Assessing Whole Effluent Toxicity Testing (WETT) Laboratories

TNI WETT Expert Committee

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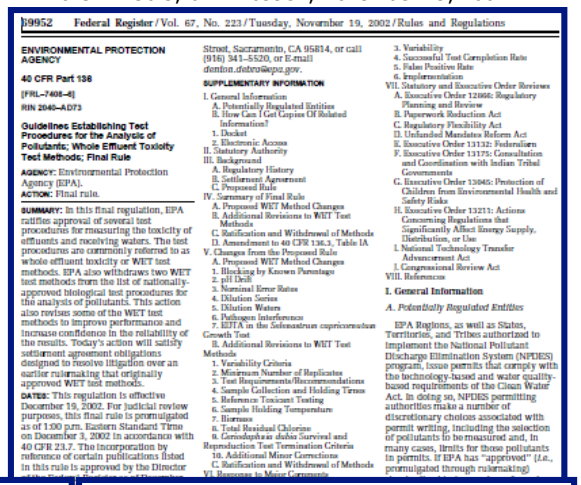
What is a WET Test?

- An important component of EPA's integrated approach to protect surface waters from pollutants.
- WET is "the aggregate toxic effect of an effluent measured directly by a toxicity test for acute and chronic effects".
- WET tests are used to determine the toxicity of an effluent over a certain period of time.
- Whole effluent toxicity is measured as opposed to chemical specific toxicity.
- Typically included in NPDES permits.

WET Effluent Test Methods

- Part 136 of the Clean Water Act (CWA): EPA promulgated 16 methods for acute and short term test species to use to estimate acute and chronic toxicity.
- 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.

40 CFR 136.3; 67 FR 69952, November 19, 2002



69952 Federal Register / Vol. 67, No. 223 / Tuesday, November 19, 2002 / Rules and Regulations

ENVIRONMENTAL PROTECTION AGENCY

40 CFR Part 136

[FRL–7408–6]

RIN 2040–AD73

Guidelines Establishing Test Procedures for the Analysis of Pollutants; Whole Effluent Toxicity Test Methods; Final Rule

AGENCY: Environmental Protection Agency (EPA).

ACTION: Final rule.

SUMMARY: In this final regulation, EPA ratifies approval of several test procedures for measuring the toxicity of effluents and receiving waters. The test procedures are commonly referred to as whole effluent toxicity or WET test methods. EPA also withdraws two WET test methods from the list of nationally-approved biological test procedures for the analysis of pollutants. This action also revises some of the WET test methods to improve performance and increase confidence in the reliability of the results. Today's action will satisfy settlement agreement obligations designed to resolve litigation over an earlier rulemaking that originally approved WET test methods.

DATES: This regulation is effective December 19, 2002. For judicial review purposes, this final rule is promulgated as of 1:00 p.m. Eastern Standard Time on December 3, 2002 in accordance with 40 CFR 23.2. The incorporation by reference of certain publications listed in this rule is approved by the Director

Stroot, Sacramento, CA 95814, or call (916) 341–5520, or E-mail denise.stroot@epa.gov.

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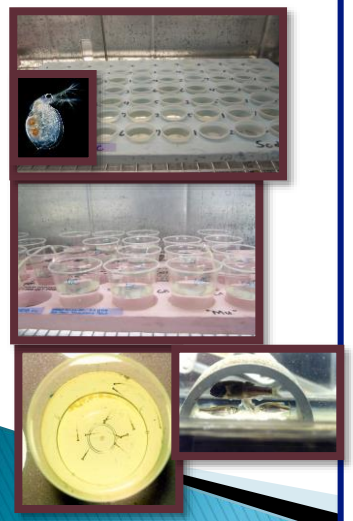
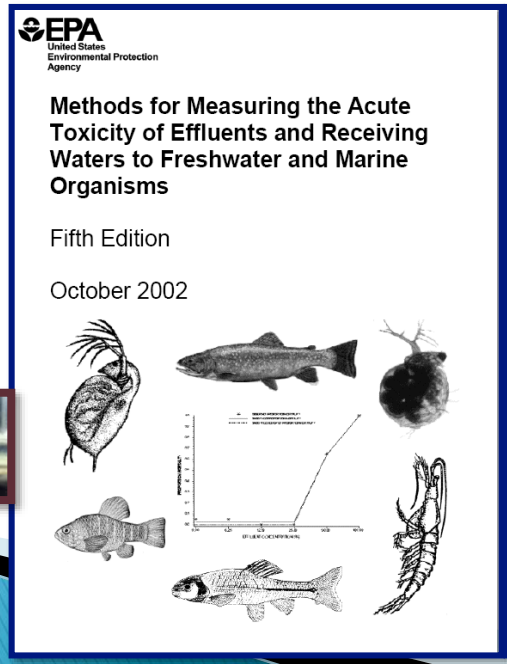
I. General Information

A. Potentially Regulated Entities

EPA Regions, as well as States, Territories, and Tribes authorized to implement the National Pollutant Discharge Elimination System (NPDES) program, issue permits that comply with the technology-based and water quality-based requirements of the Clean Water Act. In doing so, NPDES permitting authorities make a number of discretionary choices associated with permit writing, including the selection of pollutants to be measured and, in many cases, limits for those pollutants in permits. If EPA has "approved" (*i.e.*, promulgated through rulemaking)



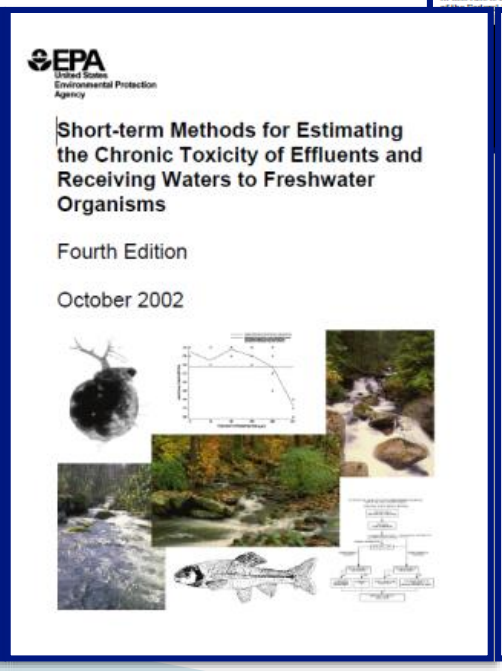


EPA United States Environmental Protection Agency

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

Fifth Edition

October 2002

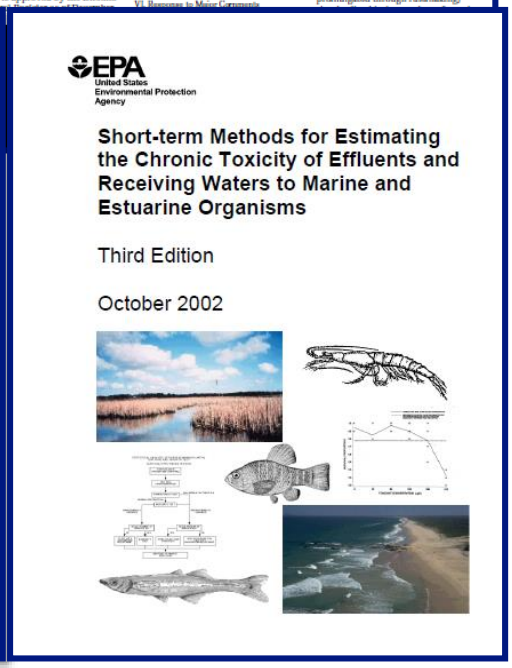


EPA United States Environmental Protection Agency

Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

Fourth Edition

October 2002



EPA United States Environmental Protection Agency

Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms

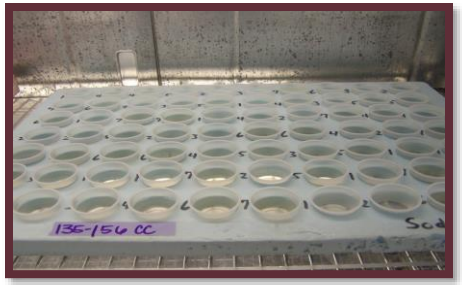
Third Edition

October 2002

Type of Tests

- Acute Tests
  - Endpoint is mortality.
  - Test duration is typically 24, 48 or 96 hours.

