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AQUATIC TOXICITY OF DECONTAMINATING SOLUTIONS DS-2/DS-2P

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RESEARCH AND TECHNOLOGY DIRECTORATE

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Aberdeen Proving Ground, MD 21010-5423

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13. ABSTRACT *(Maximum 200 words)*

The U.S. Army is working on reducing the possibility of human health effects from exposure to the 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. The proposed formulation (DS-2P) replaces EGME with propylene glycol monomethyl ether. This report provides initial environmental screening results using *Daphnia magna*, fathead minnow, *Photobacterium phosphoreum*, and earthworm.

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PREFACE

The work described in this report was authorized under Project No. 10464806DF97, Sales Order No. 2FK4. This work was started in May 1993 and completed in December 1993.

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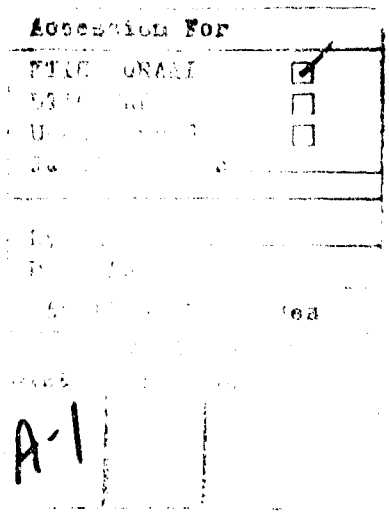

QUALITY ASSURANCE

This study, conducted as described by Protocol 22093000X053, 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	20 Jan 93	21 Jan 93
Stock preparation	9 Mar 93	9 Mar 93
Data & Final Report	9 Jun 94	9 Jun 94

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 9 Jun 94

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AQUATIC TOXICITY OF DECONTAMINATING SOLUTIONS DS-2 / DS-2P

1. INTRODUCTION

The Army is working on reducing the possibility of human health effects from exposure to the 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 abnormalities in laboratory animals [1]. Eliminating this component will reduce the chance of human health risks and also make transporting and disposal of the solution less restrictive.

The proposed replacement formulation has propylene glycol monomethyl ether (PGME) replacing EGME. Table 1 lists the formulation of DS-2 and the proposed mixture (DS-2P).

Table 1. DS-2 and DS-2P Formulation

Components	Percent by Weight*
DS-2	
Diethylenetriamine	70
Sodium Hydroxide	2
Ethylene glycol monomethyl ether	28
DS-2P	
Diethylenetriamine	70
Sodium Hydroxide	2
Propylene glycol monomethyl ether	28

* The percent by weight is an average of the manufacturers specification range.

Base line environmental data are needed in order to assess the impact of decon solutions to the environment when used in the field or if accidental spills occur. This study investigates the aquatic toxicity of the DS-2 and DS-2P mixtures on *Daphnia magna*, (water flea), *Pimephales promelas* (fathead minnow), Eisenia foetida (earthworm) and *Photobacterium phosphoreum*, (luminescent marine bacterium). The reduction in toxicity of the decon mixtures over time was also investigated using the Microtox Assay (MTX). Toxicity comparisons will determine if replacing EGME with PGME reduces toxic effects of decontamination solutions to the test organisms. The information from this study will support future environmental assessments needed in determining field use rates and disposal management.

Although a number of aquatic organisms are available for short term testing, we chose the daphnia and fathead minnow as the primary test

organisms. These two species are used nationwide, therefore an extensive data base exists for toxicity comparisons. Also, both are inexpensive to culture in the laboratory and can be maintained indefinitely. These factors reduce the variability between organisms and provide the basis for consistantly reliable data.

The MTX provides representative species from a different level of biological organization that add to the toxicological predictive power of the screening tests to be performed. The exposure of *P. phosphoreum* (luminescent marine bacterium) to toxicants will typically decrease light output in proportion to increased 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, no organism culturing required, reliability (standardization) and sensitivity. Commercially introduced in 1979, the MTX continues to be studied and evaluated for an array of applications, including toxicity screening of complex effluents, pure compounds, soil sample screening and bioremediation.

One of the first areas impacted during field use and accidental spills is soil, therefore, toxicity studies using the earthworm will yield information on how soil dwelling organisms may be affected from exposure to decon mixtures. The earthworm study determines acute effects (mortality) and subchronic effects (weight loss).

2. METHODS AND MATERIALS

All testing conformed to Environmental Protection Agency (EPA) [2,3] and American Society for Testing and Material (ASTM) [4] guidelines. These studies were conducted under Good Laboratory Practice (GLP), and conformed to all inter-agency standard operating procedures.

