N00164.AR.000468
NSWC CRANE
5090.3a

DEPARTMENT OF THE NAVY
CRANE DIVISION
NAVAL SURFACE WARFARE CENTER
300 HIGHWAY 361
CRANE, INDIANA 47522-5000

IN REPLY REFER TO:
5090
Ser 095/9187

22 SEP 1999

U.S. Environmental Protection Agency, Region V
Waste, Pesticides, & Toxics Division
Waste Management Branch
Illinois, Indiana, and Michigan Section
Attn: Mr. Peter Ramanauskas (DW-8J)
77 West Jackson Blvd.
Chicago, IL 60604

Dear Mr. Ramanauskas:

Crane Division, Naval Surface Warfare Center (NAVSURFWARCENDIV Crane) submits for review and approval three (3) copies of the Revised Pages and Response to Comments for Appendix G, Toxicity Test Procedures, for the Full-Scale Quality Assurance Project Plan for Full Scale Bioremediation Operations as enclosure (1). Enclosure (2) is the required certification statement.

NAVSURFWARCENDIV Crane point of contact is Ms. Christine D. Freeman, Code 09511, telephone 812-854-4423.

Sincerely,

JAMES M. HUNSICKER
Manager, Environmental Protection Department
By direction of
the Commander

Encl:
(1) Revised Pages & Response to Comments for FS QAPP Appendix G, Toxicity Test Procedures
(2) Certification Statement

Copy to: (w/o encls)
ADMINISTRATIVE RECORD (w/encls)
COMNAVSEASYSCOM (SEA OOT)
MK Cleveland (A. Fosdick)
SOUTHNAVFACENGCOM (Code 1864)
TOLTEST Crane

NSWC Crane
Crane, Indiana

Comment - Resolution

US EPA Region 5 Additional Comments from August 1999 on Appendix G of Quality Assurance Project Plan for the Bioremediation Facility

A. REPLIES TO SPECIFIC RESPONSES FROM COMMENT-RESOLUTION, DATED 2/12/99

1.0 INTRODUCTION

Comment 1: Page G-3, paragraph 1 — "...document that the treated soil meets toxicity remedial goals." — What are the stated goals and objectives of these earthworm toxicity tests? What are the toxicity levels that you will allow for no further action, versus what levels will indicate that further clean-up measures must be taken? These should typically be stated and determined/ agreed upon before analytical testing begins.

Response 1: "...The goal of the earthworm toxicity test is to show that the compost concentration which causes 50% mortality (LC-50) in the treated windrow is less than the LC-50 of the control windrow..."

Reply to Response 1 : No, the LC-50 of the treated windrow does not only need to be less than that of the control windrow. It needs to be less than the LC-50 of the artificial soil prepared in the laboratory, and not just the control windrow, since the control windrow is also being tested through these tests for it's own possible toxicity. The soil portion of the control windrow compost is "uncontaminated", but we need to be sure that the windrow treatment procedure in and of itself is not toxic. And, the question continues to be how much "less than" is acceptable? Usually a number is determined and agreed upon before any testing starts.

Response to Reply: Artificial soil is a homogenious mixture of peat, clay, and silica sand which is prepared in the laboratory from clean source material. The artificial soil is used as the negative control, positive control and the dilutant for the test matrices in the determination of the LC-50. The testing protocol requires that there is less than a ten (10) percent earthworm mortality rate in the artificial soil prior to using it as the dilutant. Once this criteria has been achieved the artificial soil is than used to establish an LC-50 for a reference toxicant (KCl) and as the dilutant (6.25%, 12.5%, 25%, 50%, 100%) for each of the test matrices. Since the artificial soil is derived from clean source materials, the LC-50 of the control and contaminated windrows are never expected to have a lower mortality rate than the artificial soil.

The objectives of performing the earthworm toxicity testing at Crane was to determine if the treated contaminated compost exhibited a higher mortality rate than that of the control windrow. As a part of this study,

negative and positive controls containing the artificial soil were prepared along with a non-contaminated soil control windrow and five treated contaminated windrow samples. The results artificial soil negative control and the LC-50's for the positive control, the non-contaminated soil windrow and the treated contaminated windrow samples were evaluated to ensure that the goals in the approved FS-OPS Plan had been achieved.

3.0 REMEDIAL GOALS

Comment 3: Page G-5, paragraph 2 — "For the earthworm toxicity the objective will be to determine if the compost samples show significant mortality when compared to the reference soil." Please clarify with specifics what the term "reference soil" is meant to describe here. Does this refer to the artificial soil that will be made at the laboratory (typically, from 10% peat, 20% clay, and 70% sand) and used as dilution soil as well as soil for control organism exposures? Or, is the term "reference soil" meant to refer to non-contaminated soil from the site (plus straw and chicken manure) that will be used for dilution soil as well as control media?

Response 3: "...Reference soil is the artificial soil prepared in the laboratory, ..."

Reply to Response 3: Please make sure all documents reflects that this is the objective; i.e., comparing toxicity results of test soil with those of the artificial (lab created) soil, not the windrow control soil. Especially important to change Response 1 above to reflect this. There are different references in the QAPP where these terms (artificial soil control, and windrow control) are interchanged. They are not interchangeable and are very different substances, thus they need to be carefully noted throughout all documents.

Response to Reply: Comment noted, the QAPP and laboratory SOP will be reviewed to ensure consistent usage of artificial soil and corrections made as necessary.

4.2 ANALYTICAL TESTING QUALITY CONTROL

Comment 5: Page G-6, paragraph 2 — Must mention reference toxicant studies that are conducted, what reference toxicant is used, how often the reference toxicant tests are conducted, at what concentrations and temperature, what percent of control organisms must survive

for a test to be valid, etc. Also, there needs to be some mention as to what concentrations will be used for the actual sample tests. How many replicates will be run per test concentration? What types of deviations will be allowable? What type of water will be used to saturate test media? What mortality rates in control replicates is allowed for test to be considered valid? How will LC50 values be calculated?