- Short-Term Tests to Estimate Chronic Toxicity
  - Endpoints are growth, reproduction, mortality/immobility.
  - Test duration is 8 days or less.
    - method/species determinant of duration.



The WET methods listed below are codified at 40 CFR 136.3, Table IA

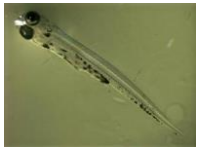
Acute Toxicity – Freshwater, Marine, and Estuarine Methods	
No.	Method Title
Acute Toxicity, Freshwater Organisms	
2000.0	Fathead Minnow, *Pimephales promelas*, and Bannerfin shiner, *Cyprinella leedsi*
2002.0	Daphnia, *Ceriodaphnia dubia*
2019.0	Rainbow trout, *Oncorhynchus mykiss*, and Brook trout, *Salvelinus fontinalis*
2021.0	*Daphnia pulex* and *Daphnia magna*
Acute Toxicity, Estuarine/Marine Organisms of the Atlantic Ocean and Gulf of Mexico	
2004.0	Sheepshead minnow, *Cyprinodon variegatus*
2006.0	Silverside, *Menidia beryllina, Menidia menidia*, and *Menidia peninsulae*
2007.0	Mysid, *Americamysis bahia*
Chronic Toxicity, Freshwater Organisms	
1000.0	Fathead minnow, *Pimephales promelas*, larval survival and growth
1001.0	Fathead minnow, *Pimephales promelas*, larval survival and teratogenicity
1002.0	Daphnia, *Ceriodaphnia dubia*, survival and reproduction
1003.0	Green alga, *Selenastrum capricornutum*, growth
Chronic Toxicity, Estuarine/Marine Organisms of the Atlantic Ocean and Gulf of Mexico	
1004.0	Sheepshead minnow, *Cyprinodon variegatus*, larval survival and growth
1005.0	Sheepshead minnow, *Cyprinodon variegatus*, embryo-larval survival and teratogenicity
1006.0	Inland silverside, *Menidia beryllina*, larval survival and growth
1007.0	Mysid, *Americamysis bahia*, survival, growth and fecundity
1008.0	Sea urchin, *Arbacia punctulata*, fertilization

https://www.epa.gov/cwa-methods/whole-effluent-toxicity-methods

Acute Test Species

Codified Freshwater

Daphnia pulex or
Daphnia magna (Method 2021.0)
Ceriodaphnia dubia (Method 2002.0)
(water flea) (adults in the photos)



Pimephales promelas
(fathead minnow)
Method 2001.0

Oncorhynchus mykiss
(Rainbow trout) **or**
Salvelinus fontinalis
(brown trout)
Method 2019.0

Codified Marine/Estuarine

Americamysis bahia (formerly *Mysidopsis bahia*, mysid shrimp)
Method 2007.0

Cyprinodon variegatus
(Sheepshead minnow)
Method 2004.0

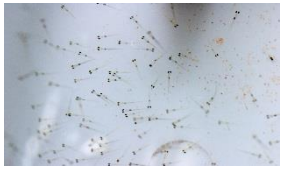
Menidia beryllina, M. menidia, M. peninsulae (silverside minnows)
Method 2006.0

Short-Term Chronic Species

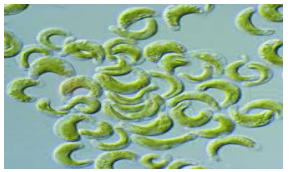
Codified Freshwater

Ceriodaphnia dubia
(water flea)
Method 1002.0

Pimephales promelas
(fathead minnow)
Method 1000.0, 1001.0



Pseudokirchneriella subcapitata
(formerly *Selenastrum capricornutum*
(freshwater algae)
Method 1003.0



Codified Marine/Estuarine

Americamysis bahia
(Mysid shrimp)
Method 1007.0

Menidia beryllina
(inland silverside minnow)
Method 1006.0

Cyprinodon variegatus
(Sheepshead minnow)
Method 1004.0, 1005.0

Arbacia punctulata
(sea urchin)
Method 1008.0

Champia parvula
(Red microalgae)
Method 1009.0;
not listed in CFR Table 1A

WET Test Methods

- The WET methods must be followed as they are written in the EPA WET test methods (2002 manuals).

- New NPDES permits and permit re-issuances incorporate the WET test methods into the permit
  - Incorporate by reference the WET methods in general permit conditions.
  - Direct reference by citing current test methods.
  - Laboratory must follow WET methods QA/QA (EPA 2002 manuals).

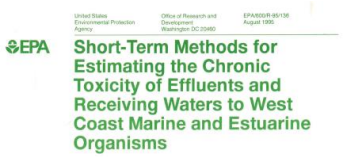
Promulgated Manual References

- Acute Test Method Manual
  - USEPA 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th Edition. EPA-821-R-02-012.
- Short-term Chronic Method Manuals
  - USEPA 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Edition. EPA-821-R-02-013.
  - USEPA 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 4th Edition. EPA-821-R-02-014.

https://www.gpo.gov/fdsys/pkg/FR-2002-11-19/pdf/02-29072.pdf

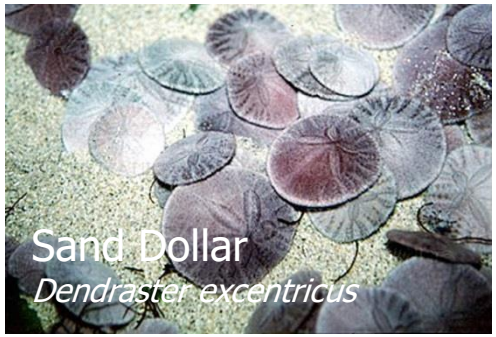
West Coast Marine and Estuarine Short-Term Methods for Estimating the Chronic Toxicity of Effluent and Receiving Waters

Common Name/Species	Biological Test Endpoint
Topsmelt, *Atherinops affinis*	growth, survival
Red abalone, *Haliotis rufescens*	larval development
Mussels, *Mytilus spp.* Oyster, *Crassostrea gigas*	larval development
Purple urchin, *Strongylocentrotus purpuratus* Sand dollar, *Dendraster excentricus*	larval development, fertilization
Mysid, *Holmesimysis costata*	growth, survival
Giant kelp, *Macrocystis pyrifera*	germ-tube length and germination



USEPA 1995. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms, 1st edition. EPA/600/R-95-136.

West Coast Marine Test Species



Limited Basis: 40 CFR 136.3; 67 FR 69952, November 2002 (direct quote)

- **Code of Federal Regulations states**:
  - EPA species/methods in the 2002 WET manuals not included for nationwide use does not prevent their use on more limited basis.
- **EPA supports their use for applications**:
  - other than for the determination of compliance with NPDES permit limits as well for limited, located or regional use where the methods have been validated by other entities.
  - EPA supports the use of the *Holmesimysis costata* acute test to measure toxicity to marine organisms of the Pacific ocean.
- **Because test procedures for measuring toxicity to estuarine and marine organisms of the Pacific ocean are not listed at 40 CFR Part 136**
  - permit writers may include requirements for the use of test procedures that are not approved at part 136 such as *Homesimysis* and other west coast WET methods (1995) on a permit by permit basis.

