

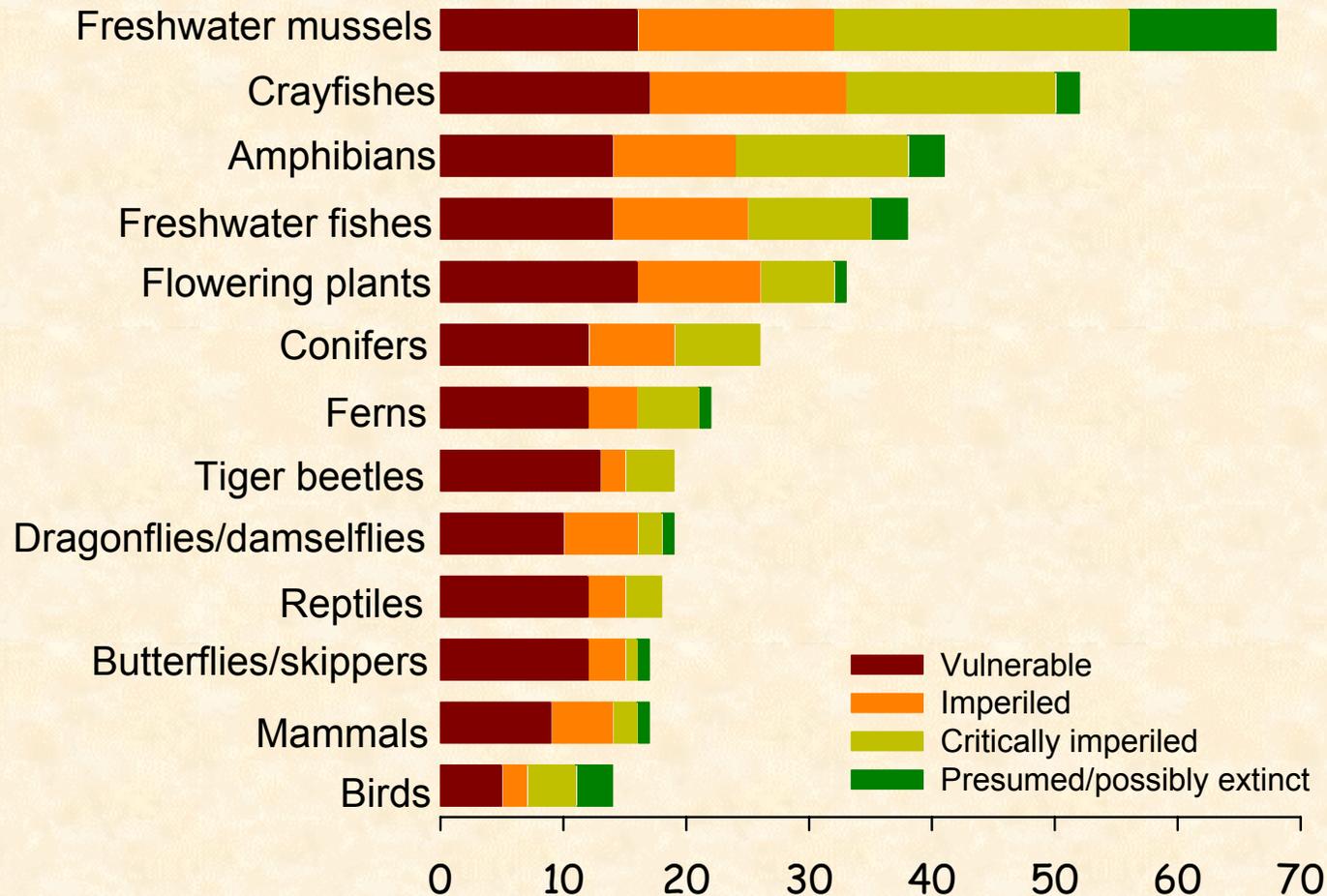
ASTM International standard guide for conducting laboratory toxicity tests with freshwater mussels (E2455-05)

Chris Ingersoll, Tom Augspurger, Chris Barnhart, Joe Bidwell, Cristi Bishop, Marsha Black, Greg Cope, Robert Bringolf, Jim Dwyer, Eugene Greer, Anne Keller, Greg Linder, Dick Neves, Teresa Newton, Rob Pepin, Andy Roberts, Cindy Roberts, Mike Salazar, Alan Samel, Chuck Stephan, John Van Hassel, Ning Wang, Tom Watters