Response 5: "...A computer program developed by C.E. Stephans (reference 3, SOP/SED/201 Page A-8) will be used..."

Reply to Response 5: The name of the computer program developed should be mentioned in all data packages when submitted, because the program name was not seen by reviewer in replacement pages provided.

Response to Reply: Comment noted. The reference to the computer program is in the standard operating procedure of the laboratory found in Attachment A. However, the computer program will be mentioned in the final data packages.

B. REPLIES TO RESPONSES FROM COMMENT RESOLUTION DATED 2/12/99 REGARDING ATTACHMENT A, LABORATORY SOPs

EARTHWORM TOXICITY TESTING SOP

Comment 2: Page A-5, paragraph II.A and B. — ...Do you have information verifying the age of purchased test organisms?...

Response 2: "...source information was included in the Final Draft issued on July 14, 1998..."

Reply to Response 2: When the biological supply company sends your shipment of organisms, you state that they will also send a written verification of species. Make sure you also get a written verification of age of organisms, to be sure they all fall within the same age range.

Response to Reply: Copies of all documentation received from the supply company will be submitted with the final data packages. Future data packages will include the culture log. Specific ages of the earthworms will be contained in the data packages were available, however, only adult earthworms with a mass greater than 300mg and a fully developed clitellum will be utilized.

US EPA Region 5 Additional Comments from August 1999 on Appendix G of Quality Assurance Project Plan for the Bioremediation Facility

Comment 6: Page A-6, paragraph III.C. — "Preparation of Test Vessels" — Four liter test vessels may be too large for conducting a test that uses 10 organisms per vessel and thus 200 g of test media per vessel. Same sized test vessels need to be used for all tests, all concentrations, and all reference tests for data to be most useful. Include the size of the actual test vessels that will be used, how they are cleaned and prepared, etc.

Response 6: The testing specifics have been incorporated into the July 14,1998 revision of the FS-QAPP,..."

Reply to Response 6: Specific size of test vessels and material make-up of the test vessels cannot be found in the 07/14/98 version of the QAPP pages submitted. Please reference the size and material of test vessels in the QAPP and SOP for earthworm toxicity.

Response to Reply: Comment noted, the SOP and QAPP will reference the one liter borosilicate glass containers with screw top covers with small holes punched in the covers to allow air to enter the exposure vessels.

Comment 7: Page A-7, paragraph III.E – "Number of Organisms" – ...As commented above, at least three replicates should be considered for each of the five different concentrations, plus a control..."

Response 7: The testing specifics have been incorporated into the July 14, 1998 revision of the FS-QAPP..."

Reply to Response 7: The QAPP revision from 07/14/98 does not specifically state that three replicates of the artificial soil will be used for worm exposure as the controls. Please clarify in QAPP and SOP that three replicates are needed for test concentrations, and three replicates for control vessels made of artificial soil. Also, if you intend to use the "control windrow" of compost made with uncontaminated soil, then it should be specified in the QAPP and SOP at this point. You must use three replicates of the control windrow, and state so in the QAPP and SOP.

Response to Reply: Comment noted. The QAPP will state that three replicates will be needed for every sample and every control including the artificial soil blank, the reference toxicant test, and the control windrow. The laboratory will not know which sample represents the control windrow and which sample represents compost soil, therefore this cannot be

identified in the SOP. The laboratory will treat each sample submitted as a unique sample analyzed in the same manner.

Comment 10: Page A-7, paragraph III.N. — "Reference Toxicant" — Provide details on reference toxicant tests either in this SOP or a separate one that addresses reference toxicant tests for earthworms....

Response 10: The testing specifics for the reference toxicant have been incorporated into the July 14, 1998 revision of the FS-QAPP, ...Like actual samples, three replicates of each reference toxicant concentration will be used with 10 organisms in each test vessel. ....

Reply to Response 10: Reviewer cannot find reference in the QAPP as to the number of replicates that will be used for all test concentrations as well as for controls. The SOP states that two replicates will be used, but since three replicates are being used for the actual test samples, it is suggested that three replicates be used for all reference toxicant tests. This will also be helpful in having enough data in the reference tests for statistical analyses.

Response to Reply: The QAPP has been revised to state that the number of replicates for all test concentrations and controls will be three replicates. The laboratory SOP stating that two replicates will be used instead of three will be corrected. All samples and controls will be analyzed with three replicates.

Comment 12: Page A-8 — DATA HANDLING — ...what types of statistical analyses will be conducted? Will bench sheets be included in reports? What types of computer programs will be used?

Response 12: ...The five samples will be averaged and compared to the LC-50 for the control windrow...

Reply to Response 12: No, the LC-50 for samples should not be compared to the control windrow, as previously mentioned in Reply to comment A.1. above. The LC-50 from sample tests needs to be compared to the artificial soil control to determine significant toxicity, not the control windrow which contains compost materials of unknown toxicity.

Response to Reply: See Response to Comment A.1 above.

C. ADDITIONAL COMMENTS ON EARTHWORM TOXICITY TESTING SOP, DATED 6/8/99.

Comment 1: I. Purpose/Objective. In sentence two, "Test concentrations will be prepared by the addition of the test soil to the control/artificial soil..." – Be sure this term means the artificial lab created soil and not the control windrow soil. If control windrow soil is also being tested as part of this toxicity study, it needs to be mentioned and specified in the SOP and QAPP.

