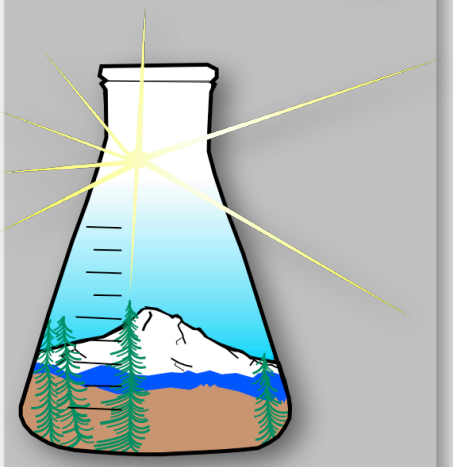
Stevens Ecology



OFFICE
1710 State Road
Mosier, OR 97040
USA

VOICE
(541) 478-0594
(866) 942-7601

INTERNET
www.stevensecology.com

EMAIL
info@stevensecology.com

Project Report

11 March, 2014

PREPARED FOR:

The Grignard Company

PROJECT NUMBER: **4569** REV.1

Biodegradability and Ecotoxicity Evaluation of Lubricants

PREPARED BY:

Todd O. Stevens, Ph.D.

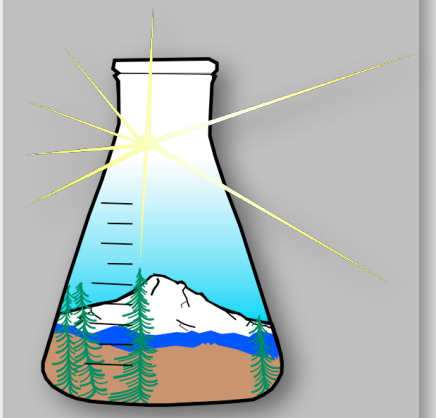
Table of Contents

List of Figures

List of Tables

Stevens Ecology

Biogeochemistry Research and Analysis



OFFICE
1710 State Road
Mosier, OR 97040
USA

VOICE
(541) 478-0594
(866) 942-7601

INTERNET
www.stevensecology.com

EMAIL
info@stevensecology.com

Project Summary and Certification

Two samples of lubricants, submitted by The Grignard Company, were tested for aerobic biodegradability by OECD method 301B, and for ecotoxicity by OECD methods 201, 202, and 203 limit tests at1000 ppm.

Sample 4569.4 (VGP Grease) was mineralized by 63% and sample 4569.5 (StranCore) was mineralized by 71% during a 28-day incubation and both can be considered "Ultimately Biodegradable." Neither of the samples were acutely toxic to algae, aquatic invertebrates, or fish at 1000 ppm concentration.

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

_____ _____

(signed) (date)

Todd O. Stevens, Ph.D.

Sample	Label	Percent Degraded 28 days	Designation	Acute Toxicity to Algae	Acute Toxicity to Daphnia	Acute Toxicity to Minnow
Reference	Canola Oil	67.2	Ultimately Biodegradable	none at 1000 ppm	none at 1000 ppm	none at 1000 ppm
4569.4	VGP Grease	63.2	Ultimately Biodegradable	none at 1000 ppm	none at 1000 ppm	none at 1000 ppm
4569.5	StranCore	71.2	Ultimately Biodegradable	none at 1000 ppm	none at 1000 ppm	none at 1000 ppm

Project Description

Two samples of lubricants, submitted by The Grignard Company were subjected to ecotoxicity testing by the OECD 201, 202, and 203 limit test methods, and to aerobic biodegradability testing by the OECD 301B open-bottle aerobic biodegradation assay. These toxicity tests challenge aquatic organisms from different trophic groups with a single high concentration of the test material to evaluate the possibility of toxic effects. The aerobic biodegradation protocol simulates an aerobic aquatic environment and is considered indicative of biodegradability in all aerobic environments. Biodegradation is measured in relationship to a reference material known to be biodegradable. The corresponding OECD guidelines provide several benchmark "pass" levels. Substances that pass the "readily biodegradable" criteria are considered likely to be completely mineralized during standard waste-water treatment. Ready biodegradability tests may also yield sufficient data for the "inherently biodegradable" and "ultimately biodegradable" designations, but are not designed specifically for that purpose.

Sample Description

Two samples of grease were received in the laboratory on 17 December, 2013. The appearance of the samples as received is shown in Figure 1. Sample 4569.4 was labeled "VGP Grease" lot number 121613B, while sample 4569.5 was identified by the company as "StranCore" lot number 121613A. Sample descriptions are shown in Table 1.

A standard reference material, known to be ultimately biodegradable, was used as a positive control. For this assay, the reference was Low-Erucic Acid Rapeseed Oil, or Canola Oil (Aldrich W530228).

