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Independent University Study to Assess the Performance of a Humate Amendment for Copper Detoxification at the H-12 Outfall at Savannah River Site

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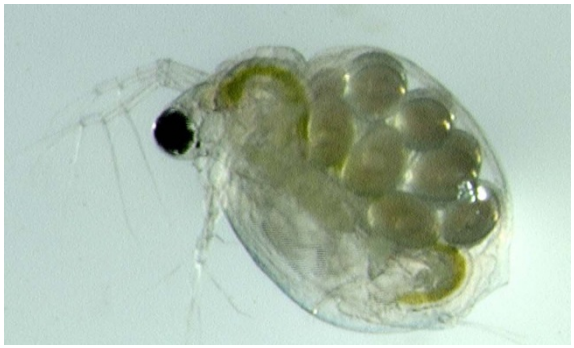

Ceriodaphnia dubia

Daphnid cultures at University of South Carolina – Aiken (USCA)

Cover photograph – USCA student Joshua King at the H-12 Outfall on the US Department of Energy Savannah River Site, South Carolina

1.0 EXECUTIVE SUMMARY

The overarching objective of this study was to evaluate the effectiveness of the copper detoxification process that is in place at the Savannah River Site H-12 Outfall. The testing was performed in two phases; Phase 1 assessed the safety and potential for intrinsic toxicity of the humate amendment being used at the H-12 Outfall, Borregro HA-1, as well as an alternative amendment sodium humic acid. The second phase assessed the effectiveness of Borregro HA-1 in mitigating and reducing toxic effects of copper.

The Phase 1 tests demonstrated that the Borregro HA-1 amendment caused no adverse effects to *Ceriodaphnia dubia* (*C. dubia*) or *Daphnia ambigua* (*D. ambigua*) at concentrations that are significantly above those that are realistically expected in the H-12 outfall stream. Notably, the addition of Borregro HA-1 does not add toxic levels of potassium to the water. Based on the data, the two amendments tested during Phase I, Borregro HA-1 (a potassium humate) and a laboratory grade sodium humic acid, have a comparable toxicity profile in any potential application scenario.

Phase 2 testing demonstrated that the Borregro HA-1 amendment mitigated both the acute and the chronic toxicity of copper in simulated outfall water. LC_{50} values for both indicator species were significantly higher in the treatments that included Borregro HA-1 during acute toxicity testing. While mortality and reproductive effects were observed in the chronic toxicity tests, these effects were generally consistent with, or somewhat less than, those predicted by the Biotic Ligand Model (BLM) that was used as the basis for the detoxification system design.

The toxicity testing validated the viability using humate to mitigate the toxicity of low-levels of copper, particularly for outfalls where the original source water is low in organic carbon – such as groundwater or rain water. The data confirmed that operating such a system based on the BLM should help protect sensitive aquatic species such as daphnids in the receiving stream.

In both phases, all organism cultures (both species) were grown in representative H-12 simulant water and this water was used for all dilutions and toxicity exposures. As a result, the data provide a relatively clear understanding of the toxicity effects of the various toxicants of interest. This type of test protocol, in which key parameters were individually varied while important baseline water chemistry parameters (e.g., calcium and magnesium "hardness") were maintained at/near expected field conditions, was effective in providing useful information and is recommended for future studies of this type.

2.0 PURPOSE

The overarching objective of this study was to evaluate the effectiveness of the copper detoxification process that is in place at the Savannah River Site H-12 Outfall. This system amends the outfall water with a dissolved organic carbon (DOC) in the form of potassium humate. The humic and fulvic acids in this naturally-sourced DOC bind with copper and reduce its availability to impact organisms in the receiving stream.

The research protocols were designed to provide information regarding the toxicity of H-12 outfall water to sensitive organisms, specifically *Daphnia* ("water flea") species. This work builds on results from previous studies including Millings et al. (2008), Looney and Millings (2009) and Millings et al. (2013). In the current work, a step-wise series of testing was performed to: 1) document that the detoxification amendments are safe and not causing toxicity, 2) confirm the desired detoxification, 3) develop information to help resolve toxicity uncertainties, and 4) provide information to support future operations (protocols, dosing, amendment modification, etc.).

The testing included both acute and chronic toxicity tests with two species of daphnids, *Daphnia ambigua* (*D. ambigua*) and *Ceriodaphnia dubia (C. dubia).* The test design was designed to provide for a stable bulk water chemistry (i.e., conditions that are representative of the H-12 outfall) and focused on testing one potential toxicant at a time. To meet these criteria, all organism cultures (both species) were grown in representative of H-12 simulant water. This water was used for all dilutions and toxicity exposures.

This research is a collaborative effort between the Savannah River National Laboratory (SRNL) and the University of South Carolina in Aiken (USC-Aiken). The principal scientific investigators at USC Aiken were S.M. Harmon (faculty) and J.D. King (undergraduate student and Magellan Scholar). The research was supported by the US Department of Energy and the University of South Carolina Office of Undergraduate Research. This report comprises two parts, an SRNL high level summary and an appended copy of the USC-Aiken technical report with the detailed method descriptions and raw results.

3.0 BACKGROUND

Outfall H-12 is a NPDES permitted outfall located in the center of the Savannah River Site in Aiken, SC (Figure 1). The outfall collects nonprocess cooling water, cooling tower and air compressor blowdown, steam condensate, retention basin water, well water flushes and overflow, and stormwater. The H-12 Outfall discharges into an un-named ephemeral tributary of Fourmile Branch. The water at the H-12 Outfall contains measurable levels of copper (5 to 10 μg/L typical and 25 μg/L nominal maximum). In combination with the other water quality parameters in the outfall, the measured copper levels are high enough to adversely impact aquatic organisms in the receiving stream. Notably, the historical geochemical conditions result in projected copper-related toxicity to small invertebrates, such as daphnids, that serve as a key food source for other stream organisms. Based on the overall water chemistry of the H-12 Outfall, target copper levels less than 6 μg/L were proposed to limit potential toxicity and potential adverse effects on the near-field receiving tributary.

A number of alternatives were evaluated to address the potential toxicity of H-12 effluent in the receiving tributary. These alternatives included three “traditional” water treatment technologies – ion exchange, wetland treatment, and peat bed treatment – all of which act to remove copper from the wastewater. The low target metal concentration and variable outfall conditions presented a challenge for these treatment technologies. Costs and energy use for most of these alternatives were high, secondary wastes would be generated, and in the case of wetland treatment, sufficient land was not available near the industrial facilities. Water management alternatives that did not include treatment were also evaluated, including direct discharge to a larger stream, injection wells, process

water discharge reduction, and re-negotiation of discharge limits. These options were rejected for various reasons including cost, regulatory responsiveness, robustness, and/or poor performance.

