

## **CHAPTER 2.7 - Dilution Water and Test Controls**

**The purpose of this chapter is to address the use of receiving waters as diluent and controls in WET tests, discuss some problems that have been noted with receiving water controls in the past, and discuss how the DNR may interpret a WET test when a dilution water has not met test acceptability criteria.**

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### **Dilution Waters Used in WET Tests**

In Wisconsin, whole effluent toxicity (WET) tests are intended to measure the aggregate effect of all toxic contaminants in an effluent and the extent to which the chemicals are biologically available to the organisms living near the discharge. By requiring the use of receiving water as the test diluent in most tests, the WET test protocols found in the "State of Wisconsin Aquatic Life Toxicity Testing Methods Manual, 2<sup>nd</sup> Edition" (Methods Manual) attempt to account for many of the site-specific factors (e.g., bioavailability, pH, hardness, alkalinity, etc.) that may impact the determination of whether toxicity will be manifested in the environment. The Methods Manual requires that receiving water be used for dilution in chronic tests and in acute tests where a ZID has been approved. Test methods allow (but do not require) the use of standard laboratory waters for dilution in acute tests where compliance is determined at end of pipe (100% effluent) conditions. (See Section 4.4.)

Choosing the dilution water can be an important step in the WET testing process. Using laboratory water may decrease costs associated with the sampling and shipping of receiving water samples. However, WET tests which use lab water for dilution may overstate the effects of an effluent on the receiving water. The bioavailability of a toxic substance is influenced by a number of factors. Scientists have recognized the ability of natural waters to affect traditional toxicants such as metals by altering their chemistry and decreasing the toxicity that is elicited in the environment. The naturally occurring materials that tend to complex and detoxify certain materials are usually absent from lab waters. Therefore, using a standard lab water may not provide test conditions representative of the actual discharge situation. The use of receiving water for dilution increases the environmental relevance of WET testing by simulating effluent/receiving water interactions in the test.

### **What if the Receiving Water Control "Fails"?**

Unfortunately, though use of natural receiving waters for dilution in WET tests increases their environmental relevance, it also increases the complexity of the test and may occasionally present other problems related to background effects. There are a few reasons why a receiving water may not meet certain criteria in a WET test. Failure of a receiving water to meet test acceptability criteria does not necessarily mean that the receiving water is toxic. It is important to note that "toxicity" in an effluent is defined as  $\geq 50\%$  mortality (in an acute test) or  $\geq 25\%$  effect on reproduction or growth (in a chronic test). Receiving water control acceptability criteria are much lower than this (e.g.,  $\geq 10\%$  mortality in an acute test;  $\geq 20\%$  in a chronic test).

The following is a list of factors that may introduce toxicity or contamination into a receiving water control, causing it to show less than perfect performance: 1) the sample was collected in the mixing zone of the permittee's or another discharge, 2) a storm event has washed pollution into the waterbody, 3) the sample was collected in a container that was not properly cleaned and conditioned, 4) a physical structure (i.e., dam or bridge) is leaching

toxins into the waterbody, 5) natural bacteria in the receiving water may cause adverse effects to laboratory organisms (discussed in greater detail below), or 6) the presence of "natural" toxicants (e.g., cyanobacteria) in the receiving water.

### Poor Receiving Water Performance in Past Tests

In WET tests, organisms are exposed to a series of effluent concentrations for a specific time period in order to estimate the effluent's toxicity. Receiving water is often used for dilution, in order to simulate what may happen in the environment when the effluent is introduced. A receiving water control (an exposure of test organisms to dilution water with no effluent added) is required in each test, to monitor the suitability of the dilution water. An additional control is also required, using laboratory water, to monitor the health of the organisms, test conditions, and handling procedures.

According to the Methods Manual, in order for an acute WET test to be acceptable, organism survival in receiving water and laboratory water controls must  $\geq 90\%$ ; for a chronic test to be acceptable, control survival must  $\geq 80\%$  (see Sections 3.8 & 3.9) As shown in Table 1 below, receiving water controls in chronic tests completed between 1988-1998 showed a surprisingly high failure rate.

**TABLE 2.7.1**  
**Control Failure Rates in WET Tests 1988-1998**

| TEST TYPE | TOTAL TESTS | UNACCEPTABLE RECEIVING WATER CONTROLS | UNACCEPTABLE LAB WATER CONTROLS |
|-----------|-------------|---------------------------------------|---------------------------------|
| ACUTE     | 2,308       | 78 (3.4%)                             | 43 (1.9%)                       |
| CHRONIC   | 1,497       | <b>382 (26%)</b>                      | 44 (2.9%)                       |

Close examination of those chronic tests where unacceptable receiving water controls were noted revealed the following common characteristics:

1. Lowered survival in receiving water controls in fathead minnow (*Pimephales promelas*) 7-day chronic tests but not in concurrent chronic tests with *C. dubia* or concurrent acute tests with either organism.
2. High variability in survival among replicates. It was not uncommon for mortality in the receiving water control and/or lower effluent concentrations to range from 0 to 100% among replicates.
3. The dose response is often non-monotonic. That is, mortality is not always highest in the highest sample concentrations. In tests where unacceptable receiving water controls were noted, the receiving water controls and the lower effluent concentrations often showed similar lowered survival and high replicate variability while higher effluent concentrations did not show effects.
4. Mortality is often first noted in receiving water controls and lower effluent concentrations on day 4 of the chronic test, but not before (and not in the 4 day acute test). Once mortality is noted in an individual replicate, most, if not all of the fish in that replicate succumb before the test is completed.
5. Presence of fungal growth on the gills of dead and/or dying fish. This fungal growth may be attributed to *Saprolegnia* sp. and may have been a secondary infection.