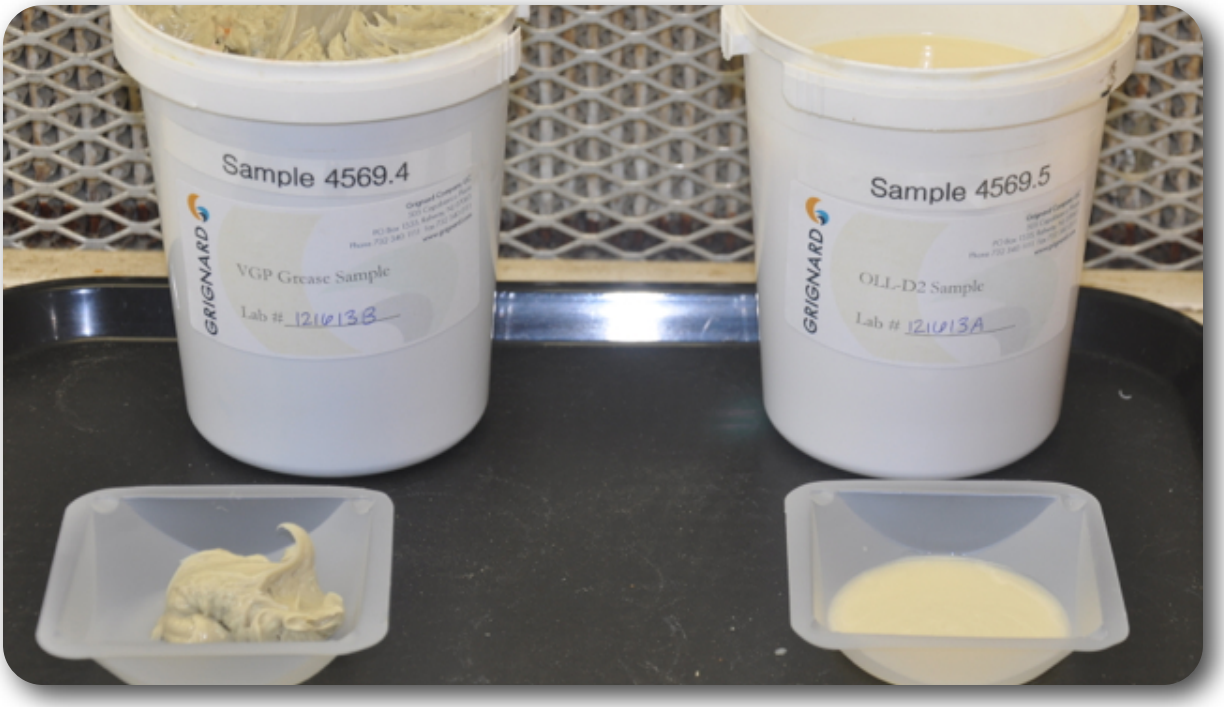


Figure 1. Samples As Received

Sample Preparation

Sample 4569.5 was homogenized with a mechanical paint-stirrer for two minutes before use. Aliquots of all samples were submitted to Midwest Microlabs, LLC (Indianapolis, IN) for elemental analysis. C, H, and N were determined by combustion at 990°C with an elemental analyzer. Oxygen was determined by Pyrolysis via the Unterzaucher method. Results are shown in Table 1, and were used to calculate biodegradation from inorganic carbon production. Density was determined gravimetrically. This accounted for 94% and 83% respectively of the sample mass. The rest presumably consisting of calcium (as per customer disclosures) but this was not analyzed.

			Elemental Composition, Percent									
sample	**Label**	**Density**	**C**	**H**	**O**	**N**	**S**	**P**	**Cl**	**Na**	**Ash**	**total**
Reference	Canola Oil	0.87	78.07	11.68	10.22	0.20	0.00	0.00	0.00	0.00	0	101
4569.4	VGP Grease	0.93	69.72	11.10	12.93	0.00	-	-	-	-	0	93.75
4569.5	StranCore	0.91	26.23	9.80	47.23	0.00	-	-	-	-	0	83.26
water		1.00	0.00	11.11	88.89	0.00	0.00	0.00	0.00	0.00	0	100

Table 1. Properties of Project Samples

Substrates could not be diluted in water to make stock solutions, nor were they soluble in other common laboratory solvents. To enable quantitative dilution of the samples suspensions were prepared of of sample 4569.4 in hexane and of sample 4569.5 in ethyl acetate. Aliquots were then added to microcosms while the suspensions were stirred. Solvent alone was added to control microcosms in a similar way. Microcosms were placed in the 45°C incubator and solvents were allowed to evaporate over night.

Seed material, the source of microorganisms for this assay, was collected from the municipal sewage treatment plant in The Dalles, Oregon. Aerobic inoculum was "mixed liquor" aerated effluent. Inoculum was transported to the laboratory, diluted to achieve 3 g l^{-1} suspended solids, and aerated for five days at 25°C, to reduce background metabolizable carbon.

