Nautilus Environmental

Aquatic Toxicity Evaluation of a Chemical Product Material: ZF-5

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Data Quality Assurance:

- Nautilus Environmental is a state certified laboratory under the California Department of Health Services – Environmental Laboratory Accreditation Program (ELAP), Certificate No. 1802.
- All test results included in this report have met internal Quality Assurance requirements, as well as all minimum acceptability criteria for test controls under the EPA protocol requirements.
- All data have been reviewed and verified.

Verified by:_____ Date:_____

INTRODUCTION

A bioassay test using the Red Abalone *Haliotis rufescens* was performed to determine whether a chemical product would produce any adverse effects on the aquatic environment. The Red Abalone was chosen, as it is one of the more sensitive marine invertebrate test species, and is a good representative of the marine environment. The test looked at the most sensitive life-stage of the abalone, which is the first 48-hours of larval development, and looked at whether the larval shell develops normally or abnormally. The abalone were not exposed directly to the chemical product, but rather were exposed to seawater that was filtered through the chemical product. The primary purpose of this study was to determine whether any toxic substances would leach out, as seawater was filtered through the product. At the end of the 48-hour test exposure, the abalone larvae were examined under a microscope to determine normality. A statistical difference in the percentage of normal verses abnormal larvae, when comparing the sample concentrations to the laboratory control, would indicate toxicity due to the chemical material. Testing was conducted between September 10 and 12, 2010 at the bioassay laboratory of Nautilus Environmental (Nautilus), located in San Diego, California.

MATERIALS AND METHODS

The study was performed in accordance with the EPA protocol "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms" (EPA/600/R-95/136, August 1995). Testing also followed the general guidelines developed by the EPA's Office of Prevention, Pesticides and Toxic Substances (OPPTS) for conducting aquatic laboratory tests with chemical products to determine ecological effects (OPPTS - Series 850; EPA Publication 712-C-96).

TEST MATERIAL

The chemical product was shipped to the testing laboratory by FedEx delivery service and was received on September 7, 2010. The product was identified as "ZF-5" filtration system. The actual material being tested consisted of a charcoal-grey, fine powder substance, which was within an enclosed chamber. Seawater was allowed to filter through this chamber, coming in direct contact or exposure to the powder material. Seawater filtered through this material at a rate of approximately one drop every two seconds. The seawater was collected underneath, after filtering through the chamber, and this water was used for preparing all sample concentrations.

LABORATORY CONTROL WATER

All testing was conducted using natural seawater obtained from the ocean intake system located at the Scripps Institution of Oceanography in San Diego, California. The seawater was transported to the laboratory in a large water truck and held in a re-circulating system with an in-line 20-µm fiber filter and a chiller unit. This seawater was used in all phases of testing, including the source of dilution water for all sample preparations. A laboratory control, consisting of just the natural seawater, was also tested and used for quality assurance purposes. All sample material results were analyzed and compared to this laboratory control to determine whether there were any significant differences.

TEST DESIGN AND SAMPLE PREPARATION

The sample material was a very fine, light weight powder that is insoluble and will float on surface water. Due to the nature of this substance, it was determined to produce a test design that would reflect the volume of the material, rather than the weight. Therefore, concentrations of the material were calculated in equal volume "parts." More specifically, we measured out an equal part of the sample material to an equal part of seawater. The units were measured as one part sample per one million parts seawater, or parts per million (ppm).

Since we were not testing the sample material directly in water, but rather the water that filters through the sample material, we had to calculate the volume of water that flowed through a fixed volume of the powder material. The testing laboratory was informed that each powder chamber that comes with the ZF-5 filtration system contains 331.1 cubic centimeters of powder. This volume is equivalent to 331.1 milliliters (ml). Therefore, by pouring 331.1 ml of seawater through the filter, we obtain a 1 to 1 ratio, and the final water collected is at a concentration of 1,000,000 ppm. This became the stock solution used for testing. We diluted this stock by a factor of ten to obtain the highest test concentration, which was 100,000 ppm. We continued diluting by a factor of ten to obtain the final test design. Therefore, the final concentrations used for testing were 100,000 ppm, 10,000 ppm, 1,000 ppm, 100 ppm, 10 ppm, and 1 ppm of the sample. We also tested a 0 ppm, which was seawater not exposed to the filter (lab control). Each concentration was tested with 5 replicates. Each replicate consisted of a 30-ml glass vial with 20 ml of the sample concentration added. After all the test vials were prepared, they were placed into a holding tray in random order, and placed in an environmentally-controlled test chamber until the test was initiated. See Table 1 for a summary of the test methodology.

Table 1. Summary of Test Parameters for the Red Abalone Bioassay Test.

