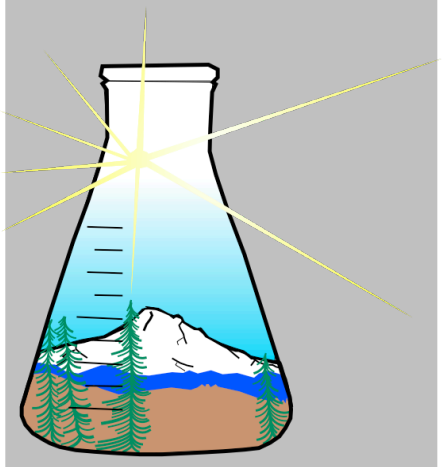
Stevens Ecology



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Project Report

August 10, 2010

PREPARED FOR:

The Grignard Company
Rahway, NJ

PROJECT NUMBER: 2930 REV 1

Biodegradation and Ecotoxicity testing of "Strancore" by OECD 302B, and OECD 202 Protocols

PREPARED BY:

Todd O. Stevens, Ph.D.

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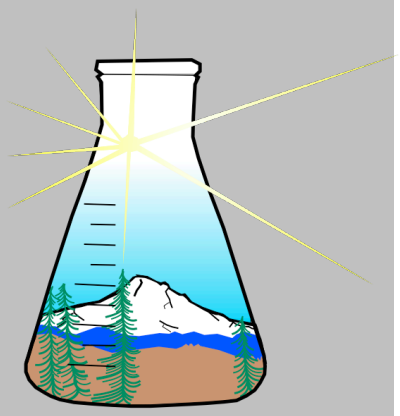

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Project Summary and Certification

A sample of liquid material labeled Strancore (our sample number 2930.1) was subjected aerobic ultimate-biodegradability testing using the OECD 302B protocol, and compared with ethylene glycol as a reference. The material was also tested for acute toxicity to fathead minnow (*P. promelus*) in an OECD 203 limit test.

The organic matter in Sample 2930.1 was > 98% degraded in 40 days incubation, although up to 10% may have been lost by abiotic mechanisms. The material appeared to meet the OECD criteria for "Ultimately Biodegradable."

In a 96-hour limit test, the material displayed no acute toxicity to *P. promelus*. Therefore, it may be considered non-toxic to fish at 100 mg · l^{-1}.

These conclusions are based on the following report of research thStrancoreat was conducted under my supervision.

_____ _____

(signed) (date)

Todd O. Stevens, Ph.D.

SAMPLE	LABEL	PERCENT DEGRADED	DEGRADATION RATE (PER DAY)	ESTIMATED HALF-LIFE	ESTIMATED PERSISTANCE*
2930T	Strancore	98.8 (± 12)	0.0319	9.5 days	31 to 63 days
2930R	ethylene glycol	101.2 (± 12)	0.0482	4.3 days	20 to 28 days

Project Description

One sample of material, submitted by The Grignard Company, was subjected to ecotoxicity testing using the OECD 203 minnow acute toxicity test, and ultimate biodegradability testing by the OECD method 302B DOC die-away test. The acute toxicity limit test challenges a group of fish with a single high dose of material for 96 hours to determine whether toxicity can be observed at that concentration. Substances that pass the test criteria may be considered "non-toxic" to the organisms at that and lower concentrations. The DOC die-away test uses a pre-acclimated inoculum and constant agitation to stimulate biodegradation. Substances that pass the test criteria are considered to be "ultimately biodegradable."

Sample Description

Samples of product were received in the laboratory on 3 June, 2010. Sample 2930 was received as a laboratory sample bottle labeled *OLL D2 Lab # 051410*. Customer-supplied documentation identifies this sample as "Strancore." The physical appearance was a viscous cream-colored liquid. Unless mixed, there appeared to be some separation of liquid phases. The appearance of the samples is shown in Figure 1.

Figure 1. Sample 2930 As Received.

A standard reference material, known to be biodegradable, was selected. Reference samples consisted of ethylene glycol, (Sigma 102466) as a reference or positive control. Unamended microcosms served as negative controls.

Sample Preparation

The sample material was used as received.

Task 1. Ultimate Biodegradability OECD 302B.

This experiment measured the disappearance of test material from shake flasks containing a pre-acclimated inoculum. Chemical oxygen demand (COD) was used a a measure of dissolved sample material.