Specialized Toxicity Tests

- Soils toxicity.
- Drilling fluid testing – fluids used during drilling operations (cooling and lubricating the drill bit, well control, etc.).
- Drilling mud testing – a mixture of the drilling fluids with the soil extracted during drilling operations.
  - Organisms used for this testing can be either swimming or burrowing organisms.
    - For example, the freshwater amphipod, *Hyalella azteca.*

Freshwater Sediment Methods

Short-term and Chronic tests with invertebrates:

- Midge, *Chironomus dilutus.*
  - Survival and growth at 10 days.
  - Survival, growth, reproduction, hatchability.

- Amphipod, *Hyalella azteca*
  - Survival and growth at 10 days.
  - Survival, growth, reproduction.

Sediment Test References

- USEPA 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates. 2nd Edition. 600 R-99-064.
  - Revision underway and expected summer of 2017.

- NPDES General Permit 290000 for discharges from the Offshore Subcategory of the Oil and Gas Extraction Category for the Western Portion of the Outer Continental Shelf of the Gulf of Mexico off the coasts of Louisiana and Texas.

Data Analysis and Review

Data Analysis

- **Types of Data**
  - Binomial data, such as survival.
  - Continuous data, such as growth, reproduction, cell count, *etc.*

- **Acute tests**
  - Point estimate techniques.
    - Probit, Spearman Karber, Trimmed Spearman Karber

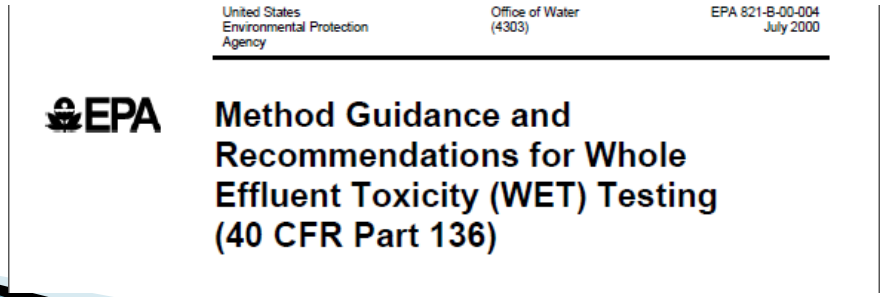
- **Chronic tests**
  - Point estimate techniques to determine the ICp (IC25).
    - Linear interpolation
  - Hypothesis tests to determine the concentration that is significantly different than the control.
    - Parametric analysis or
    - Nonparametric analysis

Test Endpoints

- LC50 – Lethal Concentration
  - the concentration of sample that kills 50% of the test organisms.
- NOEC (NOEL) – No Observed Effect Concentration (Level)
  - the highest effluent concentration that is not significantly different from the control based on statistical analysis.
- LOEC (LOEL) – Lowest Observed Effect Concentration (Level)
  - the lowest effluent concentration that is significantly different from the control based on statistical analysis.
- ICp – Inhibition Concentration percentage, i.e., IC25%
  - the effluent concentration that shows an increase in toxicity to 25% of the organisms observed for the biomass values which are combined effects of survival/growth, survival/reproduction, survival/fecundity.

Data Review

- EPA promulgated methods require that each test:
  - must have 5 concentrations and a control for effluent testing.
  - must meet the test acceptability control (TAC) for each method.
- At the end of each test:
  - Every test must be reviewed and analyzed using EPA's statistical procedures in the EPA manuals (2002, Section 10.2.5.1).
  - Every test must be reviewed for the concentration-response relationships for all NPDES WET multi-concentration tests to ensure that calculated test results are interpreted appropriately .


United States Environmental Protection Agency | Office of Water (4303) | EPA 821-B-00-004 July 2000

EPA

Method Guidance and Recommendations for Whole Effluent Toxicity (WET) Testing (40 CFR Part 136)

(EPA 2000; effluent manuals (Section 10.2.6) And 40 CFR Part 136.3; 67 FR 69952)

All Tests Must be Reviewed Following Method Guidance

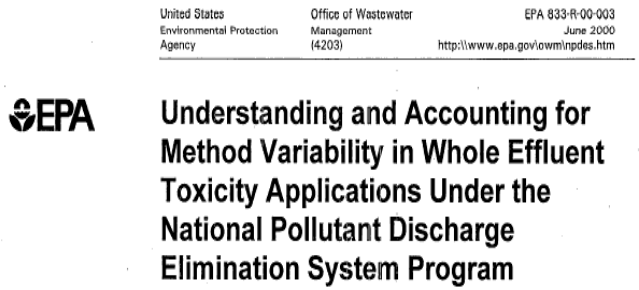
- Manuals state:
  - For the NPDES program, the point estimating techniques are the preferred statistical methods in calculating end points for effluent toxicity tests (9.5.1).

- CFR 2002 states:
  - EPA recommends the use of point estimation techniques (e.g., LC, ICp) over hypothesis testing (NOEC) approaches for calculating endpoints for effluent toxicity tests under the NPDES Permitting Program.

EPA Implemented . . . (WET rule, 67 FR 69952)

- Variability criteria (upper and lower PMSD bounds) as a test review step:
  - Required when NPDES permits require sublethal WET testing endpoints expressed using hypothesis testing endpoints (NOEC/LOEC) and the effluent has been determined to have no toxicity at the permitted receiving water concentration (WET rule, Section VI.B.).

- Within test variability for 5 methods must be reviewed (cf., CFR and Manual Sections 10.2.8.2).
  - 1000.0 Fathead minnow, *Pimephales promelas*, larval survival and growth
  - 1002.0 Daphnia, *Ceriodaphnia dubia*, survival and reproduction
  - 1003.0 Green alga, *Selenastrum capricornutum*, growth
  - 1006.0 Inland silverside, *Menidia beryllina*, larval survival and growth
  - 1007.0 Mysid, *Americamysis bahia*, survival, growth and fecundity


United States Environmental Protection Agency | Office of Wastewater Management (4203) | EPA 833-R-00-003 June 2000 http:\\www.epa.gov\owm\npdes.htm

EPA

Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination System Program

PMSD Bounds For The 5 Methods

Test Method	Endpoint	Lower PMSD bound	Upper PMSD Bound
1001.0 Fathead minnow larval growth and survival test	Growth (biomass)	12	30
1002.0 *Ceriodaphnia dubia* Survival and Reproduction Test	Reproduction	13	47
1003.0 *Selenastrum capricornutum* Growth Test	Growth	9.1	29
1006.0 Inland Silverside Larval Growth and Survival Test	Growth (biomass)	11	28
1007.0 Mysidopsis bahia Survival, Growth, and Fecundity Test	Growth (biomass)	11	37

Lower and upper PMSD bounds were determined from the 10th and 90th percentile, respectively, of PMSD data from EPA's WET Interlaboratory Variability Study (EPA 2001 Vol I & II). In the 2000 guidance, the PMSD values were from the data set of reference toxicant tests.

PT Toxicity Testing & The DMR-QA

- Monitoring the quality of data used to assure the integrity of the NPDES program.
- Permittees/Labs report any "Not Acceptable" values
  - evaluated to determine the cause of the deficiency and ensure corrective action.
- DMR-QA testing uses the method that most closely resembles the testing condition required by the permit:
  - 20°C tests done at 25°C .
  - A renewal test is conducted as non-renewal.

- If a laboratory receives a "not acceptable" result, corrective action is needed (root cause analysis, etc.).

TST – Test of Significant Toxicity

- Additional approach for testing and analyzing toxicity data.
- EPA 833-R-10-003, June 2010 NPDES TST Implementation Document
  - Changes the Null / Alternative Hypotheses
  - Traditional Analysis
    - Null = sample mean ≥ control mean (*i.e.*, not toxic)
    - Alternative = sample mean < control mean (*i.e.*, toxic)
  - TST Analysis
    - Null = sample mean ≤ b x control mean (*i.e.*, toxic)
    - Alternative = sample mean > b x control mean (*i.e.*, not toxic)
- With TST, default is that the sample is toxic, and dischargers prove they are not toxic through testing.
- Once the WET test has been conducted (using multiple effluent concentrations and other requirements as specified in the WET test methods), the TST approach can be used to analyze valid WET test results to assess whether the effluent discharge is toxic.

