the DS-2 mixture [ethylene glycol monomethyl ether (EGME)] has been determined to cause birth defects, fetotoxicity, and bone marrow problems in laboratory animals.[1] Eliminating this component will reduce the possible human health risks that may be associated with EGME. Eliminating EGME would also make the transport and disposal of the solution less restrictive.

A candidate solution named Decontaminating Agent: Multipurpose (DAM) has been proposed. The following table lists the components and the approximate percentages that make up the DAM formulation.

Table 1. DAM Components

N-cyclohexyl-2-pyrrolidinone (CHP)	≈50%
Calcium hypochlorite (HTH) in Water*	≈50%

*8 g HTH was added to 50 mL water.

Base line environmental data are needed to assess the components' impact to the environment when used in the field or if spills occur during transportation. This study investigates the aquatic toxicity of the DAM mixture and the individual components on *Daphnia magna* (the water flea) and on *Photobacterium phosphoreum* (a luminescent marine bacterium). The 48 hr acute *D. magna* and the 5/15 min Microtox Assays provide the quickest and least expensive means for a first tier environmental toxicity screening. The reduction in toxicity of the DAM mixture over time will also be investigated using the Microtox Assay.

There are a number of aquatic test organisms available for short term testing. We have chosen the daphnia as the primary test organism for several reasons. Daphnia are used nation wide, therefore toxicity comparisons with previous studies are available. Daphnia are inexpensive to culture in the laboratory and can be maintained indefinitely, therefore reducing the variability between organisms providing more reliable results.

The Microtox Assay provides representative species from a different level of biological organization that adds to the toxicological predictive power of the screening tests to be performed. The exposure of a luminescent marine bacterium, *P. phosphoreum*, to toxicants will typically lower light output in proportion to toxicity, allowing for a dose response relationship to occur.

Interest in this assay is based on the following advantages: quick assay time, low cost, small sample size needed, lack of organism culturing requirements, reliability (standardization), and sensitivity. The Microtox Assay has been and continues to be consistently studied and evaluated since its commercial introduction in 1979 for an array of applications, which include toxicity screening complex effluents, pure compounds, soil sample screening, and in bioremediation.[2-8]

In this study, the Microtox Assay serves to provide both end points of toxicity for the individual DAM components and of the mixture as compared to results from daphnia assays and relative toxicity of the mixture over time to observe toxicological fate.

2. METHODS AND MATERIALS

All testing conformed to current Environmental Protection Agency[9,10] and American Society for Testing and Materials[11] guidelines. These studies were conducted under Good Laboratory Practices, and conformed to all interagency standard operating procedures.

The DAM solution was prepared fresh for every phase of testing. Eight grams of HTH and 50 mL of water were placed into a 100-mL volumetric flask and shaken to dissolve the HTH. If the HTH did not disperse evenly into the water column the flask was immersed into an ultra sonic water bath. The CHP was then added to the 100-mL mark and shaken to provide a uniform mix. Samples were taken from the flask within 15 min of being mixed and diluted accordingly for each phase of the study.

2.1 Daphnia Assays.

The *D. magna* were obtained from Dr. Freida Taub (University of Washington, Seattle, WA) and reared for the past 9 years in this laboratory using methods described by Goulden et al.[12] Daphnia stock cultures were fed a mixture of vitamin enriched *Ankistrodesmus falcatus*, *Selenastrum capricornutum*, and *Chlamydomonas reinhardi*. Daphnia culture media was derived from well water, which was passed through a treatment system containing limestone pH adjustment, iron removal, carbon filtration, and UV sterilization. The well water was monitored

for 92 commonly found ground water pollutants every 4 months by Watercheck National Testing Laboratories, Inc.