To resolve the issue, the Savannah River National Laboratory (SRNL) developed a new "detoxification" approach to treat the outfall water. The detoxification system amends outfall water with natural organic matter to bind up to 25 µg/l copper rather than remove it, thereby mitigating its toxicity and protecting sensitive species in the ecosystem. The amendment used in the system is potassium humate, a relatively soluble natural organic material consisting primarily of humic and fulvic acids that is traditionally used as a soil conditioner in organic farming.

The detoxification process is based on the EPA recommended Biotic Ligand Model (BLM) that was finalized in 2007. The BLM estimates allowable copper in an outfall based on water quality parameters such as pH, dissolved organic carbon (DOC), and percentage of the DOC that is contributed by humic acid. In particular, the BLM predicts how DOC in the water binds with copper in the water, limiting the availability of copper to interact with "biotic ligands," such as gill membranes, thus reducing impacts to aquatic organisms such as daphnids. A secondary benefit of the proposed system is that the resulting water chemistry of the outfall more closely mimics the local natural streams.

SRNL used the EPA's Biotic Ligand Model Windows Interface, version 2.2.1 (HydroQual, 2007) to calculate predicted copper toxicity to aquatic organisms (EPA 2007, a,b). The baseline BLM runs were performed by setting the pH, alkalinity and other parameters to cover the expected range of those parameters at the outfall. The modeling results confirmed that an addition of DOC in concentrations similar to the natural levels in receiving stream could reduce copper toxicity in the outfall water (Millings et al., 2008), allowing the goal of the proposed NPDES permit to be met even if copper is present at concentrations greater than 6 µg/l.

Since Outfall H-12 had been able to meet the older copper limit of 25 µg/L, the objective set for the DOC addition system was to detoxify copper up to this concentration. BLM results showed that, for humate amendments, DOC values of approximately 22 mg/L or less would render 25 µg/L copper non-toxic at all outfall pH values >5.9 (Millings et al., 2008). Higher pHs would require lower levels of added DOC. Based on the BLM modeling and laboratory testing of several alternative humate amendments, design equations were developed to support engineering a system

with amendment metering using a programmable logic controller and variable flow pump (Looney and Millings, 2009). The design equations defined the operational parameters (sensors and mathematical relationships) needed to achieve the toxicity reduction objectives. Based on these studies, the specific humate amendment brand selected was Borregro HA-1; this particular humate material was also used for follow-on toxicity testing.

In 2009, SRS implemented the innovative copper detoxification system at the H-12 Outfall. Follow-up testing (Millings, 2013) showed that the full-scale system was adding the amount of DOC consistent with the design model – DOC concentrations in the outfall were at expected levels for the pH and the process control equation. Additionally, results from toxicity tests indicated that the outfall water is protective of design-based copper concentrations. The tests also showed no copper related toxicity in the H-12 Outfall water. Some toxicity was observed, however, and the results were difficult to interpret and confounded by changing water conditions within the testing protocols – for example, *C. dubia* were cultured/diluted with water hardness levels (approximately 100 mg/L as $CaCO_3$) that were higher than the outfall (approximately 15 mg/L as $CaCO_3$) and *D. ambigua* were cultured/diluted with water hardness levels that were lower (approximately 5 mg/L as $CaCO_3$). Thus, factors influencing toxicity, such as hardness (i.e., Ca and Mg), varied in the tested mixtures of outfall water and culture-diluent water in the Millings (2013) studies, introducing potential uncertainties in the results. To aid and simplify data interpretation, we emphasized use of consistent conditions in the toxicity testing herein. The current research studies used the same bulk chemistry conditions (closely representing all of the key constituents in the H-12 Outfall) for the cultures of both daphnid species and diluent solutions. The toxicity tests were then performed by varying a single parameter at a time.

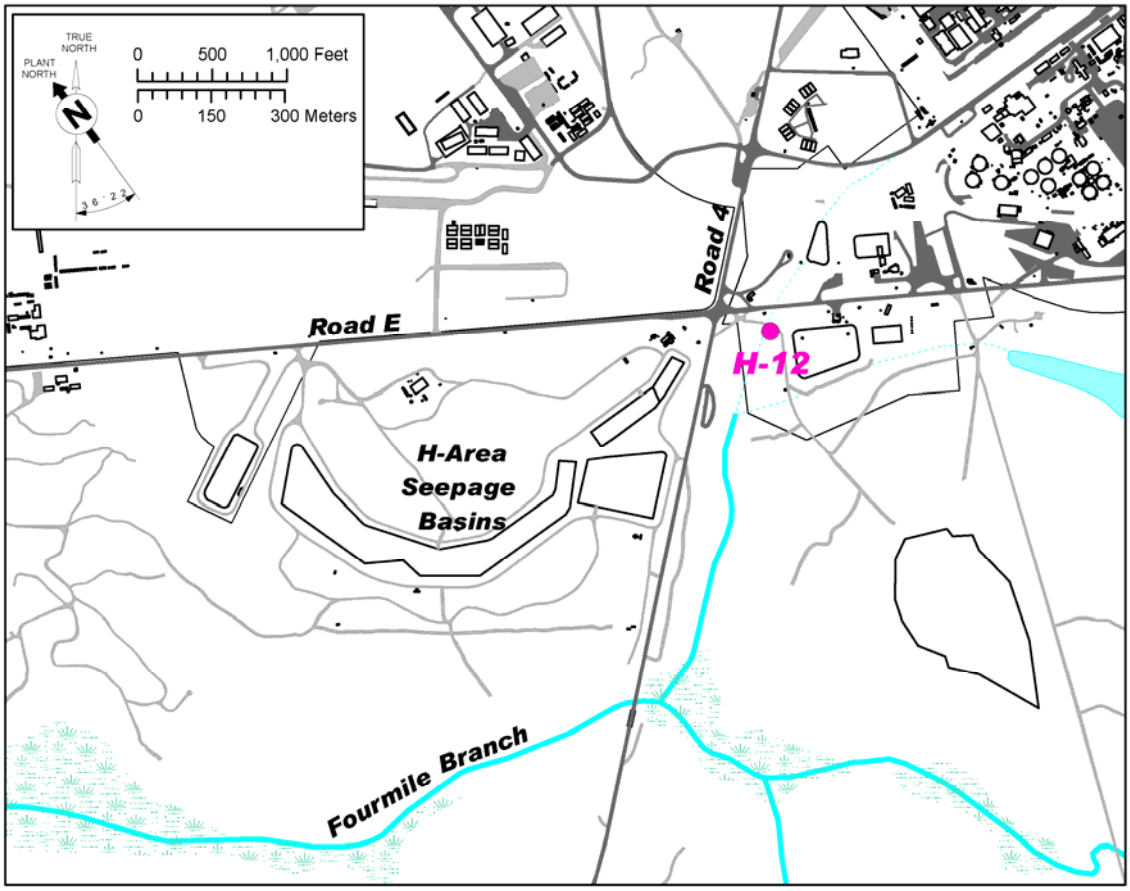

Figure 1. Map Showing Location of the H-12 Outfall

4.0 TEST STRATEGY

Daphnia ambigua and *Ceriodaphnia dubia* were cultured in simulated outfall water typical of the H-12 Outfall water chemistry following methods outlined by EPA (2002 a,b). As discussed above, this water also served as the control and dilution water for the various acute and chronic toxicity tests. The testing was performed in several phases. The type of testing (acute and/or chronic) and specific objective of each phase are described below – the technical details of the testing are provided in the appended university report.