USEPA Mussel Toxicity Testing Workshop
Chicago, IL, August 23 and 24, 2005



Organisms listed as vulnerable, imperiled or extinct in the United States

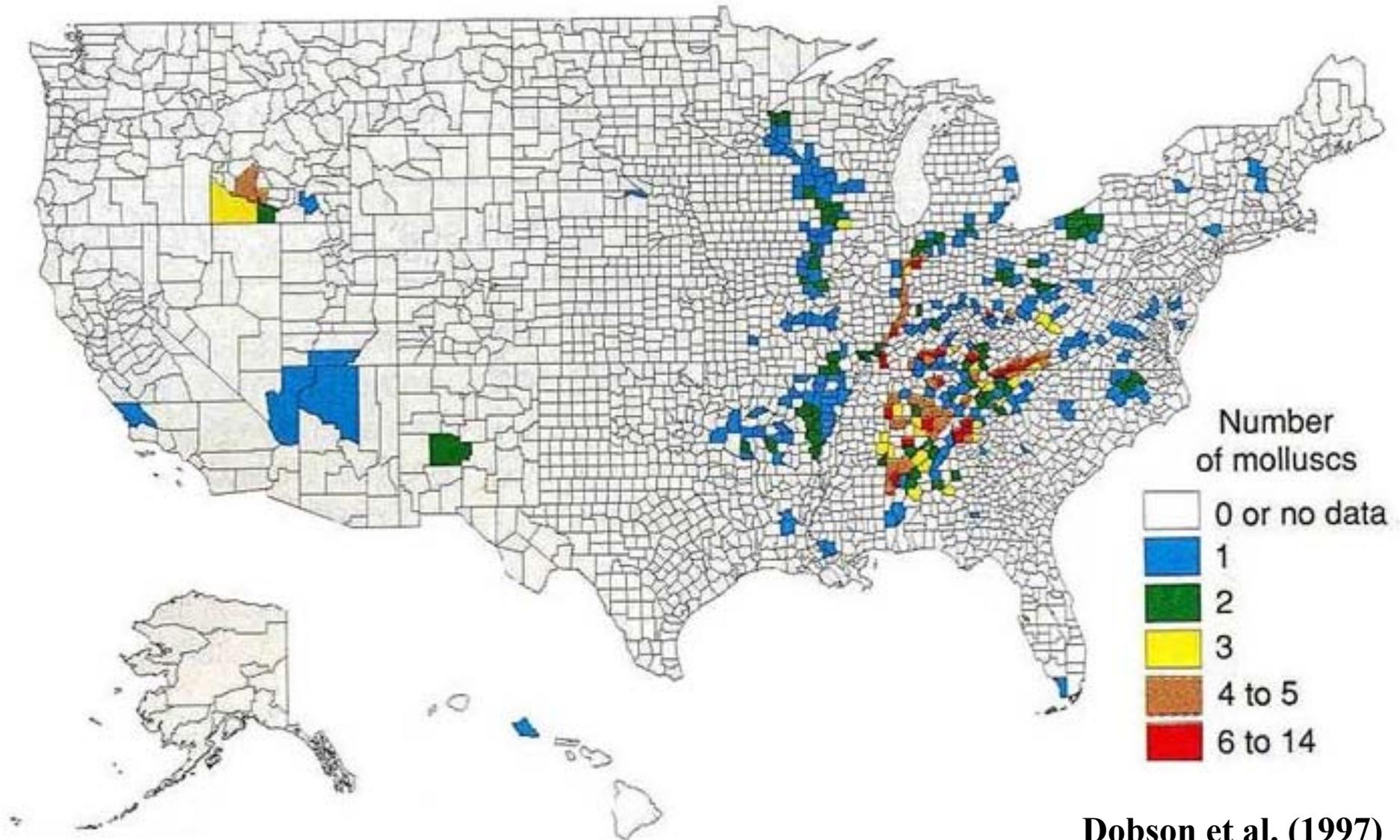


Potential causes for the decline of mussels in North America

- Greater than 70% of native unionid species in the United States listed as endangered, threatened, or of special concern
 - Habitat alteration
 - Introduction of exotic species
 - Over-utilization
 - Disease
 - Predation
 - Pollution



Geographic distribution of endangered mollusks



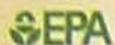
Dobson et al. (1997)

Overview

- ASTM International and Committee E47 on Biological Effects and Environmental Fate
- Methods outlined in Standard E2455-05:
 - Acute toxicity tests with glochidia
 - Acute toxicity tests with juvenile mussels
 - Chronic toxicity tests with juvenile mussels
- Test acceptability criteria including control survival
- Intra- and inter-laboratory precision in ammonia or copper toxicity tests with glochidia or juvenile mussels

ASTM International

- Global forum for developing consensus-based standards
- Focus on materials, products, systems, and services
- Founded in 1898 by engineers and chemists
- Over 30,000 members worldwide
- Comprised of technical experts in the field
- More than 100 countries represented



United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati, OH 45268

EPA/600/4-85/013
March 1985

Research and Development

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Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms

(Third Edition)

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ANNUAL BOOK OF ASTM STANDARDS

2003

SECTION ELEVEN

Water and Environmental Technology



Volume 11.05

Biological Effects and Environmental Fate;
Biotechnology; Pesticides



Revision Issued Annually

ASTM Standards

- Developed as:
 - Guides
 - Specifications
 - Test methods
- Technical documents developed by consensus
- Developed and used used voluntarily

