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# USEPA Research on Mussels and Magma: Developing Toxicity Testing Methods And Guidance for New Species for Effluents and Ambient Waters

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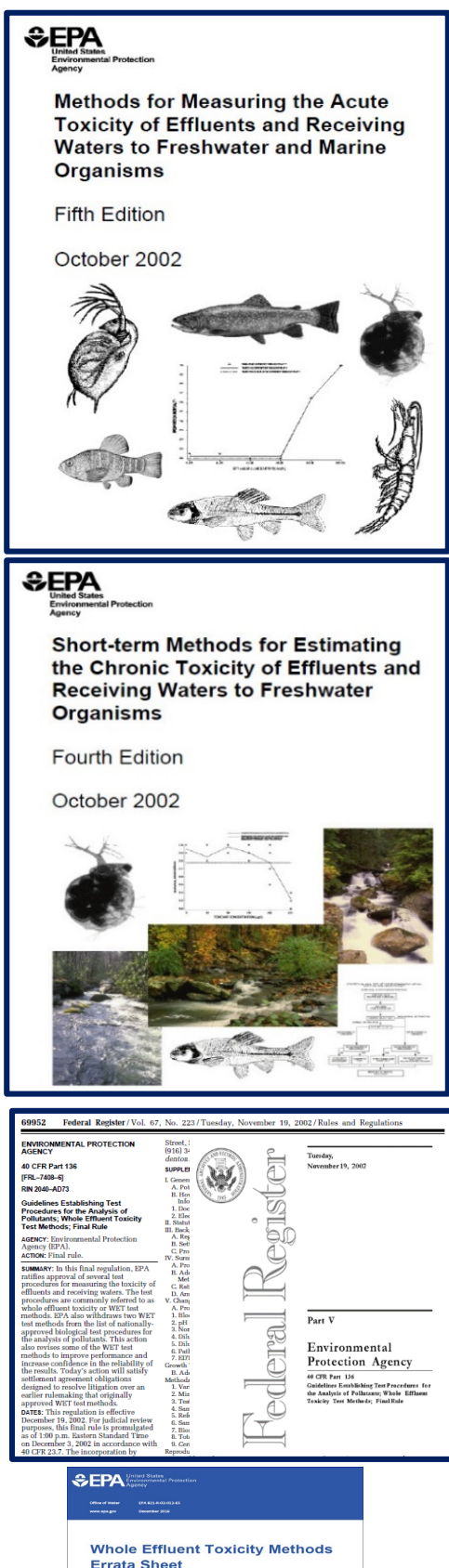
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## EPA'S TOXICITY TEST METHODS

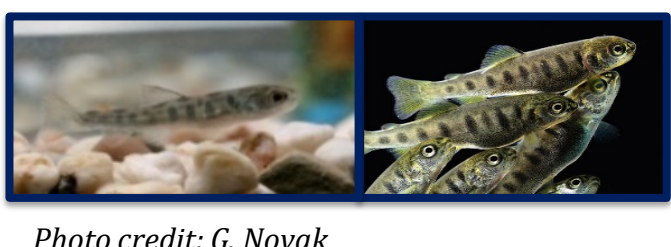
- EPA has established aquatic toxicity test methods for conducting water quality assessments on both effluents and receiving waters that are used all over the U.S. for determining possible impacts to aquatic life, and some are also used by other countries for the same purpose.
- EPA has numerous toxicity methods for acute and short-term chronic test species\* used for states across the U.S. (freshwater tests) and for states along the Atlantic Ocean and Gulf of Mexico (estuarine/marine tests) (Photos). All of EPA's effluent toxicity test methods ([www.epa.gov/cwa-methods/whole-effluent-toxicity-methods](http://www.epa.gov/cwa-methods/whole-effluent-toxicity-methods)) provide guidance so that the toxicity data generated are representative of the sample(s) assessed, and produce valid and scientifically defensible data. This is accomplished by having both mandatory and recommended provisions in the toxicity methods. EPA's toxicity manuals provide standardized test methods which include quality assurance/quality control procedures that support scientific defensibility.
- In this poster, we provide details for methods that are under development within the Office of Research and Development Laboratories in Duluth, MN and Cincinnati, OH. The current focus is the development of an effluent test protocol for a mussel for acute and short-term chronic tests and the addition of a short-term chronic method for the cladoceran, *Daphnia magna*. The current species for both the freshwater acute and short-term chronic test methods for effluents & ambient waters are shown below (CFR Table IA 40 CFR 136.3)