The inoculum was prepared from activated sewage sludge, collected from a municipal waste water treatment plant owned by the City of The Dalles, Oregon, and from soil collected from a wooded area near the laboratory (Wamic silt loam series). A 500 ml aliquot of sludge and 10 g screened soil were mixed with 500 ml mineral salts medium (see below) and 100 µl of the test material. This mixture was incubated on a rotary shaker for 7 days. Another aliquot of test material was added and incubation continued another 7 days. The acclimated solids were washed three times in mineral salts medium (see below). Solids were separated by centrifugation at 10,000g and re-suspended in fresh medium three times to remove excess dissolved organic material.

Treatments included media with test material, media with reference material, or media alone. A poisoned control treatment was used as a test of potential abiotic mechanisms of loss of the test material, such as evaporation or sorption.

Experimental Protocol

A mineral salts medium was prepared that contained, per liter:

KH_2PO_4	0.0850g
K_2HPO_4	0.02175g
$Na_2HPO_4{\cdot}2H_2O$	0.03340g
NH_4Cl	0.0050g
$CaCl_2{\cdot}7H_2O$	0.0364g
$MgSO_4{\cdot}7H_2O$	0.0225g
$FeCl_3{\cdot}6H_2O$	0.0025g

Test vessels were 2-L flasks that contained 900 ml of mineral salts medium, 100 ml washed conditioned inoculum and test material. This resulted in a suspension containing approximately 0.2 g solids per liter. Substrates were added to microcosms to achieve approximately 250 mg/L COD.

Test material was determined to have a COD of approximately 0.51 $mg \cdot l^{-1} \cdot \mu l^{-1}$. The reference material had a COD of approximately 1.11 $mg \cdot l^{-1} \cdot \mu l^{-1}$.

TREATMENT	PURPOSE	AMENDMENT	AMOUNT	ADDED COD	NUMBER
2930T	Test Material	sample 2930	500 µl	255	3
2930R	Reference Material	ethylene glycol	250 µl	278	3
2930C	Negative Control	none	0.00	0	3
2930S	Sterile Control	sample 2930 + sodium azide	500 µl + 1 gm	255	3

Table 1. Design of Aerobic Biodegradation Experiment

The test material did not immediately disperse into the solution, so each microcosm was rapidly agitated at high shear with a magnetic stir-bar for five minutes to achieve an initial homogenous suspension.

At each time point, 1 ml was removed from each flask (while maintaining agitation) by pipette. Samples were diluted with deionized water and added to a tube of COD reagent (Environmental Express, Mt. Pleasant, SC). Tubes were inverted several times, then incubated at 150°C for 1 hour. The optical absorbance of each tube was measured at 420 nm and used to calculate COD by comparison with standards.

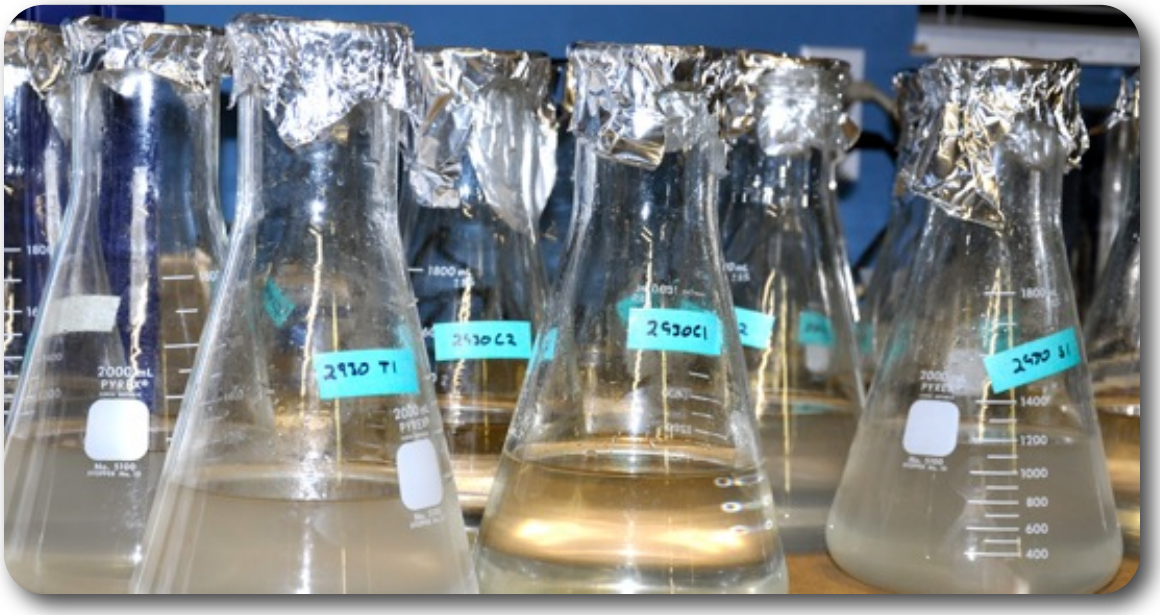

Figure 2. Test vessels at time zero

Results

COD began declining immediately in reference microcosms, indicating a viable inoculum. There was an apparent initial decrease, then increase in COD of the test substance, in both live and sterile test microcosms. This was probably due to gradual dissolution of the test material over the first few hours. Thereafter, degradation was observed in a more normal manner. COD was slightly higher in the sterile microcosms due to additional background from the sodium azide sterilant. Approximately 10% of the test material was lost from solution in the sterile controls, suggesting some loss to sorption or volatilization.