The test beakers were placed into a temperature controlled room at 20 °C with a light-dark cycle of 16:8 hr with 315 ft candles of light. Two replicates per concentration, containing 10 daphnia less than 24 hr old, in a total of 100 mL of test solution was used. The pH and dissolved oxygen measurements were taken at the start of testing. At 24 and 48 hr, daphnia were checked for mortality. If the daphnia were not actively swimming, they were touched with a pasture pipet. If there was no response or the daphnia could not swim actively for 15 s, it was considered immobilized. The EC_{50} (the effective concentration at which 50% of the organisms are immobilized) values were computed using the probit analysis as prepared by Kessler.[13] The EC_{50}s were also tabulated graphically using a least square regression analysis verifying all probit results.

2.2 Microtox Assay.

Materials used in the Microtox Assay, lyophilized *Photo-bacterium phosphoreum* at approximately 100,000,000 per vial (Reagent), 2% sodium chloride solution (Diluent), 22% sodium chloride solution (Microtox Osmotic Adjustment Solution, MOAS) for adjustment of osmotic pressure of concentrated samples not requiring pre-dilution with Diluent, were supplied by Microbics, Inc.

The choice of using the Basic Extended Dilution test[14] version of the Microtox Assay was based on the requirement of an EC_{50} end point and the ability of the test to encompass a large range of concentrations in which the EC_{50} may be found.

Typically, the Basic Extended assay is conducted within the temperature controlled (15 °C) wells of a photometer (Microtox Analyzer), which consists of 12 sample solutions, serially diluted by a factor of 2, with 3 controls. A corresponding set of tubes filled with diluted Reagent (following a 15 min temperature stabilization period) are read at time zero for initial light output (I_o). Aliquots from the corresponding tubes of diluted sample are added and mixed. Light output is then measured at predetermined times (t), usually 5 and 15 min. Due to the natural decay of light output over time, the timed readings are normalized using the "Blank Ratio (BR)," which is the ratio of the light output of the control at time t to light output of control at time 0. The BR is applied to I_o's to correct for drift and effects of diluting the organisms. Light lost to light remaining is calculated, and further data reduction produces an EC_{50} (the effective concentration at which there is a 50% reduction in light output).

Depending on toxic response over time and quality of data as determined by confidence factors, either the 5- or 15-min EC_{50} is used for comparisons. If it is observed that the values and confidence limits for each time interval are approximately equal, it is customary to use the 5-min EC_{50}. Should data show an increased toxic response for the 15-min reading, and if the 95% confidence range is similar to the 5-min data, the 15-min data is used for comparative purposes. Data for this study is given at t = 5 min because all data generated at 15 min show slight decreases in toxicity, which indicates that the full effect of the toxicants occurred within 5 min.

Calcium hypochlorite and N-cyclohexyl-2-pyrrolidinone were each mixed separately in volumetric flasks to desired stock concentrations using 2% sodium chloride (Microtox Diluent). All stocks were made fresh 15 min prior to start of assays with the exception of those used in assays determining toxicity reduction over time. Solutions of calcium hypochlorite were placed in an ultrasonic water bath for approximately 3 min to brake dissolve particles.

The DAM mixture was prepared in a glass volumetric flask as previously described and allowed to sit for approximately 2 weeks under standard laboratory conditions of 9 hr light/15 hr dark, with a temperature of 21 $\pm$ 3 °C. Aliquots were taken at time 0, 7, and 14 days for use in determining the toxicity over time.

3. ANALYTICAL DETECTION METHODS

The compound CHP is a heterocyclic organic, clear nonvolatile liquid, which is also water soluble. The CHP used for the standard in this study was obtained from GFA Chemical Corporation (lot# 18/19). Compound purity was 98% minimum as determined via gas chromatography (as area %). A primary standard was prepared by dispensing a known volume of CHP directly into a 100-mL volumetric flask and diluted to volume with distilled water. Working standards ranging from 200 to 800 µg/mL in distilled water were made by volumetric dilution of the primary standard.

