What's the Buzz?
EPA-ORD is Developing Method Guidance for More Species for Effluent and Ambient Toxicity Testing Methods

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USEPA Office of Research and Development

SETAC North America Annual Meeting
Toronto Canada

Topics

- Background
- Rationale for new taxa
- Phase One - species/test methods
- Overview of each method
- Method validation plans
- Intra and Interlaboratory plans
- Phase Two – species/test methods

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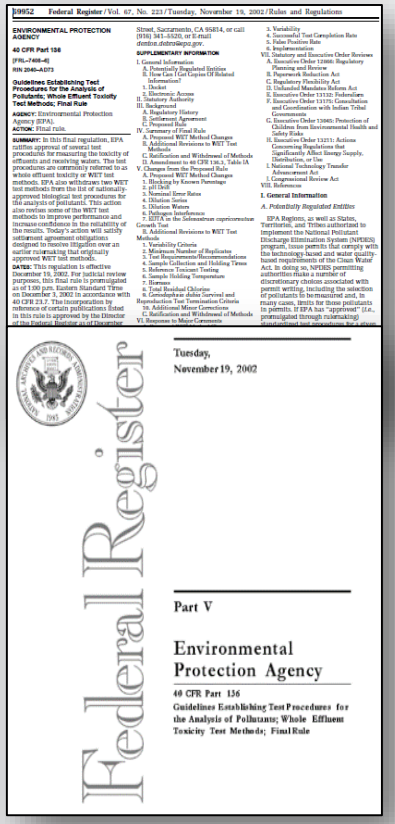
WET Effluent Test Methods



- EPA finalized the Part 136 of the Clean Water Act (CWA) rulemaking and incorporated the methods by reference in 2002.
  - EPA promulgated 16 methods for acute and short term test species to use to estimate acute and chronic toxicity, see Table IA in the 40CFR.
  - EPA's test methods must be followed as they are written, methods are 'codified' in regulation.
  - NPDES permits and permit re-issuance incorporate the method/manuals into the permit; along with clarifications and errata.

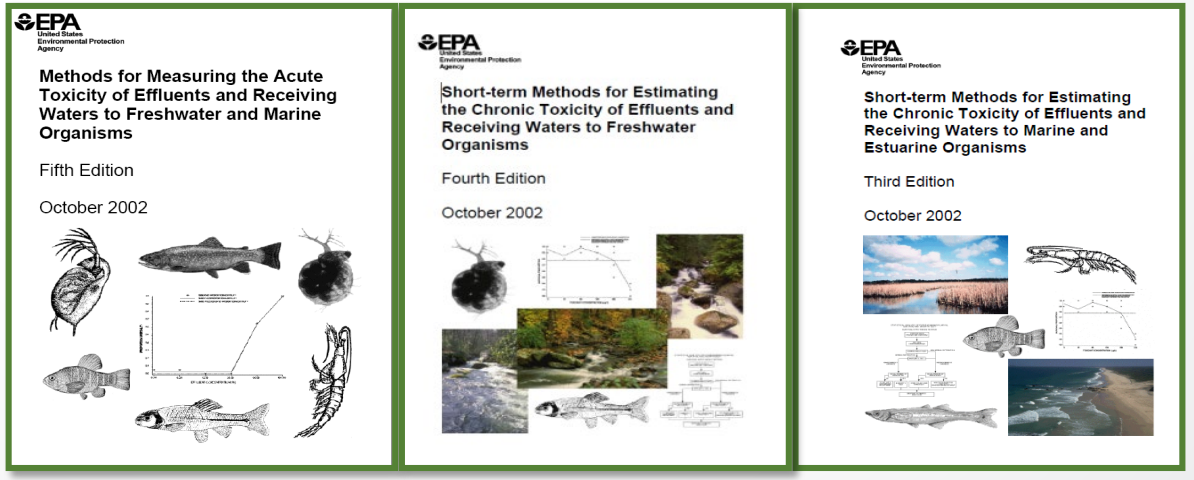
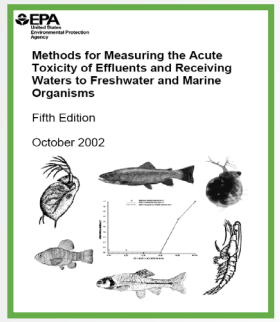
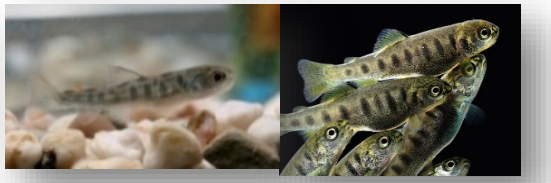


Table IA 40 CFR 136.3 Freshwater Marine Acute & Short-term Chronic Test Methods for Effluents & Ambient Waters