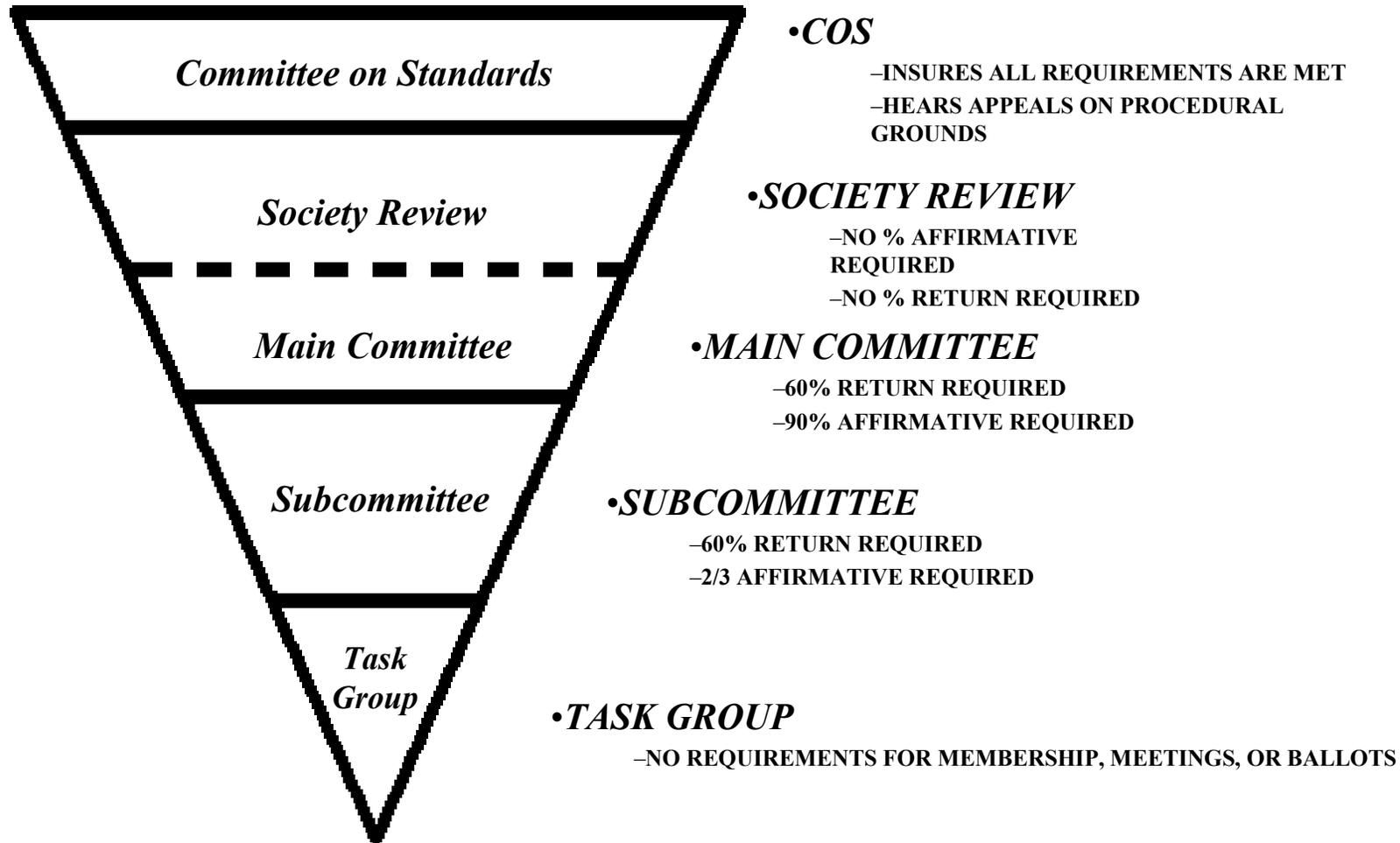
ASTM Committee participation

- Technical experts from industry, academia, government, non-government organizations (NGOs)
- Committees are open to all
- All members have an equal voice, equal vote
 - One vote per “voting interest”
- Process leads to worldwide acceptance of standards

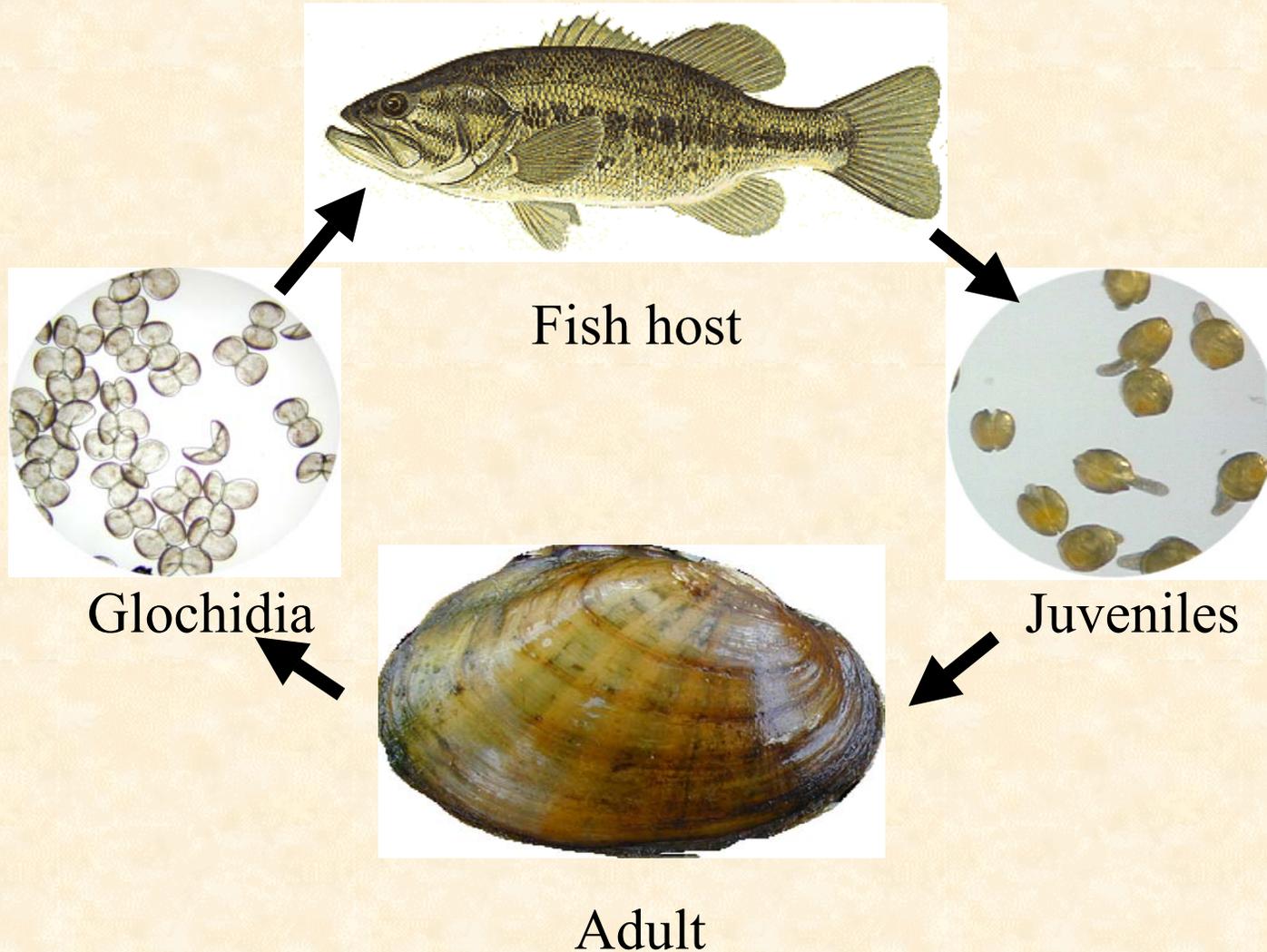
ASTM Committee E47 on Biological Effects and Environmental Fate