Traceability in WET Testing

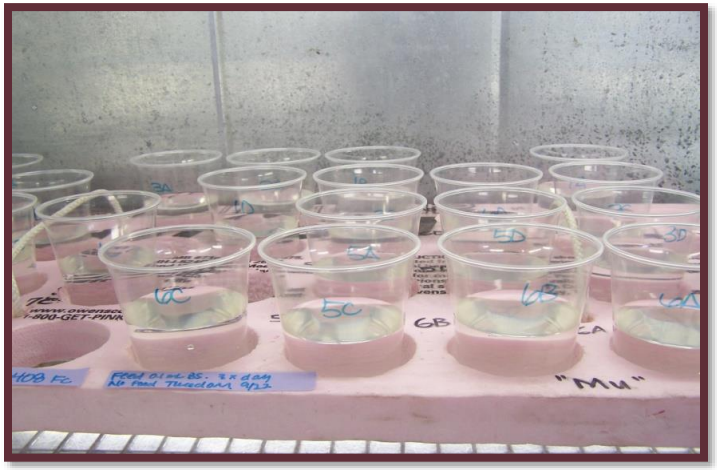
Definition Of Traceability

- "The property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties."

- International Vocabulary of Basic and General Standard Terms in Metrology. ISO, Geneva, Switzerland 1993 (ISBN 92-67-10175-1).

Definition Of Traceability

- Traceability refers to the completeness of the information in every step of the experiment.



Elements of WET Traceability

- Standard Operating Procedures (SOPs).
- Personnel Training.
- Test Organism Health.
- Test Design.
- Documentation.

Goals In Traceability

- Completeness Of the Information Regarding Every Step In a Process.
- Documentation Of Recorded Information.
- Generation Of Reproducible And Defensible Data.

Standard Operating Procedures (SOP)

A Good WET SOP Must:

- Provide all the information necessary to perform a task.
- Be a stand alone document.
- Provide quality information.
- Include references to relevant documents.
- Include a list of revisions to track edit history.
- Include a signature log for all personnel.

All SOPs in an organization should have the same structure.

Personnel Training

Training Records Should Include:

- General Laboratory checklist.
- Analyses specific checklist.
- Demonstration of Capability Certification Statement.
- Initials and Signature Documentation.
- On-going Training Attendance.
- Initial Demonstration of Capability (IDOC).
- Continuing Demonstration of Capability (CDOC).
- Ethics Policy Agreement.
- Job Responsibilities Checklist.

ALL ANALYSTS TRAINED SIMILARLY USING
LAB-DEFINED PROTOCOLS, SOPs.

Test Organism Health

- Detailed records are maintained for cultures.







Documentation Should Include

- Randomization records.
- Culture health records.
- Temperature documentation (e.g., Incubator ID linked data).
- Food source ID and feeding regimen records.
- Water batch ID and preparation records.

Example of Brood Board (BB) Tracking for *Ceriodaphnia dubia*:

	1	2	3	4	5	6	7	8	9	10
Cup ID	51-3	51-4	51-6	51-10	51-20	51-25	51-28	51-38	51-49	51-50
Day 1	0	0	0	0	0	0	0	0	0	0
Day 2	0	0	0	0	0	0	0	0	0	0
Day 3	0	0	4	4	0	4	0	0	3	0
Day 4	5	4	0	0	4	0	4	3	0	4
Day 5	8	8	9	10	8	8	10	7	8	9
Day 6	0	0	0	13	0	10	0	0	12	0
Day 7	13	12	11	0	11	10	14	12	0	11
Mean	**26**	**24**	**24**	**27**	**23**	**22**	**28**	**22**	**23**	**24**
Mortality Date										

Documentation for Neonates

- Identify the brood board number.
- Identify the parent female.
- Maintain records proving that the parent female has been deemed valid for use in testing.
- Identify the time period in which they were born (*i.e.*, 8 hour window).
- Maintain records that the neonates were fed properly prior to use in testing.

Standard Reference Toxicant Testing

- Standard reference toxicant testing (SRT) is performed with a known toxicant to determine trends in culture health.
  - Minimum of monthly for all organisms raised in-house.
  - Concurrent with permitted test on all organisms that are wild-caught.
  - Concurrent with permitted test on all organisms obtained from a commercial supplier, unless the supplier provides control chart data from at least five monthly reference toxicant tests.
  - Regardless of above, laboratory must perform at least one acceptable SRT per month for each test conducted that month.

Standard Reference Toxicant Testing

- If a routine RT test fails to meet the test acceptability criteria, it must immediately be repeated.

- Every toxicity test report should identify the most recent reference toxicant test.

Experimental Design: Randomization

- The distribution of test organisms among test chambers and the arrangement of treatments and replicate chambers.
- Purpose is to avoid situations where test organisms are placed serially into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.
- Chronic manuals require random templates.

Experimental Design: Randomization in Chronic *Ceriodaphnia dubia* Test (Method 1002.0)

- Test cups are randomized on boards so that one treatment does not benefit from variation in light or temperature.
- Health of the neonate is highly dependent upon the health of the parent female.
  - For each test, neonates are assigned randomly, using a procedure that ensures that one replicate in each test treatment gets neonates from the same parent female (called “blocking by known parentage”) .
- Must have documentation of blocking procedure.

Figure 1: Examples of a Test Board and Randomizing Template

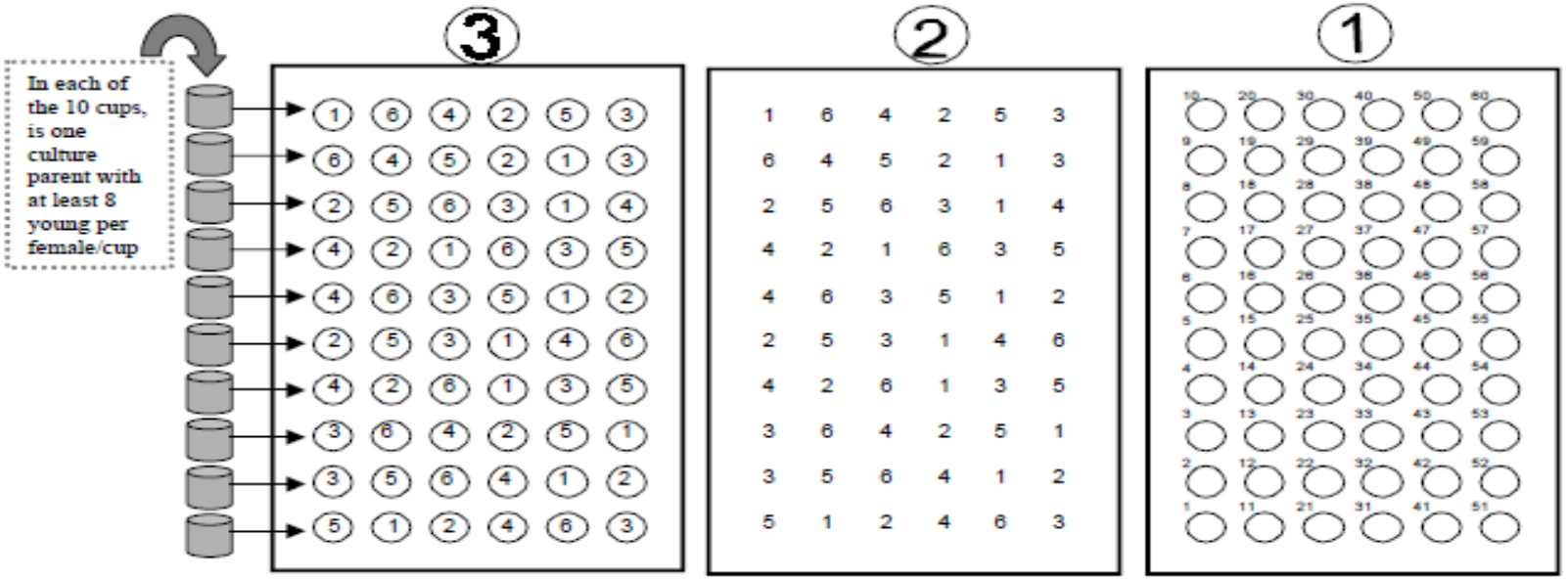


Figure 1. Examples of a test board and randomizing template:
1) test board with positions for six columns of ten replicate test chambers with each position numbered for recording results on data sheets,
2) cardboard randomizing template prepared by randomly drawing numbers (1-6) for each position in a row across the board, and
3) test board with random locations showing from template.

