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ASSESSING THE FEASIBILITY OF AN IN VITRO CYTOTOXICITY METHOD TO DETECT HARMFUL UBIQUITOUS CHEMICALS

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

//SIGNED//

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The objective of this work was to assess the feasibility of accomplishing aqueous extracts of soil samples and determining if the extracted solution induced adverse effects in the human myelomonocytic cell line, HL60. Dosing of HL60 cells was accomplished over a 24-hour period using 100% of extracted media from standard soil samples containing known contaminants. Assessments of viability, apoptosis, reduced thiols, and mitochondrial membrane integrity were accomplished by argon-ion laser flow cytometric ananlysis, using chemical labels specific for each endpoint. The in vitro cytotoxicity data was compared with the results of Microtox and Mutatox tests was well as earthworm and plant toxicity tests. In vitro cytotoxicity tests results exhibited good correlation with other tests results.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

The research reported herein was initiated and conducted at the Operational Toxicology Branch of the Air Force Research Laboratory at Wright-Patterson Air Force Base. This work was conducted between March 1998 and April 1999.

Special acknowledgments and appreciations are extended to Remediation Technologies, Inc., One Monroeville Center, Suite 1015 Monroeville, PA 15146-2121. They graciously provided the soil samples used in this study and, additionally, provided the results of baseline tests of soils. These test results characterized the chemistry and toxicity of the soil samples.

This report describes the results of a preliminary study for developing an *in vitro* cytotoxicity test methodology for screening soils for the potential to cause adverse health effects. No animals were used in the research summarized by this report.

ABBREVIATIONS

ATCC	American Type Cell Culture
CMFDA®	5-chloromethylfluorescein diacetate
°C	Degrees Celsius
ddH_2O	Deionized, distilled water
EC-50	Effective concentration that reduces Vibrio fischeri luminescence by 50%
g	Grams
G	Gravity
HBSS	Hank's balanced salt solution
HEPES	4-(2-Hydroxyethyl)-1-peperazineethanesulfonic acid
MCF	Mean Channel Fluorescence
MEIC	Multicenter for the Evaluation of *In Vitro* Cytotoxicity
min	Minutes
mL	Milliliter (10^{-3} liters)
µg	Microgram (10^{-3} grams)
µL	Microliter (10^{-6} liters)
µM	Micromoles (10^{-6} moles)
RPMI	Cell/tissue culture media developed at Roswell Park Memorial Institute

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INTRODUCTION

The U.S. has military personnel deployed to several foreign locations on missions of combat and/or humanitarian support. The primitive nature of most of these deployment sites increases the interaction of our troops with the local environment. Within the Department of Defense, there is an increasing interest in developing the tools necessary to provide an operational commander with accurate and timely evaluations of the health risks associated with the sites being considered for deployment.

Using this information in the mission planning process will allow actions to be taken to minimize mission degradation due to environmentally related adverse health effects. Intervention could take the form of alternate site selection, reduction of individual deployment time, and the use of personal protective equipment.

We want to be able to quickly answer the question, "Is this site potentially hazardous to our troops?" rather than, "What potentially toxic substances are at this site?" As such, our ultimate goal is to develop a biologically based assay to evaluate the functional toxicity of an environmental sample. To this end, we are focussing our efforts on changes in cellular activity of mammalian cell lines following exposures to extracts from environmental samples.

In this study, our goal was to assess the potential for detecting the presence of a hazardous contaminant or multiple contaminants in a crude soil extract, characterize the contaminant(s) in terms of cellular effects, and have initial results within 48 hours. We were not concerned with identification of the contaminant(s).

This report presentsthe initial efforts to develop quick screening procedures using *in vitro* mammalian cell lines to assess the potential toxicity of environmental samples such as soil, water, and air. Future research will be designed based on these results.

MATERIALS AND METHODS

Soil Samples: Two soil samples were obtained from each of three contaminated sites. The sites were identified as U, V, and W. The two samples associated with each site were: 1) a sample from the contaminated area which was given the identifying suffix of T (*e.g.*, UT), and 2) a sample from a clean site (any contaminants identified were within acceptable levels) near the contaminated site was given the identifying suffix of C (*e.g.*, UC). A total of six samples were obtained: UT, UC, VT, VC, WT, and WC.

Sample UT was clayey silt. The chloride levels (1,102 mg/kg) were high and the metal content was low. The primary contaminant was a diesel or a similar refinery cut.

Sample VT was silty sand. The metal content was low and it was contaminated with diesel or a similar refinery cut.

Sample WT was silty sand. It had a low metal content and contained diesel or a similar refinery cut.

Samples UC, VC, and WC served as additional controls for the test soils.

Cell Line: The HL-60 cell line was obtained from American Type Cell Culture (ATCC Number: 45501), 10801 University Boulevard, Manassas, VA 20110-2209. These cells were derived from a 36-year-old female diagnosed with acute promyelocytic leukemia and are monoblastoid in morphology.

Cells were maintained in two 75 mL culture flasks with RPMI 1640 containing 10% Fetal Bovine Serum, and 1% Penicillin at 37° C in 5% CO_2. The RPMI 1640 contained additional supplements of glutamine, glucose, sodium bicarbonate, HEPES, and sodium pyruvate. Cell cultures were periodically tested for mycoplasma and monitored for bacterial contamination.

Fluorochromes: Annexin-V-Alexa 568® was obtained from BOEHRINGER MANNHEIM Corporation, 9115 Hague Road P.O. Box 50414, Indianapolis, IN 46250-0100. BOBO-1-Iodide®, CMFDA®, Rhodamine-123, and propidium iodide were obtained from Molecular Probes, 4849 Pitchford Avenue, Eugene, OR 97402-9165.

Flow Cytometer: FACScan®, Becton Dickinson, Immunocytometry Systems, 2350 Qume Drive, San Jose, CA 95131-1807. Argon-Ion laser.

Extraction: Soil samples (10 grams) were mixed with 15 mL of RPMI 1640 cell culture media (supplemented with 10% Fetal Bovine Serum and 1% Penicillin) in a 50 mL conical centrifuge tube and placed on a shaker for 120 min at 300 rpm. Samples were then centrifuged at 1900 X g for 10 min, and sterile filtered into a 50 mL conical filter tube containing a 0.22 micron filter. The approximate yield of extract media was 10 - 13 mL. Extract media pH was adjusted to 7.2 – 7.3 , conductivity determined, and refiltered.

HL60 Dosing Procedure: HL60 cell cultures were removed from culture flasks and placed in a sterile 50 mL conical tube for washing. Cells were first washed (x1) in normal growth media. After washing, cells were diluted to approximately 1.33 x 10^6 cells/mL in 100% extracted test media and plated (in triplicate) (1 x 10^6 cells/well, 750 µl) in a sterile 24 well plate. Plated cells were placed in a humidified 5% CO_2 incubator at 37° C for 24 hours. A negative control and a positive control of 500 µM cycloheximide were included with each assay. Each soil and control was plated on individual plates in order to avoid any potential for cross contamination. After the 24-hour incubation period, cells were removed from each well, washed with HBSS, and tagged with fluorochrome to determine the relative amount of apoptosis, viability, reduced thiols, and mitochondrial membrane potential.

Tagging with Annexin-V-Alexa 568®/BOBO-1-Iodide®:

Reagents: Incubation Buffer: 10 mM HEPES, 140 mM NaCl, 5mM CaCl2 in ddH_2O, pH 7.4. BOBO-1-Iodide Stock Solution: 50 µg/mL (ddH_2O). Annexin-V-Alexa 568®/BOBO-1-Iodide® Working Solution: 20 µL Annexin-V-Alexa 568® reagent and 20 µL BOBO-1-Iodide® stock solution in 1 mL of Incubation Buffer. Hanks Balanced Salt Solution (HBSS).

Procedure: Wash 10^6 HL60 cells once in 1 mL HBSS and centrifuge at 300 X g for 5 min. Decant supernatant and resuspend cell pellet in 100 µL of Working Solution. Incubate cells at room temperature for 10 – 15 min. Resuspend cells in 750 µL of Incubation Buffer, vortex, and accomplish analysis on flow cytometer.

Tagging with CellTracker Green CMFDA (5-chloromethylfluorescein diacetate):

Reagents: CMFDA Working Solution: Add 11 µL of dimethyl sulfoxide to one 50 µg vial of CMFDA reagent. Add this solution to 20 mL of HBSS to obtain 5 µM CMFDA.