- Formed in 1980 as the first ASTM environmental committee
- 222 members in 6 Subcommittees:
 - E47.01: Aquatic Assessment and Toxicology
 - E47.02: Terrestrial Assessment and Toxicology
 - E47.03: Sediment Assessment and Toxicology
 - E47.04: Environmental Fate of Chemical Substances
 - E47.05: Risk Assessment, Communication and Management
 - E47.06: Terminology and Technical Services
- 81 standards with 5 new standards in development

ASTM Balloting Process



Life cycle of freshwater mussels



ASTM standard guide for conducting laboratory toxicity tests with freshwater mussels (E2455-05)

- Task group established: August 2004
- ASTM standards used to develop E2455-05:
 - ASTM Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians (E729-96 (2002))
 - ASTM Standard guide for conducting early life-stage toxicity tests with fishes (E729-98 (2004))
 - ASTM Standard test method for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates (E1706-05)

ASTM standard guide for conducting laboratory toxicity tests with freshwater mussels (E2455-05)

- Subcommittee ballot: December 2004
- Concurrent Main committee and Subcommittee ballot: May 2005
- Approved standard: July 2005
- Proposed revision to standard: August 2005
 - Ongoing ballot

ASTM standard E2455-05

- Scope
- Reference documents
- Terminology
- Summary of standard
- Significance and use
- Apparatus
- Hazards
- Dilution water
- Test material
- Test organisms
- Quality control and quality assurance
- Experimental design
- Analytical methodology
- Calculation of results
- Report
- Precision and bias
- Keyword

ASTM standard E2455-05 (cont.)

➤ Test organisms

- 10.1 Life history of freshwater mussels
- 10.2 Test species and life stage
- 10.3 Age
- 10.4 Source
- 10.5 Care and handling of organisms in the laboratory
- 10.6 Feeding
- 10.7 Disease treatment
- 10.8 Acclimation
- 10.9 Quality

ASTM standard E2455-05 (cont.)

➤ Quality control and quality assurance

11.1 Introduction

11.2 Performance-based criteria

11.3 Facilities, equipment, and test chambers

11.4 Test conditions

11.5 Quality of test organisms

11.6 Quality of food

11.7 Test acceptability

11.8 Analytical methods

ASTM standard E2455-05 (cont.)

- Quality control and quality assurance

11.9 Calibration and standardization

11.10 Replication and test sensitivity

11.11 Demonstrating acceptable performance

11.12 Record keeping

ASTM standard E2455-05 (cont.)

Annex A1: Guidance for conducting water-only toxicity tests with early life stages of freshwater mussels

A1.1 Significance

A1.2 Test conditions for conducting water-only toxicity tests with glochidia

A1.3 Test conditions for conducting water-only toxicity tests with juvenile mussels

A1.4 Conducting a test

A1.5 Additional information on experimental design and interpretation of data

Guidance in E2455-05 on *in vivo* vs. *in vitro* transformation of juvenile mussels

- 10.5.4.2 “Juvenile mussels cultured *in vitro* should **not be used to conduct toxicity tests** unless it has been demonstrated that the sensitivity of the juvenile mussels cultured *in vitro* is similar to the sensitivity of juvenile mussels cultured *in vivo*.”

Guidance in E2455-05 on *in vivo* vs. *in vitro* transformation of juvenile mussels

- Less lipids and glycogen in mantle cells of the juvenile mussels transformed *in vitro* compared to *in vivo* (Fisher and Dimock 2002, Hudson et al. 2003)
- Juvenile mussels transformed *in vivo* were less sensitive to thermal and hypoxic stresses compared to *in vitro* (Fisher 2002)

Guidance in E2455-05 on *in vivo* vs. *in vitro* transformation of juvenile mussels

- Juvenile *U. imbecillis* transformed *in vitro* were less sensitive compared to *in vivo* in 24-h exposures to sodium dodecylsulfate; however, sensitivity to cadmium or ammonia was similar (Summers 1998)
- Juvenile mussels transformed *in vitro* were more sensitive compare to *in vivo* in exposures to copper (Warren and Klaine 1994)

Table A1.1. Summary of test conditions used to conduct toxicity tests with glochidia of freshwater mussels.