### Toxicity, **acute**, fresh water organisms, LC<sub>50</sub>, percent effluent (CFR Table IA 40 CFR 136.3)



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



Rainbow trout, *Oncorhynchus mykiss*, & brown trout *Salvelinus fontinalis*  
48 or 96 h  
Acute Method **2019.0**

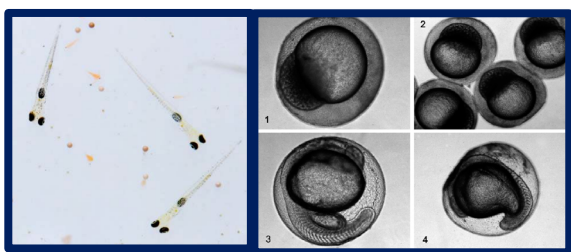


Cladoceran, *Ceriodaphnia dubia*  
48 h  
Acute Method **2002.0**



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

### Toxicity, **chronic**, fresh water organisms, NOEC or IC25, percent effluent (CFR Table IA 40 CFR 136.3)



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



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



Green algae,\*\*\*  
*Selenastrum capricornutum*  
96 h static/one sample  
Short-term Chronic Method  
**1003.0**

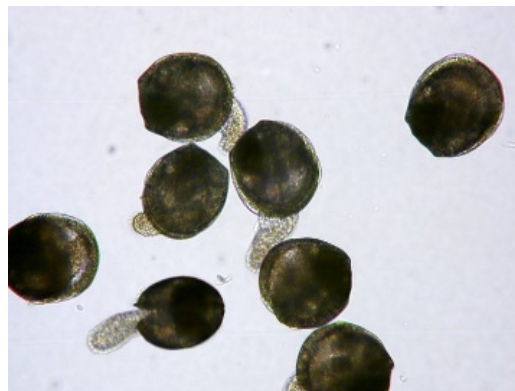
- \* The EPA method manuals (available at [www.epa.gov/cwa-methods/whole-effluent-toxicity-methods](http://www.epa.gov/cwa-methods/whole-effluent-toxicity-methods)) are incorporated by reference into the US Code of Federal Regulations (CFR) Rulemaking (<https://www.govinfo.gov/content/pkg/FR-2002-11-19/html/02-29072.htm>)
- \*\* The micro alga has been renamed, *Raphidocelis subcapitata*, formerly *Selenastrum capricornutum* (as shown above and in the published manuals and CFR rule publication).
- \*\*\* For an interim it was also named *Pseudokirchneriella subcapitata*. All names are synonymized.

## TWO TEST METHODS UNDER DEVELOPMENT

Adult *D. magna* with eggs in brood pouch (right) and neonate (below)



Newly metamorphosed ~2-day-old Fatmucket (0.25 -0.3 mm length)



~2 month old fatmucket (1-2 mm length)

### Cladoceran, *Daphnia magna* sublethal test

- Daphnids are sensitive species and have been demonstrated to have reproducible results in a laboratory setting.
- Species is frequently used for toxicity testing for chemicals and for acute tests with effluents.
- As the *D. magna* chronic test, a three-brood test, is a 21-d test, this method focuses on developing growth test for *D. magna* at 25°C (rather than 20°C). Tests will be initiated with <24 h old young and the development of the method will include assessing growth at 4-d and 5-d. The growth endpoint will be a growth measurement rather than the three brood young production.

### The Fatmucket - *Lampsilis siliquioidea*

- Widely distributed in North America.
- Long-term brooder, available through the year for culture/testing. The fatmucket appears to be among the most sensitive of all aquatic species to some contaminants.
- It is relatively easy to culture in the laboratory
- Sensitive and reproducible results are obtained in a laboratory setting.
- ASTM Standard Methods include
  - 24 h acute test with glochidia (viability)
  - 96 h acute with juvenile mussels (survival)
  - 28-d exposure with juvenile mussels for the growth and survival effects
- Method development tests will be initiated with juveniles (1- to 3-wk-old) and after 7-d, growth rates are determined.