Toxicity, **acute, fresh water** organisms, LC_{50}, percent effluent



Fathead minnow, *Pimephales promelas* & Bannerfish Shiner, *Cyprinella leedsi*
48 or 96 h
Acute Method 2000.0

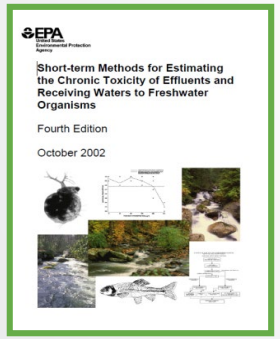


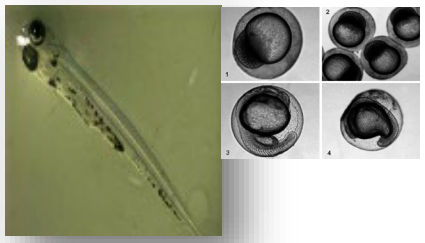
Rainbow trout and Brown Trout
Oncorhynchus mykiss & Salvelinus fontinalis
48 or 96 h
Acute Method **2019.0**

Cladoceran
Ceriodaphnia dubia
48 h
Acute Method **2002.0**

Cladocerans,
Daphnia magna & Daphnia pulex
48 h
Acute Method **2021.0**

Toxicity, **chronic, fresh water** organisms, NOEC or IC25, percent effluent





Fathead minnow
Pimephales promelas
7-d, daily renewal or Teratogenicity Test 8-d
Short-term Chronic Methods **1000.0 & 1001.0**

Cladoceran
Ceriodaphnia dubia
7-d renewal, daily
Short-term Chronic Method **1002.0**

Green algae
Selenastrum subcapitata
96h static/one sample
Short-term Chronic Method **1003.0**
(known as *Raphidocelis subcapitata)*

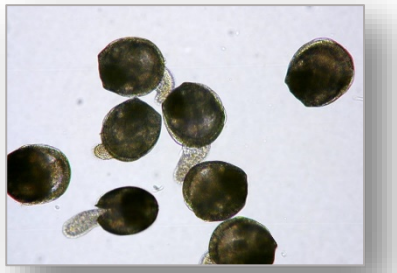
Rainbow trout ~28 d old Photo by George Novak

Method Manuals are Incorporated by reference, "Methods for Freshwater &/or Marine Acute and Short-term Chronic Tests", cf., www.epa.gov/cwa-methods/whole-effluent-toxicity-methods

Species Selection for Application to WET

- Identification of the specific WET methods to be by Office of Wastewater Management (OWM) and identified as a need by the EPA Water Divisions
- Toxicity testing standard methods are needed in the evaluation of effluents and ambient waters
  - point sources,
  - resource extraction (i.e., waters with elevated ions and/or conductivity),
  - agricultural activities (i.e., waters with complex pesticide mixtures).
- Initial WET test development and validation will focus on refining the development of assays with
  - Mussel, fatmucket (*Lampsilis siliquoidea*)
  - Cladoceran, *Daphnia magna* – 4-d Short Term Chronic

Method Development: Freshwater Invertebrate: Mussel Species, Fatmucket (*Lampsilis siliquoidea)*



Newly metamorphosed
~2-d-old fatmucket
(0.25-0.30 mm length)

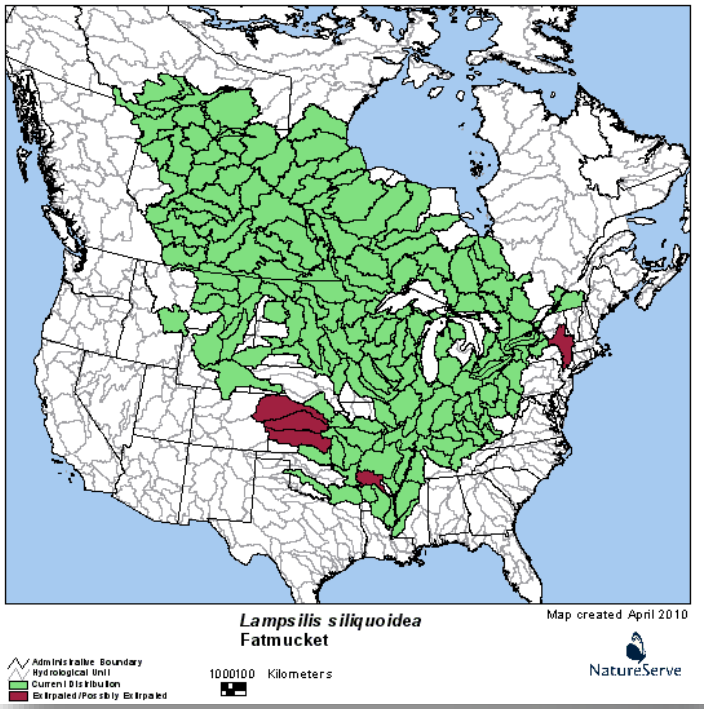
~2-month-old fatmucket
(~2 mm length)