Task 1. Aerobic Biodegradation by ASTM D 5864 / OECD 301B

This experiment measured the mineralization of the test sample to CO_2 in "open" aerated microcosms that simulated an aerobic aquatic environment, with microorganisms seeded from a waste-water treatment plant. This is considered representative of most aerobic environments that are likely to receive waste materials.

Samples were incubated at 25°C in a mineral salts medium containing mature activated sewage solids in an apparatus that provided continuous aeration, agitation, and trapping of emitted CO_2 (Figure 2). Treatments included media with test material, media with reference material, or media alone.

Compressed air, free of CO_2, was continuously sparged through each test vessel, then bubbled through a chain of three CO_2 traps, containing .05M NaOH. As microorganisms from the inoculum gradually degraded the test substrates, the resulting CO_2 was swept into the trap bottles and converted to carbonates. The carbonate accumulated in the traps was measured periodically to determine Biodegradation.

Experimental Protocol

An inoculum of activated "mixed liquor" sewage effluent was obtained from a municipal sewage treatment plant in The Dalles, Oregon. This material was conditioned in the laboratory as described above. The inoculum was diluted 1:100 in a mineral salts solution containing, per liter:

KH_2PO_4	0.00850g
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

Substrates were added to microcosms in amounts that yielded approximately 25 mg of carbon per liter, as shown in Table 2. Slight variations are due to corrections for density of the samples.

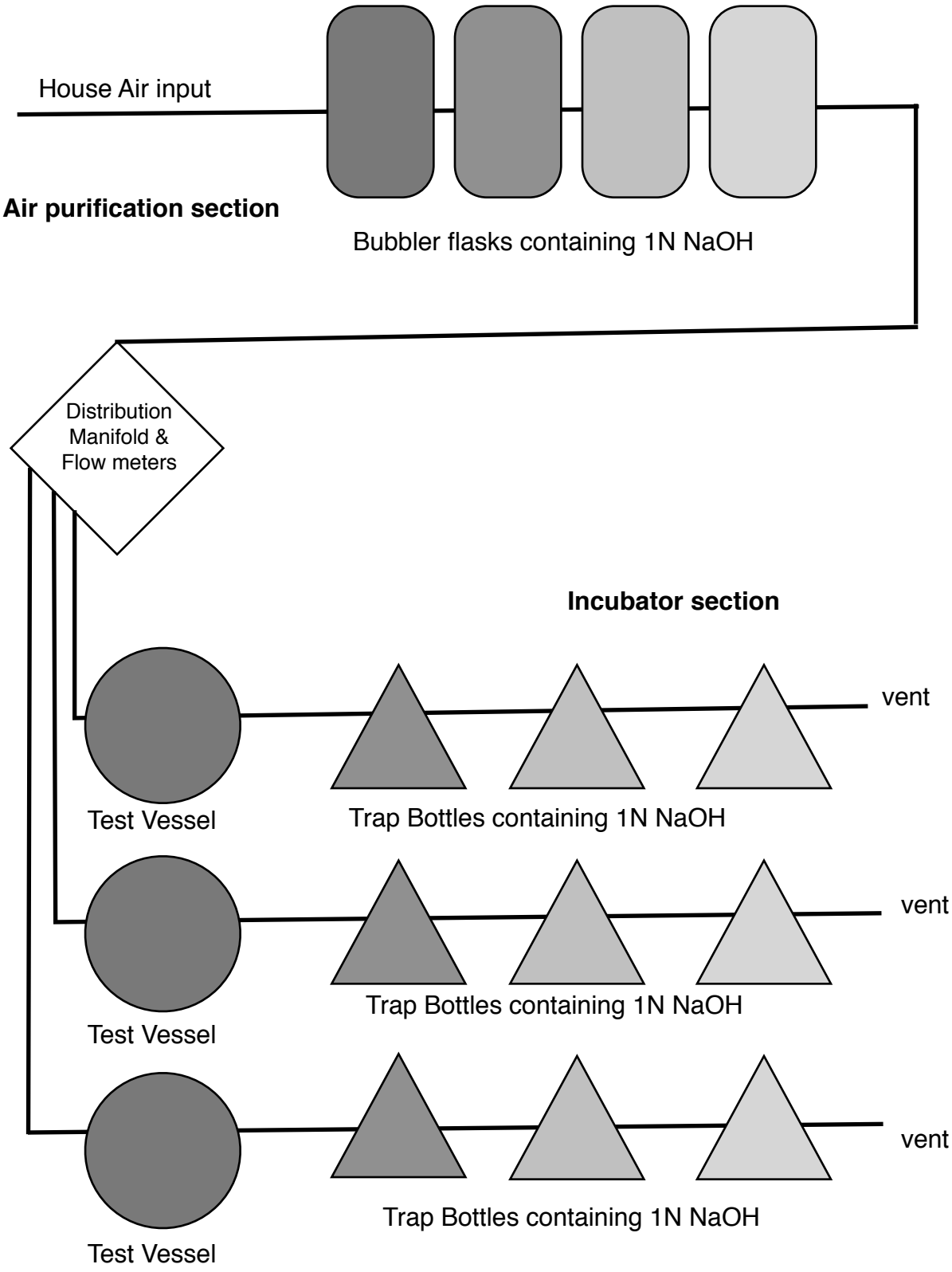


Figure 2. Schematic drawing of experimental apparatus.

