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"Whole Effluent Toxicity (WET) Test Method Changes" Comment Clerk (WETEU-IX)
Water Docket (4101)
Environmental Protection Agency
Ariel Rios Building
1200 Pennsylvania Avenue, NW
Washington, DC 20460

Subject: Comments on Proposed Changes to WET Methods Manuals

The Wisconsin Department of Natural Resources (WDNR) and University of Wisconsin-Madison's State Lab of Hygiene (SLH) have reviewed the proposed changes to the USEPA whole effluent toxicity methods manuals. We appreciate the time and effort spent by USEPA staff and others in trying to improve these documents and address outstanding issues. Our comments relating to technical aspects of these documents are attached. We appreciate the opportunity to comment on the technical issues associated with these proposed changes here in writing and at the WET Testing Laboratory Stakeholder Meeting in Chicago on January 8th. We appreciated the manner in which this stakeholder meeting was conducted. There was an excellent exchange of information, ideas, concerns, etc., with everyone respecting everyone else's opinion; all of the cards were laid on the table and we believe that all involved in the meeting benefited. Special thanks go to the knowledgeable and experienced staff, especially Florence Fulk, Teresa Norberg-King, and Robert Brent (from DynCorp), who designed and facilitated this meeting. They are always consummate professionals who represent the Agency well.

Our comments were both mailed as hard copies and emailed to OW-docket@epa.gov on January 11, 2002. A hard copy was sent in order to save some time and effort in printing at your end and to insure that documents were received in a readable format; an additional electronic format was sent to make development of a comments response document easier. Any questions regarding the comments or attached documents can be directed to Kari Fleming at (608) 267-7663 or flemik@dnr.state.wi.us.

Sincerely,

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1. **Fathead Minnow Chronic Test Methods - Pathogen Control.** The WDNR and SLH commend USEPA for making modifications to their methods which acknowledge the potential for the "pathogen problem" in fathead minnow chronic tests, however we feel that these methods should be presented as an alternate method for use when surface waters are used for dilution in the test, rather than requiring parallel tests in each individual discharge situation.

   While the proposed methods, requiring parallel tests when a pathogen problem is suspected, may be practical for once-through cooling water effluents, this option is not at all practical for situations like those in Wisconsin where surface waters are used in all tests for dilution and the "pathogen problem" occurs frequently and unpredictably. In Wisconsin, the effects described above have occurred during all months of the year and over 25% of chronic tests conducted between 1988-1998 showed these symptoms. Of a total of 124 different surface waters used as diluents in these tests, 91 (73.3%) showed these effects during one or more tests (see "FHM Chronic Pathogen Guidance"). So, it appears as if this phenomenon occurs frequently when surface water is used as the diluent, is independent of the surface water used, and is totally unpredictable. Since this phenomenon is hard to predict and has been shown to occur throughout Wisconsin with a variety of effluents and surface waters, requiring parallel tests for every discharger that has to do chronic tests would be an enormous and unnecessary burden to each permittee.

   Research done at the SLH from 1998-2000 (see attached "Pathogen Poster Text", "Pathogen Poster Summary" and "FHM Chronic Pathogen Guidance") to determine the cause(s) of the "pathogen problem" found that pathogenic organisms (e.g. *Flexibacter spp.*, *Aeromonas hydrophila*, and others) could be found everywhere in tests where a surface water was used as the diluent (in controls and effluent treatments in the test, in culture waters, fathead minnows, food, etc.) and that attempts to remove them from the test were unsuccessful. This suggests that the problem is not due to organisms in the surface water samples or the presence of some unknown pathogen, but rather conditions in the WET test that cause these organisms to flourish and negatively effect test organisms. Since research suggests that this phenomenon is created by WET test conditions and because the use of these new methods (using smaller test chambers with 2 fish per chamber) appear to be successful at removing the "pathogen problem" (see attached "FHM small cups" and "FHM large cups") from the chronic fathead minnow test without modifying the potential toxicity of effluent or surface water samples, the WDNR and SLH request that USEPA modify their methods to allow this as an alternate test procedure, rather than a TIE type method.