Adult fatmucket

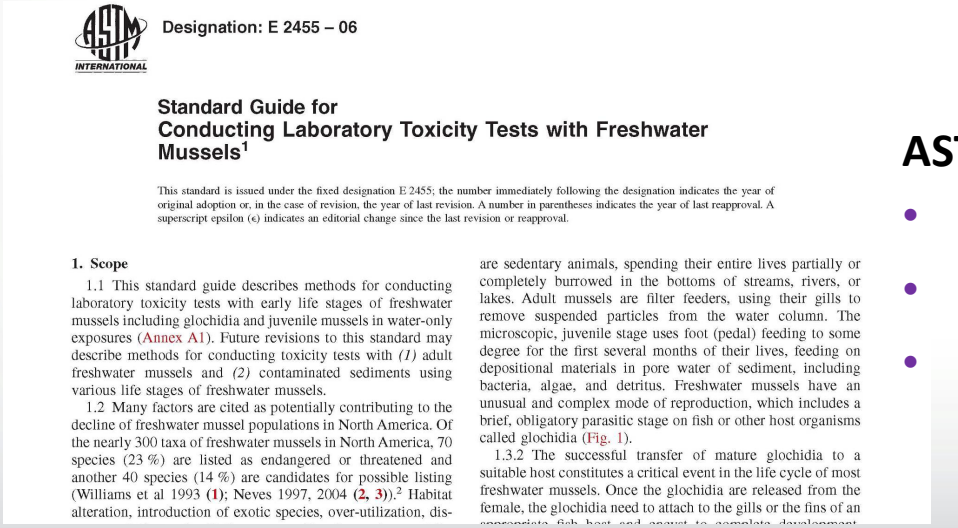
- Mussels are widely distributed in North America and are long lived, living from 2 to several decades
- Unusual and complex mode of reproduction.
  - A brief, obligatory parasitic stage on fish or other host organisms called glochidia.
  - Glochidia and juvenile mussels are ecologically and physiologically different from adult mussels, protection of habitat quality of adult life stages may not be protective of glochidia or juvenile life stages of freshwater mussels.
  - Distributions of adult mussels are dependent both on the presence of host fish and on microhabitat conditions.
- Long-term brooder, available through the year for culture and testing and the fatmucket can be cultured in the laboratory

Photos courtesy of C. Barnhart

Freshwater Invertebrate, Mussel: Fatmucket, *Lampsilis siliquoidea*



- Fatmucket
  - Demonstrated to be among the most sensitive of the various aquatic species to some contaminants, including ammonia, chloride, sulfate, potassium, copper, nickel, and zinc.
  - Has been successfully used in toxicity testing to support water quality criteria development



Designation: E 2455 – 06

Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels[1]

This standard is issued under the fixed designation E 2455; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This standard guide describes methods for conducting laboratory toxicity tests with early life stages of freshwater mussels including glochidia and juvenile mussels in water-only exposures (Annex A1). Future revisions to this standard may describe methods for conducting toxicity tests with *(1)* adult freshwater mussels and *(2)* contaminated sediments using various life stages of freshwater mussels.

1.2 Many factors are cited as potentially contributing to the decline of freshwater mussel populations in North America. Of the nearly 300 taxa of freshwater mussels in North America, 70 species (23 %) are listed as endangered or threatened and another 40 species (14 %) are candidates for possible listing (Williams et al 1993 (**1**); Neves 1997, 2004 (**2, 3**)).[2] Habitat alteration, introduction of exotic species, over-utilization, dis-