Procedure: Wash 10^6 HL60 cells once in 1 mL HBSS (no FBS) and centrifuge at 300 X g for 5 min. Decant supernatant and resuspend cell pellet in 1 mL of 37° C CMFDA Working Solution. Incubate for 30 min at 37° C. Centrifuge at 300 X g for 5 min and decant supernatant. Add 1 mL of HBSS, vortex, incubate for 30 min at 37° C. Centrifuge at 300 X g for 5 min and decant supernatant. Resuspend cells in 1mL HBSS, vortex, and accomplish analysis on flow cytometer.

Tagging with Rhodamine-123/Propidium Iodide

Reagents: Rhodamine-123 Working Solution: 0.1 mM (ddH_2O). Propidium Iodide Working Solution: 20µg/mL (ddH_2O). RPMI. HBSS.

Procedure: Wash 10^6 HL60 cells once in 1 mL HBSS and centrifuge at 300 X g for 5 min. Decant supernatant and resuspend cell pellet in 1 mL of HBSS. Add 5 µL of Rh123 Working Solution, vortex, incubate in dark at 37° C for 20 min. Centrifuge at 300 X g for 5 min

and decant supernatant. Resuspend cells in 1 mL HBSS, vortex, and accomplish analysis on flow cytometer.

Flow Cytometry:

Viability/Apoptosis: Apoptosis and viability were determined by simultaneous analysis of Annexin-V-Alexa 568® and BOBO-1-Iodide® positive cells. BOBO-1-Iodide® is a nuclear staining fluorochrome absorbed across cellular membranes of necrotic cells. Annexin-V-Alexa® binds to phosphatidyl serine expressed at the outer membrane surface of cells actively undergoing apoptosis.

BOBO-1-Iodide® (excitation 462 nm, emission 482 nm) positive cells were analyzed with the FL1 detector. 10,000 cells were analyzed for viability by direct measurement of fluorescence and exclusion of BOBO-1-Iodide® positive cells. Viability was expressed as a percentage of 10,000 cells.

Annexin-V-Alexa 568® (excitation 488-596 nm, emission > 600 nm) positive cells were analyzed with the FL3 detector. 2,500 gated live cells (>95% viability) were analyzed for apoptosis by direct measurement of fluorescence and expressed as a percentage of 2,500 viable cells.

Reduced Thiols: CMFDA® (5-chloromethylfluorescein diacetate) is a membrane-permeant probe which is hydrolyzed by intracellular esterases yielding 5-chloromethylfluorescein. This product is conjugated to the thiol group of reduced glutathione by glutathione S-transferase, and excess unconjugated product diffuses into the extracellular medium. Product may also conjugate with thiol groups of intracellular proteins yielding background fluorescence, therefore, this is considered to be a semi-quantitative screening procedure.

10,000 gated live cells (>95% viability) were analyzed by measuring mean channel fluorescence (MCF) on the FL1 detector. MCF of dosed cells was expressed as a percentage of the MCF of the negative control.

Mitochondrial Membrane Potential (MMP): Rhodamine-123 is absorbed by mitochondria of viable cells. Adverse changes in the mitochondrial membrane results in leakage of Rh-123 from the mitochondria and cell into the extracellular medium. MMP is determined by measuring Rh-123 fluorescence of live and dead cells. Cells were counter stained with propidium iodide for identifying and quantifying dead cells.

2,500 gated live cells (>95% viability) were analyzed for Rh-123 fluorescence by measuring MCF on the FL1 detector. Rh-123 MCF of necrotic cells was also measured. MMP was determined by subtracting dead cell MCF from live cell MCF (dosed cells) divided by live cell MCF minus dead cell MCF (no dose cells).

Microtox® Testing:

Supernatant Studies: 5 grams of soil was mixed with 7.5 mL of Microtox® diluent (H20 with 2% NaCl) in a sterilized 40 mL glass vial. The mixture was placed horizontally on an orbital shaker at 200 rpm for 2 hours. After shaking, the mixture was centrifuged at 1200 rpm for 20 min at 4°C. Exposure of the Microtox® organism (Vibrio fischeri) was then conducted on four concentrations (100%, 50%, 25%, and 12.5%). Results are expressed as effective concentration at which 50% reduction in luminescence occurs (EC-50).

Soil Slurry Studies: 7 grams of soil was placed in a 50 mL glass beaker. 35 mL of Microtox® diluent (H_2O with 2% NaCl) was added to the sample and mixed. The slurry was tested at 9 concentrations (100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, and 0.38%). Results are expressed as per supernatant studies.

Mutatox® Testing:

Soil extraction was accomplished at a ratio of 1:5 (soil:water). Two sets of ten dilutions (100%, 50%, 25%, 12.5%, 6.25%, 3.13%, 1.57%, 0.78%, 0.39%, and 0.2%) were prepared. Rat liver S9 fraction was added to one set of dilutions. Exposure of dark mutants of Vibrio fischeri to each dilution was then accomplished with and without S9 fraction. Bioluminescence (light level reading) is then measured at each dilution.

Statistics: Two tailed paired t-testing ($p < 0.05$) was used in comparing results. For each of the four biomarkers, the control soil was compared to the negative control, the test soil was compared to the negative control, and the test soil was compared to the control soil. For apoptosis, the % Annexin-V-Alexa 568® positive cells out of 2,500 live cells was used for the comparisons. For viability, the % BOBO-1-Iodide® negative cells out of 10,000 total cells was used. To compare the thiol reduction, the % CMFDA® positive cells out of 10,000 live cells was used. The % Rhodamine-123 positive cells out of 2,500 live cells was used to test for differences in mitochondrial membrane potentials.

RESULTS

Baseline Toxicity Testing: Baseline toxicity characterization of the soil samples was obtained and is presented in Table 1. All test soils show some degree of toxicity for both plants and earthworms. Soils UT and VT were both Microtox® positive with the EC-50 extrapolated to 94% and 99.8% extract concentrations respectively. Soil WT was Microtox® negative.

Sample	Earthworm Toxicity Test	Plant Toxicity Test	Microtox® Toxicity Test* (EC-50)
UT	Mod – High	Very High ++++ - +++++	Positive (94%)
VT	Nil - Low	Pronounced ++++++	Positive (99.8%)
WT	Low – Mod	High +++ - ++++	Negative (Non Toxic)

Table 1. Baseline Toxicity Characterization. *Accomplished in triplicate.

Microtox® Testing of the Extract Supernatant: The results of Microtox® testing of supernatants indicate a calculated 50% decrease (EC-50) in luminescence of the Microtox® organism at concentrations of: 10.7%(UT), 10.2%(VT), and 81.6%(WT). (Figure 1). Supernatants of samples UC, VC, and WC tested negative.

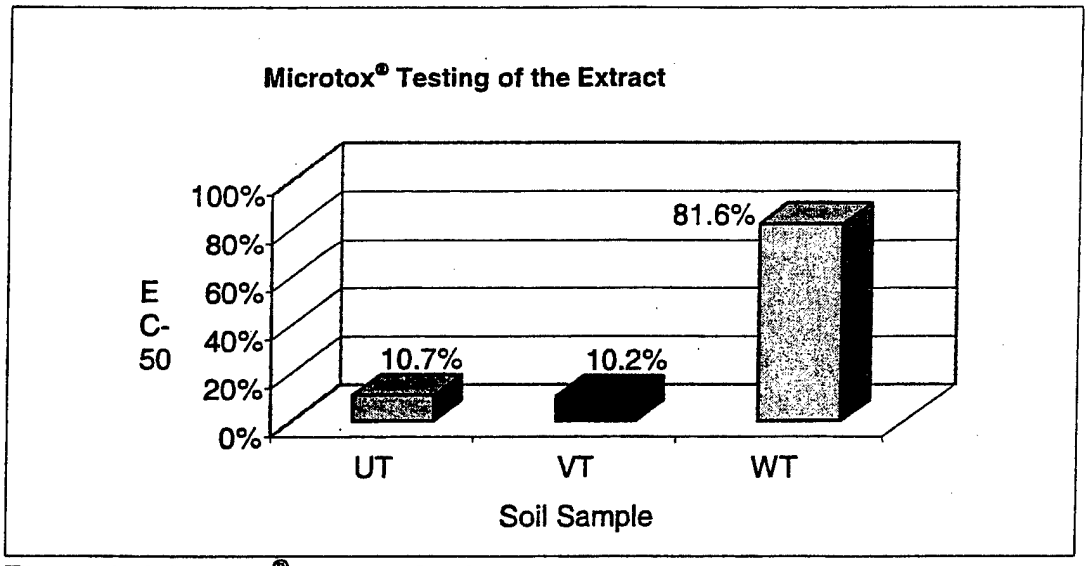

Figure 1. Microtox® testing of soil extract supernatants.