While some of these characteristics were not always observed in tests with unacceptable receiving water controls, items 1,2 and 5 together were the most common.

## Chemical Toxicity vs. Biological Contamination

The characteristics listed above suggest that the lowered survival in receiving water controls was not due to chemical toxicity. In tests where chemical toxicity is shown, higher effects are expected in higher effluent concentrations, along with very low between-replicate variability. However, in the tests described above, the opposite was often true - lower effluent concentrations would show higher effects and high between-replicate variability would be present. These types of "reverse dose-response" patterns are usually associated with contaminated samples (e.g., due to collection with unclean equipment, lab errors, etc.). Other evidence pointing away from chemical toxicity includes the fact that the *C. dubia* species was not affected while the fathead minnow was. *C. dubia* is known to be more sensitive than the fathead minnow to most chemicals (ammonia being the most well known exception). The fact that the fish is affected and *C. dubia* isn't eliminates many chemical toxicants as suspects for causing this phenomenon.

Additional information comes from other states who have noted similar problems. Some laboratories, outside of Wisconsin, have performed toxicity identification evaluations on receiving water samples which showed similar effects to be pathogenic (Downey, et al, 2000; Kszos, et al 1997; Grothe, et al, 1996). Attempts to associate lowered survival with chemical toxicity were not successful in these TIE trials. Sample manipulations designed to remove metals, organics, and other chemical classes did not improve survival or lower between-replicate variability in receiving water samples with these symptoms. Further toxicity characterization of similar samples showed that only sterilization was effective in eliminating or reducing mortality. Autoclaving, pasteurization, addition of antibiotics, filtration and irradiation with ultraviolet light all improved survival and lowered between-replicate variability in affected receiving water samples. Another set of experiments showed that when living fish carrying fungus were removed from test beakers, the remaining fish were much more likely to survive (Downey, et al, 2000; Kszos, et al 1997).

In Texas, eight power plants observed similar effects as noted above in once-through cooling waters and receiving water controls. There, the effects occurred most often during late fall to early spring. Tennessee also reported this phenomenon in a number of streams. Data from New England indicate that shallow, slow running, highly urbanized streams were most likely to experience this phenomenon and a seasonal effect was also noted. Data from Massachusetts and Rhode Island show some rivers which consistently produce this phenomenon, others which show it periodically and some which do not show it at all (Downey, et al, 2000).

In Wisconsin, this phenomenon seemed to occur whenever surface waters were used in testing, throughout the year, and on a wide variety of streams and lakes (including headwaters, outstanding resource waters, & other locations where no point or non-point impacts were expected). As shown in Table 2.7.1 above, of a total 1,497 chronic *P. promelas* tests performed between 1988-98, 26% showed unacceptable survival in receiving water controls. Of a total of 124 receiving waters used in these tests, 91 (73.3%) showed these effects during one or more tests. These receiving waters range in size from large rivers to shallow, intermittent streams; and also occurred with waters taken from lakes, pools, and impoundments. Due to the numerous differences between the physical, chemical, anthropological, and climatic influences on all of these receiving waters, it is highly unlikely that the same chemical toxicant would be found in all of these locations.

Collectively, the factors listed above provide strong evidence which suggests that a microbiological interference, rather than chemical toxicity, was responsible for unacceptable receiving water controls.

### A Solution to the Problem

Research was conducted at the University of Wisconsin-Madison's State Lab of Hygiene (SLH) from 1998-2000 to determine why receiving water controls were performing poorly (Geis, 2003). The SLH conducted tests on 18 receiving water sites. Initially, microbiological work was done to isolate pathogenic organisms from receiving

waters, the fish and their food. This work showed that pathogens (e.g. *Flexibacter spp.*, *Aeromonas hydrophila*) could be found everywhere and that attempts to remove them from the lab (e.g. through decontamination of the fish and their food) were unsuccessful. This suggested that the problem was not the presence of bacterial organisms in certain samples, since these bacteria were always present, but rather conditions in the receiving water and WET test that caused these organisms to flourish (e.g., optimum nutrients, light, temperature, food, etc.).