Days Incubation	2930T	2930C	2930S	2930R
0	241	31	266	314
0.5	362	27	345	382
1	361	27	403	350
7	229	30	419	167
14	205	31	386	78
21	145	30	373	42
28	75	29	369	28
35	49	31	367	30
40	38	34	366	30

C = control
R = reference
T = Test Material
S = Sterile Control

mean values, n= 3

Table 2. COD in Test Vessels

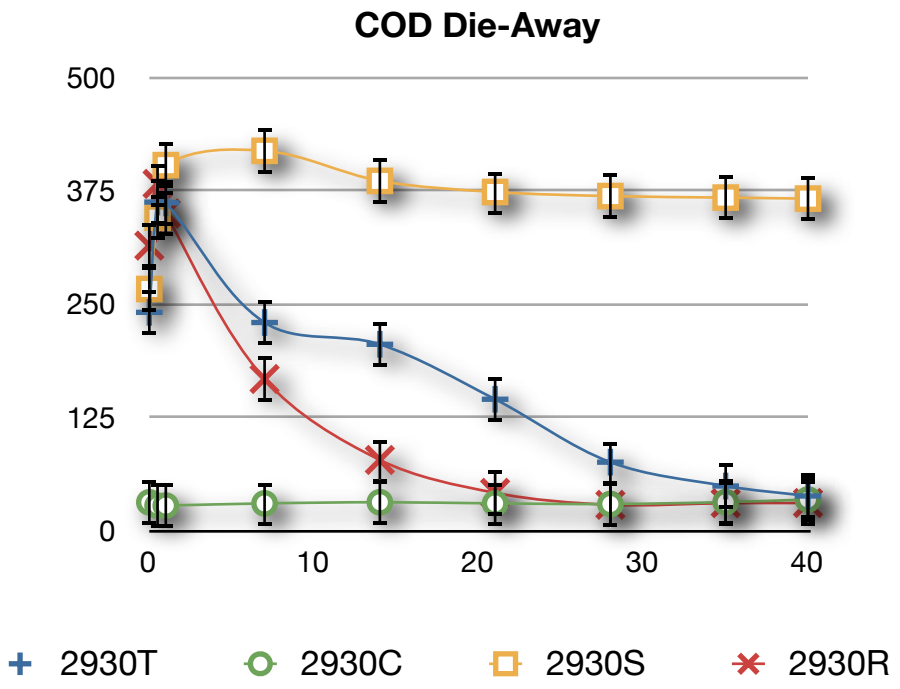

Figure 3. COD Die-Away Data. Data are means, n = 3

Biodegradation was calculated by subtracting the COD of control microcosms and dividing by the COD on day 1. These results are shown in Table 3. The organic fraction of the test sample was approximately 98% biodegraded during 40 days incubation. However, the vessels containing the sample were noticably more turbid than the other vessels, even at the end of the incubation.

Days Incubation	2930T	2930S	2930R
0	37.2	37.3	12.1
0.5	-0.3	15.4	-10.2
1	0.0	0.0	0.0
7	40.3	-3.6	57.4
14	47.8	5.6	85.5
21	65.6	8.5	96.4
28	86.2	9.5	100.3
35	94.7	10.5	100.4
40	98.8	11.6	101.2