Microtox® Testing of Soil Slurry: The results of Microtox® testing of soil slurries indicate an EC-50 of: 1.38%(UT), 1.40%(VT), and (0.047%)(WT). (Figure 2). Control soils UC, VC, and WC were not tested by the slurry method. Greater toxicity was exhibited in the soil slurries when compared to extracts. This is especially notable in the WT soil.

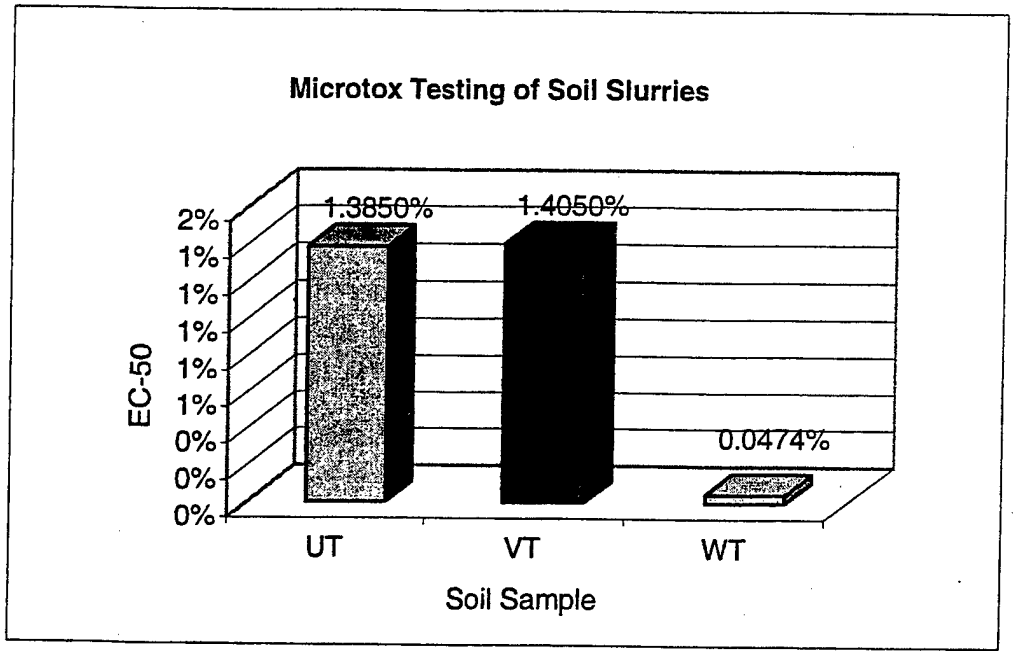

Figure 2. Microtox® testing of soil slurries

Mutatox® Testing: Testing of soils in the absence of rat liver S9 fraction indicated no direct mutagenic effect by any of the soil samples, as evidenced by very low to no light emission over the range of dilutions. In the presence of S9 fraction all soils exhibited strong light emission, indicating the presence of one or more metabolites with mutagenic effects. Light emission in the presence of S9 fraction decreases at higher concentrations due to toxic effects of contaminants in the extracts (Figures 3 – 5).

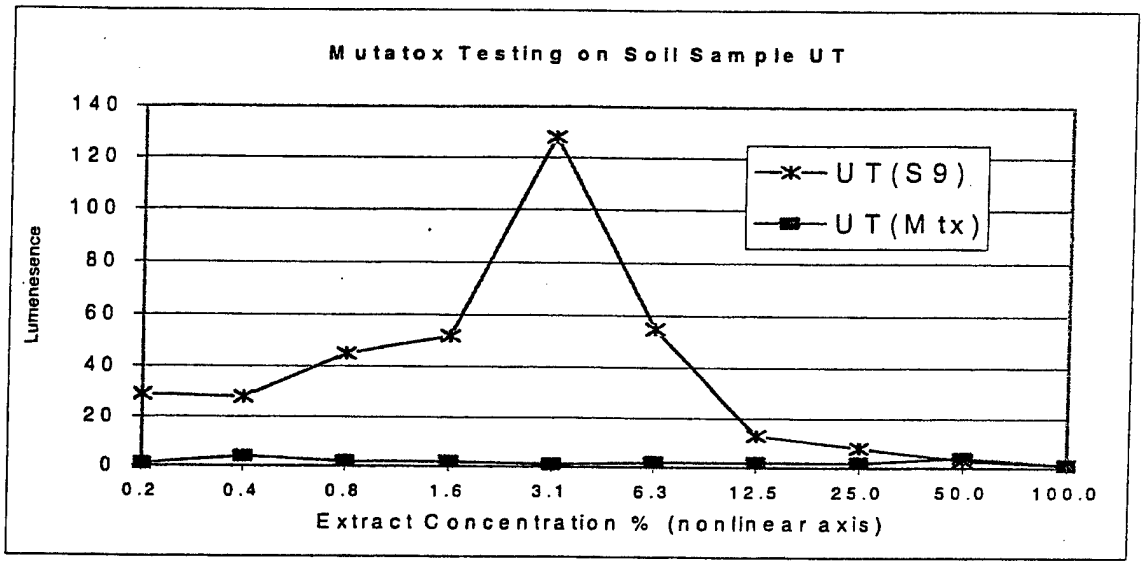

Figure 3. Mutatox® testing of soil sample UT. (S9) = Rat liver S9 fraction. (Mtx) = no S9 fraction.

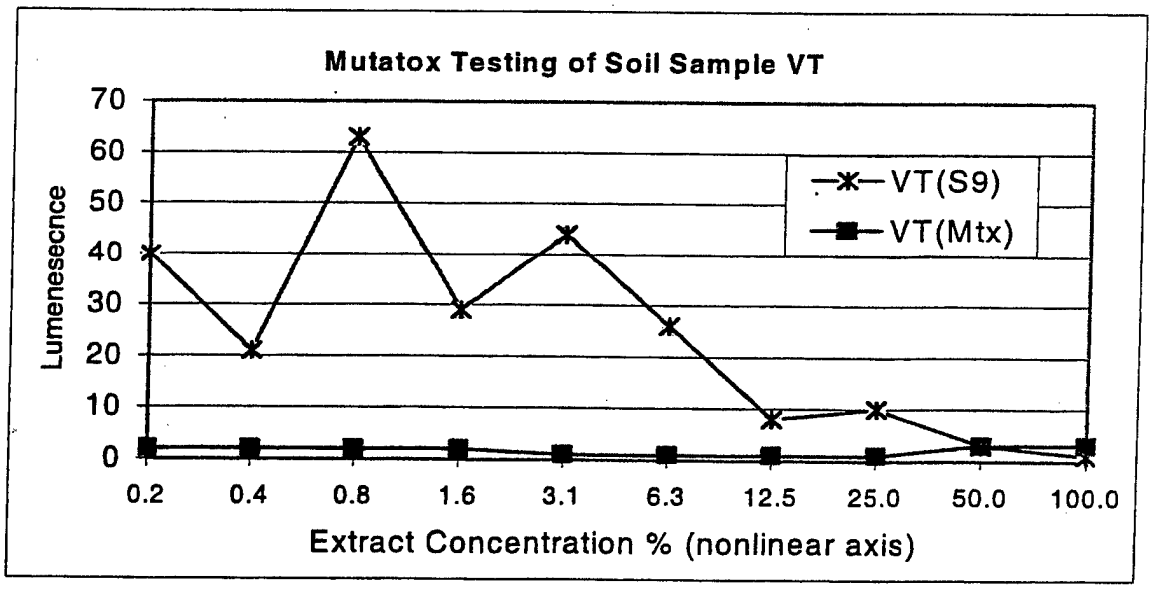

Figure 4. Mutatox® testing of soil sample VT.