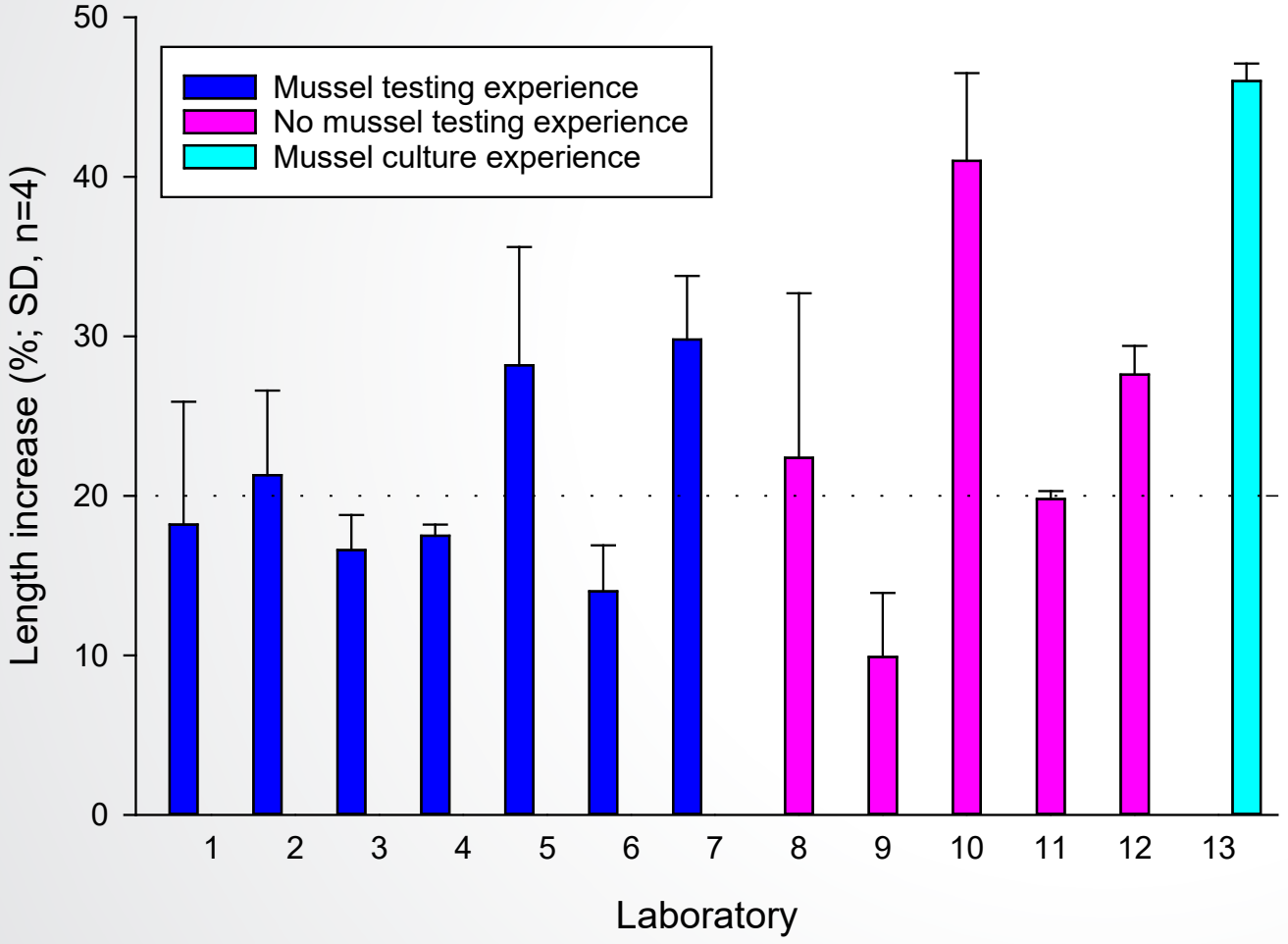
are sedentary animals, spending their entire lives partially or completely burrowed in the bottoms of streams, rivers, or lakes. Adult mussels are filter feeders, using their gills to remove suspended particles from the water column. The microscopic, juvenile stage uses foot (pedal) feeding to some degree for the first several months of their lives, feeding on depositional materials in pore water of sediment, including bacteria, algae, and detritus. Freshwater mussels have an unusual and complex mode of reproduction, which includes a brief, obligatory parasitic stage on fish or other host organisms called glochidia (Fig. 1).

1.3.2 The successful transfer of mature glochidia to a suitable host constitutes a critical event in the life cycle of most freshwater mussels. Once the glochidia are released from the female, the glochidia need to attach to the gills or the fins of an

ASTM mussel standard methods include:

- Acute 24-h with glochidia (endpoint: viability)
- Acute 96-h with juvenile mussels (survival)
- Chronic 28-d with juvenile mussels (survival, growth)

Interlaboratory Evaluation of the 7-d mussel growth test



- Results from a project with USGS-Columbia.
- Found variation in growth among the labs depending on experience level with mussels:
  - 1- to 3-week-old fatmucket grew substantially over 7-d period, average length increases 20-30%

Method Development: *Daphnia magna* 4-d

Test Overview

- Static-renewal, 4 d
- 25 ± 1°C
- Survival and growth (mean dry weight) (required)
- ≥ 90% or greater control survival and growth 10X initial dry weight (required)

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DEVELOPMENT AND VALIDATION OF A DAPHNIA MAGNA FOUR-DAY SURVIVAL AND GROWTH TEST METHOD

