

**BIOASSAY REPORT
CHRONIC AND ACUTE
BIOASSAYS CONDUCTED
April 26 through May 1, 2006,
May 3 through 7, 2006
and May 22 through 27, 2006**

Prepared for

**RAY POLAND AND SONS
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INTRODUCTION

CH2M HILL conducted a series of bioassays and analytical testing from April 26 through May 27, 2006, on a sample provided by Ray Poland and Sons, Washington.

The bioassay tests were conducted using the water flea (*Daphnia magna*), the rainbow trout (*Oncorhynchus mykiss*), and the green algae (*Raphidocelis subcapitata*, formerly known as *Selenastrum capricornutum*).

The analytical tests conducted were Chemical Oxygen Demand (COD) and Biological Oxygen Demand (5 day and 30 day BOD).

SUMMARY OF RESULTS

The test results are summarized in Table 1 below. Individual test data are summarized in the Results and Discussion section below.

Table 1 Summary of Test Results 0.4% End Use Product			
Bioassay Testing			
Test	Endpoint	Value	95% Confidence Limits
<i>Daphnia magna</i> - Acute	LC50	37,613 ppm	34,818 to 40,631 ppm
Rainbow trout - Acute	LC50	9,600 ppm	6,400 to 12,800 ppm
Green Algae - Chronic	IC50	3,517 ppm	2,838 to 4,070 ppm
Analytical Testing			
Test	Duration	Value	% of Total Product Biodegradation
Chemical Oxygen Demand (COD)	na	3012 mg/L	na
Biological Oxygen Demand (BOD)	5 day	1118 mg/L	37.1 %
Biological Oxygen Demand (BOD)	30 Day	1554	51.6 %

METHODS AND MATERIALS

TEST METHODS

The acute tests were performed according to: *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, USEPA Office of Water (2002); EPA-821-R-02-012; and *Laboratory Guidance and Whole Effluent Toxicity Test Review Criteria*, Washington State Department of Ecology (revised December 2001) Pub# WQ-R-95-80.

The chronic test was performed according to: *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, Fourth Edition, (EPA 2002); EPA-821-R-02-013; and *Laboratory Guidance and Whole Effluent Toxicity Test Review Criteria*, Washington State Department of Ecology (revised December 2001) Pub# WQ-R-95-80.

TEST ORGANISMS

For the testing initiated on April 26 and 27: The *Daphnia magna* were obtained from CH2M HILL's in-house cultures and were less than 24 hours old at test initiation. The rainbow trout were obtained from Trout Lodge, Sumner, Washington, and were 24 days old after swim up at test initiation. The *Raphidocelis subcapitata* were obtained from CH2M HILL's in-house cultures and were 6 days old at test initiation.

For the testing initiated on May 3: The *Daphnia magna* were obtained from CH2M HILL's in-house cultures and were less than 24 hours old at test initiation. The rainbow trout were obtained from Trout Lodge, Sumner, Washington, and were 30 days old after swim up at test initiation. The *Raphidocelis subcapitata* were obtained from CH2M HILL's in-house cultures and were 5 days old at test initiation.

For the testing initiated on May 22 and 23: The *Daphnia magna* were obtained from CH2M HILL's in-house cultures and were less than 24 hours old at test initiation. The rainbow trout were obtained from Trout Lodge, Sumner, Washington, and were 18 days old after swim up at test initiation. The *Raphidocelis subcapitata* were obtained from CH2M HILL's in-house cultures and were 4 days old at test initiation.

All organisms tested were fed and maintained during culturing, acclimation, and testing as prescribed by the EPA. The test organisms appeared vigorous and in good condition prior to testing.

DILUTION WATER

The dilution water used was reconstituted, moderately hard water with a total hardness of 80 to 100 mg/L as CaCO₃ and an alkalinity of 60 to 70 mg/L as CaCO₃. The dilution water used for the algae test was reconstituted, moderately hard water with nutrients, including EDTA, added.

SAMPLE COLLECTION

A single sample, labeled "06186A – Baum's Novacool UEF", was shipped via Federal express by Ray Poland and Sons personnel on April 18, 2006. The sample was received by CH2M HILL on April 20, 2006. All Chain of Custody for sample collection is provided in Appendix C.