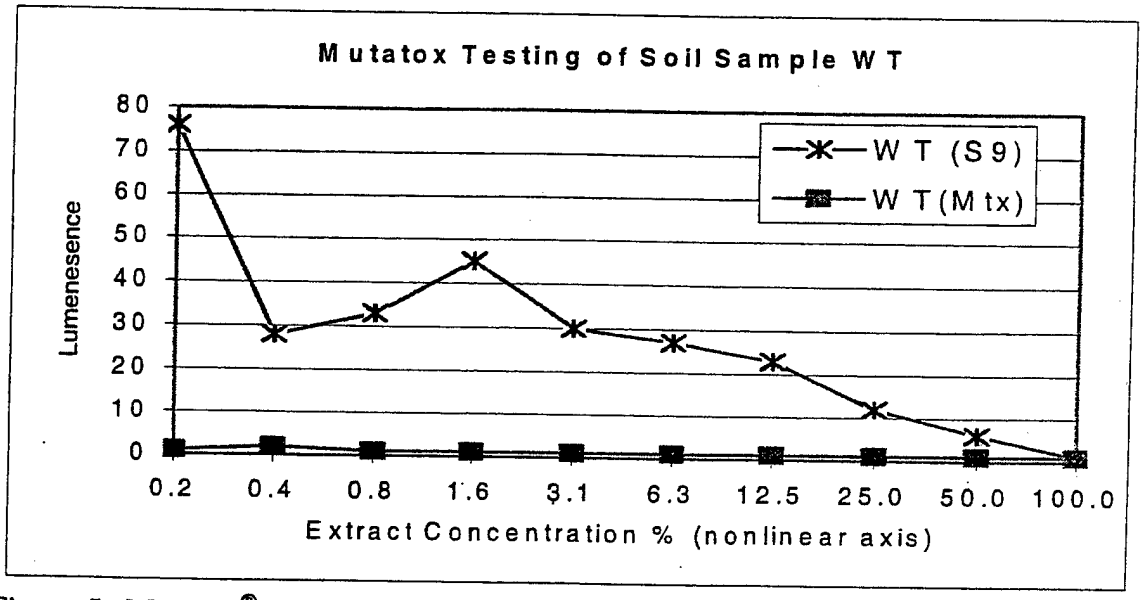

Figure 5. Mutatox® testing of soil sample WT.

Cytotoxicity Testing: Cytotoxicity testing was performed using extracts from all six soil samples (Figure 6). The control soils (UC, VC, and WC) were not significantly different from the negative control. Cytotoxicity exhibited by samples UT and VT were significantly different from respective control soils (UC and VC) and the negative control. There was no significant difference between test soil WT when compared to its control soil (WC) and the negative control.

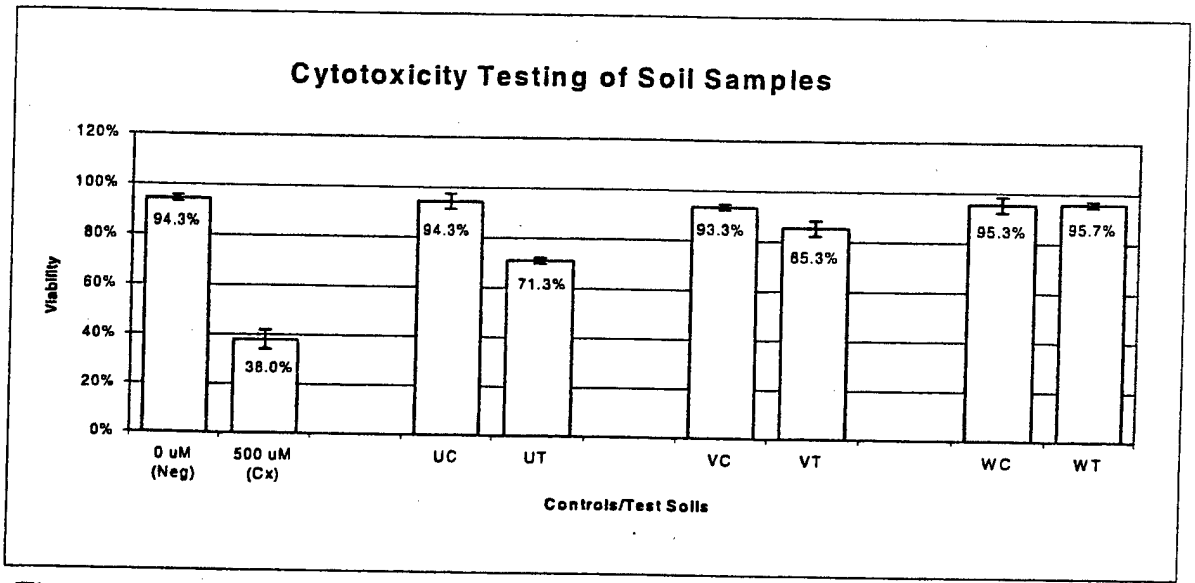

Figure 6. Cytotoxicity testing of control and test soil samples. Error bars are ± 2 standard deviations. Neg = Negative Control (normal cell culture media). Cx = Positive Control (cycloheximide).

Cytotoxicity testing indicated soils UT and VT had significant toxic effect on HL60 cells. This is consistent with the baseline toxicity characterization results as well as additional Microtox® testing of supernatants and slurries. Test soil WT result is consistent with the baseline characterization Microtox® result. It is important to note that additional Microtox® testing of WT was positive in the supernatant at very high concentration (82%). Also, the WT slurry Microtox® testing was positive at the lowest concentration (0.05%).

Overall results suggest greater bioavailability of contaminants in soils UT and VT as compared to WT. *In vitro* cytotoxicity testing yielded no information with respect to potential indirect mutagenic effects such as the Mutatox® test in which all soils were positive.

Apoptosis and Oxidative Stress Markers: Additional endpoints of apoptosis, reduced thiols, and mitochondrial membrane potential were accomplished in order to assess intracellular events. UC, UT, VC, and VT soils appear to contain a factor(s) resulting in adverse cellular effects. WC and WT test results indicate no significant adverse effects. Figures 7 – 9 present results of the above endpoints. Although viability has been previously discussed, results are again presented with the other endpoints for reference.

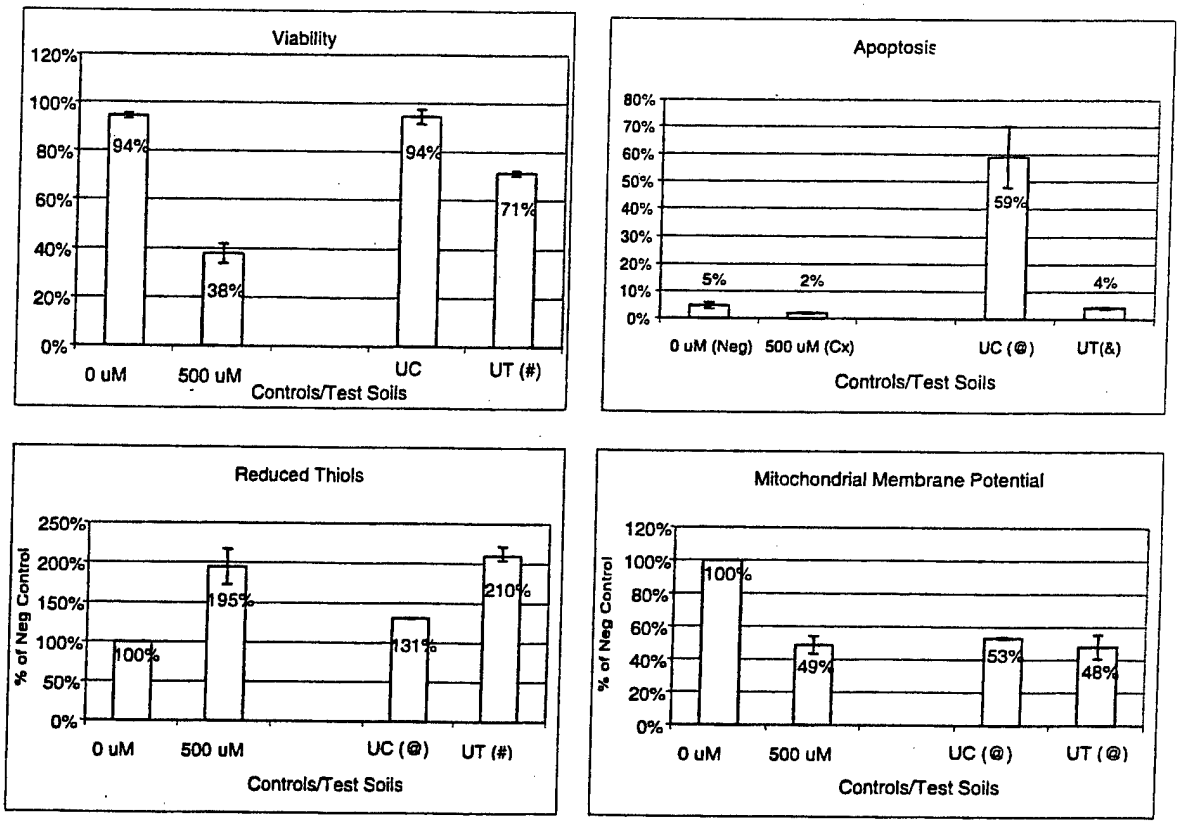

Figure 7. Viability, apoptosis, thiol reduction, and mitochondrial membrane potential for soils UC and UT. Error bars are ± 2 standard deviations. # indicates significant difference compared to negative control and control soil. & indicates significant difference compared to control soil. @ indicates significant difference compared to negative control.