Since this research suggested that the problem lay in the method itself, the SLH switched its focus from trying to identify the organisms at fault to how to change test methods to eliminate the phenomenon. The laboratory began testing different manipulations of the test set-up in order to try to eliminate this pathogenic effect. Analysis of the data showed that manipulations like filtering or irradiating the receiving water, using older (48-h) fish, using clean test cups each day of the test, and using smaller test cups with fewer fish per cup all significantly reduced the occurrence of the effect (reinforcing the theory that these effects are pathogenic and not toxicological). The use of smaller test chambers (30 mL cups) with 2 fish per chamber was significantly better at reducing the effect than all of the other treatments. It was also the simplest modification, which did not have as much potential to alter the chemical nature of effluent samples.

The Department asked WET laboratories to pilot this method from about 1999-2004. In all tests where this new method was used, no receiving water controls were unable to meet test acceptability criteria. Because of the overwhelming success of this research and subsequent test pilot, the Department modified its chronic fathead minnow test methods in the "*State of Wisconsin Aquatic Life Toxicity Testing Methods Manual, 2<sup>nd</sup> edition*", to require the use of the new smaller test chambers/2 fish per chamber method. New federal methods from USEPA in 2002 also reflected some changes due to the SLH's findings.

### **Action Following a Test With An Unacceptable RW Control**

In the past, the majority of tests where poor receiving water performance has been noted were most likely caused by the "pathogen effect". Since this phenomenon is not expected to occur when using the new fathead minnow chronic test methods, the Department will no longer excuse "inconclusive" tests that are a result of poor control survival. In some cases, these tests may have to be repeated.

Other sources can contribute to control problems as well. If proper test methods are used and the receiving water control still does not meet test acceptability criteria, the permittee should evaluate the situation to see if there are any obvious factors which may be contributing to the poor performance. For instance, the permittee should check to see that proper sampling protocols were used:

Chapter 1.1 of the WET Guidance Document: *"Equipment to be used for WET test sampling should be cleaned appropriately, in order to insure that samples do not have the potential for interfering biological organisms.... If a facility's sampler or a portable sampler is used, all tubing should be replaced with new tubing (including the pump head tubing). If this is not possible, all tubing should be cleaned according to the requirements in the State of Wisconsin Aquatic Life Toxicity Testing Methods Manual..."*

Section 3.12 of the Methods Manual: *"All sample containers that are reused shall be cleaned according to the following procedures, except where sampling equipment may not be compatible with acids or acetone, in which case the manufacturer's recommended cleaning procedures should be followed:*

- 1. Soak 15 minutes and scrub with detergent in tap water, or clean in an automatic dishwasher.*
- 2. Rinse twice with tap water.*
- 3. Rinse with 10% HCl or 10% HNO<sub>3</sub> (v:v) to remove scale, metals, and bases. **Caution:** HNO<sub>3</sub> is a strong oxidizer and may react and combust with acetone.*
- 4. Rinse twice with tap water.*
- 5. Rinse once with liberal amounts of fresh, full-strength, reagent grade acetone (or an alternate solvent approved for use by the DNR) to remove organic compounds. Use a fume hood or canopy.*
- 6. Rinse three times with distilled or deionized water."*

**Receiving water samples should be treated with the same care as effluent samples. All buckets, funnels, or other equipment used to collect receiving water samples should be new or cleaned according to the requirements specified above, in order to avoid the introduction of interfering organisms or contaminants.**

Other actions the permittee can take is to check if the sample was taken near another discharge, a dam or other physical structure, or another potentially toxic source. When poor receiving water performance has been noted, it may be necessary to identify another location on the same receiving water or another, similar surface water within the same basin or watershed that can be used. Every attempt should be made to identify a waterbody with similar physical and chemical characteristics (e.g., pH, alkalinity, hardness) as the receiving water. The receiving water control results, subsequent sampling evaluation, and change in sampling location (if applicable) should be noted in the test report or accompanying cover letter.

In situations where no alternative receiving water can be identified, it may be necessary to substitute laboratory water as the primary control water and diluent. If the receiving water has shown poor performance repeatedly and no obvious cause or contributing factor can be found, and no alternative receiving water location can be identified, subsequent tests of the discharge may be completed using laboratory water as the primary control water and diluent (after receiving written approval from the Department). A receiving water control should be set in conjunction with the test, as the secondary control, so receiving water performance can be monitored. If after subsequent tests the receiving water performance appears to have improved, the Department may again require the receiving water to be used as the primary control water and diluent. Acceptable laboratory control water can be synthetic (reconstituted) or natural uncontaminated ground or surface waters collected from another source. When laboratory water is used as the primary test control or diluent water source, hardness (as CaCO<sub>3</sub>) must be adjusted as required in the Methods Manual (Section 4, part 4.3).

#### **References:**

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Geis, Steven W.; Kari Fleming, Amy Mager and Lou Reynolds. 2003: "*Modifications To The Fathead Minnow (Pimephales Promelas) Chronic Test Method To Remove Mortality Due To Pathogenic Organisms*". Environmental Toxicology and Chemistry: Vol. 22, No. 10, pp. 2400–2404.

Grothe, Donald R. and Johnson, Daniel E.. 1996. *Bacterial Interference In Whole Effluent Toxicity Tests*. Environmental Toxicology and Chemistry, Vol. 15, No. 5, pp. 761-764.

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