   We would also like to see the method revised to allow a new minimum of 20 fish per treatment when using the alternative 30 ml beaker method. Requiring 20 fish per treatment allows the test to be performed in a standard *Ceriodaphnia dubia* brood board and the time required for test transfer remains comparable to the current method. Upon test completion, the 10 cups are randomly paired and these fish weighed, resulting in replicate weights from four fish. (Pooling is necessary to achieve weights in the sensitivity range of the balance.) The result is five replicates per test treatment. While the CV is generally greater when weighing fewer fish (because single fish mortalities have a greater effect on the average weight with fewer fish), we have found the weight CVs to be comparable to the *C. dubia* chronic test. Also, when the pathogen effect occurs, CVs are better in 30 ml beakers in side by side tests with the current method (see attached "Pathogen Poster Text", "Pathogen Poster Summary" and "FHM Chronic Pathogen Guidance").

   Another subjective argument for allowing 20 fish per treatment is that this alternative method is already an improvement in the statistical power of the test over the current method. A basic principle of statistics says that increasing replicates is better than increasing individuals within a replicate. We have been performing fish chronic tests using the alternative method since September 2000 and have included comparisons of CV for both methods (see attached "FHM small cups" and "FHM large cups"). Side by side tests were performed prior to that (see attached "20 fish vs. 40 fish" - to be sent in the week of January 14th).

2. **pH Control.** The WDNR and SLH agree with USEPA's decision to recommend pH control in WET tests, however, these methods should be applied to acute tests, as well as chronic. In fact, the WDNR and SLH

feel that is appropriate to require pH control, using CO_2 entrapment methods, in all acute tests where compliance is determined at end-of-pipe. The WDNR currently requires that all effluents containing ammonia above levels of concern perform acute tests using CO_2 entrapment methods (see Section 4.17.2 of the attached "WDNR Methods Manual Edition 1") and has plans to extend this to require that all static-renewal acute tests be conducted in a CO_2 atmosphere that maintains the pH at a level no lower than the measured effluent pH at the time of discharge (see Section 4.15.6 of the attached "Proposed New Edition of WDNR Methods Manual"). In proposed WDNR methods, static and static-renewal chronic tests are not required to be conducted in a CO_2 atmosphere, but if CO_2 is used, the pH is required to be maintained at a level no lower than the receiving water pH.

The WDNR and SLH prefer CO_2 entrapment methods over others for controlling pH drift, due to the potential for chemicals added to adjust pH to be toxic themselves or to interfer with the toxicity of other chemicals in the effluent. We believe that flow-through tests are simply not practical or possible in most situations and can be quite costly to the permittee, and are therefore not a good option for removing pH drift in acute tests. The WDNR has required static-renewal acute and chronic tests since the late 1980's and our experience is that static-renewal methods are not sufficient to avoid pH drift, either.

3. **USEPA interlaboratory variability study and the *Selenastrum* chronic test.** The WDNR and SLH have reviewed the results of the USEPA interlaboratory variability study, and the results reported seem to reflect the experience and current performance (test completion, precision, and false positive rates) of Wisconsin certified labs (including contract labs and the SLH), for the *C. dubia* and fathead minnow acute and chronic tests. However, the WDNR and SLH have had significantly better luck with the *Selenastrum* chronic test. The WDNR and SLH have been working since 1997 to "fix" *Selenastrum* test methods and the methods that have been developed, including the use of microplates and other modifications, have shown significantly better test completion rates (88%) than those reported in the interlab study report. These new methods utilizing microplates instead of flasks also significantly reduce test time and cost (see attached "Selenastrum Test Costs" and "Algae Research Paper").

   During the WDNR/SLH study, discussions with other states and stakeholders with experience using current USEPA *Selenastrum* methods suggested that intratest variability was the biggest concern. Historically, labs had shown difficulty meeting acceptability criteria for control variability ($\leq$20% CV in control treatments). The WDNR and SLH found that many of the factors that influence variability can be controlled by eliminating “gray areas” (e.g., to use EDTA or not, hand-shaking vs. continuous shaking, number of replicates, etc.) within EPA methods. When these were controlled, criteria could be met easily. SLH pilot tests averaged well over the growth minimum required, with an average of 12% CV (well below the 20% CV acceptability criteria & similar to CVs for fathead minnow and *C. dubia* tests) (see attached "SLH Algae Tests 2000-2001").