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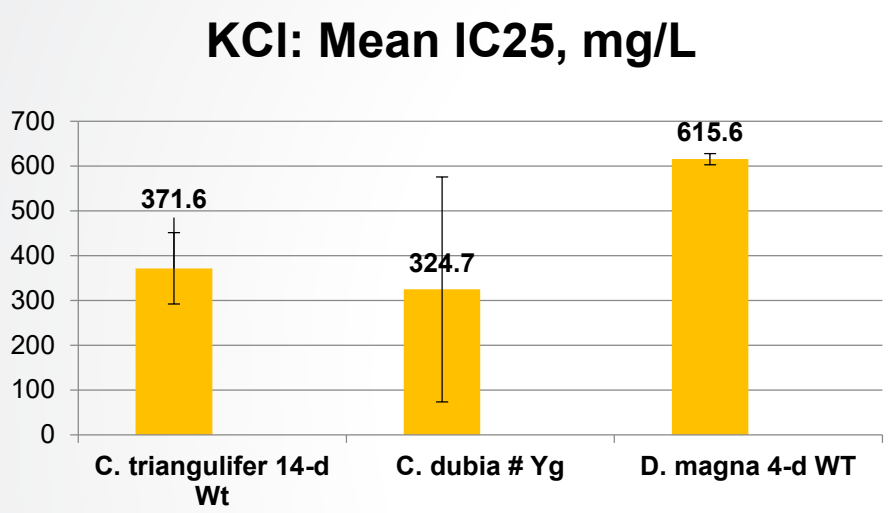
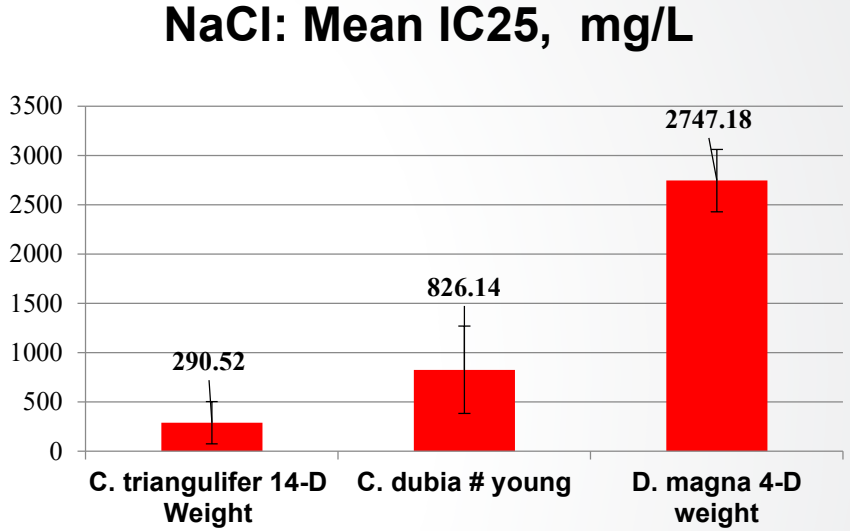
(Received 26 June 2008; Accepted 11 November 2008)

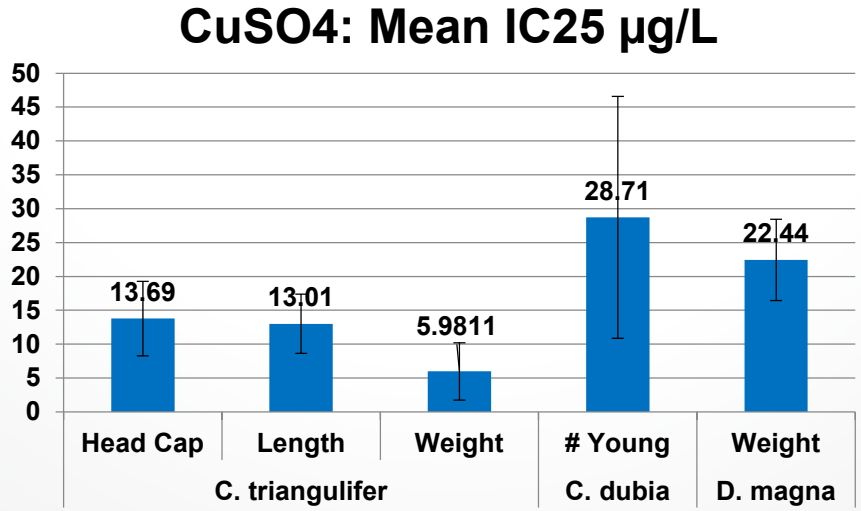
Abstract—Zooplankton are an important part of the aquatic ecology of all lakes and streams. As a result, numerous methods have been developed to assess the quality of waterbodies using various zooplankton species. Included in these is the freshwater species Daphnia magna. Current test methods using D. magna involve acute lethality test methods ranging from 24 to 96 h in duration and chronic test methods with durations of 21 to 28 d. Whereas the current acute and chronic test methods are useful, a need exists for a shorter-duration test method that will provide a chronic or subchronic endpoint with this species. In the present study, a 4-d, static–renewal survival and growth test was developed for use with D. magna. The test results were compared to per for mance criteria and results from 7-d survival and reproduction tests with Ceriodaphnia dubia to determine the level of comparability between the two methods. Results from the 4-d D. magna survival and growth test method indicated that this method will produce consistent results with various reference toxicant materials and provide data that are both reproducible and useful for detecting potential toxicity in aquatic environments.