Table 3. Biodegradation (percent) in Project microcosms

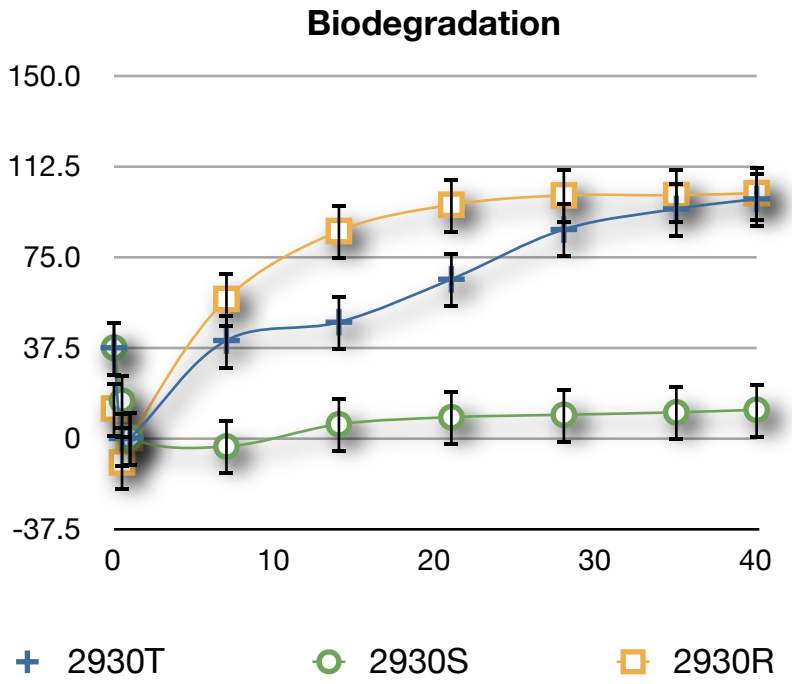

Figure 4. Biodegradation Results Based on COD Die-Away

Discussion of Biodegradability Results

Because the reference material was degraded by more than 70% in the first 14 days, the assay satisfied the criteria for a valid test of biodegradation under OECD 302.

The OECD guidelines require 70% loss of COD during the incubation to be considered "ultimately biodegradable." The COD of sample 2930 declined by 98% in 40 days incubation. If one considers that sterile controls lost approximately 10%, then the true biodegradation figure may have been about 88%, which still passes the benchmark. Therefore, by these criteria, the sample can be considered "ultimately biodegradable."

Biodegradability criteria are designed for pure substances. For products that are mixtures, greater degrees of biodegradation (e.g. 90%) are sometimes required. Degradation of sample 2930 exceeded this amount, and so should satisfy such criteria. However, residual turbidity suggests that an inorganic ingredient may remain. Biodegradability generally only applies to the organic fraction of a sample. Therefore, some caution should be used in formulating biodegradability claims. For example one possible approach to biodegradability statements is subtracting the proportion by mass of any inorganic ingredients and making any claims as "XX percent ultimately biodegradable." The remaining mass might be described as "XX percent stable minerals."

The data from the experiment were used to estimate degradation rate and half-life, as shown in Table 4. A straight-line extrapolation was used to estimate a "best case" persistence time and a half-life model for a "worst case." Bear in mind this test use an inoculum specifically pre-conditioned to the test substance, so degradation in real world situations would certainly be slower.

SAMPLE	LABEL	PERCENT DEGRADED	DEGRADATION RATE (PER DAY)	ESTIMATED HALF-LIFE	ESTIMATED PERSISTANCE*
2930T	Strancore	98.8 (± 12)	0.0319	9.5 days	31 to 63 days
2930R	ethylene glycol	101.2 (± 12)	0.0482	4.3 days	20 to 28 days

Table 4. Biodegradation Summary

* neglects fate of any inorganic ingredients

Task 2. Ecotoxicity to Aquatic Organisms, OECD 203.

This experiment measured the effect of test material on survival of *Pimephales promelas*, the fathead minnow in a limit test using protocol OECD 203.

A limit test was designed to determine whether or not mortality or toxic effects were observable at 100 mg L^{-1}. It does not provide actual measurements of parameters such as EC50, but suggests whether they lie above or below the tested concentration. It also does not provide information about possible chronic toxicity effects.

Toxicity to Minnow - P. promelas

Stocks of *P. promelas* are maintained in our vivarium, and were present on the premises for at least six months prior to the test. Seven individuals were transferred to each of three 12-L polycarbonate tanks. Tanks contained 1 L clean washed pea gravel, and an aeration-driven under-gravel circulation device. Temperatures were maintained at 22°C. A cycle of 16 hrs light, 8 hrs dark was maintained with natural sunlight (filtered by 60% shade cloth) supplemented by fluorescent light. Fish were fed daily for seven days prior to addition of substrates, and then not fed for the duration of the test.

TREATMENT	PURPOSE	AMENDMENT	SUBSTRATE CONCENTRATION	NUMBER OF INDIVIDUALS
C	negative control	none	0 mg l-1	7
TD	limit test	Sample 2930	100 mg l-1	7

Table 5. Experimental Design OECD 203 Limit Test

At time zero, substrates were added to the tanks. To aid dispersal of the sample, a hypodermic syringe and 22 ga. needle was used to draw up a small amount of tank water and forcibly mix it with the sample, which was then injected into the water. Some of the sample settled to the bottom and gradually dispersed into the water over a period of approximately twelve hours. The test tank became slightly turbid, as compared to the control tank.

