CLOSED BOTTLE TEST

WILDLIFE INTERNATIONAL LTD.

PROJECT NUMBER: 486E-105A

AUTHORS Edward C. Schaefer Douglas R. Haberlein

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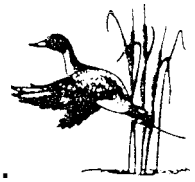
STUDY COMPLETION DATE: October 29, 1998



WILDLIFE INTERNATIONAL LTD.
8598 Commerce Drive Easton, Maryland 21601 (410) 822-8600

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SUMMARY

LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT
Wildlife International Ltd. Easton, MD 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER
486E-105A

STUDY
Closed Bottle Test

TEST CONCENTRATION
2 mg/L

TEST DATES
Biological Phase Start - June 30, 1988
Biological Phase Termination - July 28, 1998

LENGTH OF EXPERIMENTAL PHASE
28 Days

TEST SUBSTANCE
RG-2400®

PERCENT BIODEGRADATION
0%

INTRODUCTION

This study was conducted by Wildlife International Ltd. at the Wildlife International Ltd. biodegradation facility in Easton, Maryland. The test was conducted from May 06, 1998 to July 28, 1998. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 486E-105A in the archives located on the Wildlife International site.

A variety of chemicals reach the sea by direct discharge or via estuaries and rivers. Because of the growing awareness of the need to protect the marine environment against increasing loads of chemicals and the need to estimate the probable concentration of chemicals in the sea, test methods for biodegradability in seawater have been developed. A positive result indicates that there is a potential for biodegradation in the marine environment. However, a negative result does not necessarily preclude such a potential, but that additional testing may be needed.

OBJECTIVE

The objective of the study was to measure dissolved oxygen uptake over a 28-day period and express it as a percentage of the theoretical oxygen demand (ThOD) or chemical oxygen demand (COD).

EXPERIMENTAL DESIGN

The test contained an inoculum control group, a reference group, and a treatment group. The inoculum control, reference, and treatment groups contained ten replicate test chambers. The inoculum control was used to measure the dissolved oxygen consumption of the inoculum and was not dosed with a carbon source. The reference chambers were dosed with canola oil, a substance known to be biodegradable, at a concentration of 2 mg/L. The test chambers within the treatment group were used to evaluate the test substance at 2 mg/L Measurements of oxygen consumption were performed on two test chambers from the control, reference and treatment groups on days 0, 7, 14, 21, and 28.

MATERIALS AND METHODS

This study was conducted based on the procedures outlined in the protocol. "Biodegradability in Seawater by the Closed Bottle Test Method" (Appendix I). The protocol was based on the procedures specified in the OECD Guideline for Testing of Chemicals, Guideline 301D (1).

Test Substance

The test substance, **RG-2400®**, was received at Wildlife International Ltd. on April 29, 1998 and was assigned Wildlife International Ltd. identification number 4455 The test substance was an off-white grease, and was stored under ambient conditions.

The test substance was administered to the treatment group vessels by direct weight addition. Direct weight addition is the most appropriate route of administration of substances that are relatively insoluble in water.

The chemical oxygen demand (COD) of the test and reference substances was measured in triplicate and the results expressed as grams of COD per gram of test substance. The procedures used for the determination of the COD were based on Hach Method Number 8000 (2).

Reference Substance

Pure Wesson canola oil was used as a reference substance to check the activity of the inoculum. The canola oil used in this test was obtained from Food Lion # 1289 (Easton, MD) and was assigned lot number 10-7-97. The reference substance was administered to the reference group test chambers by direct weight addition.

Test Inoculum

Natural seawater was obtained at Indian River Inlet, Delaware. The seawater was aerated for approximately 12 days prior to use to reduce the level of degradable organic material present. Prior to use the pH. salinity, and dissolved organic carbon concentration of the seawater was measured. A standard plate count was performed on the inoculum. Plates were incubated at 20 $\pm$ 3°C for approximately 48 hours.

Test Medium

During aeration of the seawater and just prior to use, the following standard reagent solutions were added:

- mL of phosphate buffer solution APHA, pH 7.2
- mL of calcium chloride solution APHA, 2.75%
- mL of magnesium sulfate solution, APHA, 2.25%
- mL of ferric chloride solution, APHA, 0.025%

The dissolved oxygen concentration of the dilution water was 9.3 mg O_2/L prior to use.

Test Apparatus and Conditions

The test chambers were 300-mL BOD bottles. The test was conducted at 20°C $\pm$ 3°C. Test chambers were identified by project number, Wildlife International Ltd. test substance ID, test concentration, and bottle number.

Preparation of Test Chambers

Adequate amounts of test substance and reference substance necessary to achieve a concentration of 2 mg/L were added to the appropriate bottles.

