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ACUTE TOXICITY OF ZI-18% USING FRESHWATER ALGAE, *DAPHNIA MAGNA*, AND RAINBOW TROUT (NON-GLP OECD METHODS)

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EXECUTIVE SUMMARY

The acute toxicity of ZI-18% to freshwater algae (*P. subcapitata*), freshwater Daphnids (*D. magna)*, and Rainbow Trout (*O. mykiss*) was assessed using the following methods:

OECD, 2011. Alga, Growth Inhibition Test. Guidelines for Testing Chemicals. Section 2: Effects on Biotic System. Test Method **OECD 201**.

OECD, 2004. *Daphnia* sp., Acute Immobilisation Test. OECD Guidelines for Testing Chemicals, Section 2: Effects on Biotic Systems. Test Method **OECD 202**.

OECD, 1992. Fish, Acute Toxicity Test. OECD Guidelines for Testing of Chemicals, Section 2: Effects on Biotic Systems. Test Method **OECD 203**.

Tests were conducted under static conditions to assess the effects of the Test Item on the organisms under controlled conditions. Test durations for these acute tests ranged from 48 hours to 96 hours.

The Test item was delivered to Maxxam as a prepared liquid suspension, with an estimated active matter component of 18%. The Test Item was tested “as is” and test solution concentrations were based upon the whole Test Item and not the active matter component. The Test Item was soluble and no additional solubilising techniques were required for the toxicity tests. In addition to the toxicity tests, the Test Item was also analysed for specific gravity and for total dissolved solids (TDS) to calculate the active matter component.

A total of two Test Items were prepared for Maxxam for toxicity testing. The first was prepared on June 09, 2014, and received on June 17, 2014. A preliminary range-finding test was conducted using *Daphnia magna*. However, it was noted that the Test Item had become non-homogenous and testing on this Test Item was cancelled. A new Test Item suspension was prepared on August 18, 2014 and received on August 19, 2014. All of the range-finding and definitive tests presented in this report were tested using the August 18, 2014 Test Item suspension.

Range-finding studies were conducted for all of the acute tests. The highest test concentration used in both the range-finding and definitive studies was 100 mg/L, as recommended by each of the OECD methods used in this study. No toxicity was observed in these preliminary range-finding tests, and therefore, the definitive tests were performed using the same top concentration of 100 mg/L; however, a narrower range of test concentrations was used. Similarly, there was no toxicity observed in the definitive studies.

The toxicity data obtained in this study are considered acceptable as the test validity criteria for the control group of each test were met and the water quality measurements were within the tolerance limits of the test organisms.

A summary of acute toxicity results obtained from the definitive tests are outlined in Table 1. The toxicity values are based on nominal test concentrations (mg Test Item/L). The chemical analyses results are summarised in Table 2 (See Appendix F for detailed results).

Table 1: Acute Toxicity Results for ZI-18%

Test Method	**OECD 201**		**OECD 202**	**OECD 203**
Organism	*P. subcapitata*		*Daphnia magna*	Rainbow Trout
Endpoint	Cell Yield	SGR**	Immobilisation	Survival
NOEC/ LOEC*	100 / >100	100 / >100	100 / >100	100 / >100
IC_{25} (mg/L)* (95%CL)	>100 (N/A)	>100 (N/A)	N/A	N/A
EC_{50} (mg/L)* (95% CL)	N/A	N/A	>100 (N/A)	N/A
LC_{50} (mg/L)* (95% CL)	N/A	N/A	N/A	>100 (N/A)

*NOEC, No Observable Effect Concentration; LOEC, Lowest Observable Effect Concentration; IC25 is the concentration which inhibited growth by 25%; EC50 is the concentration in which 50% of the organisms were effected (immobilised); LC50 is the concentration in which 50% of the organisms were lethally effected (mortality).
**Specific Growth Rate

Table 2: Chemical Analysis Results for ZI-18%

Test Method	**Mean Specific Gravity**	**Total Dissolved Solids (mg/L)**
ZI-18%	1.12	165,000

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1 OECD 201 – ACUTE ALGAL GROWTH INHIBITION TEST

The 72-hr algal growth inhibition test was conducted on ZI-18% (see Appendix A for Test Item information) using freshwater algae (*Pseudokirchneriella subcapitata*) according to the OECD method, OECD 201, Freshwater Alga and Cyanobacteria, Growth Inhibition Test (OECD 2011).

A preliminary range finding study was conducted over the nominal range of 0 (negative control), 0.01, 0.1, 1.0, 10, and 100 mg Test Item/L. The range-finding results showed that there was no reduction on algal growth throughout the test concentrations when compared to the control. The range-finding IC25 value for the Test Item was visually estimated to be >100 mg Test Item/L (see Appendix E for range-finding results). The definitive test was conducted using the same top concentration with a narrower set of concentrations: 0 (negative control) 6.25, 12.5, 25, 50, and 100 mg Test Item/L. The results indicated that the estimated IC25 value for the Test Item remained at >100 mg Test Item/L (See Appendix B for definitive results).