N-cyclohexyl-2-pyrrolidinone can be analyzed by gas or liquid chromatography. High performance liquid chromatography was the method of choice since the samples were already in an aqueous state and could be injected directly onto a reverse phase column (Unisphere - PBD, 4.6 x 250 mm). The mobile phase consisted of 50% acetonitrile and 50% water at a flow rate of 2.0 mL/min. Detection was in the far ultraviolet at a wavelength of 220 nm, since no strong chromaphores are present for CHP at higher wavelengths. Sample quantitation was achieved by comparing the area response of the sample to a regression line

established from injections of known standard concentrations and their respective areas.

When calcium hypochlorite is placed in solution, the oxidation of the molecule liberates chlorine. Therefore, the determination of HTH in solution was determined by the amount of available chlorine in solution. Available chlorine determinations were completed via idometric titration using thiosulfate as titrant (ASTM Method D 2022).[15] Aliquots of the DAM solution (100 μL) were taken and diluted to 100 mL (dilution 1). One milliliter samples were removed from the diluted solution and again diluted to 100 mL (dilution 2). To the dilution 2 stock, 5 mL of acetic acid and 0.5 mg of potassium iodide were added and mixed. The final solution was immediately titrated using 0.1 N $Na_2S_2O_2$. When the iodine color disappeared, the titration was completed. The percentage of available chlorine was calculated using Equation 1.

$$\text{Available Chlorine \%} = [\ V\ N\ A\ K\ ]\ /\ W \times 100 \qquad (1)$$

where

V = volume of titrant
N = normality of the thiosulfate solution, (M/L)
K = constant for Ca(OCl)2, (35.75)
A = conversion factor (1L / 1000 mL)
W = weight of sample (g)

4. RESULTS

The concentrations of HTH and CHP in 100% DAM solution were 80 g/L and 544 g/L, respectively. The pH of 100% DAM solution was 13. However, the dilutions used for testing were high enough that pH effects on the organisms were not a factor. Free chlorine in the DAM mixture was monitored for 100 hr. At time 0, the chlorine levels were approximately 60%. After approximately 24 hr, less than half the original free chlorine remained in solution (Figure). Equilibrium was reached at 93 hr with approximately 1.6 % free chlorine, which equates to 1.2 mg/L chlorine.

Table 2 shows the acute toxicity of the DAM mix and individual components as determined by the Microtox Assay (5-min exposure) and the *D. magna* bioassay (48-hr exposure). Clearly, the HTH is the most toxic component of the DAM mixture. The HTH is more than two orders of magnitude more toxic to the bacteria than CHP, and over three orders of magnitude more toxic to daphnia than CHP.