   Based on our research, we recommend the following be changed, in order to reduce test variability and improve test completion rates: 1) *Allow the use of microplates*, either in place of or in addition to flasks, 2) *Require the use of EDTA.* We believe EDTA should be required because EDTA promotes growth and reduces variability when included in algal test media. Some feel that methods should not recommend EDTA since it may chelate metals. However, we've found that amounts used don’t remove any more toxicity than natural chelating compounds found in ambient waters, invertebrate species (e.g., *C. dubia)* are usually more sensitive to most metals and will account for effluent toxicity when tests are done with a battery of organisms, and the compounds that are most likely to cause toxicity to algae should not be affected by EDTA (since they’re organic in nature) (see Table 1 in attached "Selenastrum Implementation Guidance"). 3) *Remove centrifuging requirement.* USEPA Methods recommend centrifuging and resuspending cells when preparing the algal inoculum. We found that elimination of this step lowered test variability without reducing test performance. 4) *Require at least 4 replicates.* EPA Methods currently require a minimum of 3 replicates; we believe a minimum of 4 replicates improves test precision (easily done on microplates). 5) *Require continuous shaking.* Our study showed that hand shaking resulted in lower growth and higher

variability than if a shaker table was used. 6) *Recommend use of reconstituted water in test solutions.* EPA methods recommend that Type I water be used in test solutions; our study showed that variability was reduced if a standard reconstituted water was used instead. 7) *Shorten media expiration window.* EPA methods state that unused media may be stored up to 6 months prior to use; our study showed that it was better to discard unused media that was > 2 months old. (see attached "Selenastrum Implementation Guidance" and section 4.21.5 of the attached "Proposed New Edition of WDNR Methods Manual").

4. **Concentration-Response Relationships.** The WDNR and SLH agree that review and documentation of the concentration-response relationship is a crucial step in the review of WET test results, since the concentration-response can be used to identify anomalous results and reduce false positives/negatives. The corresponding concentration-response guidance is excellent, too, but should remain as guidance and not be written into the methods manuals, so that some room is left for the best professional judgment of reviewing staff. The WDNR and SLH recommend that language be added that requires the concentration-response to be presented graphically in test reports. Plotting the replicate and mean data for the control and effluent concentrations gives a visual picture of the concentration-response, variability of the data, and any suspicious data or potential outliers. Graphing of test data has been required on WDNR report forms for about 2 years and this has made review of concentration-response relationships much easier for laboratory and regulatory staff. We've also found that by presenting a "picture" of the data, less experienced permittees and regulatory staff can more easily understand the toxicity data. To make graphing of data easier for labs and permittees, the WDNR has developed an electronic report form which automatically graphs data that is entered by the user (see attached "WET Test Report Form").

5. **Clarification of Requirements vs. Recommendations.** In order to make sure that all readers understand the USEPA's intent for each provision of the methods manual, we recommend that terms such as "must", "shall", "should", "can", "may", etc. be used consistently and be clearly defined in the methods manuals. The WDNR has done this in their state-specific methods manual and has found that enforcement, lab certification/auditing, and defense of specific conditions is much easier when items are clearly understood by all parties involved. For consistency sake, we recommend using the ASTM definitions for these terms.

6. **DO supersaturation/aeration of samples prior to testing.** Many permittees in Wisconsin have noted problems when aeration prior to testing (to reduce supersaturation) causes effluent pH levels to drift upwards before testing, which can result in ammonia toxicity during tests that may not have occurred under normal discharge conditions. pH control during the test often does not mitigate this problem, since pH drift occurs before testing and in-test pH control simply maintains this higher pH. The WDNR acknowledges that aeration is often necessary to reduce supersaturation after samples are warmed to test temperature. However, because it was unclear whether supersaturation would negatively impact test organisms, the WDNR asked the SLH to conduct tests with and without aeration and supersaturation. The SLH has conducted effluent and ambient tests in this manner for about 9 months and data collected during this period suggests that supersaturation does not seem to cause mortality or stress to the fathead minnow or *C. dubia* (data available upon request). Therefore, we recommend that aeration prior to testing not be allowed and the requirement that D.O. be < 100% saturation prior to testing be removed from the methods (see section 4.15 of the attached "Proposed New Edition of WDNR Methods Manual").