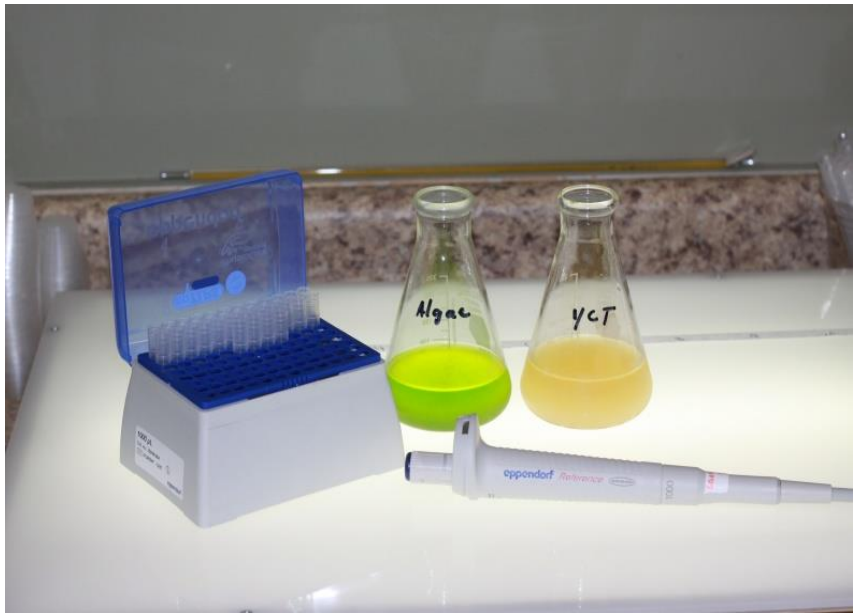
In practice: test board 1 is placed on top of the 2) the randomizing template, and the test organisms are assigned from one brood cup to each treatment within a given block. Following placement of test chambers, test organisms are allocated using blocking by known parentage.

Documentation

- Temperature control linked to temperature documentation, or
- Temperature monitoring system reports, such as HOBO temperature loggers.

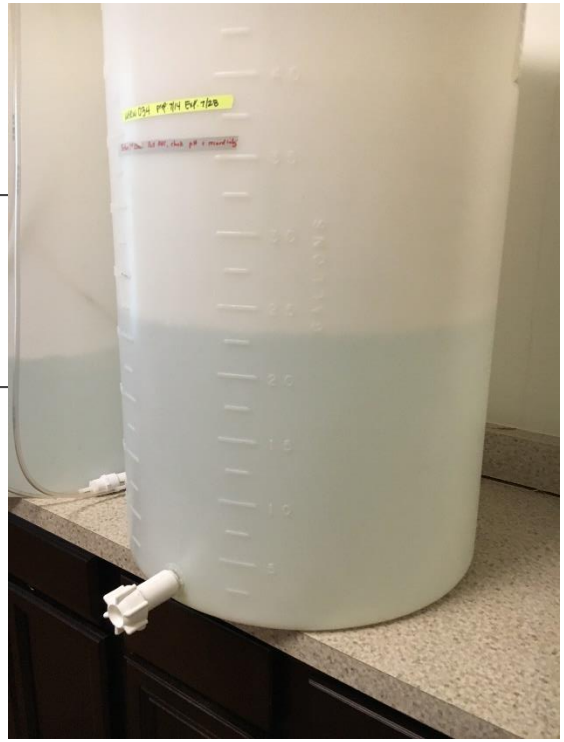
Documentation

- Food source ID / feeding regimen.



Documentation

- Water batch ID and preparation records.



Documentation

- Test Dilution Preparations
  - Dissolved oxygen (DO) should not be supersaturated or have a DO concentration below 4.0 mg/L.
    - Aerate samples after heating from 4^0 C to 25^0 C, before preparing test dilutions.
  - If the sample contain wild organisms, it must be filtered prior to use.
  - Any sample manipulations **MUST** be
  - documented.

Assessing WETT Laboratories

WETT vs. Chemical Laboratories

- Similarities:
  - Following referenced method.
  - Documentation (DOC, SOPs, Preparation logs, Reagent logs, Temperature, Balances, Instruments, *etc.).*
  - Error correction policy reviews.
  - Traceability: reagents, standards, organisms, food, water.
  - Instrument calibration and maintenance records.
  - Calculation reviews.
  - Quality Control Charts.

WETT vs. Chemical Laboratories

- Differences:
  - Organism review: taxonomic verification, culturing, organism age for tests, *etc.*
  - Synthetic/reconstituted water preparation.
  - Light cycles, intensities.
  - Standard Reference Toxicant tests (SRT).
  - Statistical analyses review per EPA methods.
  - Chemical data (*i.e.*, D.O., pH, salinity, conductivity, etc.) are supporting documentation only.

Demonstration of Capability (DOC)

Demonstration of Capability (DOC) 2009 TNI

- Initial Demonstration of Capability (IDOC).
- Each analyst shall meet the quality control requirements as specified in Section 1.7.1.2.
  - NELAC 2003 Appendix D2 or TNI 2009 V1M7 §1.6 (EL-V1M7-2009).
- Positive and Negative Controls.
  - SRTs and control organism performance.
- Continuing DOC (CDOC).
- Documented procedure describing ongoing DOC.
- Analysts must meet QC requirements of the method, Lab SOP, client specifications, and the standard.
- QC sample data must be reviewed to identify patterns for individuals or groups and make correct actions.

Standard Reference Toxicant (SRT) Tests

- SRTS are used for evaluating the health and sensitivity of organisms over time and for documenting initial and ongoing laboratory performance.
  - Initially need 5 or more acceptable SRTs for each test method, species, and endpoint.
  - When 2 species are tested at 2 temperatures for Method 2021, there must be 4 control charts for the LC50 for that method:

    Daphnia pulex @ **20°C & 25°C**

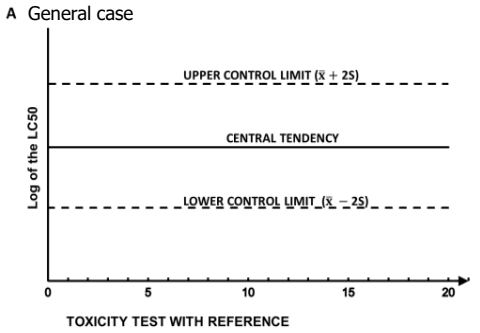
    Daphnia magna @ **20°C & 25°C**
- Appropriate negative controls must be tested at the frequency and duration specified in the test method.
- Use criteria obtained from control charts to determine group or analyst capability.
- Analyst DOC may be performance within established control limits or results obtained are the same as a trained analyst.