	Conditions	Recommended test conditions
1	Species tested	NA
2	Test type	Static, renewal, or flow-through (depending on chemical tested)
3	Test duration (h)	6, 24 (up to 48 depending viability of glochidia)
4	Temperature, C	20
5	Light quality	Ambient lab light
6	Light intensity	100 to 1000 lux
7	Photoperiod	16L:8D
8	Test chamber	100-mL glass chamber (minimum)
9	Test solution volume (ml)	75 (minimum)
10	Glochidia collection	flush gills with syringe

Table A1.1. Summary of test conditions used to conduct toxicity tests with glochidia of freshwater mussels (cont).

	Conditions	Recommended test conditions
11	Age of test organisms (h)	<24
12	No. organisms per test chamber	about 500 (1000 for repeated sampling during a toxicity test)
13	No. replicate chambers per treatment	3, counting a subsample with about 100 glochidia from each replicate
14	Feeding	None
15	Aeration	None, if dissolved oxygen is maintained above acceptable concentration.
16	Dilution water	Depends on experimental design
17	Water quality	DO, pH, ammonia, hardness, alkalinity, conductivity
18	Endpoint	Survival (valve closure with NaCl)
19	Control survival (%)	>90 (must)

Table A1.4. Summary of test conditions used to conduct toxicity tests with juvenile freshwater mussels.

	Conditions	Recommended test conditions
1	Species tested	NA
2	Test type	Static, renewal or flow-through (depending on duration of exposure and chemical tested)
3	Test duration (d)	Acute: <4 Chronic: 21 to 28
4	Temperature, C	20
5	Light quality	Ambient lab light
6	Light intensity	100 to 1000 lux
7	Photoperiod	16L:8D
8	Test chamber	Static or renewal: 50-mL beakers (minimum) Flow-through: 300 mL beakers (minimum)
9	Test solution volume (mL)	Static or renewal: 30 (minimum) Flow-through: 200 (minimum)
10	Procedure for obtaining juveniles	Fish host

Table A1.4. Summary of test conditions used to conduct toxicity tests with juvenile freshwater mussels.

	Conditions	Recommended test conditions
11	Age of test organisms (day)	Acute: <5 Chronic: 60 to 120
12	No. organisms per test chamber	Acute: 5 (minimum) Chronic: 10 (minimum)
13	No. replicate chambers per treatment	Acute: 4 (minimum) Chronic: 3 (minimum)
14	Feeding	Acute: none Chronic: Algae
15	Aeration	None, if dissolved oxygen is maintained above acceptable concentration.
16	Dilution water	Depends on experimental design
17	Water quality	DO, pH, ammonia, hardness, alkalinity, conductivity
18	Endpoints	Survival (foot movement), growth (length)
19	Control survival (%)	Acute: >90 (must) Chronic: >80 (should)

Table A1.3. Test acceptability requirements for toxicity tests conducted with **glochidia** isolated from freshwater mussels

- A. It is recommended for conducting 24-h toxicity tests with glochidia isolated from adult mussels that the following performance criteria be met:
1. Age of glochidia should be less than 24-h old at the start of the toxicity test. Viability of glochidia isolated at the beginning of a toxicity test must be greater than or equal to 80% (preferably greater than or equal to 90%).
 2. Average survival of glochidia in the control at the end of a test must be greater than or equal to 90%.
 3. Hardness, alkalinity, and pH in the dilution water should not vary by more than $\pm 10\%$ during the exposure and dissolved oxygen should be maintained above 4 mg/L.

Table A1.3. Test acceptability requirements for toxicity tests conducted with **glochidia** isolated from freshwater mussels (cont)

- B. Performance-based criteria for culturing and handling of glochidia or adult mussels include the following:
1. Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (e.g., NaCl or CuSO₄, Section 15.4). Data from these reference-toxicant tests can be used to assess genetic strain or life-stage sensitivity of test organisms to select chemicals.
 2. Laboratories should track survival of adult mussels in the cultures. Records should also be kept on procedures used to collect and hold adult mussels.
 3. Laboratories should record the following water-quality characteristics of the cultures at least quarterly: pH, hardness, alkalinity, and ammonia. Dissolved oxygen in the cultures should be measured weekly. Temperature in the cultures should be recorded daily.
 4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.

Table A1.3. Test acceptability requirements for toxicity tests conducted with **glochidia** isolated from freshwater mussels (cont)

C. Additional requirements:

1. All organisms in a test must be from the same source and should be acclimated for about 2 h to the dilution water before the start of a toxicity test. It is desirable to composite samples of glochidia obtained from at least three female mussels to start a toxicity test.
2. All test chambers (or compartments) should be identical and should contain the same amount of dilution water. Individual test organisms should be impartially assigned to test chambers (or compartments). Treatments should be randomly assigned to individual test chamber locations.
3. Negative-control and appropriate solvent controls must be included in a test. The concentration of solvent used must not adversely affect test organisms (Section 9.2.4). The concentration of an organic solvent used in the preparation of a stock or test solution should not exceed 0.5 mL/L. A surfactant should not be used in the preparation of a stock or test solution.