Of significant note is that apoptosis in the control soil (UC) was very high, with the test soil (UT) being similar to the negative control. Reduced thiols were slightly increased in cells exposed to the control soil UC extract, whereas cells exposed to the test soil UT exhibited a strong increase in reduced thiols. Given that the viability of these cells was approximately 99% when gated by flow cytometry, the results suggest a factor in the UC soil which is not toxic at the 24 hour time point, yet clearly the cells have been programmed for death. The surviving cells exposed to the UT soil extract indicate virtually no apoptosis, a strong increase in reduced thiols, and decreased mitochondrial membrane potential. This suggests an oxidative stress mechanism for cell death and may indicate that although cytotoxic after 24 hours exposure, cells are able to recover from the effects of contaminants in the test soil UT. Conversely, although not initially cytotoxic, indications of oxidative stress in cells exposed to the control soil UC extract may be attributable to apoptosis.

Results of test soil UT cytotoxicity tests compare well with other toxicity tests previously discussed. In summary, all tests of the test soil UT indicate potential for both direct toxic effect due to contaminant(s) and potential for metabolism of mutagenic product(s) from contaminant(s). All control soil (UC) cellular endpoints except cytotoxicity differed significantly from the negative control. Possibilities for this may include loss of nutrients from media into the soil matrix during the extraction process or effects due to acceptable low levels of contaminant(s) in the control soil.

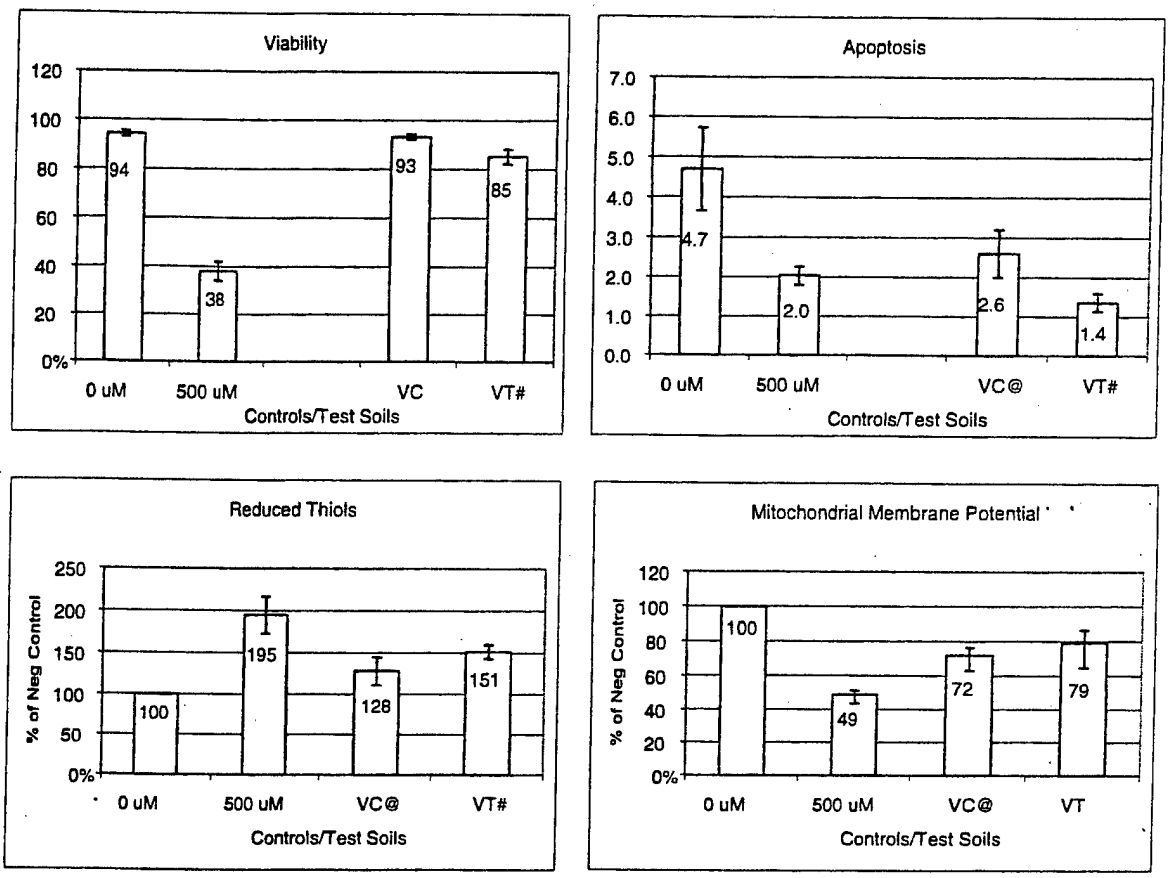

Figure 8. Viability, apoptosis, thiol reduction, and mitochondrial membrane potential for soils VC and VT. Error bars are ± 2 standard deviations. # indicates significant difference compared to negative control and control soil. @ indicates significant difference compared to negative control.

Results of test soil VT cytotoxicity tests compare well with other toxicity tests previously discussed. Even though cytotoxicity is evident at the end of the 24-hour time-point, the decrease in apoptosis and increase in reduced thiols suggest cells may be able to overcome direct effects due to contaminants. However, test soil VT was Mutatox® positive, indicating in addition to direct effects the potential for metabolism of mutagenic product(s) from contaminant(s).

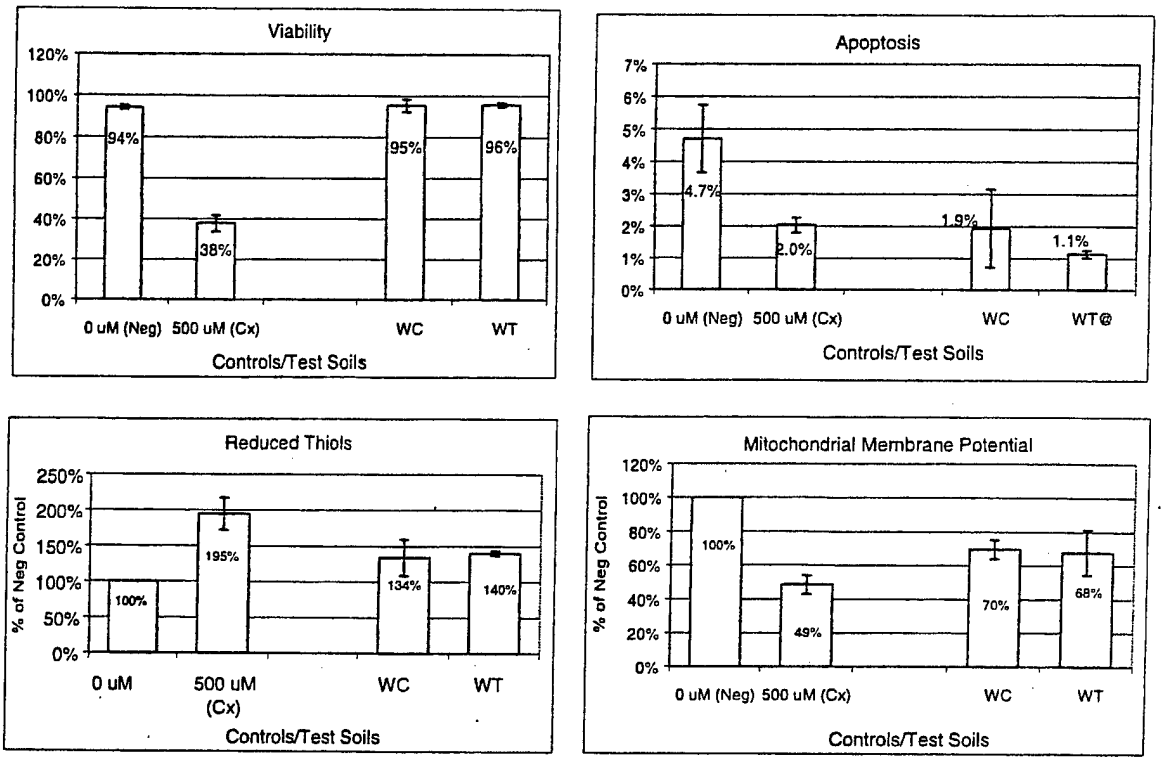

Figure 9. Viability, apoptosis, thiol reduction, and mitochondrial membrane potential for soils WC and WT. Error bars are ± 2 standard deviations. @ indicates significant difference compared to negative control.