TREATMENT	AMENDMENT	AMOUNT	NUMBER	PURPOSE
4569C	none	-	3	control
4569R	25 mg Canola oil	23.9 mg	3	reference
4569.4T	VGP Grease	25 mg	3	test
4569.5T	StranCore	25 mg	3	test

Table 2. Design of Aerobic Biodegradation Experiment

Three flasks were prepared for each treatment, each containing 1 liter of inoculated medium and a test substrate as shown in Table 2. To each flask, a train of three CO_2-trap bottle was connected.

At each time point, the lead trap bottle was removed from the train, and the remaining two flasks moved up one position. A fresh trap bottle was added to the end of the chain. The sacrificed bottle was sealed with a butyl rubber stopper and and acidified to pH <2 by the injection of 0.5 ml 10% H_2SO_4 with a hypodermic needle and syringe. After agitation and equilibration for one hour, headspace samples were removed with a gas-tight syringe and hypodermic needle, and analyzed by gas chromatography. The instrument used was a Hewlett-Packard 5880A equipped with dual packed columns (carboseive II, Supelco, Inc.) and a two-channel thermal conductivity detector, or a Carle GC 8700 equipped with a packed column (carboseive II, Supelco, Inc.) and a single thermal conductivity detector. Carrier and reference gases were helium. Instruments were calibrated using mixed-gas standards. The amount of CO_2 produced in each microcosm was used to calculate the percentage of the test substrate that was mineralized by microorganisms.

$$\%D = \frac{(TIC_t - TIC_b)}{TOC} \times 100$$

Where TICt = mg inorganic carbon in test bottle at time t
TICb = mg inorganic carbon in blank bottles at time t
TOC = mg organic carbon added initially to the test vessel

At the end of the incubation, the test vessels were acidified with 2 ml of acid solution, to volatilize any carbonates sequestered in solution. After four hours, the carbonate present in all remaining trap bottles was determined as described above, and the results added together for the final time point.

Figure 3. Method 301B Microcosms in the Incubator

Results

Mineralization of test and reference substances began immediately after the start of incubation, as determined by accumulation of inorganic carbon in the trap bottles (Figure 4).

The amount of CO_2 produced by controls was minimal but began to increase in the final week of the experiment. This amount was subtracted from CO_2 produced in test and reference microcosms and compared with the theoretical CO_2 yield to calculate percent biodegradation as shown in Figure 5.

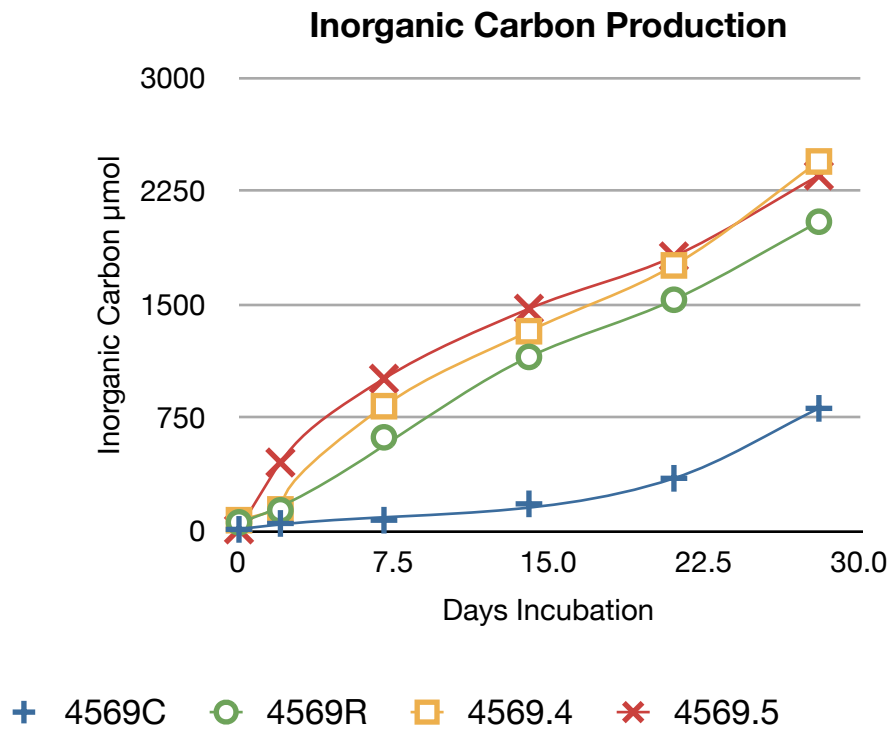


Figure 4. Inorganic carbon production.