Response 1: The control windrow soil has been identified in the QAPP, however the lab will not know which soil is from the control windrow and which is from the treated compost windrow, therefore, no distinction of these samples can be made in the SOP. The lab will be using artificial soil as the dilutant for creating all test concentrations.

Comment 2: V. Test System. A. Test Organism. 1. "The earthworm, *Eisenia Foetida*, will be used for this test; only adult worms will be used...." -- What is the age of the adult worms being used? You should make sure all worms used in a single test are within a certain range of each other.

Response 2: All earthworms will be obtained from the supplier with paperwork stating approximate age of the shipment if available. All worms used in a single test will be used from the same shipment and will represent the same age range. Modifications will be consistent with Response B.2 above.

Comment 3: V. Test Organism. A. Test Organism. 3. (Regarding rearing and maintenance regime), "...Bedding is changed every 4-6 months..." – Bedding should be changed at least every two to three months. Waiting longer than that, especially since your rearing bins have approximately 350 worms in each, may cause overcrowding and thus your testing organisms will be of poor quality. They will tend to be very small and susceptible to various poisonings.

Response 3: Comment noted. Bedding will be changed every two to three months. Organisms used for these tests were not held at the laboratory for a duration longer than three weeks, therefore overcrowding would not be a problem. In addition, worm size was specified in the SOP which would eliminate the very small worms mentioned above.

Comment 4: VI. Test Procedure. A. Preparation of Test Concentrations – No mention is made of

the use of control windrow compost during toxicity testing. Are there plans to use the control windrow in testing? If so, you must use three replicates. Also, how would this control windrow compost be identified, i.e. how will you be comparing the results to results from other concentrations and that of the artificial control soil?

Response 4: The laboratory will not know which compost is from the control windrow and which compost is from the treated contaminated windrow. The sample will be identified in a consistent manner as specified in the approved Appendix G of the FS-Operational Plan.

Comment 5: VI. Test Procedure. C. Preparation of Test Vessels – No mention is made of the size of jar being used for test vessels. Also, should mention the type of material the test vessels is made out of, and if the vessels are re-used for different tests. If vessels are reused, you must state how they are cleaned before use in toxicity testing and reference testing.

Response 5: Comment noted. Text has been revised. Modifications will be made consistent with Response B.6 above.

Comment 6: VI. Test Procedure. F. Characterization of Size, Age, or Absorption of Test Substance – Minimum and maximum size for individual worms should be stated. Also, age should be given, even if only approximate, i.e. 2 months old, 3 months old, etc.

Response 6: Comment noted. Text has been revised.

Comment 7: VI. Test Procedure. G. Test Endpoint – State the computer program being used for analyses.

Response 7: Comment noted. Text has been revised. Modifications will be made consistent with Response A.5 above.

Comment 8: VI. Test Procedure. I. Control Vessels – Besides the artificial soil (negative control), should identify that will be testing a control windrow (with three replicates) if this is being conducted.

Response 8: The laboratory will not know which compost is from the control windrow and which compost is from the treated contaminated windrow. The sample will be identified in a consistent manner as specified in the approved Appendix G of the FS-Operational Plan and analysis will be performed on every compost sample in a consistent manner with three replicates.

US EPA Region 5 Additional Comments from August 1999 on Appendix G of Quality Assurance Project Plan for the Bioremediation Facility

Comment 9: VI. Test Procedure. L. Temperature – Under what light conditions will the test be conducted? Please state in this section of SOP.

Response 9: Comment noted. Text has been revised.

Comment 10: VI. Test Procedure. M. Soil pH/ Adjustment. "...If the pH falls outside this range it may be necessary to adjust the pH within range as close to 7.0 as possible for the survival of the test organisms..." –In response to earlier comments, request was made that no pH adjustments be made during the tests. This should be reflected in the SOP.

Response 10: As a result of Comment B.3 of the EPA Comments dated 2/12/99, the provision for pH adjustments were added to the SOP. The lab has stated why they don't recommend this procedure, however the actual steps used to perform this task have been added to the SOP should they be required. Comment noted, and no pH adjustments will be made.

Comment 11: VI. Test Procedure. N. Reference Toxicant. "...two replicate test vessels and control vessels..." – Three replicates should be used for reference tests since three replicates will be used for conducting all toxicity tests on samples. This is good for consistency, comparability, and to ensure enough statistical points to run computer analyses.

Response 11: Comment noted. Three replicates were used for the reference toxicant test. Text has been revised appropriately.

Comment 12: VII. Chemical Analysis. A.2. – Again, do not adjust pH as per earlier response to comments.

Response 12: As a result of Comment B.3 of the EPA Comments dated 2/12/99, the provision for pH adjustments were added to the SOP. The lab has stated why they don't recommend this procedure, however the actual steps used to perform this task have been added to the SOP should they be required. Comment noted, and no pH adjustments will be made.

Comment 13: VIII. Endpoint Calculation. "...Data for the control vessels are not used because the program does not have the capability of adjusting for control mortality. ..." – How will mortality in the control windrow replicates be analyzed? Again, mention the computer program being used for statistical analyses and how that

information will be compared to other results.

Response 13: The control windrow will be analyzed in the same manner as samples for the treated contaminated windrow. The computer will calculate the LC-50 of the control windrow in the same manner it was calculated for every other sample from the contaminated windrow.

Quality Assurance Project Plan
For Full-Scale Operations at the Bioremediation Facility
NSWC Crane, Crane, Indiana

APPENDIX G

ERRATA SHEET

1. Remove pages G-7 through G-9 dated 2/12/99 and replace with revised pages G-7 through G-9 dated 9/22/99.

   Reason: The glass type and size of the testing vessels has been specified. The program used to calculate the LC-50 has been specified, and the number of replicates for all QC checks has been added.

