

# **Joint Exhibit 71**

## Chronic Sediment Toxicity Test with *Leptocheirus plumulosus*: Continued Difficulties and Concerns

### Update on Method Development and Pilot Testing – March 2015

SMV has been conducting pilot testing since September 2013 in order to improve the consistency of the *Leptocheirus plumulosus* chronic testing method. SMV has compared organism performance with a focus on improving survival using different sediments, water and feeding regimes. SMV has determined that the locally collected natural sediment used historically with some success for this testing is no longer suitable to generate acceptable results. In addition, SMV has yet to develop formulated sediment that is suitable for use in this testing. Developing suitable formulated marine sediment for chronic amphipod testing may take significant trial and error as there is not much information in the literature regarding this topic. Consequently, SMV attempted to find a new source of sediment that would be suitable for this testing. SMV was able to contact an environmental consulting firm in Washington that could collect and ship Sequim Bay sediment. This sediment was used as control sediment in the original method development by the EPA and has been used with some success by government laboratories to assess the toxicity of field collected sediment samples.

This plan was communicated to the EPA in a conference call on November 26, 2013. The EPA provided information on laboratories that are conducting these chronic exposures successfully. However, these laboratories were conducting exposures with field collected sediment samples as opposed conducting dosed sediment testing under GLP conditions. Overall, the information provided by the EPA was helpful and did support the decision by SMV to focus on the sediment source as the key component to conducting an acceptable exposure. Unfortunately, the survival data generated by the method validation pilot with Sequim Bay sediment was also highly variable and the same pattern of delayed mortality was observed in many replicates. Further pilot testing early in 2014 examining flaked fish food source also did not resolve the poor survival issues. In conclusion, the same issues with delayed mortality of adult organisms persisted when sediment and food source were different from those historically used at SMV.

SMV discussed these testing issues with technical staff at the US Army Engineer Research and Development Center (ERDC) in Vicksburg as this facility has had some success with the test method. Both laboratories coordinated an interlaboratory study designed to examine the effects of control sediment source, nutritional quality of food utilized and organism source on organism performance. This study terminated on April 11, 2014. The interlaboratory study was conducted according to the standard guidance with the following multifactorial experiment design:

Treatment #	Replicates	Sediment	Food	Organisms Source
1	7	Sequim Bay Control	Tetramin	ERDC
2	7	Sequim Bay Control	Tetramin	Chesapeake Cultures
3	7	Southern LA Control	Tetramin	ERDC
4	7	Southern LA Control	Tetramin	Chesapeake Cultures
5	7	Sequim Bay Control	Sera Vipan	ERDC

6	7	Sequim Bay Control	Sera Vipan	Chesapeake Cultures
7	7	Southern LA Control	Sera Vipan	ERDC
8	7	Southern LA Control	Sera Vipan	Chesapeake Cultures

Upon completion of the interlaboratory exposure, survival continued to be low/variable in all groups tested at SMV while the ACOE observed survival of >84% in all groups. None of the factors tested (sediment, organism source and food type) seem to be significantly driving the variability in the survival data. Michael Bradley (Senior Biologist at SMV) visited the ACOE facility during the initiation and maintenance of this interlaboratory test in order to observe laboratory techniques. No significant differences were noted in regards to techniques between our two laboratories. Methods remained consistent between labs with the only exception being light intensity. It came to our attention that the ACOE conducts their chronic exposures at a lower light intensity (approximately 200 lux) than referenced in the current guidance (500-1000 lux). We have been conducting our exposures according to the guidance with regards to light intensity. Looking at our historical data, there was some other anecdotal evidence that suggests light intensity may affect long term survival.

SMV conducted another pilot test (terminated on May 23) to examine survival under lower, more controlled light conditions and generated acceptable survival data of >95% with low variability amongst replicates. SMV conducted a similar pilot that terminated on July 9 in order to verify that light intensity and lighting conditions may be indirectly or directly effecting survival over the course of a chronic exposure. Unfortunately, the results of the second lighting pilot did not yield acceptable survival data and results were highly variable. Consequently, SMV is not confident with moving forward with the chronic testing we have on the schedule at this time.

Upon review of all the data collected from pilot testing to date by SMV senior scientists, the overall general trend of highly variable survival suggests that there may be issues with latent toxicity at the replicate level as opposed to issues with the main variables of the test system (i.e. sediment, organism population, food source etc). If sediment was not acceptable to support survival or a population of organisms was unhealthy, suppression in survival would be observed across all replicates. However, SMV has observed survival ranging from <10% to 100% within the same test group in many of the pilot tests. The general trend of adult amphipod mortality in the last 7-10 days of the exposure also supports the hypothesis of latent toxicity in individual replicates.