Test soil WT exhibited a significant decrease in apoptosis when compared to the negative control. This was the only significantly different endpoint exhibited by cells exposed to control soil (WC) and test soil (WT) extracts.

All cellular endpoint tests where negative with respect to adverse effects. This suggests there was no direct cellular toxicity due to contaminant. However, the test soil (WT) was Mutatox® positive, indicating the potential for metabolism of mutagenic product(s) from contaminant(s). It is also important to note that the Microtox® soil supernatant test for this soil was positive, but at a relatively high concentration (EC-50 at 82% concentration). Whereas the soil slurry test for this soil exhibited high toxicity (EC-50 at .047% concentration). This suggests low bioavailability of contaminant and may explain the lack of cytotoxicity exhibited in the cellular assay.

DISCUSSION

This work represents initial efforts in the development of a living cell-based assay for use in assessing operational areas for ubiquitous contaminants that may pose health risks to deployed troops. The use of cell-based systems for assessing toxicity of pure compounds is a common method used by toxicologists.

Overall, the results of our *in vitro* cytotoxicity testing of soil extracts compared well to commonly accepted methods of assessing toxicity of contaminants (Microtox®, Mutatox®, earth worm toxicity, plant toxicity). The additional benefits of using a cell-based system may enable obtaining additional information, which give a more definitive description of potential health risks in humans. This is of significant importance for the Department of Defense due to the wide range of deployment areas, and daily living conditions experienced by military personnel. Assessment of staging and living areas for ubiquitous chemicals could yield information that may be key in protecting troops from immediate and future health problems. By knowing there are contaminants present in a given area, troops can take precautions that may preclude acute and chronic illness. This may simply be in the form of additional hygiene requirements, avoidance, additional medical monitoring, or limitations on time troops should be in these areas. Given the nature of military operations, the presence of a contaminant(s) does not mean the area is uninhabitable, but that prudent measures should be taken to decrease potential risk to personnel.

One of many challenges posed in this work was the extraction of contaminant from soil. Once this is accomplished, the procedure becomes a routine *in vitro* cytotoxicity assay in which cells are dosed and assessed for effects. For the work accomplished here, an aqueous extraction process was decided on because removal of contaminant from soil for introduction into living organisms, even if soil were ingested, would most likely involve water. It was thought that this type of extraction would yield a better approximation of bioavailability regardless of actual concentration of contaminant present in the soil matrix. Of concern is the possibility of losing nutrients from the extraction media. This was not ascertained by chemical analysis of the media and should be accomplished in future work. However, control soils of similar composition (less contaminants) were used in an effort to assess effects due strictly to contaminant in the test soils which contained know types and quantities of contaminants. Even though soil was used for this work, extractions for other types of sample matrix (air, water, etc.) should be capable.

Continuation of and expansion of this work should be accomplished. Additional work using appropriate cell lines representative of human organ systems should be undertaken. In addition, different types of extraction processes should be investigated to determine the best approach in this area.

Interpretation and use of *in vitro* cytotoxicity testing results are a source of concern for investigators and decision-makers. What do the results mean in terms of human health risk? There is no easy answer, however, it may be best to deploy testing measures such as reported here, and continuously attempt to perfect the process in time. Responses to positive results need not be overreaction, but prudent measures based on the most accurate information obtainable should be taken in order to minimize risks to deployed personnel.

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APPENDIX

Table A1. Analytical Data Results Summary of Test Soils.

Parameter	Units	Soil Samples UT	VT	WT
General Chemistry				
Total Recoverable Petroleum Hydrocarbons	mg/Kg	4650	1450	5000
Diesel Range Organics	mg/Kg	11571	4080.8	6584
Gasoline Range Organics	mg/Kg	140	100	103.1
Chloride	mg/Kg	1102	11.1	16.2
Sulfate	mg/Kg	52.55	82.1	64.4
% Moisture, Decant-CLP	%	12.2	7.25	12.3
Ammonia Nitrogen	mg/Kg	134.5	15.1	22.5
Solids, Total (TS) Solid Matrix	%	87.8	92.75	87.7
Total Residue as Percent Solids	%	87.8	92.75	87.7
VOCs				
Acetone	ug/Kg	375	500	4.5
Toluene	ug/Kg	240		6
Ethylbenzene	ug/Kg	215		4.5
Xylenes, Total	ug/Kg	785		12.5
Naphthalene	ug/Kg	160	6150	16
Chloroform	ug/Kg		225	
2-Butanone	ug/Kg		420	
Bromodichloromethane	ug/Kg		150	
Hexane	ug/Kg		165	
SVOCs				
N-Nitrosodiphenylamine	ug/Kg	1100	420	
Bis(2-Ethylhexyl)Phthalate	ug/Kg	80		
Priority Pollutant PAHs				
Naphthalene	ug/Kg	9600	3400	260
Acenaphthene	ug/Kg	2000	470	790
Fluorene	ug/Kg	4000	1400	2000
Phenanthrene	ug/Kg	9800	1700	4600
Fluoranthene	ug/Kg	270	120	54
Pyrene	ug/Kg	1200	240	190
Chrysene	ug/Kg	230	90	
Benzo[B]Fluoranthene	ug/Kg	38		
Total Petroleum Hydrocarbons (Aliphatic)				
C8 – C10	mg/Kg	140	88	99
C10 – C12	mg/Kg	1600	480	730
C12 – C16	mg/Kg	5300	1700	2600
C16 – C21	mg/Kg	1400	580	1300
C21+	mg/Kg	290	170	160
Total Aliphatic	mg/Kg	8800	3000	4900
Total Resolved	mg/Kg	3300	1200	1900
Resolved/Total	mg/Kg	0.38	0.4	0.39

Table A1. Analytical Data Results Summary of Test Soils (cont'd).

Parameter	Units	Soil Samples UT	VT	WT
Total Petroleum Hydrocarbons (Aromatic)				
C8 – C10	mg/Kg		12	4.1
C10 – C12	mg/Kg	200	170	180
C12 – C16	mg/Kg	1700	610	960
C16 – C21	mg/Kg	830	290	580
C21-C35	mg/Kg	210	74	57
C35+	mg/Kg	41	6.8	17
Total Aromatic	mg/Kg	3000	1200	1800
Total Resolved	mg/Kg	1700	650	1000
Resolved/Total	mg/Kg	0.57	0.54	0.56
Metals				
Arsenic	mg/Kg	4.7	3.5	10.2
Barium	mg/Kg	127.5	49.7	398.5
Boron	mg/Kg	1.75	4.8	6.4
Chromium	mg/Kg	14.25	21.35	6.25
Copper	mg/Kg	12.5	64.85	4.75
Lithium	mg/Kg	9.5	11.25	5.45
Nickel	mg/Kg	13.05	23.95	12.35
Vanadium	mg/Kg	20.35	48.55	7.75
Zinc	mg/Kg	41.95	46.25	17.55

Table A2. Organic Chemistry Summary of Test and Control Soils.

Sample	Total VOCs (ug/Kg)	Total SVOC (ug/Kg)	TRPH (mg/Kg)
UC	8.0	0	21
UT	1,575	1,180	4,650
VC	1.0	320	21
VT	7,325	420	1,450
WC	6.0	67	49
WT	35.5	ND	5,000

Table A3. Inorganic Chemistry Summary of Test and Control Soils.