SAMPLE PREPARATION

The "06186A - Baum's Novacool UEF" sample was diluted to a "0.4% End Use Product" by diluting 4 ml of sample into 996 ml of deionized water. This 0.4% End Use Product stock solution represents the 1,000,000 ppm (= 100%) sample concentration. This stock solution was made daily just prior to test solutions being made.

Concentrations for the tests were made by diluting the 1,000,000 ppm stock solution into the dilution water used for each test.

TEST CONCENTRATIONS

For the testing initiated on April 26 and 27: The sample concentrations for all tests were 62500, 125000, 250000, 500000, and 1000000 ppm (= 6.25, 12.5, 25, 50 and 100%, respectively) of the "0.4% End Use Product" with dilution water for the control. For the acute *Daphnia magna* test, 40 organisms per concentration were used with four test vessels per concentration and ten organisms per vessel. For the acute rainbow trout test, 20 organisms per concentration were used with two test vessels per concentration and ten organisms per vessel. The chronic algae test was run with four replicates per test concentration using an initial cell density in each test chamber of 10,000 cells/ml.

For the testing initiated on May 3: The sample concentrations for all tests were 100, 400, 1600, 6400, 25600 ppm (= 0.01, 0.04, 0.16, 0.64, and 2.56%, respectively) of the "0.4% End Use Product" with dilution water for the control. For the acute *Daphnia magna* test, 10 organisms per concentration were used with two test vessels per concentration and five organisms per vessel. For the acute rainbow trout test, 20 organisms per concentration were used with two test vessels per concentration and ten organisms per vessel. The chronic algae test was run with four replicates per test concentration using an initial cell density in each test chamber of 10,000 cells/ml.

For the testing initiated on May 22 and 23:

The sample concentrations for the acute *Daphnia magna* test were 3125, 6250, 12500, 25000, and 50000 (= 0.313, 0.625, 1.25, 2.50 and 5.00%, respectively) of the "0.4% End Use Product" with dilution water for the control. Forty organisms per concentration were used with four test vessels per concentration and ten organisms per vessel.

The sample concentrations for the acute rainbow trout test were 3200, 6400, 12800, 25600, and 51200 (= 0.320, 0.640, 1.28, 2.56 and 5.12%, respectively) of the "0.4% End Use Product" with dilution water for the control. Twenty organisms per concentration were used with two test vessels per concentration and ten organisms per vessel.

The sample concentrations for the chronic algae test were 320, 800, 2000, 5000, and 12500 (= 0.032, 0.080, 0.200, 0.500 and 1.25%, respectively) of the "0.4% End Use Product" with dilution water for the control. Four replicates per test concentration using an initial cell density in each test chamber of 10,000 cells/ml.

MONITORING OF BIOASSAYS

For the *Daphnia magna* and rainbow trout acute tests, solutions were analyzed at initiation for dissolved oxygen, pH, and conductivity, and every 24 hours thereafter for mortality, dissolved oxygen, and pH. Conductivity was again taken at test termination. Temperature was monitored in the test chambers daily and in the incubator or waterbath continuously throughout the testing period.

For the chronic algae test, solutions were monitored for pH and temperature daily in the control and all concentrations tested. Conductivity and dissolved oxygen was measured in the control and all concentrations tested at test initiation. Total hardness and alkalinity were measured in the control, low, middle, and high test concentrations at test initiation. *Raphidocelis subcapitata* growth was determined by cell counts using a Coulter counter. Temperature was monitored in a surrogate chamber daily and in the incubator continuously throughout the testing period.

DATA ANALYSIS

The effect measured during the *Daphnia magna* and rainbow trout acute tests included survival. The statistical analyses performed were those outlined in *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, USEPA Office of Water (2002); EPA-821-R-02-012, using CETIS version 1.1.2. LC₅₀ values (the concentration of sample causing a 50 percent reduction in survival) were calculated for the acute tests using non-linear regression (Probit) analysis. When the assumptions needed for the Probit analysis could not be met, the Trimmed Spearman-Kärber method was used. When the assumptions needed for the Trimmed Spearman-Kärber could not be met, the Linear Interpolation method was used.