Table A1.3. Test acceptability requirements for toxicity tests conducted with **glochidia** isolated from freshwater mussels (cont)

- C. 4. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than 2°C. The upper or lower 95% confidence limit on individual temperatures measured in the test chambers throughout the test must not be more than 2°C above or below the mean of the time-weighted average measured temperature for the individual test chambers.
5. Calculation of an LC50 or EC50 should usually be considered unacceptable if either of the following occurred: (1) no treatment other than a control treatment killed or affected less than 37 % of the organisms and (2) no treatment killed or affected more than 63 % of the organisms.
-

Table A1.5. Test acceptability requirements for toxicity tests conducted with **juvenile mussels**

- A. It is recommended for conducting toxicity tests with juvenile mussels that the following performance criteria be met:
1. Average survival of juvenile mussels in the control at the end of a 96-h test must be greater than or equal to 90%. An insufficient number of tests have been conducted with juvenile mussels for 10 or more days to provide specific guidance on control survival in longer-term tests. However, a limited number of toxicity tests have reported control survival greater than 80% in tests conducted with juvenile mussels for 10 to 28 d. Therefore, average survival of juvenile mussels in the control at the end of a test conducted for 10 to 28 d should be greater than or equal to 80%.
 2. Hardness, alkalinity, and pH in the dilution water should not vary by more than $\pm 10\%$ during the exposure and dissolved oxygen should be maintained above 4 mg/L.

Collecting gravid mussels



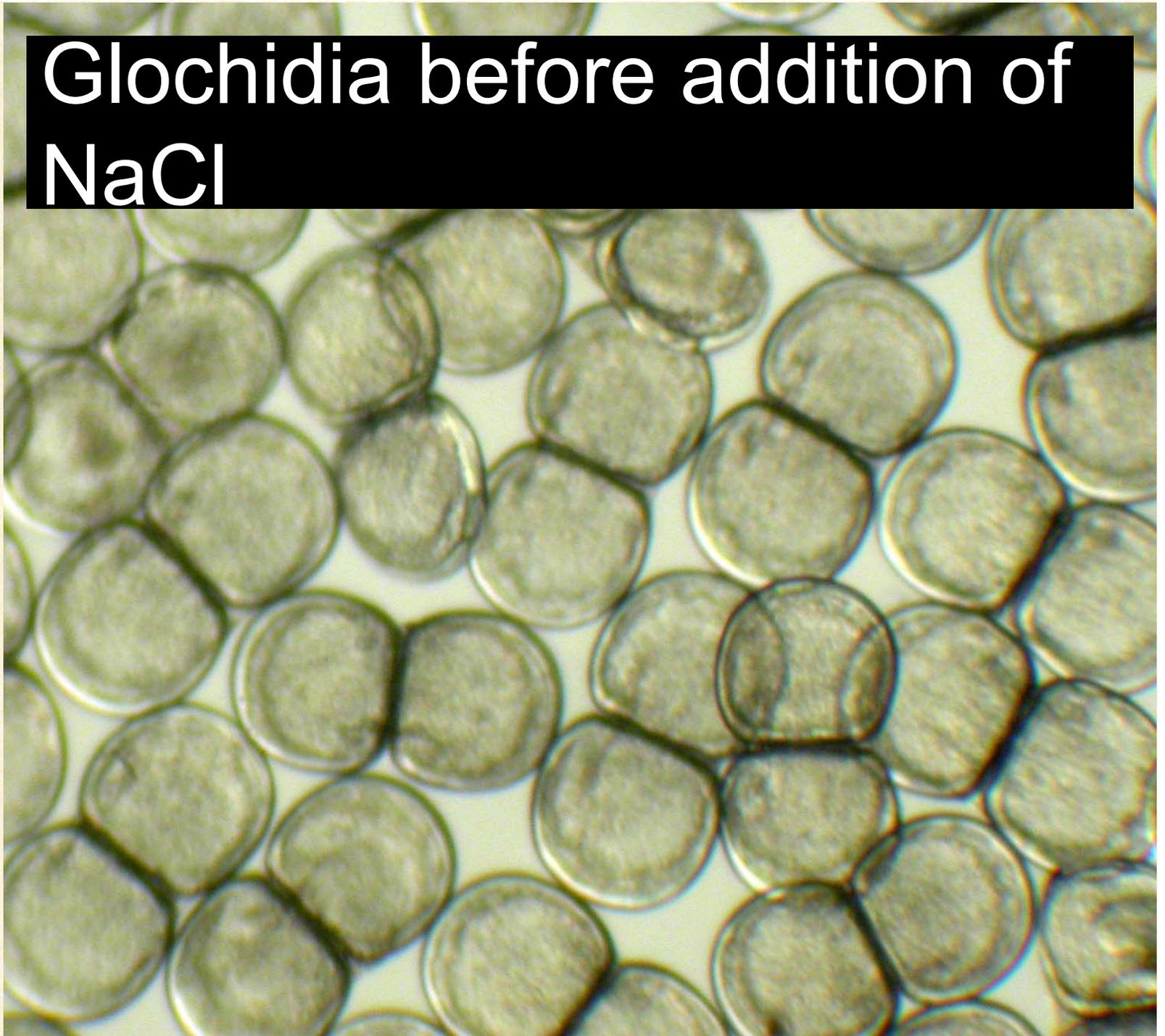
Gravid female mussels held in under flow-through conditions