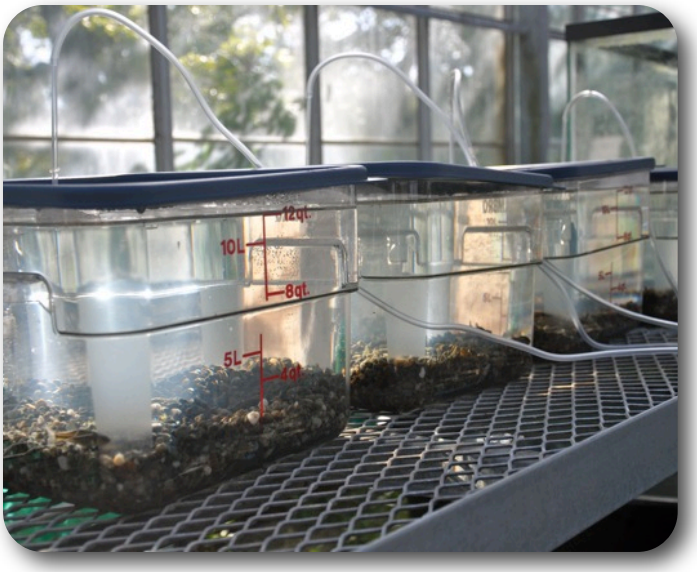

Figure 5. *P. promelas* exposure tanks

Fish were observed periodically for signs of mortality or abnormality. One individual was dead at 40 hours in the control group. No other mortalities were observed within 96 hours. No other signs of abnormality were observed, and fish behaviors were similar between control and exposure tanks.

Hours	Mortality	
	control	2930
0	0	0
3	0	0
10	0	0
22	0	0
35	0	0
40	1	0
66	1	0
96	1	0

Table 8. Mortality in *P. promelas* 100 mg $\cdot l^{-1}$ limit test.

Fish were netted into fresh tanks without samples and observed for another 96 hours. No delayed mortality was observed.

Figure 6. *P. promelas* post-exposure to test material

Discussion of Toxicity Results

At the concentration tested, the samples can both be considered non-toxic to fish with a confidence level of ≥ 99.99%. That is, the acute EC50 lies far above 100 $mg \cdot l^{-1}$. Although one mortality was observed, it was in the control group and considered not to indicate toxicity. This assay did not test for effects of chronic exposure.

Conclusions

Sample 2930 (*Strancore*) appears to be ultimately biodegradable although since it appears to contain inorganic components, caution should be exercised in formulating biodegradability statements. The material appears to have no acute toxicity to fish (*P. promelis*) at 100 $mg \cdot l^{-1}$. That is, the EC 50 is greater than 100 $mg \cdot l^{-1}$.

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Appendix 1. Raw Data

Days Incubation	dilution	T1	T2	T3	C1	C2	C3	S1	S2	S3	R1	R2	R3
0	5	0.1128	0.1744	0.1412	0.0173	0.0189	0.0191	0.1590	0.1941	0.1211	0.1701	0.1849	0.2044
0.5	5	0.2105	0.2114	0.2230	0.0151	0.0177	0.0159	0.2131	0.2018	0.1994	0.2341	0.2421	0.2044
1	5	0.2087	0.2100	0.2247	0.0171	0.0167	0.0151	0.2258	0.2389	0.2527	0.2019	0.2247	0.1959
7	5	0.1438	0.1247	0.1399	0.0181	0.0157	0.0194	0.2184	0.2487	0.2784	0.0979	0.1102	0.0894
14	2	0.3059	0.2973	0.3108	0.0442	0.0428	0.0517	0.5466	0.5514	0.6182	0.1299	0.1042	0.1122
21	2	0.2258	0.2046	0.2139	0.0444	0.0409	0.0477	0.5669	0.5417	0.5531	0.0627	0.0712	0.0509
28	2	0.1227	0.1108	0.1019	0.0450	0.0448	0.0401	0.5519	0.5291	0.5607	0.0428	0.0319	0.0513
35	2	0.0424	0.0919	0.0840	0.0514	0.0401	0.0477	0.5233	0.5519	0.5591	0.0389	0.0429	0.0523
40	2	0.0521	0.0599	0.0573	0.0522	0.0497	0.0501	0.5459	0.5227	0.5607	0.0419	0.0527	0.0401

COD measurements, optical absorbance, 420 nm