The decon solutions were received packaged in sealed 1-1/3 quart cans. The cans were opened under a nitrogen atmosphere to prevent CO_2 and moisture contact to the neat solutions. The contents were placed in screw top polycarbonate flasks and sealed with parafilm. All samples were removed from the flasks while under a nitrogen atmosphere. The solutions were tested for reactivity using the chloroform procedures [5] and proved to be within specifications.

2.1 Daphnia Assays

The *Daphnia magna* were obtained from Dr. Freida Taub [6] at the University of Washington in Seattle and reared for the past nine years in the laboratory using methods described by Goulden, *et al.* [7]. 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 six months by Watercheck National Testing Laboratories, Inc.

The test beakers were placed in a temperature controlled room at 20°C with a light-dark cycle of 16:8 hours with 315 ft candles of light. Two replicates per concentration (8.0 - 65.0 x 10^{-4} %) containing 10 (daphnia less than 24 hours old) in a total of 100 mL of test solution were used. The pH and dissolved

oxygen measurements were taken at the start of testing. After 24 and 48 hours, daphnia were checked for mortality. If the daphnia were not actively swimming, they were touched with a Pasteur pipette. If there were no response or the daphnia could not swim actively for 15 seconds they were considered immobilized. The EC_{50} (the effective concentration at which 50 percent of the organisms are immobilized) values were computed using the probit analysis as prepared by Kessler [8]. The EC_{50}s were also tabulated graphically using a least square regression analysis verifying all probit results.

2.2 Fish Assays

Adult fish were originally obtained from Kurtz's Fish Hatchery in Elverson, PA. These fish were maintained in 40 gallon glass aquaria equipped with under gravel biofilters. The culture water was the same as described in the daphnia methods. Adult fish were fed Tetramin flake food in the mornings and *Lumbriculus varigatus* (black worms) in the afternoon. This feeding regime encouraged the fish to breed continuously. Adult breeding fish are replaced annually to maintain a healthy gene pool.

Adult fish deposit eggs on the under side of clay pots. After the eggs were fertilized, they were transferred to hatchery tanks. Upon hatching, the fry were fed freshly hatched brine shrimp twice a day. The fry were used in toxicity tests after 14 days of age. Fish used in testing were all of similar age and size. The loading did not exceed 0.8 g of fish per liter of solution. If fry appeared stressed or if 5% died within 48 hours before testing, the fry would be discarded. Water temperature was maintained at 20± 1°C with 16 hours of light and 8 hours of darkness.

The test chambers were one gallon glass jars. The test chambers and glassware were scrubbed with phosphate-free soap, rinsed with tap water until sudsing had ceased, then rinsed with distilled water.

A stock solution of the toxicant was prepared and dispensed directly into the test chambers then diluted to proper concentrations. The dissolved oxygen and pH were measured before fish were transferred to the test chambers. After the fish had been added to the test chambers, a random number table was used to assign each chamber (including controls) to one of two blocks. Next, the chambers were assigned a location number to each of the treatments within that block. (See Appendix 2 for further clarification of randomizing the treatment groups.) The EC_{50} values along with the 95% confidence intervals were computed by the probit analysis method and checked graphically using the same procedure described in the daphnia methods.

2.3 Earthworm Assay

Earthworm toxicity testing utilized *Eisenia foetida* as the test organism. Survival rates and weight changes were used as indices of toxicity. Test methods used for earthworm toxicity studies were adapted from Karnak and Hamelink [9] and Neuhauser *et al.* [10].

Earthworms were originally purchased from Bert's Bait Farm, Irvine, KY, cultured in a 50/50 mixture of peat moss/potting soil and housed in styrofoam coolers at ambient laboratory temperature.

Earthworms were fed fermented alfalfa pellets obtained by placing the dry alfalfa pellets (commercial rabbit feed) in a sealed container with enough water to cover the pellets. The pellets were periodically mixed and water

added, if necessary, for a two-week period. Ideally, the amount of food added to each cooler should last the earthworms for one week. However, the exact amount added depends on the number and size of the earthworms in each particular cooler. If the food was depleted before one week, additional food was added. The contents of each cooler were mixed once per week to loosen and aerate the soil medium and evenly distribute water throughout the container. Any food remaining on top of the medium was discarded before mixing. After mixing, fresh food was added to the container.

The earthworm toxicity test involved placing 200g substrate and five earthworms into 600-mL glass beaker (two replicated per concentration 500, 1000, 2500, 5000 mg/kg). Earthworm substrate for experiments consisted of a non-sterile artificial soil and distilled water [10]. The heterogeneous parameters of a reproducible artificial soil mixture reduce the variability of the test that could occur if field soil was used. The components of the artificial soil are list in Table 2.