Phase 1a – Amendment Tests – acute toxicity testing to determine if the humate amendment being added to the H-12 Outfall is safe and not causing toxicity. This research phase also included toxicity testing of major elements, such as potassium or sodium, which are present in the baseline humate amendment or potential alternative humate amendments, respectively.

Phase 1b – Amendment Tests – chronic toxicity testing using the humate amendment being added to the H-12 Outfall to confirm the acute toxicity test results from Phase 1a.

Phase 2 – Copper Tests – acute and chronic toxicity tests to document quantitative performance of humate amendment in mitigating copper toxicity. Acute testing was performed using copper concentrations ranging from 0 to 100 μg/L in H-12 Outfall simulant (with no added humate) and in amended H-12 outfall simulant (with added humate). Three-brood chronic toxicity testing was then performed using copper concentrations ranging from 0 to 82 μg/L in amended H-12 Outfall simulant amended with humate. In both the acute and chronic copper toxicity tests using humate amendments, the humate levels used were calculated using the design control equation and were the same as those added by the full scale system (i.e., 2.62 mgC/L at a pH of 7.04).

5.0 SUMMARY OF RESULTS

The following sections provide a synopsis of results for each phase of the research. The raw data are provided in the appended university report.

Phase 1a – Amendment Tests – acute toxicity

Summary data of the acute toxicity results (estimated 48 hour LC_{50} values) are provided in Table 1. The data in the upper section of the table are for Borregro HA-1 (the reference potassium humate detoxification amendment) and a potential alternative (laboratory grade sodium humic acid). These upper section toxicity data are presented in terms of amendment concentration in units based on dissolved organic carbon in solution (i.e., mgC/L). All of the tabulated values relatively high and are similar in magnitude (70 to 100 mgC/L) suggesting that the inherent toxicity of the humate/humic acid substances is relatively low (compared to the target dose range of 0 to 30 mgC/L) and that the two tested amendments would have a comparable toxicity profile in an application scenario. To help assess if potassium is responsible for any observed amendment toxicity, the data in the lower portion of Table 1 are for a reference potassium salt solution and for Borregro HA-1. These lower section toxicity data are presented in units based on potassium concentration in solution (i.e., mgK/L). The potassium based LC_{50} values for *C. dubia* are similar to

the potassium salt solution, suggesting that potassium may play a role in the observed amendment toxicity. However, the lower potassium based LC_{50} values for *D. ambigua* and the similarity of the overall toxicity of Borregro HA-1 (potassium humate) with sodium humic acid indicates that other factors are involved as well.

Table 1. Summary Acute Toxicity Results for Humate/Humic Acid Amendments and a Standard Potassium Salt Solution (all values expressed as 48 hour LC_{50} concentrations)

	C. dubia		*D. ambigua*	
Results expressed in terms of dissolved organic carbon concentration in tested solutions				
Borregro HA-1	**70.3 mgC/L**		**75.8 mgC/L**	07/16/2014 Borregro sample
Borregro HA-1	**77.5 mgC/L**		**93.7 mgC/L**	05/15/2014 Borregro sample
Sodium Humic Acid	**74.0 mgC/L**		**>87.7 mgC/L**	
Results expressed in terms of potassium concentration in tested solutions				
Potassium salt	**60.1 mgK/L**		**98.2 mgK/L**	
Borregro HA-1	**58.7 mgK/L**		**61.8 mgK/L**	07/16/2014 Borregro sample
Borregro HA-1	**57.2 mgK/L**		**72.0 mgK/L**	05/15/2014 Borregro sample

Phase 1b – Amendment Tests – chronic toxicity

To further test the inherent toxicity of the Borregro HA-1 amendment, three-brood toxicity tests were performed in triplicate for both species using Borregro HA-1 concentrations of 0, 8, 16 and 32.5 mgC/L. There were no significant differences ($p > 0.05$) in either mortality or reproduction for either species when the controls were compared to any of the Borregro HA-1 solutions (including the highest concentration above 30 mgC/L).

Phase 2 – Copper Tests – acute and chronic toxicity tests

The data from the Phase 2 acute toxicity testing is shown in Figure 2 depicting the fraction of survivors for each specie over 48 hours on the y-axis versus copper spike concentration on the x-

axis. Two curves are shown for each specie, one with no humate and one with Borregro HA-1 added at the reference design concentration. The detoxification impact of the Borregro HA-1 is illustrated by the significant rightward shift in both graphs.