7. **Additional Chronic Test Acceptability Criteria.** In order to lower test variability and to improve the quality of tests being used for determining compliance with NPDES permits, the WDNR believes that the following additional test acceptability criteria (TAC) should be added to chronic *C. dubia* and fathead minnow tests:

   C.dubia:

   - Populations in each control must contain $\leq$ 20% males and there must be $\leq$ 20% males over all test concentrations
   - The control coefficient of variation (CV) between replicates must be $\leq$ 40% (reproduction only; reproduction CV determined with surviving females only).

- At the end of the test, ≥ 80% of the surviving *C. dubia* in the primary control must have produced their third brood

Fathead minnow:

- The control coefficient of variation (CV) between replicates must be ≤ 40% (survival and growth, growth CV determined with surviving fish only).

Along with the additional TAC in the *C. dubia* chronic test, the test termination endpoint should be changed to say: "Tests shall be terminated when 80% or more of the surviving females in the primary control have produced their third brood, or at the end of 7 days (whichever comes first). Only 3rd brood organisms should be counted."

These new TAC have also been recommended by a number of other sources, including USEPA guidance written in response to the WestCAS & Edison Electric vs. USEPA Lawsuit Settlement (control variability criteria, >80% 3rd brood criteria), National Environmental Laboratory Accreditation Program (NELAP) methods, (≤20% males in chronic *C. dubia* tests), recommendations from Society of Environmental Toxicology and Chemistry (SETAC) WET expert advisory panels and Pellston workshop participants (control variability criteria, >80% 3rd brood criteria), and advice from other states who have used similar TAC (control variability criteria, >80% 3rd brood criteria, ≤20% males in chronic *C. dubia* tests). The need for certain additional TAC (especially control variability and ≤20% males criteria) is also supported by past experience of permittees and labs in Wisconsin.

8. **Lab staff qualifications and training.** Laboratory staff experience is one of the most important factors influencing WET test and laboratory performance. Pellston workshop participants, SETAC WET expert advisory panels, and NELAP recommendations have all pointed to lab staff experience and qualifications as the single most important factor influencing WET test variability and test quality. Therefore, revisions should be made to the USEPA methods to insure that WET tests are done by staff experienced in the use or conduct of WET tests and the interpretation of data from WET tests. The WDNR has developed language for their methods manual (see section 3.17 of the attached "Proposed New Edition of WDNR Methods Manual"), in an attempt to address this. This language was adopted from NELAP requirements and the USEPA document titled "Manual for the Evaluation of Laboratories Performing Aquatic Toxicity Tests". Most other lab certification programs (e.g., analytical chemistry) have requirements for training (e.g., initial demonstration tests, documented training plans, etc.), so making this a requirement for labs performing WET tests would not be unusual or unreasonable.

Referenced and attached documents can also be found electronically (as PDF files) online at http://www.dnr.state.wi.us/org/water/wm/ww/biomon/biomon.htm, as the following:

FHM Chronic Pathogen Guidance:
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Chap2_7x_Invalid_RW_Controls_DRAFT.pdf

Pathogen Poster Text: http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Chronic_fhm_study_text.pdf

Pathogen Poster Summary:
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Chronic_fhm_study_graph_1.pdf
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Chronic_fhm_study_graph_2.pdf
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Chronic_fhm_study_graph_3.pdf

WDNR Methods Manual Edition 1: http://www.dnr.state.wi.us/org/water/wm/ww/biomon/_m_about.htm

Proposed New Edition of WDNR Methods Manual:
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Edition_2_DRAFT.pdf

Selenastrum Test Costs:
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Implementation_Discussion_Mtg_Min_12_99.pdf

Algae Research Paper: http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Algae_Research_Paper.pdf

Selenastrum Implementation Guidance:
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/Chap2_11x_Plant_Tox_Tests_DRAFT.pdf

WET Test Report Form:
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/WET_Test_Report_Form_-_Acute_&_Chronic_1.pdf
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/WET_Test_Report_Form_-_Acute_&_Chronic_2.pdf
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/WET_Test_Report_Form_-_Acute_&_Chronic_3.pdf
http://www.dnr.state.wi.us/org/water/wm/ww/biomon/WET_Test_Report_Form_-_Acute_&_Chronic_4.pdf

("FHM Small Cups", "FHM Large Cups", and "SLH Algae Tests 2000 - 2001" cannot be found on this website, but may be obtained by contacting Steve Geis at sgeis@mail.slh.wisc.edu or (608) 224-6230).