2. Remove pages A-4 through A-10g, Acute Toxicity Testing using the Earthworm, dated 6/8/99. Replace with attached pages A-4 through A-10h dated 9/18/99 revision 2 of the protocol.

   Reason: Revised Standard Operating procedure documents additional details of procedure used in toxicity testing using the earthworm, *Eisenia foetida*.

4.0
QUALITY CONTROL PROCEDURES

4.1 FIELD QUALITY CONTROL

A small control compost windrow will be constructed for each SWMU using the same amendment recipe as the full scale windrows. Clean, analyzed soil from a documented borrow source selected to be of similar soil characteristics to the contaminated soil will be used to construct the control windrow which will be approximately 10 x 10 x 5 feet high located in the same building as other windrow piles. This windrow will be constructed during the first set of full-scale windrows and maintained to simulate the full-scale windrows. This windrow will be turned at the same frequency, temperature, oxygen, and moisture content of the control windrow will be measured and recorded at the same frequency as the full-scale windrows. This control will be utilized to aid in the determination of background toxicity levels as well to serve as a guide to determine if the amendment ratios are toxic. In addition, the control pile will be analyzed for explosives at the end of cycle to verify that there is no cross contamination from other windrow piles. End of cycle for control pile will be the same number of days that the pile chosen for toxicity sample analysis was processed. Other QC field procedures for decontamination of equipment is detailed in Section 5.0 of Appendix G of the *Full-Scale Operational Plan* [MK, 1998]

The collection of field blanks, rinse water samples, and field duplicates will be performed at the same frequency as other compost samples. The specifications for compost quality control is specified in Section 4.6 on page 8 through 12 of 18 of the QAPP.

4.2 ANALYTICAL TESTING QUALITY CONTROL

Detailed standard operating procedures for each analytical test are included in Attachment A of this Appendix. These procedures outline the specific quality control measures required by the analytical method.

4.2.1 Earthworm Toxicity

Earthworm analysis will be performed using the test organism, *Eisenia Foetida* supplied from Carolina Biological Supply Company or other documented source. The condition and environment of the worms will be documented on the livestock record from receipt in the laboratory until use in this project. Worms used in this project will not have been previously submitted to any other testing. The worms will be uniform in size, at least 60 days of age, and weigh approximately 300 - 500 mg. Worms will be maintained at a temperature of 20 $\pm$ 2°C and acclimated for a period of five days prior to testing. During this time worms will be fed with Magic Worm Food. Feedings will be documented on the livestock record. Initiation of the test will begin after the five day stabilization has occurred. Ten worms will be placed in the testing vessels by sequential randomization, where no more than 2 worms will be added into each vessel at a time. Testing vessels will be one liter borosilicate glass containers with screw top

covers with small holes punched in the covers to allow air to enter exposure vessels. During the duration of the analysis, the worms will not be fed.

Each vessel will contain 200 grams of testing material (compost/artificial soil). Artificial soil prepared from a homogenous mixture of 10% sphagnum peat, 20% colloidal kaolinite clay and 70% grade 70 silica sand will be used as a blank and also as a dilutant to the compost mixture. Five varying concentrations of artificial soil and compost will be homogenized using a grinder. The concentration ranges of 6.25%, 12,5%, 25%, 50%, and 100% will be created for this analysis. In addition, three replicates for each concentration of compost will analyzed be performed to determine the LC-50 (the compost concentration that kills 50% of the worms). The LC-50 will be calculated with a computer program developed by C.E. Stephans and ASTM.

Additional QC includes the use KCl as a reference toxicant. The KCl will be utilized as a positive control to evaluate the sensitivity of each batch of organisms. Varying concentrations of KCl contaminated soil will be prepared to determine the LC-50 of the KCl contaminated soil. This value will represent the concentration of KCl which results in 50% mortality. The LC-50 will be compared to published acceptance limits as well as previous laboratory data to verify that the worms reacted at similar sensitivities as other batches of *Eisenia Foetida*. The artificial soil prepared in the lab will also be analyzed to verify that less than 10% of the worms died in the blank. These QC checks will be analyzed with three replicates for each concentration also.

Initially, and again at the completion of this analysis, the pH of the testing material will be recorded for each testing vessel. The pH will be documented and measured with a calibrated meter. The temperature of the test will be monitored continually during the analysis. The temperature should remain in the window of 22$\pm$ 2 degrees Centigrade and will not fluctuate more than 3 degrees in any twenty four hour period. Testing temperatures will be documented and measured with a thermometer that has been calibrated to an ASTM traceable thermometer. Earthworm analysis will be performed by Aqua Survey, Inc.

4.2.2 Pathogen Analysis

Pathogen analysis will be performed according to the laboratory standard operating procedure. Each sample is prepared with three dilution series, each with five replicate tubes for Salmonella and four dilution series each with five replicate tubes for Fecal Coliform as stated in the laboratory SOP. All data from the various replicates will be evaluated from the raw data. The pathogen analysis will include an empty bottle shipped to the site and returned as a blank control to verify that contamination has not occurred during transport. Pathogen analysis will be performed by Pure Earth Environmental Laboratory.

4.2.3 Microtox Analysis

The EC-50 value will be determined as the concentration of elutriate compost material which causes the effect of a 50% decrease in the light output of the microtox reagent organisms created from a strain of the marine bacterium, *Photobacterium phosphoreum*. The microtox test will be performed according to the specifications of

the proprietary method from Azur Environmental with the assistance of a computer program from Azur. The microtox test will utilize a blank to be analyzed with each assay. The blank will have a ratio of 0.75 or greater. A computer program will be utilized to determine the EC-50. This value must fall within the range of the test concentrations, and the confidence limits must closely bracket the EC-50 value. In addition, the correlation coefficient of the five data points must be greater than 0.9. If any of these values fail, the assay will be re-analyzed. Microtox tests will be performed by Aqua Survey, Inc.