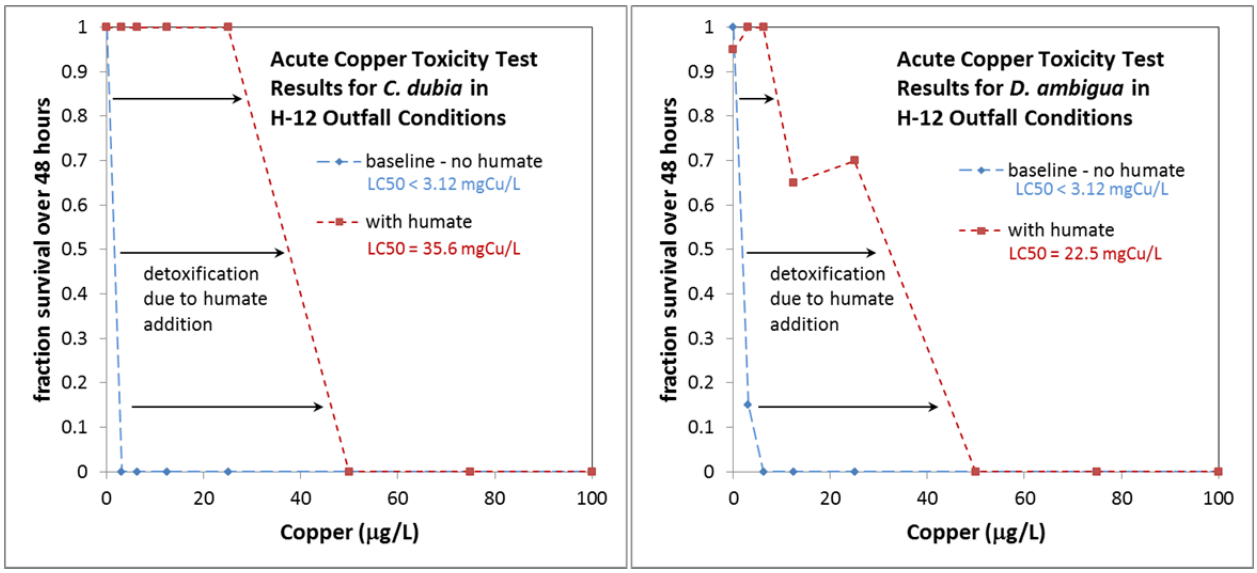

Figure 2. Summary results from the acute toxicity testing for copper in H-12 Outfall conditions

Phase 2 chronic toxicity tests, with humate, are summarized in Figure 3 (*C. dubia*) and in Figure 4 (*D. ambigua*). In each figure, the fractional survival over three broods is shown in the left graph and the average number of offspring per female is shown in the right graph.

In the *C. dubia* survival/mortality testing, there was no measured mortality in the treatments up to 60 µg Cu/L and there was significant mortality (0% survival) at the highest copper spike level (82 µg Cu/L). As in the acute tests, *D. ambigua* exhibited more sensitivity to copper exposure. For *D. ambigua*, there was no measurable mortality up to 41 µg Cu/L and significant mortality at both 60 µg Cu/L (50% survival) and 82 µg Cu/L (0% survival). The observed mortality in the chronic tests occurred at somewhat higher concentrations than the 48 hour acute tests – this may result from the daily feeding that occurs in the chronic tests and the associated reduction in stress on the organisms. Effects on reproduction generally exhibited similar trends to the survival/mortality data. For *C. dubia* there were no significant copper impacts on three brood reproduction for any treatment level up to 60 µg Cu/L. For *D. ambigua*, reproduction was modestly reduced in all treatments when

compared to the control, but reproduction was maintained at copper spike levels up to 41 to 60 µg Cu/L. In all cases, the observed mortality and adverse reproduction effects occurred at concentrations well above 25 µg Cu/L.

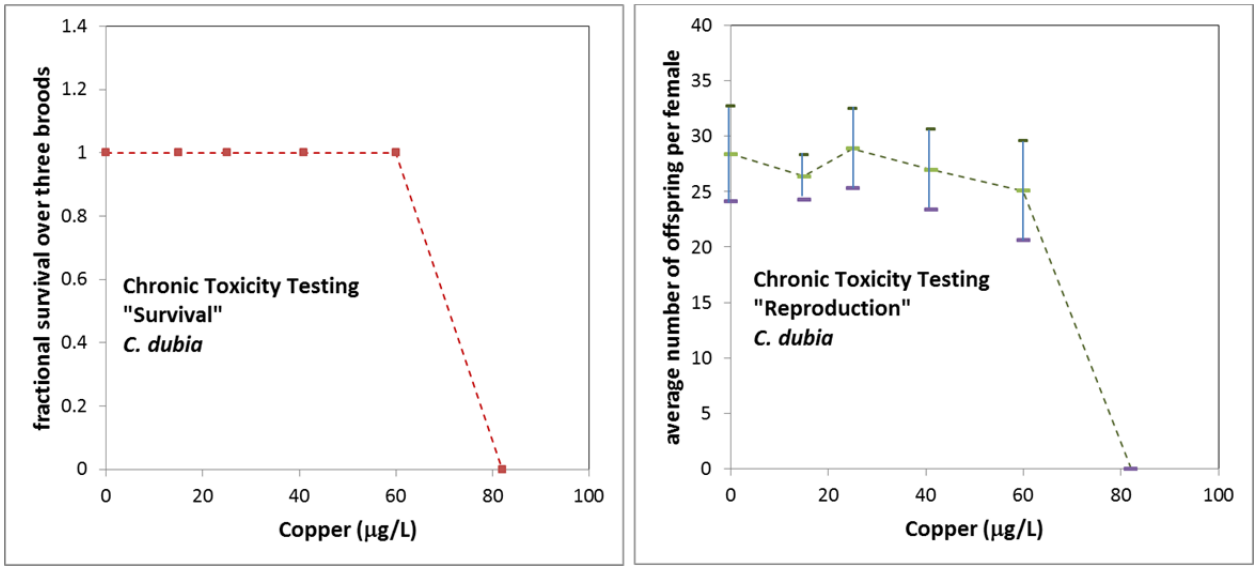

Figure 3. Summary results -- *C. dubia* chronic toxicity testing for copper in H-12 Outfall conditions

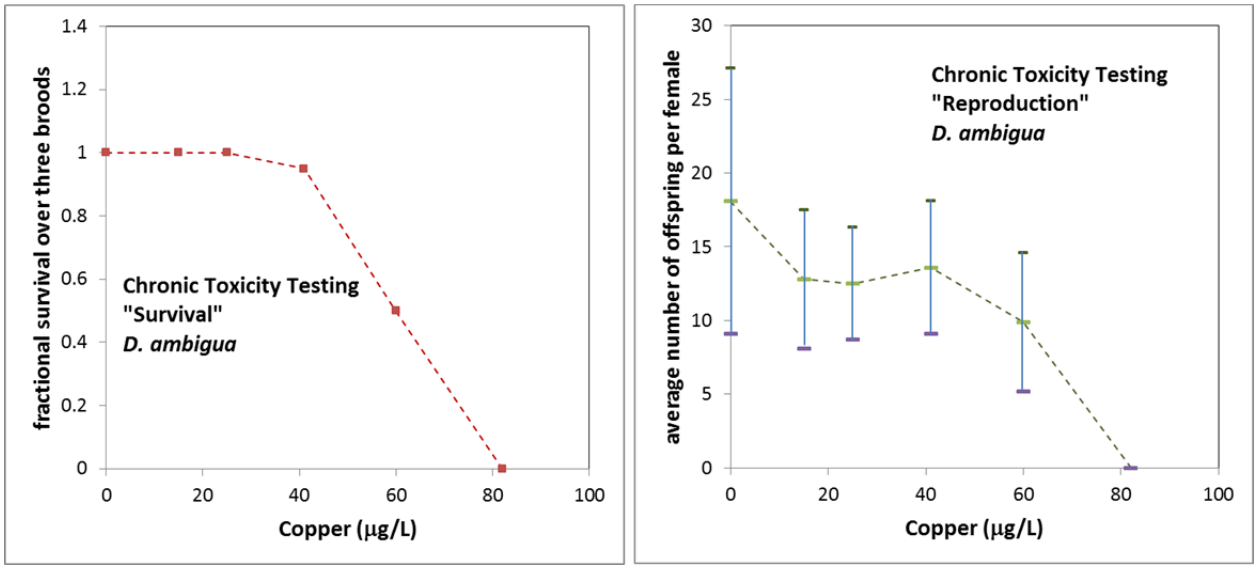

Figure 4. Summary results -- *D. ambigua* chronic toxicity testing for copper in H-12 Outfall conditions