Table 2. Components of the artificial soil used in the Earthworm Toxicity Test.

Stock Components	% by wt.
Lime	1
Finely-ground sphagnum peat moss	10
Kaolinite clay	20
Fine sand	69

The test soil was prepared by mixing (in food blender) the artificial soil with the test substance. Distilled water was slowly added and mixed until a uniform texture was established (25% soil moisture). The test soil was then divided into replicates and placed into 600-mL beakers (200 g of soil).

After the beakers were prepared, 75-100 earthworms were removed from one of the styrofoam coolers and put into a plastic pan. The earthworms were quickly rinsed in tap water and excess water drained from the pan. Five earthworms were randomly picked, quickly blotted with a paper towel, and weighed as a group. They were placed in a beaker which was then covered with nylon screen and cheesecloth secured with a rubber band. The beakers were randomly placed in plastic trays within an incubator. Water was added to the trays to increase the humidity which would reduce drying of the soil in the beakers. The incubator lights were set for continuous operation. Since the earthworms are photophobic, the light encouraged them to burrow into the soil and helped prevent them from crawling out of the beakers.

The earthworms remained in the incubator for a two week exposure period. Beakers were rearranged in the trays at the end of the first week. On day 14, the earthworms were removed from each beaker and reweighed. The earthworms were also examined for changes in color, texture, motility and general physical condition.

Weight change was evaluated using Analysis of Covariance (ANCOVA) to the Newman-Keuls paired comparison of means

2.4 Microtox Assay

Materials used in the MTX Assay included lyophilized *Photobacterium phosphoreum* at approximately one hundred million per vial (reagent), 2% sodium chloride solution (diluent) and 22% sodium chloride solution (MTX Osmotic Adjustment Solution, MOAS) for adjustment of osmotic pressure of concentrated samples not requiring pre-dilution with diluent. All materials were supplied by Microbics, Inc.

Use of the Basic Extended Dilution test [11] version of the MTX assay was based on the requirement of an EC_{50} endpoint and the ability of the test to encompass a large range of concentrations in which the EC_{50} may be found.

The Basic Extended assay was conducted within the temperature controlled (15°C) wells of a photometer (MTX Analyzer). The assay included twelve sample solutions, serially diluted by a factor of two, with three controls. A corresponding set of tubes filled with diluted Reagent (following a 15 minute temperature stabilization period) were read at time zero for initial light output (I_0). Aliquots from the corresponding tubes of diluted sample were added and mixed. Light output was then measured at predetermined times (t), usually 5 and 15 minutes. Due to the natural decay of light output over time, the timed readings were normalized using the "Blank Ratio" (BR), which was the ratio of the light output of the control at time t to light output of control at time 0. The BR was applied to I_0's to correct for drift and effects of diluting the organisms. The ratio of light lost to light remaining was 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 minute EC_{50} was used for comparisons. It is customary to use the 5 minute EC_{50} when the values and confidence limits for each time interval are approximately equal. Should data show an increased toxic response for the 15 minute reading and if the 95% confidence range were similar to the 5 minute data, then the 15 minute data was used for comparative purposes. Data for this study is given at t = 5 minutes because all data generated at 15 minutes showed only slight decreases in toxicity, indicating the full effect of the toxicants occurred within 5 minutes.

The DS-2 and DS-2P solutions were diluted separately to obtain the desired stock concentrations in volumetric flasks using 2% sodium chloride (MTX Diluent). All stocks were made within 15 minutes prior to start of assays, with the exception of the assays to determine toxicity reduction over time.

The decon solutions were prepared as previously described and aliquots were taken at time 0, 7 and 14 days for use in determining the toxicity over time. The stocks were maintained under standard laboratory conditions of 8 hours light / 16 hours dark and a temperature of 21°C ± 3° throughout the 14 day analysis. The undiluted material was subjected to the same procedures and the resulting data compared.

3. RESULTS/DISCUSSION

Overall, the daphnia were a more sensitive test organism while the earthworms were much less sensitive (having a no effect level of 5000 mg/kg). Substituting PGME for EGME did not significantly change the toxicity of the decon solution.

DS-2 and DS-2P were approximately one order of magnitude less toxic than the DAM decon solution and several orders of magnitude less toxic than malathion (Table 3). The pH of both solutions at 100% was extremely high (pH $\geq$ 13, measured using conventional glass electrode). However, the dilutions used during testing lowered the pH to tolerable limits (8.0 - 9.5) [12], thus pH was eliminated as a direct cause of toxicity to daphnia and fish.