4.2.4 TCLP and SPLP Analysis

The TCLP and SPLP test will utilize a blank for every twenty extractions that are conducted in each extraction vessel. In addition, a matrix spike will be performed with each analytical batch. Matrix spike recoveries will be compared to the analytical procedure for limits for acceptability. Both TCLP and SPLP procedures will be performed by Southwest Laboratories.

AQUA SURVEY, INC.
499 Point Breeze Road
Flemington, NJ 08822

Protocol No. GEN-5002-30
Revision No. 02
Date: 09/18/99

PROTOCOL: The Acute Soil Testing Toxicity Using the Earthworm, *Eisenia foetida*.

TEST SUBSTANCE: Soil collected from NSWC Crane

ASI NO. 98164

APPROVED BY: _____ _____
STUDY DIRECTOR DATE

APPROVED BY: Ala M Tosdick 9/21/99
SPONSOR DATE

Protocol No. GEN-5002-30
Revision No. 02
Date: 09/18/99

Approved: York Terrell — 9-15-99
York Terrell
GLP Testing Manager
Date

Approved: Jane Dowell — 9/16/99
Quality Assurance Unit
Date

Approved: — 9/16/99
James Todd
Vice President
Date

THE ACUTE SOIL TOXICITY TEST USING THE EARTHWORM, *EISENIA FOETIDA*.

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I. Purpose/Objective

This test is designed to determine the acute effect of contaminated soils on the earthworm, *Eisenia foetida*. Test concentrations will be prepared by the addition of the test soil to artificial soil. The concentration of the test substance which is lethal to half of the organisms in a concentration (LC_{50}) series during a 7-day and 14-day exposure will be calculated from observations of mortality. When no adverse effects are noted below a test concentration, the results are also expressed as a highest acute No Observed Effect Concentration (NOEC). The information from this response test can be used to predict the likelihood of an ecosystem effect if the test substance enters the terrestrial environment.

II. Justification

Evaluating contaminants for their adverse impact on the terrestrial environment is an important component of environmental hazard assessments. The present procedure evaluates the potential of contaminated soils to cause acute (14 days or less) adverse effects on the earthworm, *Eisenia foetida*. This potential is expressed as a 7-day LC_{50} and a 14-day LC_{50} value and/or as the highest acute No Observed Effect Concentration (NOEC). The effect of the test substance on the earthworm is considered indicative of its potential to affect other terrestrial organisms. The procedure described herein is similar to standard and/or generally acceptable procedures such as those described by the US FDA[1], ASTM[2], US EPA[3], OECD[4] and APHA/AWWA/WPCF[5].

III. Test Administration

A. Testing Facility

Aqua Survey, Inc.
499 Point Breeze Road
Flemington, NJ 08822

B. Sponsor

Morrison Knudsen Corporation
1500 West Third Street
Cleveland, OH 44113-1406

C. Dates of Experiment

Estimated Test Substance Exposure Initiation Date:

Estimated Test Substance Exposure Completion Date:

Estimated Study Completion Date:

D. Test Characteristics

1. ____ Range-Finding/ _X_ Definitive

2. _X_ Nominal/ ____ Measured Test Substance Concentrations

4. _3_ Number of Replicates

IV. Test and Control Substances

A. Test Substance

1. Identity:

2. ASI Number:
Batch/Lot Number:
CAS Registry No.:

B. Control Substance(s)

1. Negative Control: Untreated artificial soil

2. Reference Toxicant Test: A reference toxicant test with potassium chloride (KCL) will be performed concurrently with the test substance exposures.

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V. Test System

A. Test Organism

1. The earthworm, *Eisenia Foetida*, will be used for this test; only adult worms will be used. Adult worms weigh approximately 300 milligrams (mg) wet weight and have a distinct, fully developed clitellum.

2. Source of organism: Earthworms used for these tests will be obtained from a commercial supplier. An organism Chain-of-Custody, as well as any culturing procedures, accompany each batch of organisms and are kept on file in the archives of Non-Test Data. In addition, the receipt date, lot or batch number, condition of shipping container, worms, temperature and soil, as well as taxonomic verification of the test species, will be documented upon receipt of organisms. The following taxonomic characterization applies to the earthworm, *Eisenia foetida*:

Phylum - Annelida
Class - Oligochaeta
Order - Lumbricina
Family - Lumbricidae
Genus - *Eisenia*
Species - *foetida*

3. Rearing and Maintenance Regime: Earthworms are reared within the laboratory in plastic trays measuring approximately 34 by 28 by 14 cm. Each tray is provided with a bedding of sphagnum peat moss pH adjusted to approximately 7.0 with pure calcium carbonate (99%). Seven hundred grams (dry weight)of peat moss is added to each tray and hydrated to approximately 75% of its water holding capacity with approximately 2300 mL of deionized water. The number of earthworms that can be maintained in each tray is determined by the age and size of the worms. Seven hundred grams of bedding holds a population of approximately 350 adult worms. The trays are covered with plastic to prevent drying, however, several 1/8" air holes are punched into the plastic for air circulation. Moisture content is monitored on a weekly basis and hydrated water is added as needed. Bedding is changed every 2-3 months. Fluorescent lighting above the trays provides illumination (50-100fc) on a continuous basis. Bedding temperature is maintained at 20 ± 2°C.

Organisms are observed for signs of disease, stress, injury and mortality once each week by hand turning the soil. All observations and operations such as feeding and maintenance procedures and schedules, clinical observations, mortality and critical rearing parameter (e.g., temperature, pH, etc.) are documented in culture systems log books.