Keywords— Daphnia magna Test method Short-chronic Growth Ceriodaphnia dubia

Comparative Test Results for KCl, NaCl and Copper with *D. magna*, *C. dubia*, and *C. triangulifer*







Chronic (IC25) Results (2014) based on the average of 3 tests

Considerations and Recommendations for Validation of a New Method

- Study Plan
- Rationale
- Method Procedures
- Test Conditions
- QA/QC
- Endpoints (biological)
- Sensitivity, Side-by-Side testing with existing approved mehtods
- Intra and Interlaboratory Precision
- Sample Types, effluents and chemicals
- Nationwide Use Requirements

Second Phase: Methods for WET Testing

Potential species and test methods

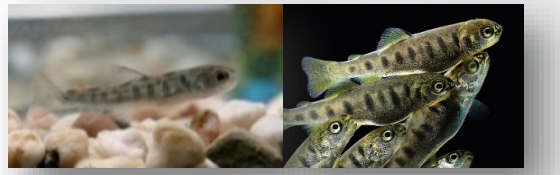
- **Mayfly (*Neocloeon triangulifer*)**
  - 96 h acute
  - short-term chronic growth test

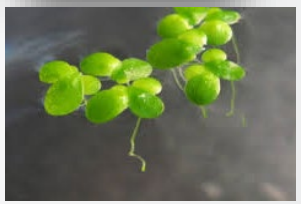
- **Trout, rainbow trout (*Oncorhynchus mykiss)*; brook trout (*Salvelinus* Fontinalis.**
  - 7-d growth test

- **Amphipod, *Hyalella azteca***
  - 96 h acute test (survival) and
  - short-term growth test (10-d, 28-42 days)

- **Midge, *Chironomus dilutus***
  - 96 h acute test (survival) and
  - short-term growth test (10-d, 20-56 days)

- **Plant species**
  - *Lemna minor* (duckweed)

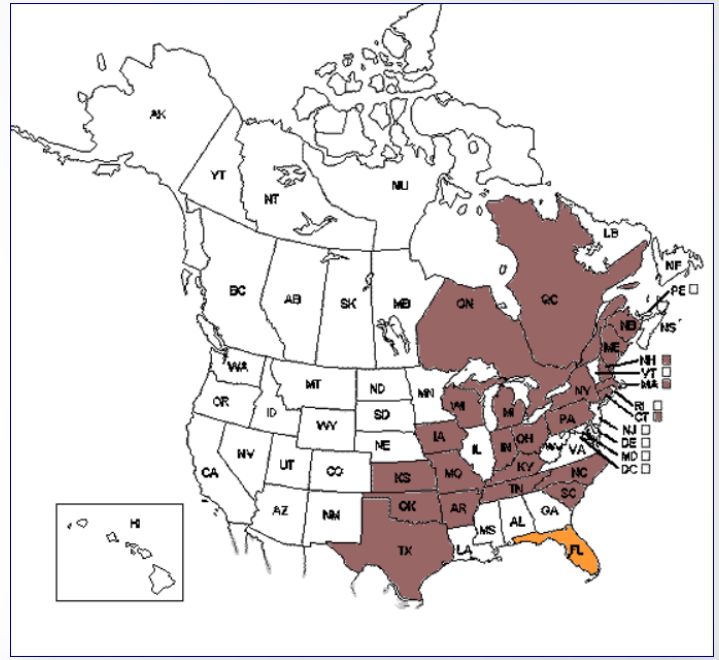




Freshwater Invertebrate, Mayfly, Neocloeon triangulifer (RARE project)

- Mayflies have been shown to be a sensitive species when tested with several metals and major ions.
  - Parthenogenic species
  - Has been demonstrated to be particularly sensitive to major ions, including chloride and sulfate that are components of water conductivity) and metals
  - Have been successfully used in toxicity testing to support water quality criteria development.

