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REPORT OF TEST

28 Day Shake Flask Ready Biodegradability Test
versus

JG-302

Conducted for:

Firefreeze World Wide, Inc.
270 Route 46
Rockaway, New Jersey 07866

Prepared by:

Daniel Cooke
Manager, Ecotoxicology

4/23/97
Date

SIGNED FOR THE COMPANY BY

Charles Tong, PhD, DABT
Director, Toxicology

4/23/97
Date

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28 DAY READY BIODEGRADABILITY

Sponsor: Firefreeze World Wide, Inc.

Address: 270 Route 46
Rockaway, New Jersey 07866

Testing Facility: SGS U.S. Testing Company, Inc.
Biological Services Division
75 Passaic Avenue
Fairfield, New Jersey 07004

Sample Description: Sample identified by Sponsor as Firefreeze World Wide product **JG-302**. Chemical composition: proprietary. Clear, colorless, slightly viscous liquid with a slight odor, soluble in water. Sample considered stable, received 3/11/97.

Project Description: 28 day Shake Flask Ready Biodegradability Test
Test dates: 3/21/97 - 4/18/97.

Procedures: SGS USTC Standard Operating Procedure MIC/28DAYSFT.012 "Biodegradability Shake Flask Test, CO_2 Evolution, 28 Days". This procedure is based on:

USEPA 796.3240 "Ready Biodegradability: Modified OECD Screening Test".
OECD 301E "Ready Biodegradability: Modified OECD Screening Test".

SUMMARY OF RESULTS:

Firefreeze World Wide product **JG-302** degraded 95.3%, by TOC reduction, within 28 days. The test substance met the degradability and microbial kinetics criteria (that the 70% "pass" level was met within 10 days after reaching 10% degradation) for ready biodegradability.

Carbon dioxide evolution data was not usable, due to carbonate interference.

The control substance, aniline, readily degraded, validating the test system.

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INTRODUCTION:

This is a report of ready biodegradability assessment performed versus Firefreeze World Wide product **JG-302**. The test was conducted in a Gledhill Apparatus (Shake Flask) system to determine the sample's biodegradability in a closed aqueous system.

By supplying the test substance as virtually the sole carbon source, the ability of the substance to be metabolized by microbes became the limiting factor. Materials that degrade under such conditions, either substantially or completely (to mineralization), within adequate time constraints can be considered "readily" or "ultimately" biodegradable.

Since the environmental conditions of the test are stringent, failure to measure degradability does not necessarily imply that the test substance is not biodegradable. Factors such as culture conditions, microbial inhibition, solubility, quantity and diversity of the microbial inoculum, and the absence of co-nutrients can affect results. Other test systems may be applied to further evaluate biodegradability.

Initial determination of organic carbon content of the batch of test substance submitted was performed by SGS USTC. **JG-302** was determined to contain approximately 3350 ppm (0.335%) organic carbon. The Sponsor stated that **JG-302** was approximately 97% water, and 3% active ingredient. Using this percentage, the active ingredients of the test substance were approximately 11% organic carbon. The results of the biodegradability test described herein deal with this organic portion of the product.

Testing was performed in accordance with SGS USTC procedures and USEPA methodologies.

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SUMMARY OF PROCEDURES: 28 Day Shake Flask Ready Biodegradability Assay

References :	USEPA 40 CFR 796.3240, "Ready Biodegradability: Modified OECD Screening Test". OECD 301E, "Ready Biodegradability: Modified OECD Screening Test". SGS USTC Protocol MIC/28DAYSFT.012, "Biodegradability Shake Flask Test, CO_2 Evolution, 28 Days".
Sample storage :	Ambient temperature, original, sealed sample container
Inoculum source :	The inoculum was collected from the activated sludge channels of a domestic sewage plant, Florham Park Sewerage Authority (NJ) on 2/27/96. Sludge was maintained in SGS USTC SCAS reactor until test initiation. Surface water was collected from SGS USTC Aquatic Laboratory fish culture systems. A soil elutriate was prepared from active soil maintained under incubation in the SGS USTC Microbiology Laboratory.
Temperature:	20 - 25°C
Illumination :	Low light conditions (to prevent photochemical breakdown or growth of algae in test flasks)
Test vessels:	2000 mL glass shake flasks (Gledhill Apparatus)
Test volume:	1000 mL
Replication :	3 replicates per treatment
Test concentration:	Approximately 20 ppm (as Carbon) of test sample
Controls :	Blank control = nutrient media only Positive control = nutrient media + Aniline (approximately 20 ppm as C)
Agitation :	Gyratory shaking at 150 ± 10 revolutions per minute
Test duration :	28 days
Chemical data :	Captured CO_2 titration at Days 3, 7, 14, 21, and 28. Total Organic Carbon (TOC) analysis by Shimadzu TOC-5000 Carbon Analyzer at Days 0, 7, 14, 21, and 28.