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4. Diet: Earthworms are maintained on a diet of magic worm food, obtained from Magic Products, Inc., Amhurst Jct., Wisconsin, 54407. Food is prepared as a slurry by adding deionized water to the magic worm food using approximately a 1:1 v/w ratio. This slurry is applied to the top of the bedding of rows of about 1/8" thick, using a 60 mL disposable syringe. The cultures are usually fed twice each week but if the number of worms in a tray is low, feeding is reduced accordingly. Food remaining on the surface of the bedding from a previous feeding is removed and discarded. An estimate of the amount of food discarded will be used in deciding what level of feeding is adequate for successful growth of earthworms.

5. Acclimation: Organisms are held at test conditions (e.g., temperature, pH, illumination) during culture so that a period of acclimation is not necessary. However, if worms are obtained from an outside source just prior to testing, that will be held at test conditions for a minimum of 5 days.

B. Artificial Soil

1. Source: Soil used as substrate in this test will be artificial. It has an adsorptive capacity resembling typical loamy soils. The substrate is prepared in the laboratory from a mixture of commercially available constituents. The following constituents are mixed on a dry weight basis, to produce the artificial soil: 10% sphagnum peat moss (that portion passing through a 2.36 mm screen); 20% Kaolin clay (greater than 50% kaolinit(with particle sizes under 40 mm): 70% silica sand (97.1% particle size of 0.053 - 0.300 mm). After the sphagnum, clay and sand are mixed, calcium carbonate equal to 0.39% of the weight of the mixture is added to adjust the pH to 6.5 ± 1.5 su at which time the artificial soil is homogenized in a 5-gallon plastic bucket, using a hand drill fitted with a stainless steel mixing blade.

2. Quality: The use of artificial soil virtually eliminates the problem of variability between different natural soil types and should contain no contaminants that might interfere with the outcome of the study.

VI. Test Procedure

A. Preparation of Test Concentrations

Test soils are screened through a one-quarter inch stainless steel screen. The screened soil is then mixed in a 5-gallon plastic bucket using a hand drill filtered with a stainless steel mixing blade until the sample is fully homogenized. Seven hundred grams of each test concentration is prepared by adding appropriate amounts of test soil with artificial soil in a geometric series in which the ratio is 2.0 (e.g., 100, 50, 25, 12.5 and 6.25 dry weight/dry weight). Negative controls (100% artificial soil only) are also prepared. To ensure even distribution of the test

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soils with artificial soil, the total amount (700g) for each concentration is homogenized using a blender. Two hundred grams of soil (dry weight) from each concentration is used for each of three replicate test concentrations and control, at which time the soil is hydrated to 75% of its water holding capacity using deionized water. Hydration is accomplished by using the moisture fraction and water holding capacity or both the test and artificial soil, then using a computer program developed by this laboratory to calculate the required volume of hydrated water. The computer program requires the following information:

THW_{ts} (test soil water hydration required in mL/100g) = PHYD $[(PAS*WHC_{as}) + (PWS*WHC_{ws})$

EHW_{ts} (existing test soil hydration water in mL/100 g) = $[(PAS*MF_{as}) + (PWS*MF_{ws})]$ X 100

Amount of hydration water to be added (mL/100 g of soil) = THW_{ts} - EHW_{ts}

Where PHYD = proportion of hydration required (constant = 0.75)
PAS = proportion of artificial soil in test soil
WHC_{as} = water holding capacity of the artificial soil in mL/100 g
PWS = proportion of waste sample in the test soil
WHC_{ws} = water holding capacity of the waste sample in mL/100g
MF_{as} = moisture fraction of the artificial soil
MF_{ws} = moisture fraction of the waste sample

B. Soil Moisture Content and Water Holding Capacity

The moisture content of soil is determined by pre weighing an empty aluminum weigh boat or crystallizing dish. Appropriate amount of soil is placed in the weigh boat in order to obtain a minimum of 100 g dry weight soil. The combined weight of the boat and sample equal the initial wet weight and is recorded to 0.1 g. The wet weight is calculated by subtracting the weight of the empty weigh boat from the combined weight. The weigh boat and sample are placed in a drying oven at 100±5°C for 24 hours at which time it is removed and allowed to cool in a desiccator. The weight of the boat and the dried sample are recorded as the final dry weight. The moisture content of the sample is determined according to the following formula:

$$\text{Percent moisture} = \frac{(\text{wet weight + pan (g) - dry weight + pan})}{(\text{Wet weight + pan (g) - pan})} \times 100$$

After the moisture content has been calculated the water holding capacity is determined by placing 100 G) of dry soil into a 500-mL glass beaker. One hundred mLs of deionized water is added to the sample and mixed thoroughly with a glass rod to ensure that all particles are wet and a slurry of soil and water exists. A circle of coarse porosity, qualitative, crepe filter paper is folded into eighths and placed in a 100-mm glass funnel. The filter paper is moistened with approximately 10 mL deionized water at which time the weight of the funnel including the wet filter paper is determined. Next, the slurry of soil and water is poured in the funnel; all

remaining soil in the beaker and on the stirring rod is rinsed into the funnel with deionized wate The funnel is covered tightly with aluminum foil and allowed to drain for a minimum of three (3) hours at room temperature. The time of drainage will be documented.
After the drying period the foil is removed and the funnel with drained soil is weighed to find the final weight. The final weight minus the initial weight is the number of mLs of water held by the soil. The water held, when related to the amount of soil, is the water-holding capacity and is expressed as g water/100g dry weight soil.

C, Preparation of Test Vessels

One liter borosilicate glass containers with screw top covers with small holes punched in the covers to allow air to enter the exposure vessels. The side of each replicate jar is labeled with the site soil concentration and the replicate jar number. Permanent felt-tip markers are used for labeling.