Phase 2 testing demonstrated that the Borregro HA-1 amendment mitigated both the acute and the chronic toxicity of copper in simulated outfall water. LC_{50} values for both indicator species were significantly higher in the treatments that included Borregro HA-1 during acute toxicity testing. In fact, it was difficult to discern an actual LC_{50} for copper exposures in the control -- without the Borregro HA-1 amendment -- because these LC_{50} values were so low (< 3.12 µg Cu/L). While mortality and reproductive effects were observed in the chronic toxicity tests, these effects were generally consistent with, or somewhat less than, those predicted by the Biotic Ligand Model (BLM) that was used as the basis for the detoxification system design. For example, the 41 µg Cu/L treatment was selected to represent the Criterion Maximum Concentration (CMC) value calculated by the BLM. This would have been the concentration where we expected a mortality rate around 50%. While fecundity amongst *D. ambigua* was significantly altered at this treatment, fecundity was not affected in *C. dubia*, and significant mortality was not observed for either species. Similarly, the 25 µg Cu/L treatment was where the BLM predicted chronic effects. There were no chronic effects observed in *C. dubia* at this treatment, while *D. ambigua* did display reduced fecundity as predicted by the BLM.

In both phases of the research, all organism cultures (both species) were grown in representative H12 outfall simulant water and this water was used for all dilutions and toxicity exposures (see Francisco et al., 1993). As a result, the data were relatively clean and provide a clear understanding of the toxicity effects of the various toxicants of interest. This type of test protocol, in which key parameters are individually varied while important baseline water chemistry parameters (e.g., calcium and magnesium “hardness”) are maintained at/near expected field conditions, appears to be effective in providing useful information and is recommended for future studies of this type

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APPENDED TECHNICAL REPORT

University of South Carolina – Aiken

S.M. Harmon and J.D. King

"Toxicity Studies in Support of the Use of Borrego HA-1 for the Reduction of Copper Toxicity at the H-12 Outfall at Savannah River Site"

Final Report

Toxicity Studies in Support of the Use of Borrego HA-1 for the Reduction of Copper Toxicity at the H-12 Outfall at Savannah River Site.

January 7, 2016

SM Harmon

JD King

USC Aiken Dept. of Biology and Geology

Introduction:

Previous research at DOE's Savannah River Site has led to the use of agricultural humate products, such as Borregro HA-1, for the remediation of contaminated stream water. Borregro HA-1 is a carbon-rich potassium humate substance that is usually added as a soil amendment for agricultural application. It has been hypothesized that it can also be added to an aquatic system to increase the natural dissolved organic matter (DOM) and subsequently decrease the toxicity of cationic metals. The Borregro HA-1 will provide the system with macromolecules that strongly attract cations such as copper, lead, zinc, and mercury (Weiner 2008). Once bound to these organic molecules, metals are no longer bioavailable to aquatic organisms; therefore, they become less toxic (Calace and Petronio 2004).

The H-12 Outfall at SRS has been equipped with an automated system that mixes Borregro HA-1 into the effluent-dominated stream at rates that are based upon stream flow and pH. This system was implemented in response to a proposed National Pollutant Discharge Elimination System (NPDES) permit reduction of copper from 25 µg/L to 6 µg/L at the outfall in question (Halverson et al. 2010). However, a brief series of follow-up tests performed in 2013, after implementation of the automated system, indicated potential toxicity of the effluent with Borregro HA-1 to *Daphnia ambigua*, the indicator species required by the NPDES permit for this outfall (Millings et al. 2013). It is unclear if the toxicity observed in these experiments was due to the addition of Borregro HA-1, or if it was due to other factors related to regulatory test methodologies (e.g., differences between

the makeup of culture water and the control /dilution waters used for test solutions). The observed toxicity appeared to be unrelated to copper additions. Therefore, Phase I of this study was designed to provide additional information to resolve the toxicity uncertainties and to modify the humate addition system if necessary.

Tests conducted in Phase I of this study were designed to provide additional information on the toxicity of effluents from the H-12 Outfall and builds on results from the 2013 study (Millings et al. 2013). A step-wise series of tests were proposed to develop the information needed to resolve toxicity uncertainties. The test matrix included acute toxicity tests with *Daphnia ambigua* and *Ceriodaphnia dubia* and was designed to focus on testing one potential toxicant at a time rather than multiple variables. Phase I tests included:

- 48-hour acute toxicity tests in simulated outfall water with elevated potassium concentrations;
- 48-hour acute toxicity tests in simulated outfall water with varying sodium humic acid concentrations;
- 48-hour acute toxicity tests in simulated outfall water with varying potassium humate (Borregro HA-1) concentrations; and
- 7-day chronic toxicity tests in simulated outfall water with varying concentrations of Borregro HA-1.

Phase II was launched in December 2014 and was designed to further explore the remediative effects of Borregro HA-1 when combined with copper in simulated outfall water. As with Phase I, toxicity testing in Phase II was conducted with both *C. dubia* and *D. ambigua*. Phase II tests included:

- a 48-hour acute toxicity test on copper added to simulated outfall water with and without Borregro HA-1 additions; and
- a 7-day chronic toxicity test on copper added to simulated outfall water with a Borregro HA-1 addition.

This research is a collaborative effort between the Savannah River National Laboratory (SRNL) and the University of South Carolina Aiken (USC Aiken). This testing is research-oriented for the

purpose of resolving toxicity uncertainties related to the H-12 Outfall and associated humate addition system. As such, tests may be modified from traditional methodologies or standard practices used in certified toxicity labs. These tests are not intended to meet regulatory compliance standards.