The EC_{50} values and 95% confidence limits for DS-2P and DS-2 are listed in Table 3. The 95% confidence limits overlap when comparing the toxicity of both solutions.

Stock solutions of DS-2 and DS-2P were prepared by diluting into water and allowed to stand for several days under ambient laboratory conditions. Also, neat samples were allowed to stand under the same conditions. At day 0, 7 and 14, samples were taken and subjected to MICROTOX assays to monitor the change in toxicity. Over a 14 day period, the toxicity of DS-2 and DS-2P did not change (Table 4).

Table 3. Result from MICROTOX studies investigating the effects of time on the toxicity of DS-2 and DS-2P.

	P. phosphoreum 5 min. EC_{50} (Vol/Vol %)		
	Day 0	Day 7	Day 14
DS-2P	2.8×10^{-3}	3.5×10^{-3}	3.2×10^{-3}
DS-2	3.9×10^{-3}	4×10^{-3}	4.6×10^{-3}
DAM, [13]	5.3×10^{-4}	5.6×10^{-3}	8×10^{-3}

The specification sheets for the decon solutions require packaging in a moisture and carbon dioxide free atmosphere to prevent degradation. The test results showed no change in toxicity over a 14 day period. This suggest one of three possibilities: the decon solutions did not degrade when exposed to moisture, the degradation occurred instantly and the toxicity of non-degraded decon solution could never be obtained in an aquatic environment, or the materials were degraded before opening due to improper packaging.

The activity of the decon solutions met specs after being divided (under nitrogen) into smaller samples, therefore eliminating the possibility of improper packaging.

When diluted to approximately 50 % with water, DS-2 degrades instantaneously [14]. Assuming DS-2P reacts similarly to DS-2 when added to water, complete degradation may have occurred before toxicity end point were reached.

The most probable reason for not seeing a change in toxicity over time is the instantaneous degradation of the decon solution when added to water. Even though the MICROTOX end points are reached in 5 minutes, a change in toxicity was not observed.

It is assumed the degradation of DS-2P is instantaneous (similar to DS-2 in water) and the toxicity estimates presented in this paper are associated with the by-products produced when adding the decon solutions to water.

4. CONCLUSION

The substitution of PGME for EGME did not significantly change the toxicity of the decon solutions to the test organisms.

Over time, the toxicity of DS-2P did not change. Therefore it is assumed that the toxicity estimates of the decon solutions are directly related to the degradation by-products produced when added to water. Fate studies are needed to confirm the reaction that occurs when adding DS-2P to water.

The decon solutions scored a ranking of 9, highly toxic (out of a scale of 0 - 9, 9 being the most toxic), on the Chemical scoring System for Hazard and Exposure Identification [17].

If used in the open environment, efforts should be made to provide as much containment of decon solutions as possible to prevent runoff into the surrounding ecosystem.

Table 4. Toxicity Comparison Between DS-2P and DS-2

	Daphnia 48 hr EC50 (95% CI) Vol/Vol %	Fathead Minnow 96 hr EC50 (95% CI) Vol/Vol %	P. phosphoreum 5 min EC50 (95% CI) Vol/Vol %	Earthworm 14 day EC50 (95% CI) mg/kg
DS-2P	1.4×10^{-3} (1.2 - 1.7)	2.8×10^{-2} (2.0 - 3.0)	4×10^{-3} (2 - 9)	N.E. *
DS-2	1.7×10^{-3} (1.3 - 2.1)	2.2×10^{-2} (2.0 - 3.0)	3×10^{-3} (2 - 4)	N.E.
DAM [14]	5.0×10^{-5} (4.8 - 5.2)	-----	5×10^{-4} (4 - 7)	-----
Malathion	8.0×10^{-8}, [15]	8.0×10^{-4}, [15]	2.4×10^{-6}, [16]	

* N.E. - No effects were seen up to 2,500 mg/kg.

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SUPPLEMENTARY

INFORMATION

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Aberdeen Proving Ground, Maryland 21010-5423

ERRATUM SHEET

8 November 1994

Report No. ERDEC-TR-202

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Sandra J. Johnson

SANDRA J. JOHNSON
Chief, Technical Releases Office

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Fine sand	69

The test soil was prepared by mixing (in food blender) the artificial soil with the test substance. Distilled water was slowly added and mixed until a uniform texture was established (25% soil moisture). The test soil was then divided into replicates and placed into 600-mL beakers (200 g of soil).

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