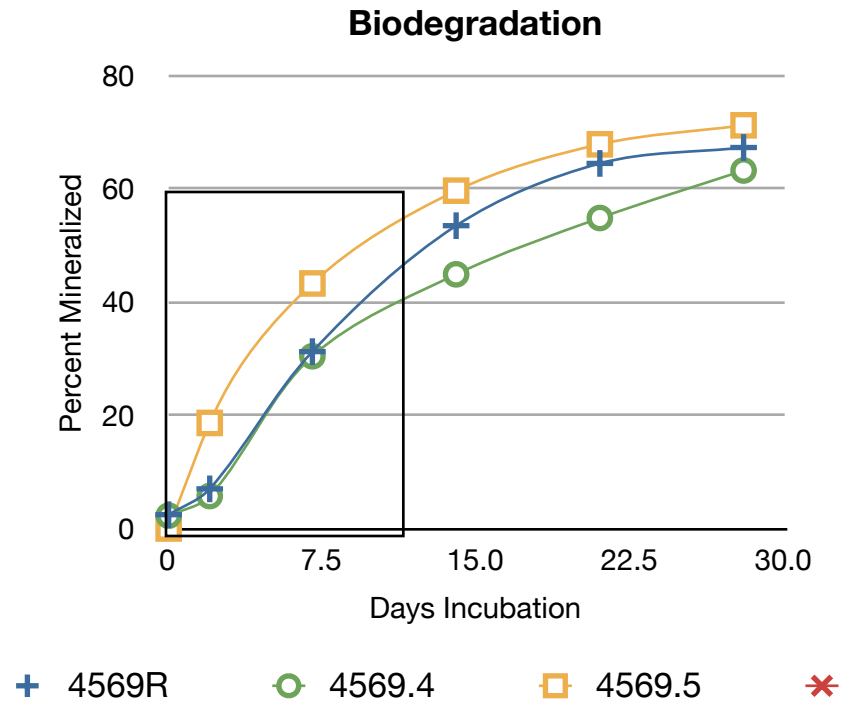


Figure 5. Calculated Biodegradation

Discussion of Results

The reference material was mineralized normally, and the inorganic carbon accumulation in control microcosms was less than 20% of that in reference microcosms until the final measurement, which was somewhat higher than usual due to degradation of material from the activated sludge. However when controls were subtracted, the reference curve appeared normal for the OECD 301B assay.

The OECD designation of "ready biodegradability" is the most stringent defined by OECD protocols. It requires at least 60% mineralization of a substance within a 10-day window during a 28-day incubation. This is depicted by the box superimposed on Figure 5. To meet the "ready biodegradability" criteria, the degradation curve must enter the box through the lower left corner and exit through the top of the box. This criteria was not satisfied for any sample in this assay, as is typical for oil samples

The OECD guidelines also provide less stringent designations. "Inherently Biodegradable" materials are those for which unequivocal evidence for mineralization is available. Typically, 20% mineralization, with no time limit is required. "Ultimately Biodegradable" materials are those for which there is a reasonable expectation that complete mineralization will eventually be achieved under optimized conditions. Typically 60% mineralization, with no time limit, and with pre-adapted cultures is required. Although this stringent experiment was not designed to test "inherent" and "ultimate" biodegradability, it can be seen that the test and reference materials passed the "ultimately biodegradable" criteria.

A summary of the results and estimated biodegradation rates are shown in Table 3. Estimated persistence was calculated for a "best case" scenario as a straight-line extrapolation and for a worst-case scenario with a half-life kinetic model. The actual rates in the environment would depend on the starting concentration, potential toxic effects at higher concentrations, and the environmental conditions. However, the data suggest that in this concentration range, the samples could require approximately 6 to 18 weeks for complete mineralization.

Note that these assays assume that the material being tested is a pure substance. The assay may not provide information about minor components present at a few percent or less. The OECD guidelines permit the treatment of mixtures of closely related substances - such as oils - to be considered as pure substances.

Sample	Label	Percent Degraded	Biodegradation Rate	Estimated Half-Life	Estimated Persistence	Designation
Reference	Canola Oil	67.2	0.0240	17 days	43 -113 days	Ultimately Biodegradable
4569.4	VGP Grease	63.2	0.0226	19 days	44 - 126 days	Ultimately Biodegradable
4569.5	StranCore	71.2	0.0254	16 days	39 - 106 days	Ultimately Biodegradable

Table 3. Biodegradation Summary

Task 2. Ecotoxicity to Algae, OECD 201.

This experiment measured the acute effect of test material on growth of the green alga *Pseudokirchneriella subcapitata* using a modification of protocol OECD 201.

The experiment was done as a "limit test" which challenged the organisms with a single high dose of the sample. Note that the specified dose was ten times the normal dose for OECD limit tests. If no effects are observed, the material can be considered "non toxic," however if effects are observed, a dose-response experiment would be required to measure toxicity parameters.