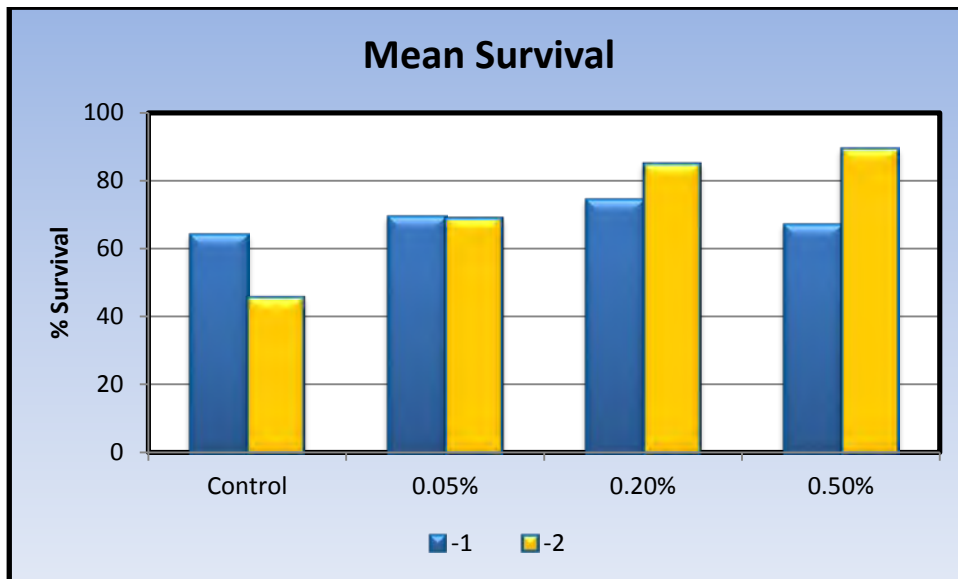
A possible cause for this mortality is glassware contamination. However, SMV has omitted this as a probable cause as the glassware used for this testing has been handled in the same manner as glassware used in other testing across our facility and similar issues with survival have not been observed in testing with other organisms. SMV believes that the latent toxicity could be coming from nitrite toxicity that is a function a variable bacterial population present in each replicate. Replicates may be building up a specific bacterial community toward the later stages of the 28 day test that convert the ammonium present in the vessel to nitrite. However, completion of nitrification process may not be possible if certain replicates lack the bacteria that further transform nitrite into nitrate leading to a buildup of nitrite in a replicate which could cause toxicity in adult amphipod toward the later stages of the exposure. The imbalance in a bacterial population may be due to the inherent bacterial population in the sediment or overlying water utilized in testing.

This issue of latent toxicity due to variability in the bacterial population within each replicate is beyond the scope in which the current EPA guidance document will be helpful in rectifying the issue. SMV will continue to put this

testing on hold until further pilot testing can be conducted to determine if nitrite toxicity is an issue and if other test conditions are exacerbating this toxicity. SMV plans to take an approach similar to a Toxicity Identification Evaluation in order to verify if nitrite toxicity in replicates is the cause of the observed delayed mortality.

SMV has since completed two identical pilot studies since this last communication to investigate the nitrogen cycle issues described in the above mentioned statement. These pilots included a number of treatments with one such treatment being the use of overlying water inoculated with nitrifying bacteria. In both pilots, this treatment yielded survival in the 71-77% range with some moderate variability (SD = 10-20%). Control replicates and other treatment groups exhibited results similar to those historically observed with significant mortality toward the latter stages of the exposure with high variability among replicates. These results seem to demonstrate that the addition of the nitrifying bacteria had a positive impact on survival but this treatment needs to be further investigated to consistently achieve >80% survival with lower variability.

A second set of identical pilot exposures were terminated later in October 2014. The objective of the latest pilot exposures was to investigate the addition of nitrifying bacteria to the overlying water at different concentrations/rates in an attempt to further improve the survival data. A control was set up with no addition of nitrifying bacteria to the overlying water while the treatments consisted of adding nitrifying bacteria at 0.05%, 0.20% and 0.50% of the overlying water volume. Survival results were as follows for the two pilot exposures:



While the data is still inconsistent across the pilots, the results of the second pilot exposure demonstrate a clear increase in survival with a theoretical increase in the nitrifying bacteria population. This evidence suggests that further pilot work focusing on the bacterial population of the overlying water and the nitrogen cycling within the test system is needed to improve consistency of the survival results.

SMV presented much of this pilot work at the NASETAC meeting in Vancouver and discussed the ongoing issues with this methodology with research scientists outside of our organization. An ad hoc advisory group included both industry and government scientists formed during a special meeting regarding this test method. SMV will be heavily involved in this advisory group with the objective to ring test the method through the participation of a number of contract and government laboratories. In addition, SMV plans to continue independent pilot testing in order to optimize the methodology for dosed sediment testing. SMV is currently targeting Quarter 3 of 2015 to



restart chronic sediment testing with *Leptocheirus plumulosus*. Once testing is restarted, the backlog of studies can be prioritized and the schedule can be better assessed for each individual studies.

Sincerely,

Christian Picard  
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