The effect measured during the algae test was algae growth response. For the chronic test, statistical analyses performed were those outlined in *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, Fourth Edition, (EPA 2002); EPA-821-R-02-013, using CETIS version 1.1.2. IC₅₀ values (the concentration of sample causing a 50 percent reduction in biological measurement, e.g. growth) were calculated for growth effects in the chronic tests using Linear Interpolation analysis.

RESULTS AND DISCUSSION

TEST RESULTS

The raw data sheets for all tests are presented in Appendix A.

The results for the *Daphnia magna* acute test initiated on April 27 are summarized in Table 2:

Table 2			
Summary of Acute Results			
Initiated on April 27			
Percent Survival			
Concentration (ppm)	0 hr	24 hr	48 hr
<i>Daphnia magna</i>			
Control	100	100	100
62,500	100	0	0
125,000	100	0	0
250,000	100	0	0
500,000	100	0	0
1,000,000	100	0	0

The *Daphnia magna* test results indicated an LC₅₀ value (the concentration of sample causing a 50 percent reduction in survival) of 31,250 ppm (95% confidence limits are listed as 31,250 to 31,250 ppm). Both the LC₅₀ value and the confidence limits may be unreliable due to poor data bracketing of the calculated LC₅₀ value.

The results for the *Daphnia magna* acute test initiated on May 3 are summarized in Table 3:

Table 3 Summary of Acute Results Initiated on May 3 Percent Survival			
Concentration (ppm)	0 hr	24 hr	48 hr
<i>Daphnia magna</i>			
Control	100	100	100
100	100	100	100
400	100	100	100
1,600	100	100	100
6,400	100	90	70
25,600	100	90	60

The *Daphnia magna* test results indicated an LC₅₀ value of 28,682 ppm (95% confidence limits are 11,735 and 1,645,534 ppm). Both the LC₅₀ value and the confidence limits may be unreliable due to poor data bracketing of the calculated LC₅₀ value

The results for the *Daphnia magna* acute test initiated on May 22 are summarized in Table 4:

Table 4 Summary of Acute Results Initiated on May 22 Percent Survival			
Concentration (ppm)	0 hr	24 hr	48 hr
<i>Daphnia magna</i>			
Control	100	100	95
3,125	100	100	100
6,250	100	100	100
12,500	100	100	100
25,000	100	100	100
50,000	100	75	15

The *Daphnia magna* test results indicated an LC₅₀ value of 37,613 ppm (95% confidence limits are 34,818 and 40,631 ppm).

For all *Daphnia magna* tests, the dissolved oxygen concentrations remained at 4 mg/L or greater throughout the test period. Test temperatures remained in the range of 20±1°C. The tests proceeded without interruption or incidence that could have affected test results.

The results for the rainbow trout acute test initiated on April 26 are summarized in Table 5:

Table 5 Summary of Acute Results Initiated on April 26 Percent Survival					
Concentration (ppm)	0 hr	24 hr	48 hr	72 hr	96 hr
<i>Oncorhynchus mykiss</i>					
Control	100	100	100	100	100
62,500	100	0	0	0	0
125,000	100	0	0	0	0
250,000	100	0	0	0	0
500,000	100	0	0	0	0
1,000,000	100	0	0	0	0

The rainbow trout test results indicated an LC₅₀ value of 31,250 ppm (95% confidence limits are listed as 31,250 to 31,250 ppm). Both the LC₅₀ value and the confidence limits may be unreliable due to poor data bracketing of the calculated LC₅₀ value.

The results for the rainbow trout acute test initiated on May 3 are summarized in Table 6:

Table 6 Summary of Acute Results Initiated on May 3 Percent Survival					
Concentration (ppm)	0 hr	24 hr	48 hr	72 hr	96 hr
<i>Oncorhynchus mykiss</i>					
Control	100	100	100	100	100
100	100	100	100	100	100
400	100	100	100	100	100
1,600	100	100	100	100	100
6,400	100	100	100	100	100
25,600	100	0	0	0	0

The rainbow trout test results indicated an LC₅₀ value of 16,000 ppm. The 95% confidence limits are listed as 16,000 to 16,000 ppm, but are unreliable due to poor data bracketing of the calculated LC₅₀ value and the limitations of the statistical analysis (Linear Interpolation method). A conservative estimate of the 95% confidence values would be 6,400 to 25,600 ppm.