Methods

Daphnia ambigua and *Ceriodaphnia dubia* were cultured in simulated outfall water typical of the H-12 Outfall water chemistry (Table 1) following methods outlined by EPA (2002 a,b); however, a non-toxic laboratory simulated version of H-12 outfall water was used instead of the typical reconstituted waters recommended by EPA. Each liter of simulated H-12 outfall water included the following reagents dissolved in ultrapure deionized water: 17.22 mg $CaSO_4$, 4.68 mg NaCl, 4.20 mg $NaHCO_3$, 1.90 mg $MgCl_2$, and 0.60 mg KCl. Simulated H-12 outfall water was made in 20-L batches and aerated at least 12 hours before use. This water also served as the control and dilution water for the acute and chronic toxicity tests described below. The final chemical analysis of this water can be found in Appendix 1.

Phase I Acute Testing

Forty-eight hour static acute tests were performed according to test conditions and protocols described by US EPA (2002a). Neonates of each species were exposed to a control treatment and an ascending series of five or six treatment concentrations prepared by spiking the simulated H-12 effluent water with an appropriate volume of concentrated toxicant solution (potassium, Borregro HA-1, or sodium humic acid). Log order range-finding tests were conducted initially to aid in the selection of the final test concentrations. Two replicates, each containing ten neonates, were prepared for each concentration. Replicates consisted of 250-ml containers filled with 100 ml of test solution. Simulated H-12 outfall water (described above) served as the control solution and dilution water for these tests. Concentrated working stock solutions of potassium were prepared by dissolving KCl and K_2SO_4 in the simulated H-12 outfall water described above. Test vessels were placed in an environmental chamber under controlled photoperiod (16L:8D) and temperature 25 ± 2°C. Mortality served as the endpoint for these toxicity tests, and results were expressed in terms of the 48-hour LC_{50} (the toxicant concentration lethal to 50% of the test organisms). LC_{50} values and the corresponding

95% confidence intervals were computed using the Trimmed Spearman Karber Method (Hamilton et al. 1977). Samples of Borregro HA-1, sodium humic acid, and potassium stock solutions were preserved and analyzed via ICP-MS at SRNS to determine actual toxicant concentrations in test solutions. Total organic carbon concentrations in Borregro HA-1 and sodium humic acid solutions were measured using a GE 5310C Laboratory TOC Analyzer. Toxicity results were calculated based on measured (actual) concentrations of both carbon and potassium.

Phase I Chronic Testing

Three-brood chronic toxicity testing with Borregro HA-1 followed protocols described by US EPA (2002b) with modifications as recommended by Francisco et al. (1993) and the North Carolina Department of Environment and Natural Resources (NCDENR 2010). These tests used the Borregro HA-1 sample that was collected on 07/16/14, and both daphnid species were tested at 0, 8, 16, and 32.5 mg C/L. Test concentrations were selected by approximating one-half of the LC_{50} from the acute tests described above, and using this as the highest concentration. Twenty replicates, each containing one neonate, were prepared for each chronic test. Replicates were comprised of polypropylene cups with 20 mL of test solution. Test organisms were fed daily, and test solutions were renewed twice during the test, on days 3 and 5. Tests were conducted in an environmental chamber with a controlled photoperiod (16L:8D) and temperature 25 ± 2°C. Chronic tests were repeated three times and the data combined prior to statistical analyses using Fisher's Exact Test for significant mortality and ANOVA and Dunnett's t-tests to compare reproduction between the controls and the test solutions.

Phase II Copper Toxicity Testing

Acute and chronic copper toxicity tests for Phase II were conducted following the general methods and using the simulated outfall water described above. Copper in the form of $CuSO_4$ was added to Nanopure water to produce a 1000 mg Cu/L stock solution that was then used to add Cu^{2+} into test solutions at the appropriate concentrations. Copper concentrations in the acute test ranged from 0 to 100 µg Cu/L, while copper concentrations in the chronic toxicity test included 0, 15, 25, 41, 60, and 82 µg/L. This series included concentrations that would reflect the Final Acute Value (FAV; 82 µg/L), the Chronic Maximum Concentration (CMC; 41 µg/L), and the Criterion Continuous Concentration (CCC; 25 µg/L) as determined by the Biotic Ligand Model (BLM) when applied to the H-12 outfall (Looney and Millings 2009).

In toxicity tests that included Borregro HA-1 additions, the Borregro HA-1 was added to simulated outfall water to achieve a final dissolved carbon concentration of 2.62 mg C/L. This concentration was calculated using the H-12 system's control equation (Millings et al. 2008) when run at a pH of 7.05, the pH measured in the simulated outfall water. After the addition of copper and Borregro HA-1, test solutions were allowed to equilibrate for at least one hour before the introduction of test organisms.

Results

Phase I - Resolving Uncertainties Related to the Toxicity of the Humate Additions

Results of the Phase I 48-hour acute testing are summarized in Table 1. In the case of potassium, the 48-hour LC_{50} values were 60.1 and 98.2 mg K/L for *C. dubia* and *D. ambigua,* respectively. The 48-hour LC_{50} values for the two samples of Borregro HA-1were expressed in terms of mg C/L. These values were 70.3 and 77.5 mg C/L for *C. dubia* and 75.8 and 93.7 mg C/L for *D. ambigua*. The LC_{50} values for the sodium humic acid test were also expressed in terms of mg C/L and they included 74.0 for *C. dubia* and an unknown value > 87.7 mg C/L for *D. ambigua*. The *D. ambigua* value represents the highest concentration that could be tested due to visibility (i.e., solutions at higher concentrations were too dark for mortality observations), but there were no test organism mortalities at this concentration. Therefore, we can safely assume that the actual LC_{50} for *D. ambigua* in sodium humic acid is higher than 87.7 mg C/L, even if this cannot be tested. Because the Borregro solutions were also analyzed for cations (Appendix 1), the LC_{50} values in the Borregro solutions can also be calculated in terms of potassium concentration (Table 2). These values include 58.7 and 57.2 mg K/L for *C. dubia* and 61.8 and 72 mg K/L for *D. ambigua*.

The results of three separate chronic three-brood toxicity tests for both species exposed to Borregro HA-1 are combined and summarized in Table 3. Data for individual tests are presented in Appendix 2. There were no significant differences ($p > 0.05$) in either mortality or reproduction for either species when the controls were compared to the Borregro HA-1 solutions (Table 3).

Phase II - The Remediative Capabilities of Borregro HA-1

Results of the Phase II 48-hour acute testing are summarized in Table 4. The 48-hour LC_{50} values for the copper plus Borregro HA-1 tests were 35.6 and 22.5 µg Cu/L for *C. dubia* and *D. ambigua,* respectively. In the test with copper only (no Borregro HA-1 addition), the LC_{50} values were lower than the lowest concentration (3.12 µg/L) and could not be calculated. This also meant that a chronic test with copper, but no Borregro HA-1 addition, would not be possible in our laboratory.

