



DEPARTMENT OF THE NAVY

CRANE DIVISION
NAVAL SURFACE WARFARE CENTER
300 HIGHWAY 381
CRANE, INDIANA 47522-5000

IN REPLY REFER TO:

5090
Ser 095/9134
28 JUN 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:

The Morrison Knudsen Corporation forwarded to your office revisions, per previous comments provided by Ms. Peg Donnelly, for the Crane Division, Naval Surface Warfare Center (NAVSURFWARCENDIV Crane) Bioremediation Facility Quality Assurance Project Plan (QAPP) Appendix G. The QAPP Appendix G revisions were dated June 11, 1999. This letter is to acknowledge that submittal and to provide the required certification statement as enclosure (1).

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

Sincerely,

JAMES M. HUNSICKER

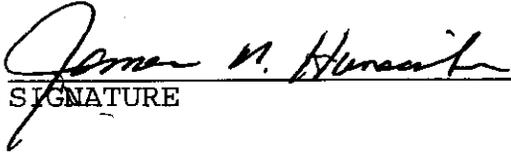
Director, Environmental Protection Department
By direction of
~~the Commander~~

Encl:

(1) Certification Statement

Copy to: (w/o encl)
ADMINISTRATIVE RECORD
COMNAVSEASYS COM (SEA OOT)
IDEM
MK Cleveland
SOUTHNAVFACENGCOM (Code 1864)
TolTest Crane

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

DIR. ENV. PROT. DEPT.
TITLE

6/29/99
DATE

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

APPENDIX G

ERRATA SHEET

1. Remove pages A-4 through A-10, Toxicity Test using the Earthworm, dated 4/16/98. Replace with attached pages A-4 through A-10g dated 6/8/99.

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

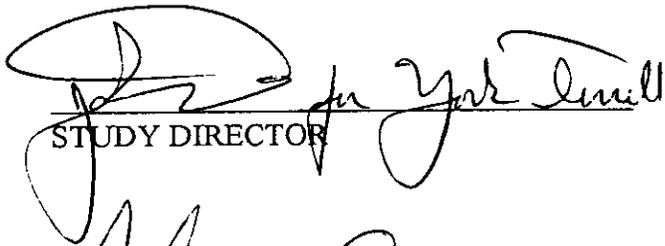
AQUA SURVEY, INC.
499 Point Breeze Road
Flemington, NJ 08822

Protocol No. GEN-5002-30
Revision No. 01
Date: 06/08/99

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

6/8/99
DATE

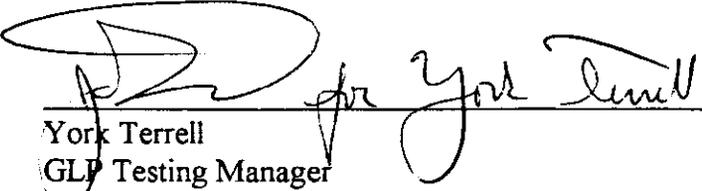
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SPONSOR

6/9/99
DATE

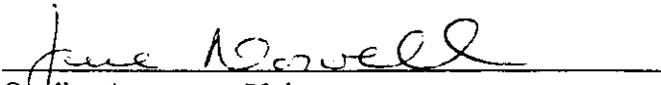
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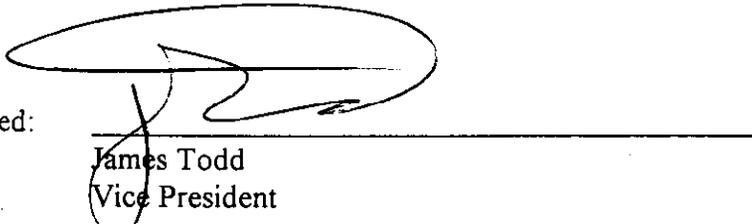
Protocol No. GEN-5002-30
Revision No. 01
Date: 06/08/99

Approved: 
York Terrell
GLP Testing Manager

6/8/99
Date

Approved: 
Quality Assurance Unit

6/8/99
Date

Approved: 
James Todd
Vice President

6/8/99
Date

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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 control/artificial soil.

The concentration of the test substance which is lethal to half of the organisms in a concentration (LC₅₀) 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 LC₅₀ and a 14-day LC₅₀ 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 FDA¹, ASTM², US EPA³, OECD⁴ and APHA/AWWA/WPCF⁵.

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/ Definitive
2. Nominal/ Measured Test Substance Concentrations
4. 3 Number of Replicates

IV. Test and Control SubstancesA. 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.

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 4-6 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.



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 a control 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% kaolinite 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 \pm 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 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

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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:

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

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

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

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 \pm 5 \pm 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:

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

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 water. 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.

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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

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.

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. 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 (FDA¹).

A computer program 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.

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 $20 \pm 2^\circ\text{C}$ via an environmental chamber and is continuously measured (hourly) using a Ryan thermometer.

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 may be 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 two 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

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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. Control Mortality

Mortality or abnormal behavior in the controls 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) Aballing≡ 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. Controls 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 E C. Measurements should be made continuously (hourly) during the entire study period.
2. pH: The Asoil 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.

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

VIII. Endpoint Calculation

A computer program developed by C.E. Stephan⁶ and ASTM⁷ is used to compute point and interval (i.e., 95 percent confidence interval) estimates of the 14-day LC₅₀ 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 (log₁₀ transformed for all methods); the number of organisms exposed; and the number of organisms that die. Data for the control vessels are not used because the program does not have the capability of adjusting for control mortality. The program computes point and interval estimates of the LC₅₀ 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 LC₅₀ 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.

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 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 LC₅₀ value, and when sufficient data have been generated, the 7-day LC₅₀ values, the associated 95 percent confidence limits, and the method(s) of calculation.
10. 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.

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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 Lumbricid 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 LC₅₀. 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.