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SUMMARY OF PROCEDURES (cont.) : 28 Day Shake Flask Ready Biodegradability Assay

Nutrient media : A defined aqueous inorganic salt medium was used, consisting of 1.0 mL of each of the following stock solutions added to 1.0 L of deionized water:

Stock Solution	Compound	Concentration (g/L)
I	KH_2PO_4 K_2HPO_4 $Na_2HPO_4 \cdot 2H_2O$ NH_4Cl	8.5 21.75 33.4 20.0
II	$MgSO_4 \cdot 7H_2O$	22.5
III	$CaCl_2$	27.5
IV	$FeCl_3 \cdot 6H_2O$	0.25
V trace elements	$MnSO_4 \cdot 4H_2O$ H_3BO_3 $ZnSO_4 \cdot 7H_2O$ $(NH_4)_6Mo_7O_{24}$ $FeCl_3$, EDTA	0.0399 0.0572 0.0428 0.0347 0.1000
VI - vitamins	yeast extract	0.15

Physical data : Temperature of the system daily, and pH of the test flasks initially.

Response : CO_2 evolution, TOC degradation.

Test acceptability : Positive control substance (aniline) must degrade ≥ 60% as measured by CO_2 evolution and/or ≥ 70% as measured by TOC reduction.

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RESULTS: Biodegradability - CO_2 Evolution

TABLE I: CO_2 Titration Data

BLANK	Titrant Value (mL)					
	Rep	Day 3	Day 7	Day 14	Day 21	Day 28
	A	0.8	0.1	0.2	0.7	0.2
	B	1.2	0.4	0.6	1.2	0.3
	C	0.8	0.5	0.6	0.7	0.5
Mean value ($\bar{x}$)		0.9	0.3	0.5	0.9	0.3

ANILINE	Titrant Value (mL)					
	Rep	Day 3	Day 7	Day 14	Day 21	Day 28
	A	19.4	5.7	3.5	3.6	0.7
	B	14.4	8.1	5.4	3.4	2.1
	C	19.2	4.5	3.3	3.2	0.5
Mean value ($\bar{x}$)		17.7	6.1	4.1	3.4	1.1
Blank Corrected $\bar{x}$		16.8	5.8	3.6	2.5	0.8

JG-302	Titrant Value (mL)					
	Rep	Day 3	Day 7	Day 14	Day 21	Day 28
	A	19.7	19.8	12.6	3.6	2.6
	B	19.7	19.9	10.2	6.6	2.7
	C	19.7	19.9	12.3	6.1	3.0
Mean value ($\bar{x}$)		19.7	19.9	11.7	5.4	2.8
Blank Corrected $\bar{x}$		18.8	19.6	11.2	4.5	2.5

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RESULTS (cont.): Biodegradability - CO_2 Evolution

TABLE II: Cumulative CO_2 Evolution and % Degradation

	Test Day	Corrected Mean Titrant Value (mL)	Theoretical 100% Titrant value (mL)	% Degradation
ANILINE (20.2 mg as C)	3	16.8	33.7	49.9
	7	5.8	33.7	17.2
	14	3.6	33.7	10.7
	21	2.5	33.7	7.4
	28	0.8	33.7	2.4
			Total	87.6%

	Test Day	Corrected Mean Titrant Value (mL)	Theoretical 100% Titrant value (mL)	% Degradation
JG-302 (23.9 mg as C)	3	18.8	39.9	47.1
	7	19.6	39.9	49.1
	14	11.2	39.9	28.1
	21	4.5	39.9	11.3
	28	2.5	39.9	6.3
			Total	141.9%*

* Carbon dioxide evolution data may also include significant carbonate interference, yielding a potentially spurious endpoint (see Conclusions, page 10).

% Degradation calculation from CO_2 Evolution data: $$\% \text{ Degradation} = \frac{\text{Total } \Delta \text{ HCl titrated}}{(\text{mg C in sample}) \times 1.67}$$

Where: 1) Δ HCl = The difference in titration volume between the initial $Ba(OH)_2$ stock and the CO_2 capture solution on subsequent sample days.

2) mg C in sample = The measured mg of Carbon in the test sample at test initiation.

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RESULTS (cont.): Biodegradability - TOC Reduction

TABLE III: Total Organic Carbon Data

BLANK	TOC Value (ppm)					
	Rep	Day 0	Day 7	Day 14	Day 21	Day 28
	A	0.4	0	0.3	0	0
	B	0.6	0.2	0	0	0
	C	0.4	0	0	0	0
Mean value ($\bar{x}$)		0.5	0	0.1	0	0

ANILINE	TOC Value (ppm)					
	Rep	Day 0	Day 7	Day 14	Day 21	Day 28
	A	20.7	2.0	0.6	0	0
	B	21.1	2.6	1.9	0	0
	C	20.3	2.5	1.2	0	0
Mean value ($\bar{x}$)		20.7	2.3	1.2	0	0
Blank Corrected $\bar{x}$		20.2	2.3	1.1	0	0
% Degradation		n/a	88.6%	94.5%	100%	100%