Table 5 summarizes the chronic test results. In the *C. dubia* test, there was significant (100%) mortality in the 82 µg Cu/L treatment. There were no significant differences in reproduction for any of the other treatments. *D. ambigua*, on the other hand, demonstrated significant mortality in both the 60 and 82 µg Cu/L treatments, and reproduction was significantly reduced in all treatments when compared to the control.

Conclusions

Based on the results of Phase I tests, it can be concluded that the Borregro HA-1 causes no adverse effects to *Ceriodaphnia dubia* or *Daphnia ambigua* at concentrations that are realistically expected in the H-12 outfall stream (0 to 30 mgC/L). It is also important to note that these concentrations of Borregro HA-1 do not add toxic levels of potassium to the stream (approximately 9.6 to 30.2 mgK/L). There were also no adverse acute effects observed in the sodium humic acid test at carbon concentrations less than 50 mg/L, indicating that this substance may be considered further as an alternative to potassium humate products if necessary.

Phase II testing demonstrated that the Borregro HA-1 additions helped to ameliorate both the acute and the chronic toxicity of copper in the simulated outfall water. LC_{50} values for both indicator species were significantly higher in the treatments that included Borregro HA-1 during acute toxicity testing. In fact, it was difficult to discern an actual LC_{50} for copper exposures without the Borregro HA-1 amendment, because these values were so low. Because we cannot reliably manage values this low (< 3.12 µg Cu/L), we could not devise chronic toxicity treatments that did not include Borregro HA-1. While mortality and reproductive effects were observed in the chronic toxicity tests, it should be noted that these effects were somewhat less than those predicted by the BLM, particularly for *C.*

dubia. For example, the 41 µg Cu/L treatment was selected to represent the CMC value calculated by the BLM. This would have been the concentration where we expected a mortality rate around 50%. While fecundity amongst *D. ambigua* was significantly altered at this treatment, fecundity was not affected in *C. dubia*, and significant mortality was not observed for either species. Similarly, the 25 µg Cu/L treatment was where the BLM predicted chronic effects. There were no chronic effects observed in *C. dubia* at this treatment, while *D. ambigua* did display reduced fecundity as predicted by the BLM. In conclusion, the chronic results indicate that, at a minimum, the simulated outfall water with Borregro HA-1 responds as expected to added toxicants such as copper. This could not be said for the simulated outfall water without the Borregro HA-1, where copper is much more toxic without the additional DOC in the test medium.

Table 1. Forty-eight hour LC_{50} values for *C. dubia* and *D. ambigua* exposed to potassium, Borregro HA-1, and sodium humic acid.

Substance of Interest	Nominal Exposure Concentration	Actual Exposure Concentration	*C. dubia* # Dead	*C. dubia* # Alive	*D. ambigua* # Dead	*D. ambigua* # Alive
Potassium (mg/L)	0	0	0	20	0	20
	6.25	6.6	2	18	3	17
	12.5	13.2	1	19	1	19
	25	26.4	0	20	1	19
	50	52.7	4	16	3	17
	75	79.1	18	2	7	13
	100	105.5	19	1	11	9
	48-hour LC_{50} (95% CI)		**60.1 mg K/L 52.9-68.3 mg K/L**		**98.2 mg K/L 76.3-126.3 mg K/L**	
Borregro HA-1 collected 07/16/14 (mg C/L)	0	0	0	20	0	20
	10	7.5	0	20	0	20
	30	25.2	0	20	0	20
	50	44.9	0	20	0	20
	75	110	20	0	17	3
	100	123	20	0	20	0
	48-hour LC_{50} (95% CI)		**70.3 mg C/L none**		**75.8 mg C/L 69.9-82.2 mg C/L**	
Borregro HA-1 collected 05/15/14 (mg C/L)	0	0	0	20	0	20
	10	8.96	0	20	0	20
	30	27.6	0	20	0	20
	50	77.6	4	16	0	20
	75	98.3	19	1	11	9
	100	106	20	0	20	0
	48-hour LC_{50} (95% CI)		**77.5 mg C/L 69.1-86.9 mg C/L**		**93.7 mg C/L 90.5-97.0 mg C/L**	
Sodium Humic Acid (mg C/L)	0	0	0	20	0	20
	10	9.5	0	20	0	20
	30	29.9	0	20	0	20
	50	50.1	3	17	0	20
	75	71.7	5	15	0	20
	100	87.7	18	2	0	20
	48-hour LC_{50} (95% CI)		**74.0 mg C/L 67.3-81.4 mg C/L**		**> 87.7 mg C/L NA**	

Table 2. Forty-eight hour LC_{50} values for *C. dubia* and *D. ambigua* exposed to Borregro HA-1 calculated in terms of potassium concentration.

Substance of Interest	Nominal Carbon Exposure Concentration	Actual Potassium Exposure Concentration	*C. dubia* # Dead	*C. dubia* # Alive	*D. ambigua* # Dead	*D. ambigua* # Alive
Borregro HA-1 collected 07/16/14 (mg K/L)	0	0	0	20	0	20
	10	9.6	0	20	0	20
	30	28.8	0	20	0	20
	50	47.9	0	20	0	20
	75	71.9	20	0	17	3
	100	95.9	20	0	20	0
	48-hour LC_{50} (95% CI)		**58.7 none**		**61.8 (58.5-65.3)**	
Borregro HA-1 collected 05/15/14 (mg K/L)	0	0	0	20	0	20
	10	10.1	0	20	0	20
	30	30.2	0	20	0	20
	50	50.3	4	16	0	20
	75	75.4	19	1	11	9
	100	100.5	20	0	20	0
	48-hour LC_{50} (95% CI)		**57.2 (52.3-62.5)**		**72 (66.6-77.7)**	

Table 3. Results of chronic three-brood toxicity tests with *Ceriodaphnia dubia* and *Daphnia ambigua* exposed to Borregro HA-1. There were no significant differences ($p > 0.05$) in either mortality or reproduction for either species when the controls were compared to the Borregro HA-1 solutions using ANOVA and Dunnett's t-tests.