Algal mixture prepared daily: adding 1-mL Nano 3600™ and 2-mL Shellfish Diet 1800™ into 1.8-L water (~500 nL cell volume/mL; ASTM 2018)



## OBJECTIVE AND APPROACHES

- The objective of this project is to develop invertebrate assays test methods for short-term chronic toxicity test methods, specifically
  - Mussel, fatmucket (*Lampsilis siliquioidea*): acute (96 h) and 7-d Short-term Test to Estimate Chronic Toxicity
  - Cladoceran (*Daphnia magna*): 4, 5 d Short Term Chronic Test to Estimate Chronic Toxicity
- Method development will include testing of several single/reference chemicals and effluents for both species along with the standard WET species at two of EPA's research labs (i.e., Duluth and Cincinnati). Interlaboratory testing with both labs for both species is planned for both short term growth methods to estimate chronic toxicity for *D. magna* and the fatmucket.
  - Both labs will test the same 5 reference materials (KCl, ZnSO<sub>4</sub>, ammonia chloride, phenol, bifenthrin) three times each, and conduct a minimum of 5 effluents from wastewater treatment plants and industrial discharges. This will be done to demonstrate that the test can be reliably performed in different labs using new test method endpoints.
  - Various test parameters will be assessed including food, water, number of replicates, culture procedures and proficiency guidance for users.
  - For the *D. magna* growth method, the test duration to be tested will include a 4-day and 5-d test period. An evaluation of both the (4 day and 5 day) methods will be assessed based on test sensitivity, test method precision and test power.
  - For the fatmucket, comparisons of the age of initial organisms will be compared with the various effluents and reference toxicants. With the age range of 1, 2 or 3 week old, measurements of the starting age will be needed to determine the growth on a consistent basis (increase from initial weights). In addition the test development will consider test sensitivity, test method precision, and test power.
- Test method development will include an assessment of the relevance and reliability of the test method protocol as well as the refinement/optimization of the method and development of test protocols.

Exposure Conditions		
<b>Test Parameter</b>	<b>Mussel, <i>L. siliquioidea</i>, Method</b>	<b>Cladoceran, <i>D. magna</i> Method</b>
<b>Test Type</b>	Static renewal	Static renewal
<b>Test Duration</b>	7-d	4-d, 5-d
<b>Temperature</b>	25 ± 1 °C	25 ± 1 °C
<b>Test chamber size</b>	4 oz cup, with layer of silica sand, sieved to 0.2 mm particles	60 ml (suggested)
<b>Test solution volume</b>	100 ml	50 ml (suggested)
<b>Test water</b>	Reconstituted water	Reconstituted water
<b>Renewal of solutions</b>	Daily renewal, ~80% replacement	Daily (required)
<b>Age of test organism</b>	1, 2, or 3 week post-metamorphosis	< 24 h old (12 h age window) (required)
<b>Organisms /replicate</b>	10 juvenile mussels /replicate	20 neonates (suggested)/replicate
<b>Replicates</b>	4 replicates /concentration	4 replicates / concentration
<b>Test Concentrations</b>	Minimum of 5 with the control	Minimum of 5 with the control
<b>Feeding</b>	Algal mixture prepared daily, ramped diet (see photo below) 2 mL / 2X day for 1- to 2-wk-olds 3 mL / 2X daily for 3-wk-olds	Algae ( <i>Raphidocelis subcapitata</i> ) & alfalfa extract (required)
<b>Endpoint</b>	Mortality (gaped shell with swollen tissue and empty shell), growth (maximum shell length)	Survival and growth (mean dry weight) (required)
<b>Test acceptability criterion</b>	≥80% survival in the controls and growth criteria (percent increase) TBD	≥ 90% or greater control survival and growth 10X initial dry weight (required)

## LOOKING FOWARD

As the method development continues, the results will be presented at scientific meetings and collaborating with scientists that are members of the new Aquatic Toxicity Testing Interest Group (ATTIG) North America Society of Environmental Toxicology and Chemistry (SETAC) which has a charter for scientific exchange on toxicity methods for effluent. The ATTIG was formed to communicate the methods and learn from the scientific community and the ATTIG is composed of volunteers members that conduct toxicity testing (academia, government, and the business sector). This is a peer to peer discussion and has been effective for moving method development for a variety of species forward in a collaborative fashion.

Longer-term additional effluent toxicity test method may be needed for other invertebrate taxonomic groups so that we can enhance our understanding of the range of species sensitivities, aid in the detection of both known and unknown chemical and biological contaminants in wastewater and ambient waters and provide procedures to aid in prioritizing problem contaminants and mixtures.



Mayfly (*Neocleonea triangulifer*)



Amphipod, *Hyaella azteca*



Midge, *Chironomus dilutus*

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