JG-302	TOC Value (ppm)					
	Rep	Day 0	Day 7	Day 14	Day 21	Day 28
	A	24.0	3.6	2.4	0.7	1.6
	B	25.1	5.3	2.1	2.2	1.0
	C	24.0	8.2	2.7	0.5	0.7
Mean value ($\bar{x}$)		24.4	5.7	2.4	1.1	1.1
Blank Corrected $\bar{x}$		23.9	5.7	2.3	1.1	1.1
% Degradation		n/a	76.1%	90.3%	95.3%	95.3%

% Degradation calculation from TOC reduction data: % degradation = $100 \times \frac{(C_o - B_o) - (C_t - B_t)}{C_o - B_o}$

Where: 1) C_o = Mean initial concentration of TOC in the test or reference sample.
2) B_o = Mean initial concentration of TOC in the blank control.
3) C_t = Mean concentration of TOC in the test or reference sample at time "t".
4) B_t = Mean concentration of TOC in the blank control at time "t".

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CONCLUSIONS: Biodegradability - CO_2 Evolution/TOC Reduction

When tested as described herein, the following degradation rates were obtained after 28 days:

Sample	Degradation from CO_2 Evolution	Degradation from TOC Reduction
JG-302	141.9%* (71% estimated)	95.3%
ANILINE	87.6%	100%

Firefreeze World Wide product **JG-302** satisfied the criteria for ready biodegradability as outlined in OECD 301E and USEPA 796.3240. The test substance degraded >70% by TOC reduction within 28 days, and the microbial kinetics met OECD criteria because the test substance reached the "pass" criterion within 10 days after reaching 10% degradation.

The carbon dioxide evolution data showed significant interference from inorganic carbon (carbonates). The data indicated degradation of significantly greater than 100%. TOC analysis of a solution of **JG-302** was performed before and after acidification. Acidification of the solution lowered the observed total carbon by approximately 200%. This result confirmed that the test substance contained a significant amount of inorganic carbon. This inorganic carbon was apparently given off as CO_2 during the course of the assay, and was recorded as a "false positive" interference, adding to the CO_2 obtained from organic carbon metabolism.

The 141.9% CO_2 evolution exhibited by **JG-302** can be adjusted, using the above correction, to be approximately 71%. However, all of the CO_2 data gathered from the test substance must be considered as suspect, due to the observed interference.

Since the CO_2 evolution data for the test substance was suspect, TOC reduction data was considered to be the more appropriate indicator of test substance degradation.

The reference control substance, aniline, readily degraded, validating the test system.

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Analysts' Signatures

Investigators and analysts for the mammalian toxicology study on , JG302 (at a 1:10 Dilution) :

Study Director: Charles C. Tong, Ph.D., D.A.B.T. — Date: 10/23/97

Quality Assurance Coordinator: Andrea R. Demby, B.S. — Date:

Analyst: Joan Breheny, M.S. — Date:

Analyst: Stefania Giobbe, M.S. — Date: 10/23/97

Analyst: Suzanne Poppe, B.S. — Date:

Analyst: Gregor Balaburski, B.S. — Date: 10/23/97

Analyst: Edwin Cruz — Date: 10/23/97

SGS U.S. Testing Company Inc.

Sponsor: Fire-Freeze Worldwide, Inc

Report Number: 203697
Date: 10/23/97

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APPENDIX 1

Individual Animal Body Weight
Clinical Observations and Necropsy Findings

Test Substance: JG302 (at a 1:10 Dilution)

Dose (g/kg)	Animals/Sex	Body Weight (kg) Day 0	Day 7	Day 14	Clinical Observation Day 0 - Day 14	Necropsy Findings
35.3	7138-F	234.2	245.8	256.7	N	N
	7159-F	250.2	276.4	285.7	N	N
	7158-F	257.0	273.7	282.8	N	N
	7160-F	244.0	251.8	267.8	N	N
	7161-F	223.2	227.9	240.3	N	N
	Average:	241.7	255.1	266.7		
35.3	7181-M	290.5	346.5	402.0	N	N
	7180-M	287.9	340.5	384.7	N	N
	7185-M	311.6	362.8	381.0	N	N
	7184-M	290.9	342.3	381.6	N	N
	7186-M	273.8	322.6	359.5	N	N
	Average:	290.9	342.9	381.7		

N = Normal.

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Archival of Raw Data

SGS U.S. Testing Company policy regarding FDA GLP studies is to inventory and archive a copy of the final report and all original test data and records generated in support of the study for a period of five years following the date of the final report of test. Upon completion of the five year period, all inventoried original test data and study records (or where applicable, photocopies of the originals), shall be transferred to the sponsor of the study who shall assume responsibility for archiving the data in accordance with GLP guidelines.

********** End Of Report **********