The results for the rainbow trout acute test initiated on May 23 are summarized in Table 7:

Table 7 Summary of Acute Results Initiated on May 23 Percent Survival					
Concentration (ppm)	0 hr	24 hr	48 hr	72 hr	96 hr
<i>Oncorhynchus mykiss</i>					
Control	100	100	100	100	100
3,200	100	100	100	100	100
6,400	100	100	100	100	100
12,800	100	100	90	0	0
25,600	100	0	0	0	0
51,200	100	0	0	0	0

The rainbow trout test results indicated an LC₅₀ value of 9,600 ppm. The 95% confidence limits are listed as 9,600 to 9,600 ppm, but are unreliable due to poor data bracketing of the calculated LC₅₀ value and the limitations of the statistical analysis (Linear Interpolation method). A conservative estimate of the 95% confidence values would be 6,400 to 12,800 ppm.

For all rainbow trout tests, the dissolved oxygen concentrations remained at 6 mg/L or greater throughout the test period. Test temperatures remained in the range of 12±1°C. The tests proceeded without interruption or incidence that could have affected test results.

The results for the algae chronic test initiated on April 27 are summarized in Table 8:

Table 8 Summary of Chronic Results Initiated on April 27 <i>Raphidocelis subcapitata</i>	
Concentration (ppm)	Growth (Cells/ml x 10⁶)
Control	3.963
62,500	0.088
125,000	0.064
250,000	0.100
500,000	0.087
1,000,000	0.131

The algae test results indicated an IC₅₀ value of 32,000 ppm (95% confidence limits are listed as 31,878 to 32,202 ppm). Both the IC₅₀ value and the confidence limits may be unreliable due to poor data bracketing of the calculated IC₅₀ value.

The results for the algae chronic test initiated on May 3 are summarized in Table 9:

Table 9 Summary of Chronic Results Initiated on May 3 <i>Raphidocelis subcapitata</i>	
Concentration (ppm)	Growth (Cells/ml x 10⁶)
Control	3.699
100	2.587
400	1.940
1,600	1.712
6,400	0.686
25,600	0.106

The algae test results indicated an IC₅₀ value of 876 ppm (95% confidence limits are listed as 107 to 2,932 ppm).

The results for the algae chronic test initiated on May 23 are summarized in Table 10:

Table 10 Summary of Chronic Results Initiated on May 23 <i>Raphidocelis subcapitata</i>	
Concentration (ppm)	Growth (Cells/ml x 10⁶)
Control	4.466
320	3.361
800	3.187
2,000	2.729
5,000	1.748
12,500	0.401

The algae test results indicated an IC₅₀ value of 3,517 ppm (95% confidence limits are listed as 2,838 to 4,070 ppm).

For all algae tests, the test temperatures remained in the range of 25±1°C. The tests proceeded without interruption or incidence that could have affected test results.

REFERENCE TOXICANT TESTS

The results of the reference toxicant tests using sodium chloride (*Daphnia magna* and *Raphidocelis subcapitata*) and sodium lauryl sulfate (*Oncorhynchus mykiss*) indicate that the test organisms were within their respective sensitivity range based on EPA guidelines (EPA 1994). The IC₂₅ values, LC₅₀ values, and control chart limits are listed in Tables 11 and 12. The data sheets for the reference toxicant tests are provided in Appendix B.

Table 11		
Acute Reference Toxicant Tests (g/L)		
Species	LC₅₀	Control Chart
<i>Oncorhynchus mykiss</i> (RBT 236)	3.8	1.6 to 5.3
<i>Oncorhynchus mykiss</i> (RBT 237)	2.5	1.6 to 5.3
<i>Daphnia magna</i>	4.4	3.6 to 5.4

Table 12		
Chronic Reference Toxicant Tests (g/L)		
Species	IC₂₅	Control Chart Limits
<i>Raphidocelis subcapitata</i> (growth) April	1.40	0.28 to 2.25
<i>Raphidocelis subcapitata</i> (growth) May	0.83	0.27 to 2.19