Isolation of glochidia from a female mussel using a syringe

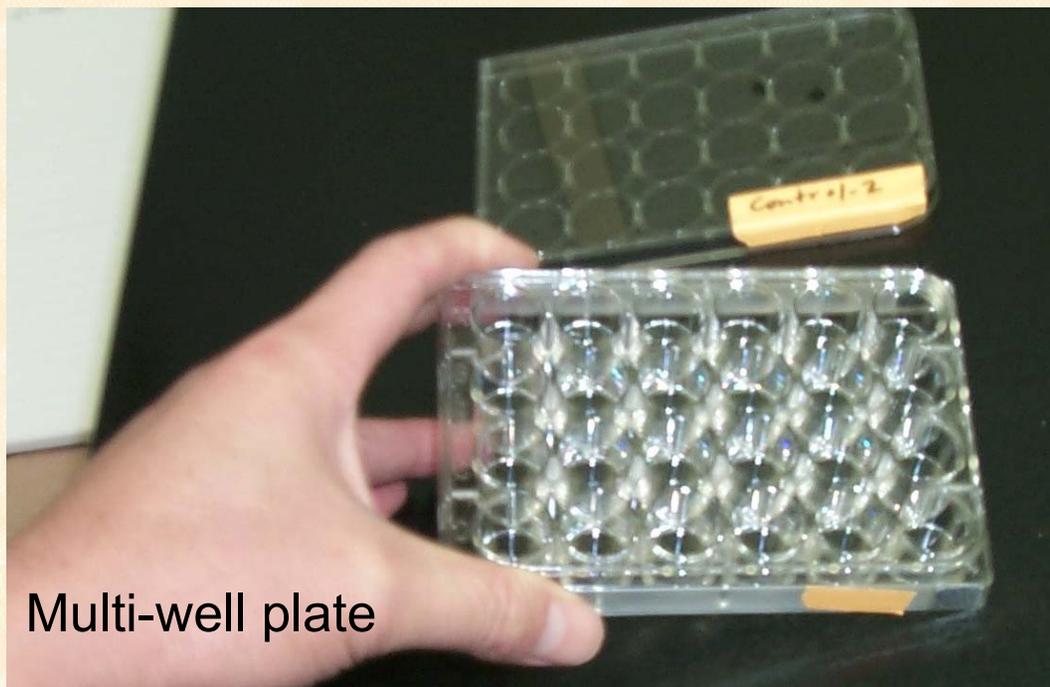


Glochidia before addition of NaCl

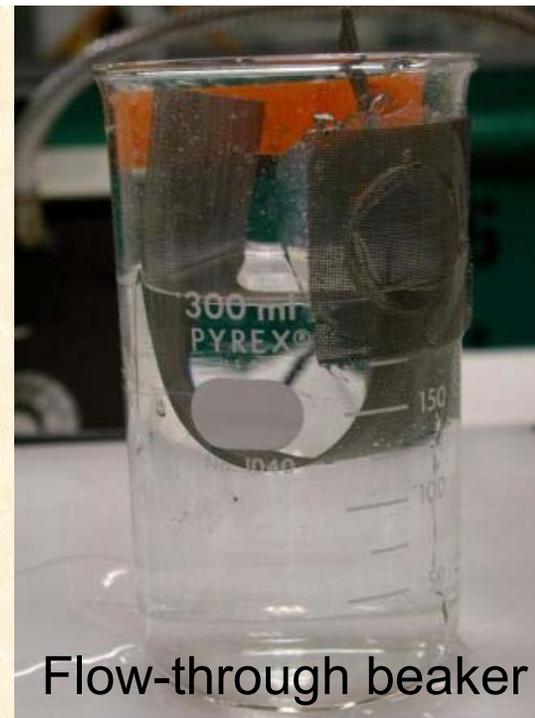




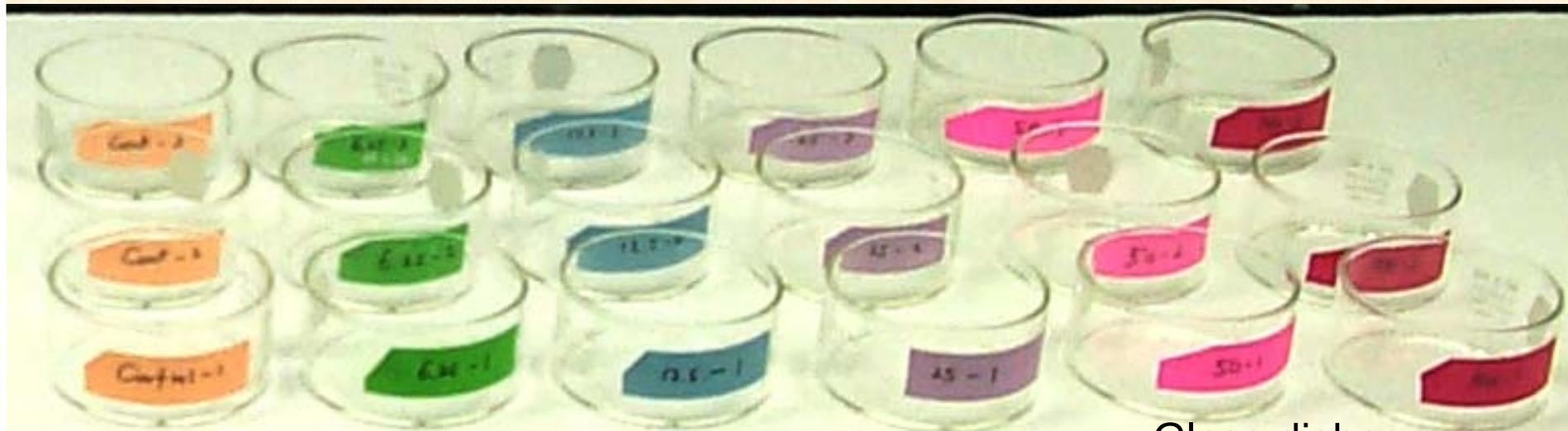




Multi-well plate



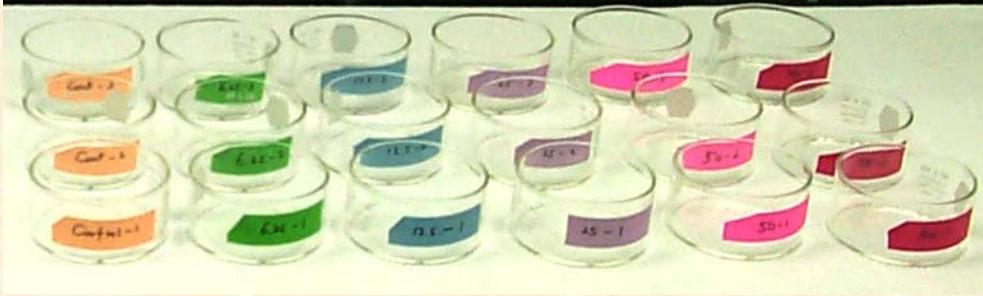
Flow-through beaker



Glass dishes

Conditions for acute toxicity test with mussels

Glochidia test



Test type: static

Test duration: 24 or 48 h

Age of organism: <2-h old

organisms/chamber: ~1000

replicates/concentration: 3

Endpoint: Survival

Acceptability: >90% survival in control

Juvenile mussels test



Test type: static renewal

Test duration: 4 or 10 d

Age of organism: <5-d or ~60-d old

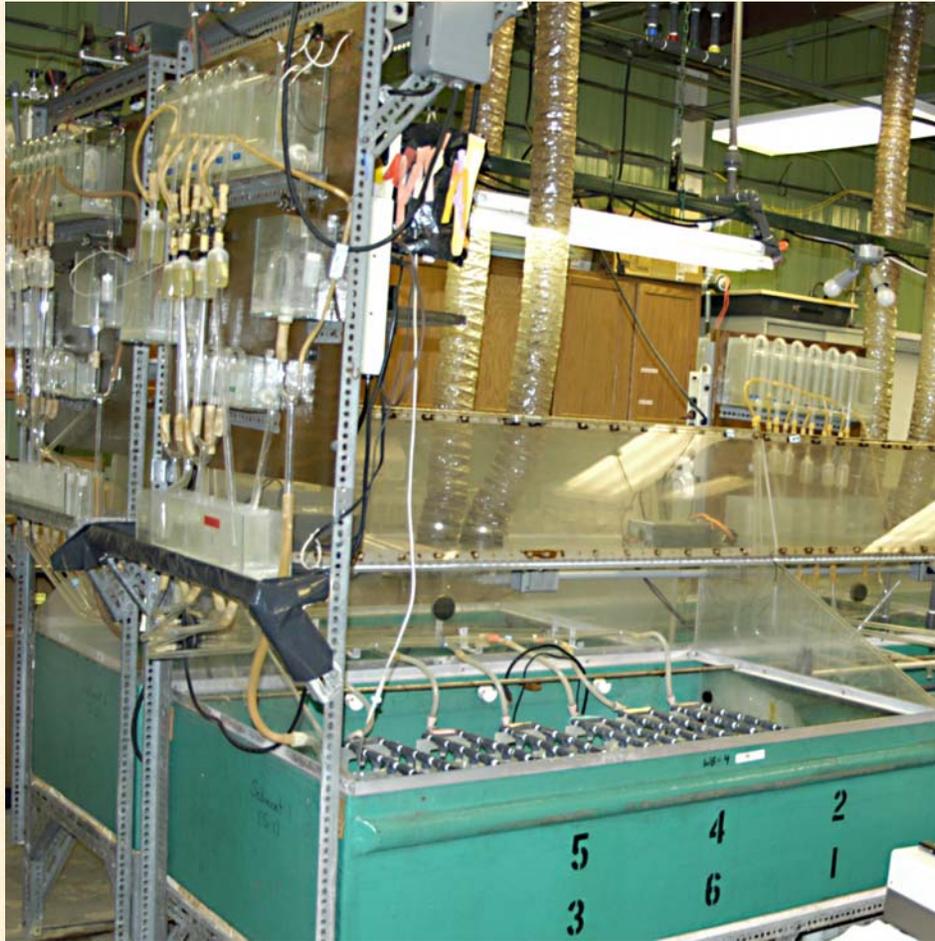
organisms/chamber: 5

replicates/concentration: 4

Endpoint: Survival

Acceptability: >90% survival in control

Conditions for chronic toxicity tests with mussels



Intermittent flow diluter system

Test type: Flow-through
Test duration: 28 d
Age of organism: 2-month old
organisms/chamber: 10
replicates/concentration: 4
Feeding: Mixture of instant algae
Endpoint: Survival and growth
Acceptability: >80% survival in control





Monitoring test

Future plans for revising E2455-05

➤ Ongoing ballot:

- Intra-laboratory toxicity test results with juvenile mussels
- Conditions for conducting flow-through tests

➤ Future ballots (additional annexes):

- Methods for conducting sediment toxicity tests with glochidia or juvenile mussels
- Methods for conducting toxicity tests with adult mussels

Questions?