The stock and test solutions were prepared in nutrient growth media (control/dilution water) using volumetric glassware. The nutrient growth medium recipe is based on the recipe recommended by Environment Canada (2007) for culturing and testing with this test species. Test solutions were not renewed during the test.

There were six replicates for the negative control and three replicates for each test treatment. Each replicate consisted of 50 mL of test solution in a 250 mL glass Erlenmeyer flask. Test vessels were inoculated with 0.5 mL of *P. subcapitata* culture that was in exponential growth phase (containing approximately 1.04×10^6 cells/mL), resulting in an initial (0-h) estimated algal density of 1.04×10^4 cells/mL in the 50 mL test volume. The test vessels were placed randomly in the test chamber and rotated daily.

The temperature of the test area was monitored daily using a min/max thermometer. At test initiation the temperature was 25°C, slightly higher than the recommended 21 – 24°C, but within the culturing temperature allowance. However, the temperature was adjusted to, and maintained at, 22°C for the remainder of the test. As the control flasks met validity criteria, it was determined that this slight variation in temperature at the beginning of the test did not negatively impact the test. The test vessels were shaken horizontally at approximately 75 rpm, under continuous light of 5806-7571 lux throughout the test.

Algae in test vessels were counted daily by way of chlorophyll fluorescence detection (Nexcelom Cellometer AutoX4 counter). In addition, visual observations were documented daily. The pH of the test solutions was measured at test initiation and test

termination and final pH readings of the test solutions differed less than 1.5 pH units. Conductivity was also measured at test initiation.

The tests were considered valid, according to the OECD 201 test criteria, the biomass (cell yield) in the control cultures increased by a factor of greater than 16 (84), the highest mean %CV for specific growth rate in the control cultures were less than 35% (19.5), and the %CV of mean specific growth rates in the control cultures were less than 7% (2.72).

A reference toxicant (positive control) test was conducted with zinc within two weeks of the definitive test and the resulting IC50 was within two standard deviations of the mean IC50 of the previous tests (Appendix B).

The 72-hr IC50 for cell yield and for specific growth rate were calculated using Linear Interpolation (ICPIN) method in the statistical program, CETIS™ (Version 1.8.7.16) (Tidepool Scientific Software Copyright 2000-2013).

Results, statistical analyses, and raw data for the definitive test as well as organism information and a reference toxicant control chart are presented in Appendix B. Results and raw data for the range-finding study is presented in Appendix E.

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2 OECD 202 – *DAPHNIA MAGNA* ACUTE IMMOBILISATION TEST

An acute immobilisation test for ZI-18% (see Appendix A for Test Item information) was conducted with *Daphnia magna* according to the OECD method, OECD 202, *Daphnia* sp., Acute Immobilisation Test (OECD 2004). This test was conducted to determine the effects of the Test Item on the daphnids by assessing the immobilisation of the test organisms over a 48 hour exposure period, under controlled conditions.

A preliminary range finding study was conducted over the nominal range of 0 (negative control), 0.01, 0.1, 1.0, 10, and 100 mg Test Item/L. The range-finding results showed that there was no immobilisation effect on the daphnids and the EC50 value for the Test Item was visually estimated to be >100 mg Test Item/L (see Appendix E for range-finding results). The definitive test was conducted using the same top concentration with a narrower set of concentrations: 0 (negative control) 6.25, 12.5, 25, 50, and 100 mg Test Item/L. The results indicated that the estimated EC50 value for the Test Item remained at >100 mg Test Item/L (See Appendix C for definitive results).

The stock and test solutions were prepared in reconstituted water (control/dilution water) using volumetric glassware. This water was prepared by adding approximately 1.1441 g $MgSO_4$, 1.3200 g $CaSO_4$, 2.1120 g $NaHCO_3$, 0.0881 g KCl, 10 mL of a 4 mg/L Vitamin B12 (as cyanocobalamin) solution, and 40 mL of a 1 mg/L selenium solution to ~20 L of deionised water. The water was aerated at test temperature at least overnight prior to use in the test. Final water hardness was 112 mg/L as $CaCO_3$ (measured by EDTA titration). Test solutions were not renewed during the test.

There were four replicates per treatment, which consisted of 5 neonates in a total volume of 200 mL test solution in a 250 mL glass beaker. The neonates were <24 h old at test initiation and were collected from a brood that had 3.2% parental mortality (<25% is required) in the 7 days preceding test initiation. The neonates were not fed during the tests, but were fed a suspension of *Pseudokirchneriella subcapitata* algae a minimum of 2 hours prior to use in the tests. The test vessels were covered with a Plexiglas sheet and no aeration was provided during the test. The tests were conducted at a daily mean water temperature of 20 ± 2°C, with a photoperiod of 16L:8D. The test chambers were monitored daily for number of immobilised or floating organisms. At test completion, the number of immobilised and dead organisms was recorded. Measurements of dissolved oxygen concentrations, temperature, and pH, were taken at the start and end of the test. Conductivity was also measured at test initiation.