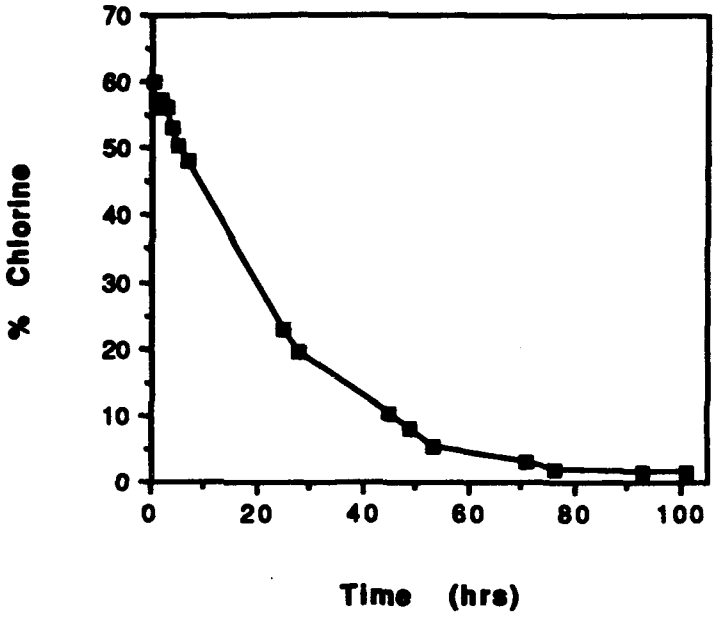

Figure. The Amount of Free Chlorine in the DAM Mix over Time

Table 2. The Toxicity of the DAM Solution and the Individual Components

			Toxicity Rating[c]
CHP	Daphnia	48 hr EC_{50} = 107 mg/L	3 (Low Toxicity)
	Microtox	5 min EC_{50} = 39.8 mg/L	†
HTH	Daphnia	48 hr EC_{50} = 0.04 mg/L	9 (Highly Toxic)
	Microtox	5 min EC_{50} = 0.25 mg/L	†
Acetone	Daphnia	48 hr EC_{50} = 10.0 mg/L	6 (Highly Toxic)
	Microtox	5 Min EC_{50} = 21,500 mg/L[16]	†
PEG[a]	Daphnia	48 hr EC_{50} > 1,000 mg/L[b]	0 (Not Toxic)
	Microtox	5 Min EC_{50} = 8,880 mg/L[16]	†
DAM Mix	Daphnia	48 hr EC_{50} = 5.00 X 10^{-5}%	9 (Highly Toxic)
	Microtox	5 min EC_{50} = 5.30 X 10^{-4}%	†

[a] polyethylene glycol (PEG)
[b] Work conducted at Chemical and Biological Defense Agency.
[c] The toxicity ranking is based on a scale of 0-9, 9 being the most toxic.[16]
†The Microtox is a relatively new assay and has not yet been worked into the scoring criteria.

The DAM mix was more toxic to both test organisms than HTH (Table 2). However, the concentrations of the individual components in the mix, at the EC_{50} values for daphnia and Microtox (Table 2), were below detectable limits for our current in-house analytical methods. Extrapolating from the concentrations of the 100% DAM mix, the concentrations of HTH and CHP were 0.4 and 2.7 mg/L, respectively, for daphnia and 4 and 27 mg/L, respectively, for Microtox. These calculations assume that there is no degradation of HTH and CHP in solution. Therefore, the actual concentrations would be less. Daphnia were more sensitive over all than the bacteria, except to CHP. The CHP was approximately 2.5 times more toxic to the bacteria than to daphnia.

The effects of time on the toxicity of the DAM mixture were investigated. The Microtox Assays were conducted over a 2-week period, with monitoring at time 0, 1, and 2 weeks. After 1 week, the DAM solution had become 10 times less toxic to the bacteria. Between week one and week two, the toxicity of the DAM solution was reduced by a factor of 1.4 (Table 3). As a comparison, the toxicity of acetone and PEG, to daphnia and *Photobacterium phosphoreum* have been included in Table 2.

Table 3. The Reduction of DAM Toxicity over Time using the Microtox Assay

Microtox (t=0)	5 min EC_{50} = 5.3 X 10^{-4}%	(4.1 - 7.0) 10^{-4}*
Microtox (t=7 days)	5 min EC_{50} = 5.6 X 10^{-3}%	(5.0 - 6.4) 10^{-3}
Microtox (t=14 days)	5 min EC_{50} = 8.0 X 10^{-3}%	(7.0 - 9.0) 10^{-3}

*95% Confidence Intervals

5. DISCUSSION

The assays conducted in this report were done under static conditions. Many researchers suggest that flow through or static renewal assays should be used in estimating the toxicity of mixtures containing chlorine, because residual chlorine is lost over time. However, the use of the DAM mixture would not be a continuous insult to the environment as in sewage treatment plant effluents. Therefore, the authors chose to conduct short-term static assays to estimate the toxicity of the DAM mixture.

The HTH was the most toxic component of the DAM solution. Even though the HTH was diluted to the point of no pH effects, the material remained toxic to daphnia and *P. phosphoreum*. The 5- and 15-min Microtox data showed the toxicity of the HTH to be reduced over a 10-min period. This is a direct

result of the oxidation/hydrolysis of HTH in solution. The CHP was only moderately toxic to the test organisms and does not pose as high a threat to an aquatic ecosystem as HTH. The HTH is the major contributing factor in the toxicity of the DAM solution.