D. Collection of Organisms for Testing

Earthworms of relatively uniform size and age are collected from a single, established stock culture and transferred to intermediate holding vessels. Sequential randomization is accomplished by allocating to each holding vessel no more than 20 percent of anyone set of test organisms at a time.

E. Number of Organisms

Ten (10) organisms per replicate test vessel will be used at each concentration and for each control. All controls and samples will be analyzed in triplicate.

F. Characterization of Size, Age, or Absorption of Test Substance

No attempt is made to characterize the test organisms as to exact age or sex. Adult earthworms which weigh approximately 300 mg wet weight and have a distinct fully developed clitellum will be used to characterize the age of the worms. The degree of absorption of the test substance by the test system will not be determined in this test. Mean weights will be determined by weighing a surrogate container of earthworms from the same lot as used for testing. Average weights are calculated and these values are used as mean test population characteristics. No other weights will be determined during this study.

G. Test Endpoint

The 14-day LC_{50} with its associated 95 percent confidence limits, and, when sufficient mortality data are noted, the 7-day LC_{50} values and their confidence limits, will be calculated. The LC_{50} value is the median lethal concentration (i.e., the concentration, in terms of initial or measured values, of test substance in substrate that results in the death of 50 percent of the test organisms within a specified time interval (e.g., 14 days). Death is defined as lack of observable movement

by a test organism when gentle mechanical stimulation is applied to the anterior end (FDA[1]). A computer program developed by C.E. Stephan[6] and ASTM[7] will be used to calculate the LC_{50} value and the associated confidence limits for each concentration series as described in Section VIII.

When possible, the no observed effect concentration, or NOEC, (the highest concentration tested at which there were no observable effects) will be determined through visual inspection of the data. However, appropriate statistical analyses (i.e., analysis of variance [ANOVA], group mean comparison, etc.) may be used.

H. Test Duration

The test will be conducted for a period of 14 days.

I. Control Vessels

Artificial soil (negative control) vessels will be prepared, in addition a positive control containing KCl as a toxicant with artificial soil will be prepared. All positive and negative controls will be analyzed in triplicate.

J. Feeding

The organisms will not be fed during this test.

K. Loading

The biological loading should not exceed one (1) organism mass per 20 g of soil.

L. Temperature

The temperature is maintained at 20 ± 2°C via an environmental chamber and is continuously measured (hourly) using a Ryan tempmonitor®. Fluorescent lighting above sample vessels provides illumination (50-100 fc) on a continuous basis.

M. Soil pH/Adjustment

Initial pH values are measured and recorded on the Earthworm Survival Data Sheet. The pH values should fall within the range of 5.0 and 9.0 SU for initiation of the test. If the pH falls outside this range it may be necessary to adjust the pH within range as close to 7.0 as possible for the survival of the test organisms. pH adjustment may be done with 5N HCL or 5N NaOH. Other normality solutions may be used. If the soil needs to be pH adjusted, a 40-g slurry may be prepared and adjusted by adding 20g soil to 20 mL deionized water. The mixture is stirred for approximately 30 minutes at which time the mixture is allowed to settle, for about one hour. At this time the pH maybe measured from the supernatant. The pH is

adjusted by adding the appropriate volumes of HCL or NaOH to the mixture until the pH of the slurry is in an acceptable range. The volume of HCL or NaOH and the normality used to adjust the pH of the slurry are recorded and then used in calculating the amount needed for adjusting the pH of the calculated wet weight of soil needed for the test.

N. Reference Toxicant

A reference toxicant test with KCL will be performed to assess the adequacy of the test conditions and to monitor the sensitivity of the test organisms. A stock solution containing 50,000 ppm of KCL is prepared by adding 10 grams of KCL in a total volume of 200 mL using DI water. Test concentrations of 625, 1250, 2500, 5000 and 10,000 ppm is prepared by placing 200 grams dry weight of artificial soil in each of three replicate test vessels and control vessels. A magnetic stir bar is used to stir the KCL until it is completely dissolved. Appropriate volumes of stock solution (i.e., 2.5, 5.0, 10.0, 20.0 and 40.0 mL for the 625, 1250, 2500, 5000 and 10,000 ppm test concentration, respectively) is added as part of the total volume of water used to hydrate the artificial soil). The total volume of water used for hydration is determined as per section VI.A. The acceptable ranges for this reference toxicant is 4321 to 8081 ppm.

O. Mortality of Negative Control

Mortality or abnormal behavior in the 100% artificial soil should not exceed 10 percent.

P. Observations

Observations performed after 24 hours of exposure is optional but may be conducted to determine if earthworms are burrowing and if any deaths have occurred on the test soil surface. Lack of burrowing in the presence of continuous light is considered a behavioral response to the toxicants and will be noted.

After seven days of exposure, the number of live worms is counted and observations of behavioral and pathological changes are recorded. The test jars are removed from the environmental chamber and emptied one at a time on a counting tray. During the worm count detailed observations are made regarding: (1) number of percentage of worms that did not burrow, (2) ulceration (3) coiling (4) "balling" together of worms (5) contraction (6) rigidity (7) elongation (8) mid segmental swelling (9) segmental constriction and (10) segmental loss. All observations are recorded on the Test Effects Report forms. Each replicate is counted and the number of mortalities is entered on the live count sheet. A worm is considered dead if it does not respond to a gentle mechanical stimulus to its anterior end. Worms decay rapidly in the moist testing environment. If worms cannot be found they are considered to have died and decomposed completely. After worms are counted, the soil is replaced in test vessels and worms are placed on the surface of the soil. After 14 days of exposure the worms are observed and counted as per day seven. Negative and positive control worms are counted on day seven and 14 as per test organisms.