The tests were considered valid, according to the OECD 202 test criteria, as less than 10% (0%) of the control neonates died or displayed atypical or stressed behaviour and the dissolved oxygen concentration in the control and test vessels at test end was higher than 3.0 mg/L (8.7 – 9.0 mg/L). A reference toxicant (positive control) test was

conducted using zinc within two weeks of the definitive test and the resulting LC50 was within two standard deviations of the mean LC50 of the previous tests (Appendix C).

The 48-hr EC50 for immobilisation was calculated using Linear Interpolation (ICPIN) method in the statistical program, CETIS™ (Version 1.8.7.16) (Tidepool Scientific Software Copyright 2000-2013).

Results, statistical analyses, and raw data for the definitive test as well as organism information and a reference toxicant control chart are presented in Appendix C. Results and raw data for the range-finding study is presented in Appendix E.

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3 OECD 203 – ACUTE FISH LETHALITY TEST (RAINBOW TROUT)

An acute lethality test for ZI-18% (see Appendix A for Test Item information) was conducted with rainbow trout (*O. mykiss)* according to the OECD method, OECD 203, Fish, Acute Toxicity Test (OECD 1992). This test was conducted to determine the effects of the Test Item on the fish by observing the survival of the test organisms over a 96 hour exposure period, under controlled conditions.

A preliminary range finding study was conducted over the nominal range of 0 (negative control), 0.01, 0.1, 1.0, 10, and 100 mg Test Item/L. The range-finding results showed that there was no effect on fish survival and the LC50 value for the Test Item was visually estimated to be >100 mg Test Item/L (see Appendix E for range-finding results). The definitive test was conducted using the same top concentration with a narrower set of concentrations: 0 (negative control) 6.25, 12.5, 25, 50, and 100 mg Test Item/L. The results indicated that the estimated LC50 value for the Test Item remained at >100 mg Test Item/L (See Appendix D for definitive results).

The stock and test solutions were prepared in fish laboratory water (control/dilution water) using volumetric glassware. Test solutions were not renewed during the test. There was one replicate per treatment, which consisted of 10 fish in a total volume of 9 L test solution in a glass vessel. The mean fish length was 4.4 cm with a mean weight of 0.59 g. The loading density was 0.65 g/L which is below the maximum loading density of 1 g/L for the test. The fish were not fed during the test and for at least 24 h prior to test initiation. The test chambers were covered with Plexiglas and minimal aeration was provided in all concentrations. The test was conducted at a daily mean water temperature of 15 ± 2°C, with a photoperiod of 16L:8D.

The test chambers were monitored daily for fish behaviour and survival. Measurements of dissolved oxygen concentrations, temperature, and pH, were taken at the start and end of the test as well as daily during the test. Conductivity was measured at test initiation.

The tests were considered valid, according to the OECD 203 test criteria, as the mortality of the control fish did not exceed 10% (0%), nor did the fish display loss of equilibrium, or atypical swimming behaviour. Furthermore, the dissolved oxygen concentration remained higher than 60% saturation (75.8 – 96.9%). A reference toxicant test is not required by the OECD 203 method; however a positive control test was conducted using zinc within two weeks of this test. The same batch of organisms was used for the reference toxicant test and the definitive test, and the resulting LC50 was within two standard deviations of the mean LC50 of the previous tests (Appendix D).

The 96-hr LC50 was calculated using the Linear Interpolation (ICPIN) method in the statistical program, CETIS™ (Version 1.8.7.16) (Tidepool Scientific Software Copyright 2000-2013).

Results, statistical analyses, and raw data for the definitive test as well as organism information and a reference toxicant control chart are presented in Appendix D. Results and raw data for the range-finding study is presented in Appendix E.

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4 CHEMICAL ANALYSES

Aliquots of the Test Item were analysed for specific gravity and total dissolved solids (TDS). Analyses were conducted according to the OPPTS 830.7300 Density/Relative Density/Bulk Density method (2002) and the Standard Methods 2540C Total Dissolved Solids Dried at 180°C (1997). Details of the results of these analyses are presented in Appendix F.

The mean specific gravity was determined to be 1.12 and the TDS was determined to be 165,000 mg/L. The calculated TDS content expressed as a percentage by weight (% w/w) is 14.7% w/w. To calculate TDS in % w/w units, the following calculation may be used:

$$\text{TDS (\% w/w)} = \frac{\text{(TDS (mg/L) / Density (kg/L))}}{10{,}000}$$