Control Charts for SRTs

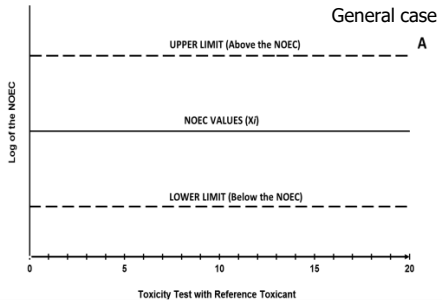
- Maintain Cusum chart.
  - Use 2 years of data or a minimum of 20 points.
  - Only the last 20 data points are used to determine acceptance criteria.
- Cusum charting plots the effect (LC, EC, IC, NOEC).
  - Anti-logarithm values or logarithm values.
  - Control chart cannot mix log values on a log scaled y-axis, or antilog values on an arithmetic scale y-axis.

Control Charts for SRTs

- For point estimates (LC, EC, IC), the log or the anti-log of the effect concentration is plotted on the chart.
  - Cumulative mean and upper and lower control limits (±2S) are re-calculated with each successive test result.

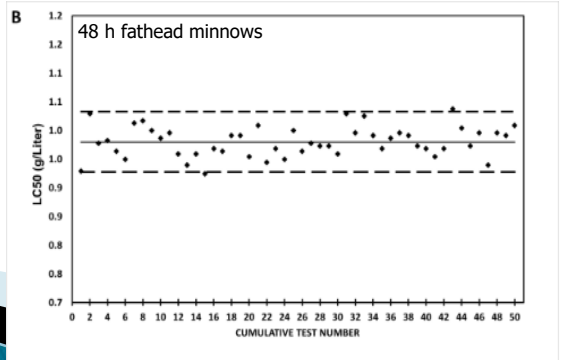


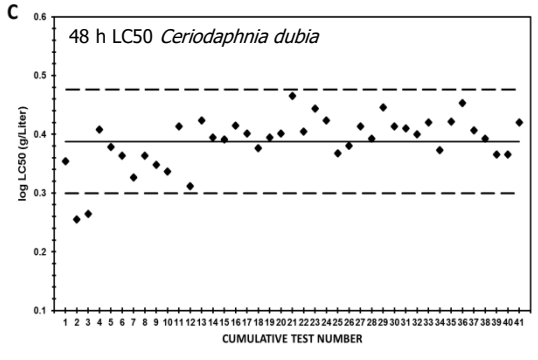
- For hypothesis test endpoints (NOECs), the log or anti-log of the NOEC from each test is plotted directly on the control chart.
  - NOEC are discrete points that cannot be averaged; therefore the cumulative mean is not used.



Examples: 48 h acute tests with sodium chloride

Note: graphs using antilogarithm values may be preferable as many graphics programs do not allow enlarging the log scale axis sufficiently





Test Acceptability

- "May", "Should"
  - Discretionary terms.
  - Provide flexibility to labs/analysts.
  - Recommendations, best practices, but do not imply requirements.

- "Must", "Shall"
  - Words of obligation.
  - Explicit requirement.

EPA Review Checklist

- Checklist for each test method:
  - Test Acceptability Criteria (TAC)
    - 'Must' – where all conditions must be met, test is invalid if aren't met.
  - Other pertinent test conditions:
    - 'Shoulds' – test may be suspect if multiple "shoulds" violated.
- TAC – is minimum for successful performance!
  - Higher control performance should be expected from laboratories.

Test Acceptability

- An individual test may be conditionally acceptable if some specified conditions are not met, depending on the degree of departure and objectives of the test.
- Depends on the professional judgment of the Technical Director and permitting authority.
- Example
  - Test anomalies may be discounted or not included in the NOEC calculations if an assigned cause is evident.

TNI Language Regarding Methods or Regulations

- 2009 V1M2 Section 5.9.3c "....The quality control protocols specified by the laboratory's SOP shall be followed (see Section 4.2.8.5 in this Standard). The laboratory shall ensure that the essential standards outlines in Technical Modules or mandated methods or regulations (whichever are more stringent) are incorporated into their method manuals. When it is not apparent which is more stringent, the QC in the mandated method or regulations is to be followed."

What To Look For During An Assessment

What To Look For

- Mishandling of test organisms
  - Good labs will have documentation of good health and procedures for insuring consistently healthy organisms.
- Missing documentation
  - Good labs will have culture logs, calibration logs, taxonomy and organism history.
- Inadequate cleaning
  - Confounding test results, many exceptions, *e.g.*, not using replicates due to death of all organisms inexplicably.
- Temperature and light controls checked.

What to Look For

- Positive control
  - Reference toxicant tests/ lab ability to achieve consistent results AND overall health of the test organisms.
- The vendor
  - Provided reference toxicant test results only show overall health of the test organisms, it does not show DOC of the laboratory.
- Lab must also show it can consistently achieve statistically the same results on different organism batches using the same toxicant.
- Control charts for every combination of variables.

What to Look For

- Taxonomy
  - the science of identification and/or verification of species of organisms.
  - must be verified annually or more often if cultures are re-started.
- Source (supplier) provides taxonomic ID when lab uses purchased organisms rather than in-house cultures.
- Taxonomic identification kept on file must include reference
  - citation and Page(s).
  - name(s) of the taxonomic expert(s).

What to Look For

- Test records
  - All data recorded timely and in a definite format from sample receipt to final report.
- Any changes or decisions pertaining to data must be recorded and reasons must be in records and report.
- Supporting Chemical Data
  - pH
  - Conductivity
  - Temperature
  - Hardness
  - Alkalinity
  - Salinity, Chlorine, *etc.*, when applicable
  - DOC records maintained
  - QA/QC in the referenced method must be followed

What to Look For

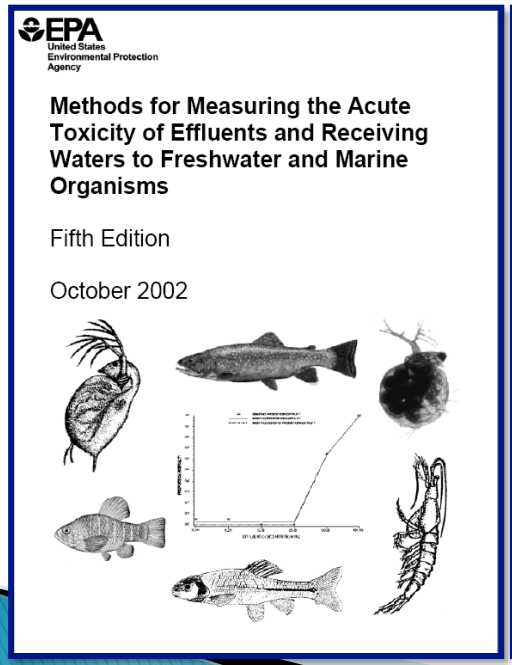
- Analysis of purified water and organism food.
- Test organism history, provides traceability.
- Test organism when using a Vendor:
  - Certificate from vendor of hatch or release date, (and time for *Ceriodaphnia dubia* chronic test organisms).
  - Temperature maintained in brood culture until shipment
  - Food given since birth/hatch/release.
- In house cultures:
  - All the same data must be maintained by the laboratory.