[Genus name changed from *Centroptilum* to *Neocloeon*]



Mayfly, *Neocloeon triangulifer* clone WCC-2 (Stroud WRC)

Mayfly, *Neocloeon (Centroptilum) triangulifer* (Ephemeroptera: Baetidae)

- EPA Cincinnati developed a culture and generated toxicity data
  - Weaver et al. (developed a culture method for *Neocloeon triangulifer* (Part 1)
  - Struewing et al compared the sensitivities of larval mayflies to several reference toxicants (NaCl, KCl, and CuSO4) (Part 2) using a 14-d test (growth endpoint).

- Studies that measured 7-d growth and survivorship in
  - NaCl 1-d, 3-d, 5-d-old larvae
  - Interlaboratory evaluation with 1-d old larvae from 5 different laboratory cultures (two U.S. EPA, USGS, University of Illinois, and Ontario Ministry of the Environment).
  - Compared 7-d vs 14 d growth IC25s

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Part 1: Laboratory culture of *Centroptilum triangulifer* (Ephemeroptera: Baetidae) using a defined diet of three diatoms

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HIGHLIGHTS

- A reproducible culture method for a parthenogenetic mayfly species for use in toxicity tests.
- The culture method has been used for over 9 generations.
- Young have been successfully tested in toxicity tests with high control survival and growth.
- Methods for culturing diatoms that can be used for culture and testing of *Centroptilum triangulifer*.

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ABSTRACT

Development of methods for assessing exposure and effects of waterborne toxicants on stream invertebrate species is important to elucidate environmentally relevant information. Current protocols for freshwater invertebrate toxicity testing almost exclusively utilize cladocerans, amphipods or chironomids rather than the more typical aquatic insect taxa found in lotic systems. *Centroptilum triangulifer* is a parthenogenetic mayfly occurring in depositional habitats of streams and rivers of the Eastern U.S. and Canada. *C. triangulifer* is an ideal stream insect for toxicity testing under field and laboratory conditions because of its short life cycle, parthenogenetic mode of reproduction, and it represents a group considered sensitive to environmental stressors. In this study, a colony of *C. triangulifer* was reared using a defined diet of three diatoms, *Mayamaea atomus var. permitis*, *Nitzschia cf. pusilla*, and *Achnanthidium minutissimum*. Percent survival (>80%), fecundity measurements (>1000 eggs) and pre-egg laying weights were used as indicators of overall colony health and fitness in our laboratory water (Lab-line) and in Moderately Hard Reconstituted Water (MHRW). Lab-line reared *C. triangulifer* had average survival rate of 92.68% for eleven generations and 82.89% over thirteen generations. MHRW reared *C. triangulifer* had an average survival rate of 80.65% for four generations and three generations of fecundities greater than 1000 eggs per individual. Pre-egg laying weight and fecundity were highly correlated and a best-fit model equation was derived to estimate egg counts for future generations. Establishment of this cultur-

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Part 2: Sensitivity comparisons of the mayfly *Centroptilum triangulifer* to *Ceriodaphnia dubia* and *Daphnia magna* using standard reference toxicants; NaCl, KCl and $CuSO_4$

Katherine A. Struewing[a,1], James M. Lazorchak[b,*], Paul C. Weaver[a], Brent R. Johnson[b], David H. Funk[c], David B. Buchwalter[d]

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[c]Stroud Water Research Center, Avondale, PA, United States
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HIGHLIGHTS

- New mayfly test species for use in toxicity tests more sensitive than standard test species.
- Procedures for conducting acute and chronic tests with a sensitive mayfly are presented.
- Testing with *Centroptilum triangulifer* may afford more protection to EPT species.

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ABSTRACT

Criteria for establishing water quality standards that are protective for 95% of the native species are generally based upon laboratory toxicity tests. These tests utilize common model organisms that have established test methods. However, for invertebrates these species represent mostly the zooplankton community and are not inclusive of all taxa. In order to examine a potential under-representation in emerging aquatic invertebrates the US Environmental Protection Agency has cultured a parthenogenetic mayfly, *Centroptilum triangulifer* (Ephemenoptera: Baetidae). This study established a 48 h acute and a 14-day short-term chronic testing procedure for *C. triangulifer* and compared its sensitivity to two model invertebrates, *Ceriodaphnia dubia* and *Daphnia magna*. Toxicity tests were conducted to determine mortality and growth effects using standard reference toxicants: NaCl, KCl and $CuSO_4$. In 48-h acute tests, the average LC50 for the mayfly was 659 mg L^{-1} NaCl, 1957 mg L^{-1} KCl, and 11 µg L^{-1} $CuSO_4$. IC25 values, using dry weight as the endpoint, were 228 mg L^{-1} NaCl, 356 mg L^{-1} KCl and 5 µg L^{-1} $CuSO_4$. *C. triangulifer* was the most sensitive species in NaCl acute and chronic growth tests. At KCl concentrations tested, *C. triangulifer* was less sensitive for acute tests but was equally or more sensitive than *C. dubia* and *D. magna* for growth measurements. This study determined *C. triangulifer* has great potential and benefits for use in ecotoxicological studies.