Sufficient test medium was then added to each bottle so that all bottles were completely full. The bottles were stoppered and capped.

Sample Analysis

The dissolved oxygen concentration was determined using a Yellow Springs Instruments Dissolved Oxygen Meter. Dissolved oxygen measurements were performed on two test chambers from the control, reference and treatment group on days 0, 7, 14, 21, and 28. Day zero samples were analyzed immediately after all bottles had been prepared.

Calculations

The average oxygen uptake exhibited by the control, reference, and treatment groups was calculated for each sampling interval. The biochemical oxygen demand (BOD) was calculated for each sampling interval using the following equation:

$$BOD = \frac{\text{mg } O_2\text{/L uptake test substance - mg } O_2\text{/L uptake blank}}{\text{mg test substance/L in vessel}}$$

The percent degradation was calculated using the following equation:

$$\% \text{ gradation} = \frac{\text{BOD (rng } O_2\text{/mg test substance)}}{\text{COD (mg COD/mg test substance)}} \times 100$$

Observations and Measurements

The temperature range recorded during the test was 18 to 20°C, within the range specified in the protocol. The seawater pH, salinity, and dissolved organic carbon concentration was 7.7, 30 parts per thousand, and 1.8, respectively. The result of the standard plate count performed on the inoculum was 2.57×10^2 CFU/mL.

The average oxygen uptake exhibited by the control, reference, and treatment group at each sampling interval is presented in Table 1. The oxygen depletion of the inoculum control was $\leq$ 2.5 mg 0_2/L. The calculated biochemical oxygen demand (BOD) and percent degradation results are also presented in Table 1.

Degradation of the test substance was not observed over the 28-day test.

The viability of the inoculum and validity of the test were supported by the results of the reference substance, canola oil, which degraded approximately 111%. An average percent biodegradation of greater than 60% was achieved by day 14, thereby fulfilling the criteria for a valid test (1).

The chemical oxygen demand (COD) results are presented in Table 2.

REFERENCES

Number 1

Organisation for Economic Cooperation and Development.
1992
Ready Biodegradability: Closed Bottle Test
OECD Guideline 301D

Number 2

Hach Water Analysis Handbook, 2nd Edition
1987
Method 8000

Table 1
Average Oxygen Uptake
Biochemical Oxygen Demand (BOD)
Percent Degradation Results

Test Substance	Cone. (mg/L)	Day	DO (mg/L)	DO Uptake	Average uptake	BOD (m g/mg)	Percent Degradation
Control	NA	0	9.2	NA	NA	NA	NA
Control	NA	0	9.2	NA			
Control	NA	7	8.6	0.6	0.7	NA	NA
Control	NA	7	8.4	0.8			
Control	NA	14	7.8	1.4	1.4	NA	NA
Control	NA	14	7.8	1.4			
Control	NA	21	6.9	2.3	2.1	NA	NA
Control	NA	21	7.3	1.9			
Control	NA	28	6.8	2.4	2.5	NA	NA
Control	NA	28	6.6	2.6			
Canola	NA	0	9.2	NA	NA	NA	NA
Canola	2	0	9.2	NA			
Canola	2	7	3.4	5.8	5.7	2.5	95
Canola	2	7	3.6	5.6			
Canola	2	14	<1	8.2	8.0	3.3	126
Canola	2	14	1.5	7.7			
Canola	2	21	<1	8.2	8.2	3.1	128
Canola	2	21	<1	8.2			
Canola	2	28	<1	8.2	8.2	2.9	111
Canola	2	28	<1	8.2			
RG-2400®	2	0	9.2	NA	NA	NA	NA
RG-2400®	2	0	9.2	NA			
RG-2400®	2	7	8.2	1.0	0.9	0.1	6
RG-2400®	2	7	8.4	0.8			
RG-2400®	2	14	7.4	1.8	1 .5	0.1	6
RG-2400®	2	14	8.0	1 2			
RG-2400®	2	21	7.1	2.1	2.2	0.1	6
RG-2400®	2	21	7.0				
RG-2400®	2	28	6.6	2.6	2.5	0.0	0
RG-2400®	2	28	6.8	2.4			

Table 2
Chemical Oxygen Demand (COD) Results

Test Substance	COD (g/g)			Average	Standard Deviation
	Replicate 1	Replicate 2	Replicate 3		
RG-2400®	1.45	2.02	1.78	1.75	10

APPENDIX I

Personnel Involved in the Study

The following key Wildlife International Ltd. personnel were involved in the conduct or management of this study:

1. **Henry 0. Krueger**, Ph.D.,
   Director of Aquatic Toxicology and Non-Target Plants

2. **Edward C. Schaefer**
   Manager, Biodegradation

3. **Doug R. Haberlein**
   Biologist, Biodegradation

4. **Sheri Trumbull**
   Technologist