Test organism	Red Abalone – *Haliotis rufescens*
Test organism source	The Cultured Abalone (Goleta, CA)
Test organism age at initiation	Single-cell embryo (1 hour post fertilization)
Test duration	48-hours
Test type	Static non-renewal
Feeding	None
Test chamber size	30 ml glass shell vial
Test solution volume	20 ml/ replicate
Number of replicates/ concentration	5
Test concentrations (ppm sample)	100000, 10000, 1000, 100, 10, 1, and 0 (lab control)
Dilution water	Natural seawater
Number of organisms/ replicate	250 to 300
Daily chemistry measurements	Temperature, pH, DO, and salinity
Test temperature	15 ± 1°C
Photoperiod	16 hours light/ 8 hours dark
Aeration	None
Test Protocol	EPA/600/R-95/136
Test endpoint	% normal shell development
Test acceptability criteria	≥ 80% normal shell development in the control

TEST ANIMALS

Bioassay testing was conducted using the Red Abalone, *Haliotis rufescens*. Mature abalone broodstock were obtained from The Cultured Abalone, a commercial mariculture facility located in Goleta, California. The broodstock animals were received six days prior to testing to allow acclimation to laboratory testing conditions. The animals were separated by sex and held in aerated holding buckets filled with natural seawater and maintained at a test temperature of 15 ± 1°C.

On the day of testing, the two buckets with male and female abalone are induced to spawn. The spawning induction process is conducted by adding a small volume of hydrogen peroxide and a Tris buffer solution to each bucket of seawater. The abalone are exposed to the chemicals for a 2.5 hour period, at which point the water is removed

and fresh seawater is added to the buckets. The abalone should start spawning within the first hour after the water renewal. The sperm and eggs are then collected and combined to produce fertilized embryos.

Approximately one hour after the fertilization process, the embryos are ready to be added to the test vials and the test initiated. A stock solution is prepared, and approximately 250 to 300 embryos are pipetted into each test vial. At this point, the embryos are still in their one-cell stage. During the course of the 48-hour test, the embryos will become trochophore larvae and then develop into veliger larvae. During the veliger stage, the organisms will swim up into the water column, creating the potential for more physical interaction between the organisms and any toxicants found in the water. For a more detailed description of the testing procedures, please refer to the EPA protocol on the 48-hour Red Abalone Test (EPA/600/R-95/136).

RESULTS

After 48 hours, the test is ended by the addition of formalin to each test vial and capping. This process will preserve the organisms in the vials so they can be examined under a microscope at a later date. Each vial is examined by counting the first 100 organisms observed at 40x magnification. Each organism is scored as normal or abnormal, by examining the abalone's shell development during the first 48 hours of life. A percentage of normal development is determined for each test vial. The final data is then entered into a statistical software program called Comprehensive Environmental Toxicity Information System (CETIS), version 1.7.0.4. The data is analyzed to determine a mean percentage of normal development for each sample concentration. Each sample concentration is then compared to the laboratory control results to determine whether there is a significant difference present, which would indicate toxicity. Analyzing the final data resulted in 90.2% normal development in the lab control, meeting test acceptability criteria (>80% required). Each of the sample concentrations tested resulted in a range of 89 to 92% normal development, which was not found to be significantly different compared to the lab control. Therefore, the No Observed Effect Concentration (NOEC) was 100,000 ppm (the highest sample concentration tested). The EC_{50} (the statistically derived concentration that causes a 50% adverse effect to the test organisms) was greater than 100,000 ppm. Therefore, we were able to determine that no toxic substances leached out into the water from this filter material, which would cause an adverse effect to the abalone. Final test results can be found in Table 2. Detailed statistical analyses and the raw bench data can be found in Appendix A.

TABLE 2. Summary of Results:

Sample ID	Test Concentration (ppm)	Mean Normal Development (%)
ZF-5	Lab Control	90.2
Product Material	1	89.8
	10	89.0
	100	89.8
	1,000	90.8
	10,000	90.7
	100,000	91.4
NOEC = 100,000 ppm	LOEC > 100,000 ppm	EC_{50} > 100,000 ppm

NOEC = the highest Concentration that produces No Observed Effect
LOEC = the Lowest Observed Effect Concentration (next concentration higher than the NOEC)
EC_{50} = the concentration that causes an adverse effect to 50% of the organisms

Quality Assurance

The lab control met the test acceptability criterion of 80% or greater normal development. Therefore, all test results are deemed valid. In addition, based on the dose response observed during testing, the calculated effect concentration is deemed reliable.

REFERENCES

EPA 1995. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. EPA/600/R-95/136, August 1995.

Code of Federal Regulations, Title 40. Environmental Test Methods and Guidelines, for testing under the Toxic Substances Control Act. 40 CFR 797 series.

Office of Prevention, Pesticides, and Toxic Substances (OPPTS), U.S. EPA. Ecological Effects Test Guidelines – Special Considerations for Conducting Aquatic Laboratory Studies. OPPTS 850 series. EPA 712-C-96, April 1996.

Tidepool Scientific Software, 2001-2002. Comprehensive Environmental Toxicity Information System (CETIS), version 1.7.0.4