Experimental Design

Stocks of *P. subcapitata* were obtained from Carolina Biological and are maintained in our vivarium. Stock cultures were used to inoculate sterile Algae Growth Medium (AGM) consisting of (per liter):

$NaHCO_3$	15mg
$NaNO_3$	25.5 mg
$MgCl_2.6(H_2O)$	12.16 mg
$CaCl_2.2(H_2O)$	4.41 mg
$MgSO_4.7(H_2O)$	14.6 mg
K_2HPO_4	1.044 mg
$FeCl_3.6(H_2O)$	0.16 mg
$Na_2EDTA.2(H_2O)$	0.3 mg
H_3BO_3	0.186 mg
$ZnCl_2$	.00327 mg
$CoCl_2.6(H_2O)$	.00143 mg
$Na_2MoO_4.2(H_2O)$	.00726 mg
$CuCl_2.2(H_2O)$	.000012 mg
pH	7.5

Inoculum was grown in the environmental chamber under constant light at 20°C for 72 hours to produce exponentially-growing cultures. Inoculum was then diluted to obtain a suspension containing approximately 10,000 cells per ml.

Test materials were placed directly into the growth chambers at 1000 ppm. Three chambers were used for each sample. Vessels were agitated and aerated with a rotary shaker at 100 rpm.

Results

Growth of algae in control vessels proceeded at typical rates. There was no significant difference between growth in controls and in the presence of 1000 ppm of sample 4569.4 or sample 4569.5. The average specific growth rate and the average yield was the same in all three sets of vessels.

Time	4569C	4569.4	4569.5
0	0.0031	0.0031	0.0029
24	0.0180	0.0183	0.0166
48	0.0721	0.0662	0.0695
72	0.1690	0.1616	0.1677
average specific growth rate	0.85	0.89	0.88
yield	0.17	0.16	0.16

Table 4. Algae Growth Measurements

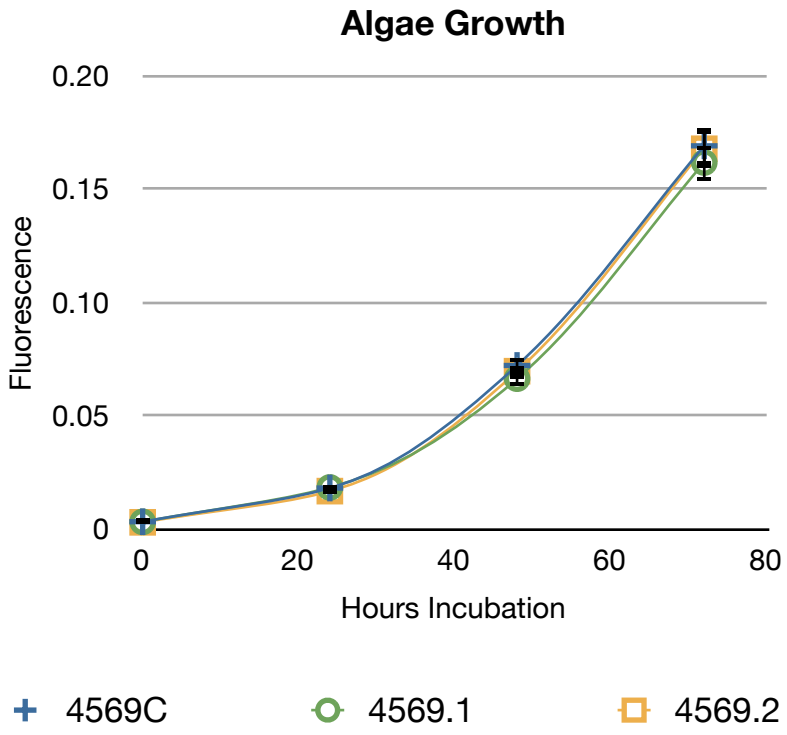


Figure 6. Effect of Samples on Algae Growth

Task 3. Ecotoxicity to Daphnia magna, OECD 202

This experiment measured the effect of test material on survival of aquatic organisms in a 48 hour acute toxicity limit test. Protocol OECD 202 was used to challenge *Daphnia magnus*, the common water flea. Note that the concentration used was ten times greater than the standard concentration for OECD limit tests.

Stock populations of *D. magna* are maintained in our vivarium and all individuals used in testing were produced on the premises and reared in the same base water used for the experiment. Survival or Inactivation was assessed by gently agitating the test vessel and counting the number of individuals that exhibited active motility. Those individuals that inertly sank to the bottom were considered inactivated or dead. Note that by this definition, "mortality" sometimes increases and decreases over time.