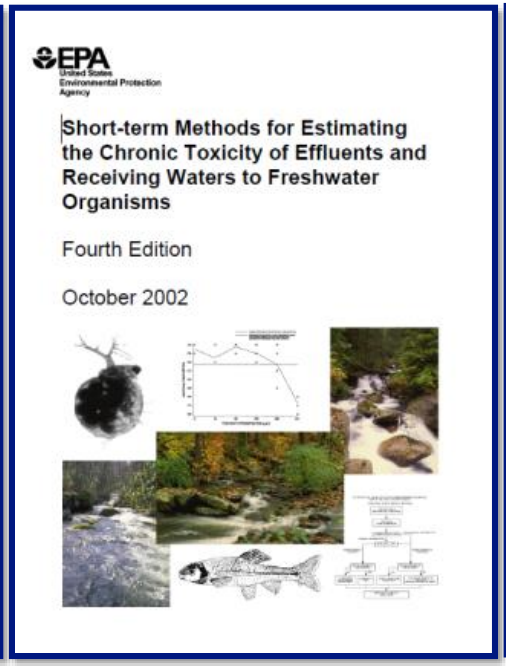
What To Look For

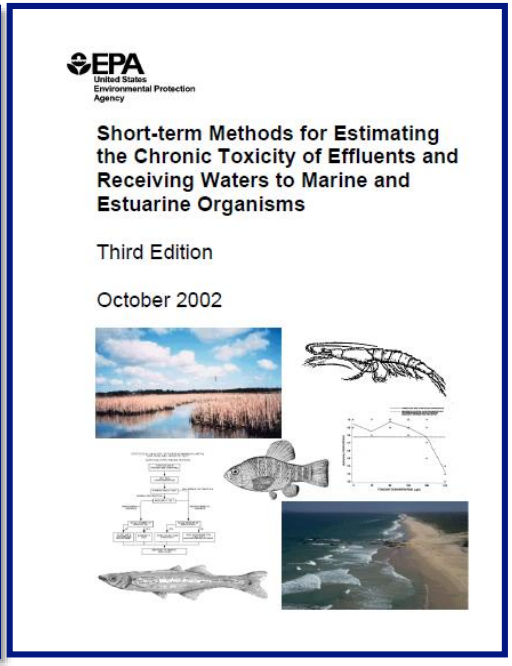
- TNI V1M2 4.13.3f: "All information necessary for historical reconstruction of data shall be maintained by the laboratory."
  - If anything listed in the reference method is missing, the assessor can cite above for the finding.
    - Serial numbers on electrodes.
    - Thermometers.
    - Missing lot number of organisms used.
    - Batch numbers of synthetic water or food used.
    - Missing records of daily feedings of the tests or cultures.
    - You get the picture!

What To Look For

- References available for use:
  - TNI Volume 1 Module 7 Checklist
  - The WETT Methods Manuals and Errata
    - https://www.epa.gov/cwa-methods/whole-effluent-toxicity-methods



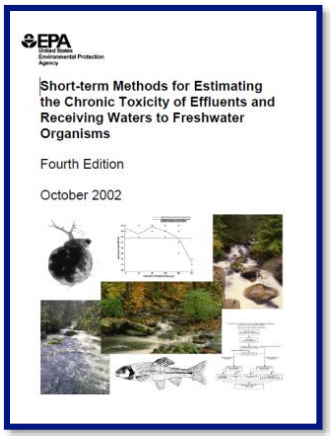




Examples of Auditor Findings

Laboratory Not Randomizing Test Chambers in the Chronic Fathead Minnow test (EPA Method 1000.0) Using a Random Template

- This is a “must” not a “should”.
- 11.3.4.5.1– “All test chambers **MUST** be randomized using a template for randomization or by using a table of random numbers.”
- 11.10.2.3 – “Randomize the position of the test chambers at the beginning of the test (see Appendix A). Maintain the chamber in this configuration throughout the test.”



Random Number Template

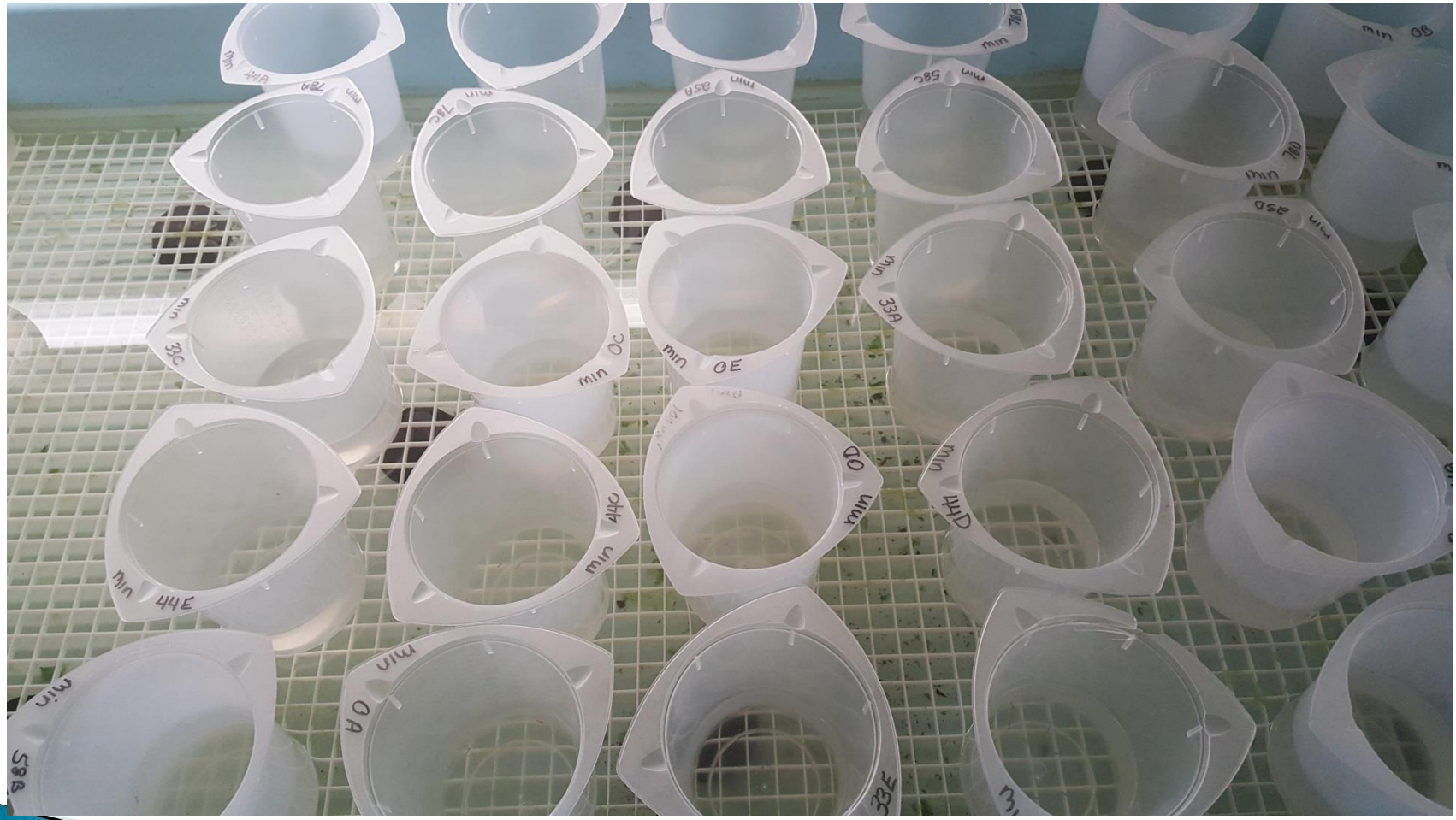
Test: LF-Larval Fish Growth and Survival Test
Species: PP-Pimephales promelas
Sample ID: LA12345
Start Date: 7/25/2016 End Date: 7/31/2016

Test ID: X6100
Protocol: EPAFW02-EPA/821/R-02-013
Sample Type: EFF1-POTW
Lab ID: LA00917

Pos	ID	Rep	Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Total Wgt(	Tare Wgt(	Wgt Count
66	14	4	12.5											
67	9	4	6.25											
68	21	1	50											
69	12	2	12.5											
70	28	3	100											
71	8	3	6.25											
72	2	2	D-Control											
73	23	3	50											
74	29	4	100											
75	1	1	D-Control											
76	20	5	25											
77	27	2	100											
78	11	1	12.5											
79	19	4	25											
80	26	1	100											
81	25	5	50											
82	13	3	12.5											
83	7	2	6.25											
84	17	2	25											
85	3	3	D-Control											
86	10	5	6.25											
87	24	4	50											
88	4	4	D-Control											
89	15	5	12.5											
90	6	1	6.25											
91	22	2	50											
92	5	5	D-Control											
93	30	5	100											
94	18	3	25											
95	16	1	25											

Comments:

Randomized Chronic Fish Test



Laboratory Failed to Use Blocking by Known Parentage When Initiating the Chronic *Ceriodaphnia dubia* Chronic Test (Method 1002.0)

- This is a "must".
- 13.10.2.2 – "The test chambers **MUST** be randomly assigned to a board using a template (Figure 1) or by using random numbers (see Appendix A). Randomizing the position of the test chambers as described in Figure 1(or equivalent) will assist in assigning test organisms using blocking by known parentage (Subsection 13.10.2.4)."