Sample	Chlorides (mg/Kg)	Sulfate (mg/Kg)	Nitrate (mg/Kg)	Phosphate (mg/Kg)
UC	ND	57	ND	ND
UT	1,102	53	ND	ND
VC	12	43	8	ND
VT	11	82	ND	ND
WC	32	883	15	ND
WT	16	64	ND	ND

Table A4. Gasoline and Diesel Range Organics Summary of Test and Control Soils.

Sample	GRO (mg/Kg)	DRO (mg/Kg)
UC	ND	ND
UT	140	11,571
VC	ND	35
VT	100	4080
WC	ND	27
WT	103	6584

Table A5. Poly Aromatic Hydrocarbons, Total Hydrocarbons, and Biomarker Summary of Test and Controls Soils.

Sample	Total PAHs (ug/Kg)	Priority Pollutant PAHs (ug/Kg)	Total Aliphatic Hydrocarbons (mg/Kg)	Total Aromatic Hydrocarbons (mg/Kg)	Biomarker Compounds (ug/Kg)
UC	220	104.9	12	13	ND
UT	472,690	27,138	8,800	3,000	244
VC	481	168.9	35	11	25
VT	153,550	7,420	3,000	1,200	244
WC	55	10.9	27	21	1.8
WT	261,814	8,184.0	4,900	1,800	246

ND = Not Detected

Table A6. Total Metals Summary of Test and Control Soils.

Sample	UC	UT	VC	VT	WC	WT
Metals (mg/Kg)						
Arsenic	5.6	4.7	4.3	3.5	6.6	10.2
Barium	132	127.5	64	49.7	557.5	398.5
Boron	4.2	1.75	4.45	4.8	4.6	6.4
Cadmium	ND	ND	ND	ND	ND	ND
Chromium	16.35	14.25	20.95	21.35	4.3	6.25
Copper	10.75	12.5	16.3	64.85	3.4	4.75
Lead	11.75	154.05	11.7	10.25	2.2	5.45
Lithium	11.8	9.5	10.55	11.25	ND	12.35
Mercury	ND	ND	ND	ND	ND	ND
Molybdenum	ND	ND	1	ND	ND	ND
Nickel	12.8	13.05	17.4	23.95	ND	ND
Selenium	ND	ND	ND	ND	ND	ND
Vanadium	33.6	20.35	37.65	48.55	3	7.75
Zinc	38.65	41.95	48	46.25	12	17.55

ND = Not Detected

Table A7. Physical Chemistry Summary of Test and Control Soils.

Sample	pH	Cation Exchange Capacity (CEC) (meq/100g)	Electrical Conductivity (mmhos/cm)	Sodium Adsorption Ratios (SAR) (units)
UC	6.5	17.00	0.37	1.20
UT	7.7	17.40	2.13	48.30
VC	7.2	12.10	0.21	0.50
VT	6.8	7.70	0.74	1.80
WC	8.3	6.30	0.96	2.10
WT	8.6	6.10	0.53	2.30

Table A8. Microtox® Testing, Raw Data.

Test Type	Soil	EC_{50} (95% Confidence Interval)
Supernatant	VC	NT
	UC	NT
	WC	NT
	VT	10.21% (4.28 - 24.39)
	UT	10.66% (5.68 - 19.99)
	WT	81.59% (28.68 - 232.12)
Slurry	VC	NT
	UC	NT
	WC	NT
	VT	1.405% (1.014 – 1.948)
	UT	1.385% (1.078 - 1.781)
	WT	0.04737 (0.04613 – 0.04865)
	WT*	0.05496% (0.04323 - 0.06987)

NT= not toxic at concentration tested

* = The WT soil was tested with two different aliquots of soil.

Table A9. Mutatox® Testing, Raw Data.

Light Level Readings Following 16-Hour Incubation							
Extract Concentration (%)	WT (MTX)	WT (S9)	UT (MTX)	UT (S9)	VT (MTX)	VT (S9)	Benzo-a-pyrene (10 ug/mL)
0.2	1	76	1	29	2	40	1
0.39	2	28	4	28	2	21	6
0.78	1	33	2	45	2	63	1
1.57	1	45	2	52	2	29	1
3.13	1	30	1	128	1	44	1
6.25	1	27	2	55	1	26	12
12.5	1	23	2	13	1	8	52
25	1	12	2	8	1	10	442
50	1	6	4	3	3	3	6600
100	1	1	1	1	4	1	148

Table A10. *In Vitro* Cytotoxicity Testing of Soil Extracts (Soil Samples UC & UT).

Viability

24 Hrs		0 uM	500 uM Cx	AC	AT
Plate #	Date				
096A/B	6-Apr-99	95%	40%	93%	71%
096C/D		94%	38%	96%	72%
		94%	38%	94%	71%
	Sum	283.00%	114.00%	283.00%	214.00%
	n	3	3	3	3
	Sample	0 uM (Neg)	500 uM (Cx)	AC	AT
	Mean	94%	38%	94%	71%
	SD	0.58%	2.00%	1.53%	0.58%
	2SD	1.15%	4.00%	3.06%	1.15%
	CV	0.61%	5.26%	1.62%	0.81%
p	vs Neg Cntrl		0.0002	1.0000	0.0006
	AC vs AT				0.0006

Apoptosis

24 Hrs		0 uM	500 uM	AC	AT
Plate #	Date				
096A/B	6-Apr-99	4.1%	2.1%	53.0%	4.3%
096C/D		5.0%	2.1%	60.0%	3.9%
		5.0%	1.9%	64.0%	3.9%
	Sum	14.10%	6.10%	177.00%	12.10%
	n	3	3	3	3
	Sample	0 uM (Neg)	500 uM (Cx)	AC	AT
	Mean	4.7%	2.0%	59.0%	4.0%
	SD	0.52%	0.12%	5.57%	0.23%
	2SD	1.04%	0.23%	11.14%	0.46%
	CV	11.06%	5.68%	9.44%	5.73%
p	vs Neg Cntrl		0.0157	0.0029	0.2638
	AC vs AT				0.0037

Reduced Thiols (RT)

24 Hrs	Date	0 uM		500 uM		AC		AT	
Plate #		MCF Live Cells	RT	MCF Live Cells	RT	MCF Live Cells	RT	MCF Live Cells	RT
096A/B	6-Apr-99	170	100.0%	294	188.5%	204	130.8%	327	209.6%
096C/D		144	100.0%	294	188.5%	204	130.8%	336	215.4%
		154	100.0%	324	207.7%	203	130.1%	318	203.8%
	Sum	468.00	300.0%	912.00	584.6%	611	391.7%	981	628.8%
	n	3.00	3	3	3	3	3	3	3
	Sample		0 uM (Neg)		500 uM (Cx)		AC		AT
	Mean	156	100.0%	304	194.9%	204	130.6%	327	209.6%
	SD	13.11	0.0%	17.32	11.1%	0.58	0.4%	9.00	5.8%
	2SD	26.23	0.0%	34.64	22.2%	1.15	0.7%	18.00	11.5%
p	vs Neg Cntrl			0.0080		0.0241		0.0039	
	AC vs AT							0.0016	

Mitochondrial Membrane Potential (MMP)

24 Hrs	Date	0 uM			500 uM			AC			AT		
Plate #		MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP
096A/B	6-Apr-99	517	24	100%	308	27	51%	316	23	54%	263	24	44%
096C/D		545	25	100%	278	26	46%	310	21	53%	294	19	50%
		553	29	100%	290	25	49%	311	20	53%	295	23	50%
	Sum	1715	78	300%	876	78	146%	937	64	160%	852	66	144%
	n	3	3	3	3	3	3	3	3	3	3	3	3
	Sample		0 uM (Neg)			500 uM (Cx)			AC			AT	
	Mean	572	26	100%	292	26	49%	312	21	53%	284	22	48%
	SD	71.8	2.6	0%	15.1	1.0	2.66%	3.2	1.5	0.37%	16.2	2.6	4%
	2SD	143.6	5.3	0%	30.2	2.0	5.32%	6.4	3.1	0.73%	36.4	5.3	7%
	CV	13%	10%	0%	5%	4%	5%	1%	7%	1%	6%	12%	8%
p	vs Neg Cntrl				0.0248			0.0258			0.0146		
	AC vs AT										0.1484		

Table A11. *In Vitro* Cytotoxicity Testing of Soil Extracts (Soil Samples VC and VT).