Test chambers consisted of glass milk-dilution bottles containing 100 ml of house tap water, or a dilution with the test sample. Because the test samples were insoluble in water, and to prevent mortality due to purely physical interactions (e.g. entrapment of Daphnia at oil/water interface,) samples were coated onto ca. 10 cm^2 squares of nylon mesh. The sample-coated nylon (or nylon alone, for controls) was submerged in the chambers. Because rapid biodegradation of the materials at 1000 ppm would deplete the oxygen in the chambers, each vessel was aerated with an aquarium pump. Aeration tubing was nested within lexan shields, since small air bubbles can also be harmful to *Daphnia*. Chambers were incubated at 22°C in subdued light.

Figure 7. Daphnia magna

Results

Mixtures of effluent and tap water were prepared in binary dilutions between 20 and 100 percent effluent. Twenty individuals were incubated in each dilution. Exposure chambers were incubated at 22°C in subdued light for 48 hours. The number of individuals displaying active motility was counted periodically to assess toxicity and create dose-response curves.

	Number of Motile Individuals		
Hours Exposure	Control	4569.4	4569.5
0	20	20	20
1	19	18	18
2	18	17	16
4	18	18	18
8	19	17	19
24	20	20	20
40	19	20	20
48	20	19	20

	Percent Apparent Mortality		
Hours Exposure	Control	4569.4	4569.5
0	0	0	0
1	5	10	10
2	10	15	20
4	10	10	10
8	5	15	5
24	0	0	0
40	5	0	0
48	0	5	0

Table 5. Effect of Samples on *Daphnia*

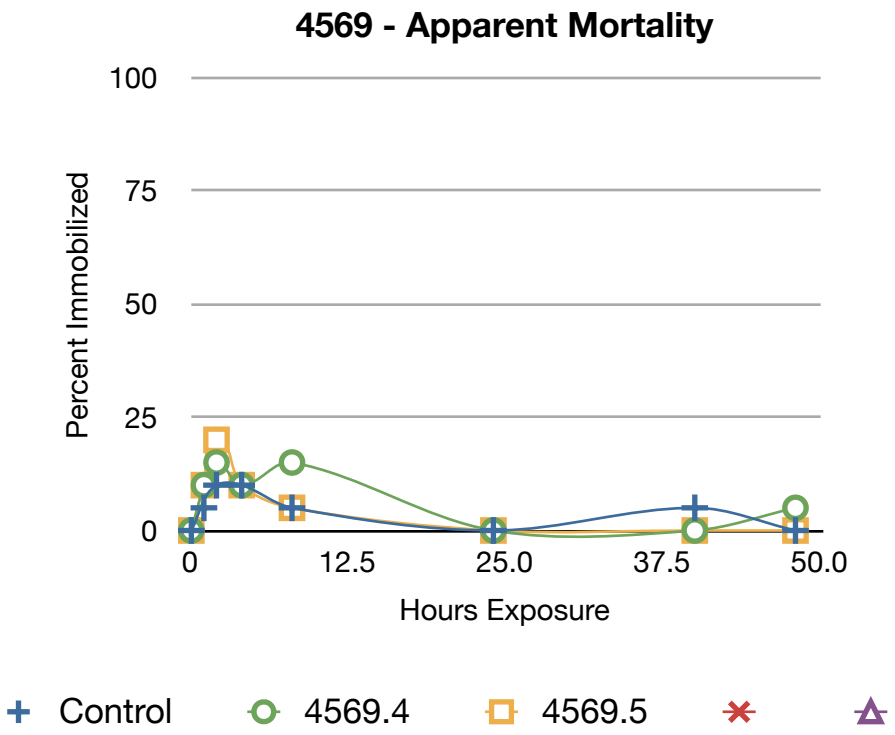


Figure 8. Toxicity of samples to *Daphnia*

There was a slight apparent mortality (immobilization) in all treatments after approximately 4 hour exposure to the samples, however the Daphnia appeared to recover from this after 24 hours, resulting in an apparent decrease in mortality. The final mortality was similar for both samples and the control, consisting of one or two individuals immobilized.

Discussion of Toxicity Results

There was no apparent effect of the two samples at 1000 ppm concentration on inactivation of *Daphnia*.

Task 4. Toxicity to Minnow - P. promelas

This experiment measured the effect of test material on survival of juvenile fish in a limit test. Protocol OECD 203 was used to challenge *Pimephales promelas*, the fathead minnow.

A limit test was designed to determine whether or not mortality or toxic effects were observable at 1000 mg L^{-1}. Note that this is ten times greater than the concentration usually used for OECD limit tests.

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
4569C	negative control	none	0 mg l-1	7
4569-4	limit test	VGP Grease	1000 mg l-1	7
4569-5	limit test	StranCore	1000 mg l-1	7

Table 6. Experimental Design OECD 203 Limit Test

At time zero, substrates were added directly to the tanks. This induced a slight turbidity in the test tanks, as compared to the control tank.

Since the test samples were partially immiscible in water, they could not be evenly dispersed and the concentrations during the test could not be readily determined.