Borregro HA-1 Concentration (mg C/L)	*C. dubia* Survival (%)	*C. dubia* Average Number of Offspring per Female ± 1 SD	*D. ambigua* Survival (%)	*D. ambigua* Average Number of Offspring per Female ± 1 SD
Control	100	27.6 ± 9.9	98	20.4 ± 8.8
8	100	27.1 ± 8.7	100	21.7 ± 9.5
16	98	32.9 ± 11.1	100	20.7 ± 7.4
32.5	97	28.0 ± 9.5	100	21.0 ± 8.0

Table 4. Forty-eight hour LC_{50} values for *C. dubia* and *D. ambigua* exposed to copper with and without Borregro HA-1 additions

Substance of Interest	Exposure Concentration	*C. dubia*		*D. ambigua*	
		# Dead	# Alive	# Dead	# Alive
Copper (µg/L) (no Borregro HA-1)	0	0	20	0	20
	3.12	20	0	17	3
	6.25	20	0	20	0
	12.5	20	0	20	0
	25	20	0	20	0
	50	20	0	20	0
	75	20	0	20	0
	100	20	0	20	0
	48-hour LC_{50} (95% CI)	**< 3.12** µg Cu/L **none**		**< 3.12** µg Cu/L **none**	
Copper (µg/L) with Borregro HA-1 (2.62 mg/L)	0	0	20	1	19
	3.12	0	20	0	20
	6.25	0	20	0	20
	12.5	0	20	7	13
	25	0	20	6	14
	50	20	0	20	0
	75	20	0	20	0
	100	20	0	20	0
	48-hour LC_{50} (95% CI)	**35.6** µg Cu/L **none**		**22.5** µg Cu/L **(18.4-27.7)**	

Table 5. Results of chronic three-brood toxicity tests with *Ceriodaphnia dubia* and *Daphnia ambigua* exposed to copper in simulated outfall water with a Borregro HA-1 addition. Significant differences from the control are noted with asterisks.

	C. dubia		*D. ambigua*	
Copper (µg/L) with Borregro HA-1 (2.62 mg C/L)	Survival (%)	Average Number of Offspring per Female ± 1 SD	Survival (%)	Average Number of Offspring per Female ± 1 SD
Control	100	28.4 ± 4.3	100	18.1 ± 9.0
15	100	26.3 ± 2.0	100	12.8 ± 4.7**
25	100	28.9 ± 3.6	100	12.5 ± 3.8**
41	100	27.0 ± 3.6	95	13.6 ± 4.5**
60	100	25.1 ± 4.5	50*	9.9 ± 4.7**
82	0*	NA	0*	NA

*Mortality was significant ($p<0.05$) based on Fisher's Exact Test.

**Reproduction was significantly lower ($p<0.05$) than that of the control based on Dunnett's t-tests.

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Appendix 1

Analytical results of the Borregro HA-1 and sodium humic acid solutions used for acute toxcity testing, as well as the simulated H-12 water used for culturing and as a control and dilution water for toxicity testing. Data are from EBL (SRS Environmental & Bioassay Lab); QA level = research data only (not certified)

Analyte	**MDL**	**Units**	**Borregro (Sample 1A) 100 mg/L (used in acute tests)**	**Borregro (Sample 1B) 100 mg/L (used in acute tests)**	**Sodium Humic Acid 100 mg/L (used in acute tests)**	**Sim H-12 H20 (used in acute tests)**
			2-Sep-14	**2-Sep-14**	**2-Sep-14**	**2-Sep-14**
Ag	1	ug/L	<MDL	<MDL	<MDL	<MDL
Al	20	ug/L	1,605	1,349	1,816	55
As	5	ug/L	11	<MDL	24	<MDL
B	2	ug/L	44	46	6	<MDL
Ba	0.5	ug/L	25	15	5	<MDL
Be	0.5	ug/L	1	1	2	<MDL
Ca	20	ug/L	9,043	9,980	4,900	4,046
Cd	0.5	ug/L	<MDL	<MDL	<MDL	<MDL
Co	1	ug/L	1	<MDL	3	<MDL
Cr	1	ug/L	4	5	9	<MDL
Cu	1	ug/L	9	2	9	<MDL
Fe	5	ug/L	1,622	313	1,911	<MDL
K	15	ug/L	95,917	100,535	8,064	480
Mg	10	ug/L	987	692	501	457
Mn	1	ug/L	41	22	7	<MDL
Mo	1	ug/L	<MDL	<MDL	<MDL	<MDL
Na	10	ug/L	21,297	21,980	27,069	3,072
Ni	1	ug/L	1	<MDL	10	<MDL
Pb	5	ug/L	<MDL	<MDL	<MDL	<MDL
Sb	5	ug/L	<MDL	<MDL	<MDL	<MDL
Se	10	ug/L	<MDL	<MDL	<MDL	<MDL
Sn	5	ug/L	<MDL	<MDL	<MDL	<MDL
Ti	1	ug/L	192	213	99	<MDL
V	1	ug/L	8	14	19	<MDL
Zn	1	ug/L	10	10	11	3

Appendix 2

Results of individual chronic three-brood toxicity tests with *Ceriodaphnia dubia* and *Daphnia ambigua* exposed to Borregro HA-1. There were no significant differences ($p > 0.05$) in mortality for either species when the controls were compared to the Borregro HA-1 solutions using a Fisher's Exact Test. Significant differences in reproduction as determined by ANOVA and Dunnett's t-tests are indicated with an "*".

Test Conducted 9/8/14 – 9/15/14

	C. dubia		*D. ambigua*	
Borregro HA-1 Concentration (mg C/L)	Survival (%)	Average Number of Offspring per Female ± 1 SD	Survival (%)	Average Number of Offspring per Female ± 1 SD
Control	100	32.1 ± 10	100	30.1 ± 5.0
8	100	27.4 ± 7.4	100	31.3 ± 6.0
16	100	31.9 ± 4.1	100	25.8 ± 6.2*
32.5	100	29.2 ± 5.8	100	25.8 ± 6.2*

Test Conducted 9/29/14 – 10/6/14

	C. dubia		*D. ambigua*	
Borregro HA-1 Concentration (mg C/L)	Survival (%)	Average Number of Offspring per Female ± 1 SD	Survival (%)	Average Number of Offspring per Female ± 1 SD
Control	100	19.6 ± 2.2	95	13.0 ± 4.1
8	100	20.9 ± 3.0	100	12.4 ± 3.2
16	95	25.9 ± 15.1	100	14.2 ± 4.8
32.5	90	19.6 ± 9.6	100	13.4 ± 4.6

Test Conducted 10/27/14 – 11/3/14

	C. dubia		*D. ambigua*	
Borregro HA-1 Concentration (mg C/L)	Survival (%)	Average Number of Offspring per Female ± 1 SD	Survival (%)	Average Number of Offspring per Female ± 1 SD
Control	100	31.2 ± 9.9	100	18.2 ± 5.9
8	100	33.3 ± 9.6	100	21.5 ± 6.6
16	100	41.0 ± 3.5	100	22.1 ± 5.9
32.5	100	35.3 ± 4.4	100	24.0 ± 6.6

*Indicates a significant difference in reproduction ($p < 0.05$) when compared to the control.