Due to its stability in water, chlorine (Cl^-) will not remain in solution long. The chlorine demand created from dissolved organics (mainly organic nitrogen) and some inorganics will react with chlorine, reducing the concentration of available chlorine in solution. The measurement of available chlorine in this study, was actually a measurement of free residual chlorine composed of chlorine, hypochlorous acid (HOCl), and hypochlorite ions (^-OCl) (Equation 2). After 24 hr, the amount of free chlorine in solution was reduced approximately 40%. At 96 hr, the amount of free chlorine was 1.2%.

$$Ca(OCl)_2 + H_2O \rightleftharpoons {}^-OCl$$
$$^-OCl + H_2O \rightleftharpoons HOCl + {}^-OH \qquad (2)$$

In the presence of organic nitrogen, hypochlorous acid tends to form chloramines. Conflicting open literature shows chloramines to be more toxic than chlorine in some cases[17] and less toxic in other cases.[18] However, these reported results were conducted using freshwater fish not daphnia. The 1-cyclohexyl-2-pyrrolidinone component of the DAM mixture contains nitrogen. Additional nitrogen, in the form of ammonia produced from respiratory by-products, provides a source of nitrogen. Therefore, the potential exists for the formation of chloramines.

Over time, the toxicity of the DAM mixture was unchanged or reduced. For example, there was no difference in toxicity to daphnia when exposed to the DAM mixture from 24 to 48 hr. The exposure of *P. phosphoreum* to the DAM mix showed the toxicity to be reduced 10 fold within 7 days and 15 fold within 14 days. If chloramines were formed in the DAM mixture, it does not appear to be adding to the toxicity of the DAM solution.

The formation of OH- causes the overall pH of the DAM mixture to reach pH values up to 13. Even with the formation of hypochlorous acid (a very weak acid), the pH remains high. To reach an EC^{50} level for daphnia, the DAM mixture was diluted to 10-5%, which resulted in a pH = 8.1, thereby eliminating the possibility of the pH contributing to the toxicity (control pH = 8.1).

The reaction of chlorine in fresh water has been thoroughly characterized; however, chlorine in salt water is a more complex situation. It has been proposed that when chlorine enters salt water it forms halogenated compounds, which

contribute to toxicity.[19] More work in the field of chlorine speciation in marine environments is needed before conclusions can be made on the mode of toxicity to marine organisms.

Katz reports free chlorine is much more toxic to fresh water organisms than to marine organisms.[20] Salt water organisms are mush less affected by chlorine due to their osmotic regulatory systems and chemical speciations of chlorine in salt water. Therefore, the impact of the DAM mixture on a marine environment may be less than in a freshwater ecosystem.

Brungs[17] conducted a study using 27 different fish species, one daphnia, and one protozoa species. He proposed that free chlorine concentrations, at intermittent exposures, not exceeding 0.04 mg/L for a period of 2 hr/day, would protect most species of fish. Based on the chlorine concentration remaining in the DAM mixture after 100 hr (0.09 mg/L), it would still exhibit affects on test organisms. However, under field conditions, many more parameters are involved that may reduce the effects of chlorine. There is a need to establish a maximum concentration and a time between releases that would allow the ecosystem to recover. Release rates should be determined for each area where the DAM solution is to be used.

6. CONCLUSION

The Decontaminating Agent: Multipurpose (DAM) mixture was toxic to daphnia even when the pH affects were eliminated. Calcium hypochlorite (HTH) was more toxic to the test organisms than N-cyclohexyl-2-pyrrolidinone. Free chlorine from the HTH was the major contributor to the toxicity of the DAM Decontamination Solution. Using the Environmental Protection Agency Chemical Scoring System for Hazard and Exposure Identification, based on the component toxicity, the DAM mixture was ranked 9 (highly toxic, on a scale from 0-9, 9 being the most toxic), which is more toxic to daphnia than acetone and polyethylene glycol (ranked 6 and 0, respectively). Over time, the toxicity (using Microtox Assays) of the DAM solution was reduced due to the lose of chlorine from the system. Caution should be taken to prevent long term excessive exposure to aquatic ecosystems.

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