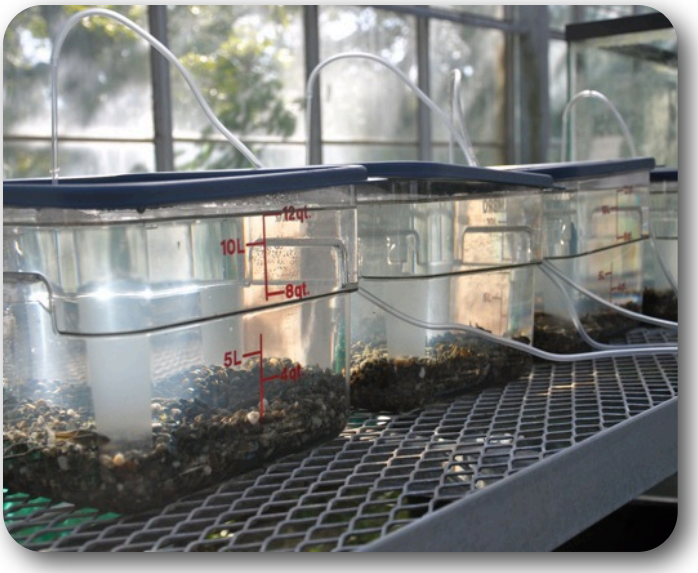


Figure 9. *P. promelas* exposure tanks

Fish were observed periodically for signs of mortality or abnormality. After 96 hours, no signs of morbidity or mortality were observed in the control tank or either of the test tanks.

Hours	Mortality		
	4569C	4569.4	4569.5
0	0	0	0
2	0	0	0
4	0	0	0
8	0	0	0
24	0	0	0
50	0	0	0
72	0	0	0
96	0	0	0

Table 7. Percent Mortality in *P. promelas* 1000 mg · l^{-1} limit test.

Figure 10. *P. promelas* individuals

Discussion of Toxicity Results

At the concentration tested, the samples appeared to have no acute toxicity to fish with a confidence level of ≥ 99.99%.

Conclusions

Sample 4569.4 (VGP Grease) was mineralized by 63% and sample 4569.5 (StranCore) was mineralized by 71% during a 28-day incubation and both can be considered "Ultimately Biodegradable." Neither of the samples were acutely toxic to algae, aquatic invertebrates, or fish at 1000 ppm concentration.

References Cited

ASTM. 2005. Standard test method for determining aerobic aquatic biodegradation of lubricants or their components. ASTM International. West Conshohocken, PA.

Bitton, G. 1999. Wastewater Microbiology. 2nd ed. Wiley-Liss. New York.

Clesceri, L.S., Greenberg, A.E., and Trussell, R.R. 1989. Standard Methods for the Examination of Water and Wastewater. 17th ed. American Public Health Association, Washington, D.C.

OECD. 2003. OECD guidelines for the testing of chemicals: Principles and strategies related to the testing of degradation of organic chemicals. Organization for Economic Cooperation and Development, Paris.

OECD. 1992. OECD guidelines for the testing of chemicals: Biodegradability in Seawater. Organization for Economic Cooperation and Development, Paris.

Wackett, L.P. and Hershberger, C.D. 2001. Biocatalysis and biodegradation. Microbial transformation of organic compounds. American Society for Microbiology, Washington, D.C.

Revision History

0	2/4/14	Original Report
1	3/11/14	Updated product name as per 3/10/14 letter from client

Appendix: Additional Data Tables

Date	Days Incubation	C	C	C	R	R	R
Jan 8, 2014	0	7.14	21.42	0	67.83	42.84	57.12
Jan 10, 201	2	64.26	32.13	49.98	135.66	139.23	132.09
Jan 15, 201	7	53.55	67.83	89.25	653.31	581.91	621.18
Jan 22, 201	14	99.96	210.63	224.91	1295.91	1142.4	1013.88
Jan 29, 201	21	381.99	342.72	317.73	1627.92	1513.68	1445.85
Feb 5, 2014	28	785.4	774.69	871.08	2113.44	1938.51	2084.88

T1	T1	T1	T2	T2	T2
57.12	28.56	124.95	7.14	21.42	0
171.36	182.07	71.4	481.95	435.54	439.11
917.49	788.97	767.55	996.03	999.6	1024.59
1395.87	1285.2	1281.63	1470.84	1499.4	1438.71
1881.39	1677.9	1710.03	1785	1827.84	1834.98
2691.78	2181.27	2459.73	2406.18	2259.81	2388.33

Daily CO2 Evolved

Days Incubation	4569C	4569R	4569.4	4569.5
0	10	56	70	10
2	49	136	142	452
7	70	619	825	1007
14	179	1151	1321	1470
21	347	1529	1756	1816
28	810	2046	2444	2351

Mean CO2 Evolved

Days Incubation	4569R	4569.4	4569.5
0	2.43	2.26	0.00
2	6.99	5.72	18.63
7	31.21	30.39	43.24
14	53.44	44.85	59.61
21	64.42	54.79	67.80
28	67.23	63.18	71.15

Mean Percent Biodegradation