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RARE PROJECT: Development/Improvement of Methods for using Mayflies in WET Tests

- Goal of assessing mayflies as a species for WET test procedures.
  - acute (4-d) and
  - short-term chronic (7-d)
- Evaluated the optimum organism age at start of test with goal of finding best combination of maximum sensitivity and minimum test duration.
- Investigated influence of various aspects of diatom culture technique on food quality and therefore mayfly growth.
- *See platform* 227 at 10 a.m. Refining culturing and effluent testing methods for the mayfly, *Neocloeon triangulifer, by DJ Soucek*

Trout, Midge, Amphipod, Plant

Trout, rainbow trout (*Oncorhynchus mykiss)*

- Rainbow trout - 3 to 6 days post swim-up fry (age, 15–25 days)
- brook trout - 12 to 20 days post-swim-up fry (age, 30–45 days).
- 15 C
- 5 fish/400 ml solution/4 reps
- 0.5 ml brine shrimp/beaker, twice daily
- 7-d growth test ‡90% Control survival and minimum growth
- 1.5X initial x dry weight


Arch Environ Contam Toxicol 53, 397–405 (2007)
DOI 10.1007/s00244-006-0227-8

Rainbow Trout (*Oncorhynchus mykiss*) and Brook Trout (*Salvelinus fontinalis*) 7-Day Survival and Growth Test Method

James M. Lazorchak · Mark E. Smith

Received: 1 November 2006 / Accepted: 2 April 2007
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Abstract A short-term method was developed in this study for conducting subchronic survival and growth renewal toxicity tests with rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). Previously published early life-stage methods for various salmonid species involve test durations of 30 to 90 days. This trout method, however, follows a previously published 7-day fathead minnow (*Pimephales promelas*) growth method. The tests performed in this study measured subchronic growth and survival effects using standard reference toxicants (ammonium chloride, potassium chloride, phenol, and zinc sulfate), receiving water, and effluent samples. The test results were compared with performance criteria and results for 7-day survival and growth tests with *P. promelas* to determine the level of comparability between the two species. The results from tests with both salmonid species indicated that this 7-day survival and growth test method using *O. mykiss* and *S. fontinalis* provides reproducible results with various reference toxicant materials and can be used successfully to detect potential toxicity in environmental samples.

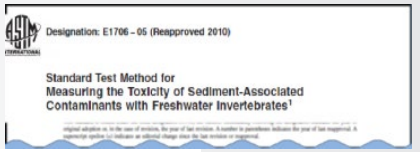
Introduction

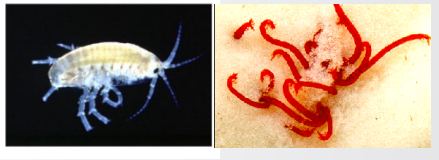
Western and New England states are concerned with the effects that contaminants may have on salmonid species (e.g., rainbow, cutthroat, brook, and brown). These species are important indicators denoting the overall biologic integrity of these regions, especially that of areas in which salmonid species constitute a significant portion of the overall fish biota.

The available early life-stage growth methods for salmonid species involve test durations of 30 to 90 days (ASTM 2006a; Environmental Canada 1998). These long durations make it difficult to conduct tests under field

Amphipod and midge

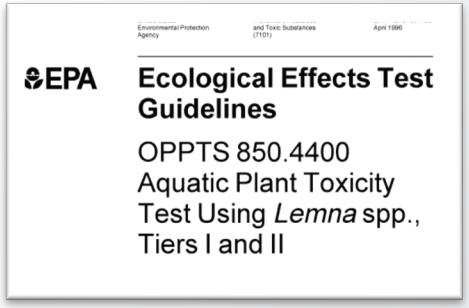
- Variations of existing methods
- 96 h and 7-day exposures
- *Hyalella azteca* and *Chironomus dilutus*
- 23 C
- Endpoints - 96 h acute test (survival) and short-term growth test





Plant species

- *Lemna minor* (duckweed)



Method Study Plans

- Refine the method procedures for both the mussels and *Daphnia magna* for acute and chronic toxicity.
  - Study Plan and Rationale
  - Method Procedures and Test Conditions
  - QA/QC
  - Endpoints
  - Sensitivity with other current test species
  - Interlaboratory precision
  - Nationwide Use requirements
- Perform testing with chemicals of various mode of action and conduct the tests in two laboratories (Duluth and Cincinnati)
  - Evaluate the test method with effluents provided by the Regions.
- Establish a workgroup from govt, academia, and private sector for incorporating options in the method development.
- Prepare test method guidance.