VII. Chemical Analysis

A. Measurements of Physical and Chemical Parameters

1. Temperature: The temperature of the exposure environmental chamber should be held at 20 ± 2° C. Measurements should be made continuously (hourly) during the entire study period.

2. pH: The soil pH measured in water should be between 5 and 9 su. Measurements are made at test initiation and termination. If the initial pH of the soil falls outside the 5-9 range, soils pH may be adjusted using 5N NaOH or 5N HCL.

3. Moisture Content: Soil moisture content should be maintained at approximately 75 percent of water holding capacity for optimum condition. This is accomplished by covering the exposure vessels with lids after the initial hydration.

Physical and chemical measurements will be recorded on the Test Effects Report form.

VIII. Endpoint Calculation

A computer program developed by C.E. Stephan[6] and ASTM[7] is used to compute point and interval (i.e., 95 percent confidence interval) estimates of the 14-day LC_{50} value. The 7-day and 14-day values should be calculated whenever there are sufficient mortality data to determine such values. The program requires the following data: the concentration of the test substance, either nominal or measured ($\log_{10}$ transformed for all methods); the number of organisms exposed; and the number of organisms that die. Data for the positive KCl sample and the negative artificial soil sample vessels are not used because the program does not have the capability of adjusting for check standard mortality. The program computes point and interval estimates of the LC_{50} value using three approaches:

1. Nonlinear Interpolation, using the concentrations that bracket 50 percent mortality, to give an approximate point estimate; and the Binomial Method, to give a conservative 95 percent confidence interval. In the case of utilizing a single concentration, use of the Binomial Method is capable of indicating (at a conservative 95 percent confidence interval) whether or not the LC_{50} value lies above or below the test substance concentration.

2. The Moving Average Method, to give both a point and interval estimate. This output is included only when two consecutive concentrations exhibit an effect.

3. The Probit Method, to give both a point and interval estimate. This output is included only when two consecutive concentrations exhibit an effect. Additionally, when the number of test substance concentrations is at least three, a chi-square statistic is included to test the goodness-of-fit for the Probit Method.

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The no observed effect concentration (NOEC) will be determined through observation and when appropriate, statistical analyses (i.e., analysis of variance [ANOVA], group mean comparison, etc.).

IX. Criteria for Test Validity

A. Control Mortality

The negative control (100% artificial soil) mortality should not exceed 10 percent.

B. Mishaps

Abnormal occurrences (i.e., laboratory accidents) that might influence the outcome of the test will be considered in determining the validity of test results.

X. Reporting and Records

A. Reporting

The report should contain:

1. Name and address of testing facility.
2. Good Laboratory Practice compliance statement.
3. The Quality Assurance Inspection Statement.
4. Dates of Study.
5. Name of Study Director and list of scientists involved in the test.
6. Details documented in this protocol, details of solution preparation, all data, and any appropriate graphs or calculation summaries.
7. All observations and results as well as any protocol deviations.
8. The 14-day LC_{50} value, and when sufficient data have been generated, the 7-day LC_{50} values, the associated 95 percent confidence limits, and the method(s) of calculation.
9. When possible, the no observed effect concentration (NOEC).

B. Records

The following records will be maintained by ASI and are available upon request..

1. All raw data (or exact copies thereof) and summaries of data, final reports, protocols and amendments will be retained for a period of 5 years with a subsequent review.

2. All Standard Operating Procedures and all Summaries of Training and Experience for all scientists involved in the testing will be retained for a period of 10 years with a subsequent review.

3. The Archive of Non-Test Data shall contain:

   a. Taxonomic verification of the test species and chain-of-custody records.

   b. Chemical characterization of the substrate.

   c. Diet receipt records and characterization results.

   d. Rearing and maintenance records for the aquatic organisms.

   These records will be retained for a period of 10 years with a subsequent review.

XI. References

1. United States Food and Drug Administration (US FDA). 1987. Environmental Assessment Technical Assistance Handbook, PB87-175345, Document 4.12, "Earthworm Subacute Toxicity". March 1987.

2. American Society for Testing and Materials (ASTM). Annual Book of ASTM Standards. 1995. Section 11, Water and Environmental Technology. Volume 11.04: Pesticides; Resource Recovery; Hazardous Substances and Oil Spill Responses; Waste Disposal; Biological Effects. Standard Guide for Conducting A Laboratory Soil Toxicity Test with Lumbercid Earthworm, *Eisenia foetida* E1676-95; American Society for Testing and Materials, Philadelphia, PA, pp. 1125-1139.

3. United States Environmental Protection Agency (US EPA). 1988. Protocols for Short-Term Toxicity Screening of Hazardous Waste Sites (USEPA/600/3-88/024). A.8.5, Earthworm Survival. March 1988.

4. Earthworm, Acute Toxicity Tests, 1984. In OECD Guideline for Testing of Chemicals. No. 207, pp. 1-9.

5. American Public Health Association/American Water Works Association/Water Pollution Control Federation. 1989. Standard Methods for the Examination of Water and Wastewater, 17th ed. American Public Health Association, Washington, DC.

6. Stephan, C.E. 1977. Methods for Calculating an LC_{50}. In: F.L. Mayer and J.L. Hamelink, Eds., Aquatic Toxicology and Hazard Evaluation, Special Technical Publication No. 634, American Society for Testing and Materials, Philadelphia, PA, pp. 65-84.

7. American Society for Testing Materials. 1988. Proposed New Standard Practice for Using Probit Analysis. ASTM Subcommittee E-47.07. Draft No. 4, June, 1988.

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I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gather and evaluate the information submitted. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, the information submitted is, to the best of my knowledge and belief, true, accurate, and complete. I am aware that there are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations.

SIGNATURE

JAMES H. HUNSICKER
Director, Environmental Protection Department
By direction of
the Commander

TITLE

9/27/99

DATE