Viability

24 Hrs		0 uM	500 uM	EC	ET
Plate #	Date				
096A/B	6-Apr-99	95.0%	40.0%	93.0%	87.0%
096E/F		94.0%	38.0%	94.0%	84.0%
		94.0%	36.0%	93.0%	85.0%
	Sum	283.0%	114.0%	280.0%	256.0%
	n	3	3	3	3
	Sample	0 uM "Neg"	500 uM Cx	EC	ET
	Mean	94.3%	38.0%	93.3%	85.3%
	SD	0.6%	2.0%	0.6%	1.5%
	2SD	1.2%	4.0%	1.2%	3.1%
	CV	0.6%	5.3%	0.6%	1.8%
p	vs Neg Cntrl		0.0002	0.2254	0.0041
	EC vs ET				0.0202

Apoptosis

24 Hrs		0 uM	500 uM	EC	ET
Plate #	Date				
096A/B	6-Apr-99	4.1%	2.1%	2.3%	1.3%
096E/F		5.0%	2.1%	2.6%	1.3%
		5.0%	1.9%	2.9%	1.5%
	Sum	14.1%	6.1%	7.8%	4.1%
	n	3	3	3	3
	Sample	0 uM "Neg"	500 uM Cx	EC	ET
	Mean	4.7%	2.0%	2.6%	1.4%
	SD	0.5%	0.1%	0.3%	0.1%
	2SD	1.0%	0.2%	0.6%	0.2%
	CV	11.1%	5.7%	11.5%	8.4%
p	vs Neg Cntrl		0.0157	0.0067	0.0066
	EC vs ET				0.0094

Reduced Thiols

24 Hrs	Date	0 uM		500 uM		EC		ET	
Plate #		MCF Live Cells	Thiols	MCF Live Cells	Thiols	MCF Live Cells	Thiols	MCF Live Cells	Thiols
096A/B	6-Apr-99	170	100.0%	294	188.5%	213	136.5%	236	151.3%
096E/F		144	100.0%	294	188.5%	187	119.9%	230	147.4%
		154	100.0%	324	207.7%	200	128.2%	243	155.8%
	Sum	468.0	300.0%	912.0	584.6%	600.0	384.6%	709.0	454.5%
	n	3	3	3	3	3	3	3	3
	Sample		0 Um "Neg"		500 uM Cx		EC		ET
	Mean	156.0	100.0%	304.0	194.9%	200.0	128.2%	236.3	151.5%
	SD	13.1	0.0%	17.3	11.1%	13.0	8.3%	6.5	4.2%
	2SD	26.2	0.0%	34.6	22.2%	26.0	16.7%	13.0	8.3%
	CV	8.4%	0.0%	5.7%	5.7%	6.5%	6.5%	2.8%	2.8%
p	vs Neg Cntrl			0.0080		0.0005		0.0102	
	EC vs ET							0.0321	

Mitochondrial Membrane Potential

24 Hrs	Date	0 uM			500 uM			EC			ET		
Plate #		MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP
096A/B	6-Apr-99	517	24	100%	308	27	51%	385	14	68%	483	18	85%
096E/F		545	25	100%	278	26	46%	405	17	71%	456	15	81%
		653	29	100%	293	26	49%	436	16	77%	401	13	71%
	Sum	1715.00	78.00	300%	879.00	79.00	147%	1226.00	47.00	216%	1340.00	46.00	237%
	n	3.00	3.00	3	3.00	3.00	3	3.00	3.00	3	3.00	3.00	3
	Sample		0 uM "Neg"			500 uM Cx			EC			ET	
	Mean	571.67	26.00	100%	293.00	26.33	49%	408.67	15.67	72%	446.67	15.33	79%
	SD	0.00	1.00	0%	0.00	1.00	3%	0.00	1.00	5%	0.00	1.00	7%
	2SD	0.00	2.00	0%	0.00	2.00	5%	0.00	2.00	9%	0.00	2.00	14%
	CV	0%	4%	0%	0%	4%	5%	0%	6%	6%	0%	7%	9%
p	vs Neg Cntrl				0.0240			0.0265			0.1964		
	EC vs ET										0.4321		

Table A12. *In Vitro* Cytotoxicity Testing of Soils Extracts (Soil Samples WC and WT).

Viability

24 Hrs		0 uM	500 uM	IC	IT
Plate #	Date				
096A/B	6-Apr-99	95.00%	40.00%	97.00%	96.00%
096G/H		94.00%	38.00%	95.00%	95.00%
		94.00%	36.00%	94.00%	96.00%
	Sum	283.00%	114.00%	286.00%	287.00%
	n	3	3	3	3
	Sample	0 uM	500 uM	IC	IT
	Mean	94.33%	38.00%	95.33%	95.67%
	SD	0.58%	2.00%	1.53%	0.58%
	2SD	1.15%	4.00%	3.06%	1.15%
	CV	0.61%	5.26%	1.60%	0.60%
p	vs Neg Cntrl		0.000245	0.225403	0.057191
	IC vs IT				0.741801

Apoptosis

24 Hrs		0 uM	500 uM	IC	IT
Plate #	Date				
096A/B	6-Apr-99	4.10%	2.10%	2.60%	1.10%
096G/H		5.00%	2.10%	1.40%	1.10%
		5.00%	1.90%	1.80%	1.20%
	Sum	14.10%	6.10%	5.80%	3.40%
	n	3	3	3	3
	Sample	0 uM	500 uM	IC	IT
	Mean	4.70%	2.03%	1.93%	1.13%
	SD	0.52%	0.12%	0.61%	0.06%
	2SD	1.04%	0.23%	1.22%	0.12%
	CV	11.06%	5.68%	31.60%	5.09%
p	vs Neg Cntrl		0.015715	0.050109	0.006316
	IC vs IT				0.156726

Reduced Thiols (RT)

24 Hrs	Date	0 uM		500 uM		IC		IT	
Plate #		MCF Live Cells	RT	MCF Live Cells	RT	MCF Live Cells	RT	MCF Live Cells	RT
096A/B	6-Apr-99	170	100.0%	294	188.5%	203	130.1%	221	141.7%
096G/H		144	100.0%	294	188.5%	232	148.7%	217	139.1%
		154	100.0%	324	207.7%	194	124.4%	344	220.5%
	Sum	468.0	300.0%	912.0	584.6%	629.0	403.2%	436.0	280.8%
	n	3	3	3	3	3	3	2	2
	Sample		0 uM		500 uM		IC		IT
	Mean	156.0	100.0%	304.0	194.9%	209.7	134.4%	219.0	140.4%
	SD	13.1	0.00%	17.3	11.10%	19.9	12.73%	2.8	1.81%
	2SD	26.2	0.0%	34.6	22.2%	39.7	25.5%	5.7	3.63%
	CV	8.41%	0.00%	5.70%	5.70%	9.47%	9.47%	1.29%	1.29%
p	vs Neg Cntrl			0.007999		0.08996		0.136038	
	IC vs IT							0.418158	

Mitochondrial Membrane Potential (MMP)

24 Hrs	Date	0 uM			200 uM			IC			IT		
Plate #		MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP	MCF Live Cells	MCF Dead Cells	MMP
96A/B	6-Apr-99	517	24	100%	308	27	51%	414	18	73%	425	16	75%
96G/H		545	25	100%	278	26	46%	401	20	70%	386	19	67%
		653	29	100%	641	380	48%	379	14	67%	350	15	61%
	Sum	1715.00	78.00	300%	1227.00	433.00	146%	1194.00	52.00	209%	1161.00	50.00	204%
	n	3	3	3	3	3	3	3	3	3	3	3	3
	Sample			0 uM			500 uM			IC			IT
	Mean	571.67	26.00	100%	409.00	144.33	49%	398.00	17.33	70%	387.00	16.67	68%
	SD	0.00	1.00	0%	0.00	1.00	3%	0.00	1.00	3%	0.00	1.00	7%
	2SD	0.00	2.00	0%	0.00	2.00	5%	0.00	2.00	6%	0.00	2.00	14%
	CV	0%	4%	0%	0%	1%	6%	0%	6%	4%	0%	6%	10%
p	vs Neg Cntrl				0.169585			0.077931			0.09732		
	IC vs IT										0.446996		