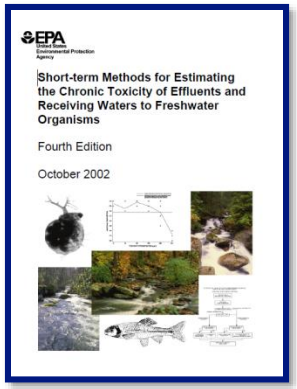


Figure 1: Examples of a Test Board and Randomizing Template

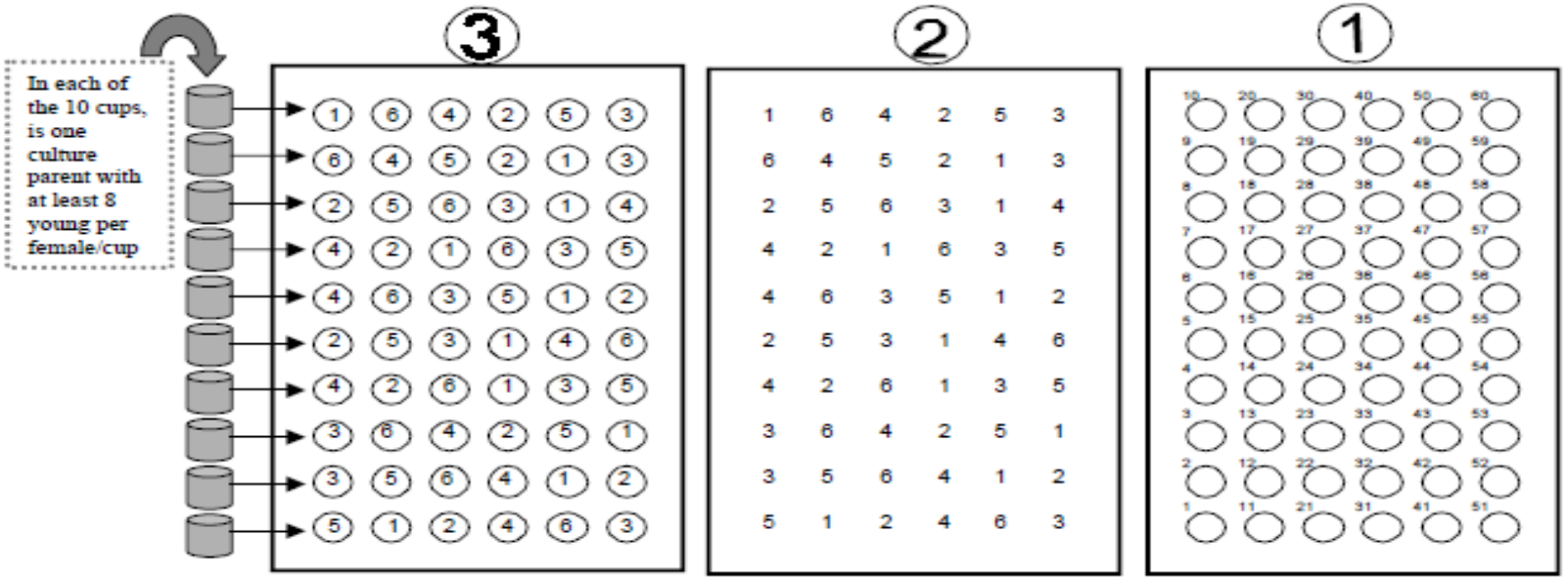


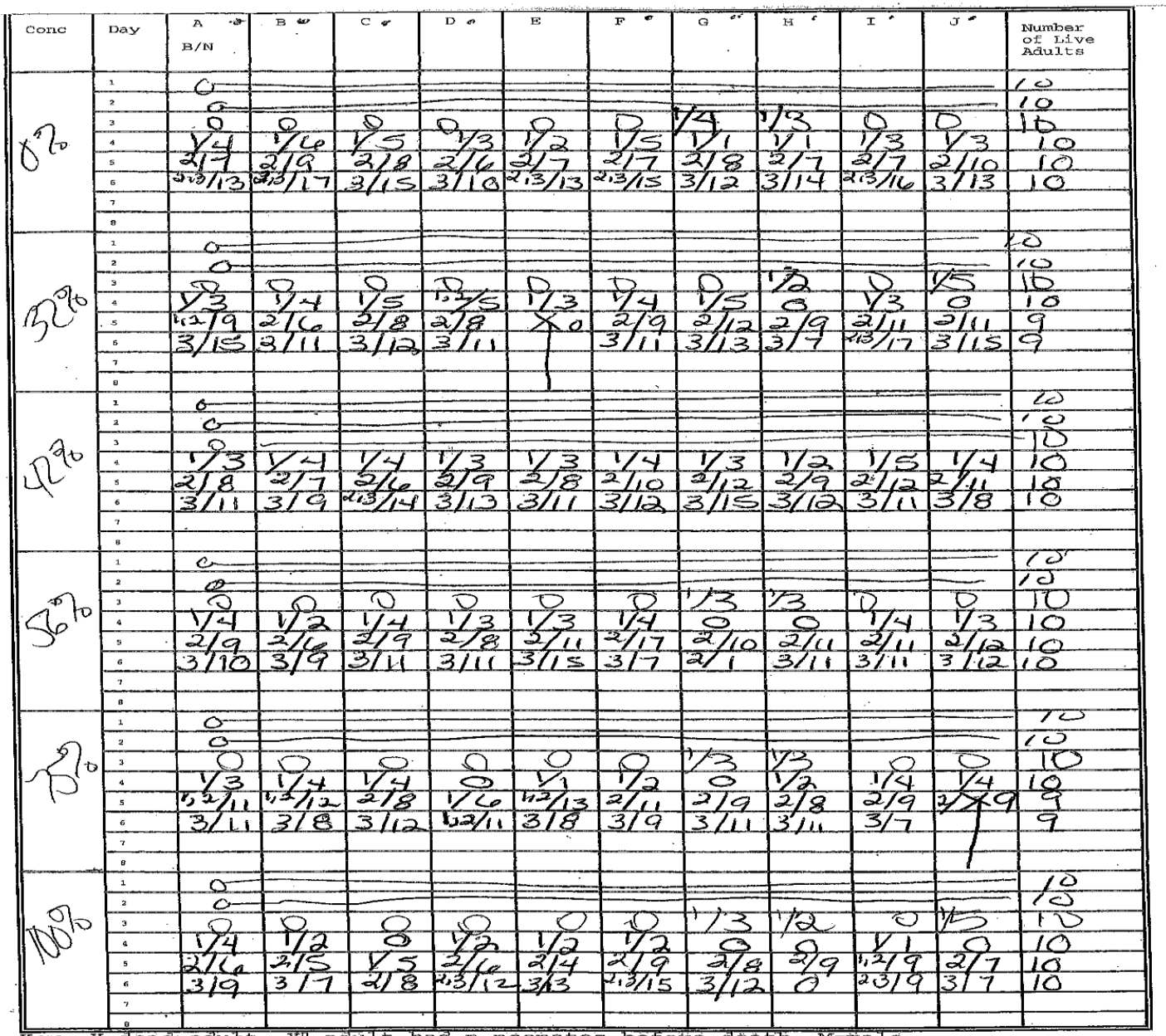
Figure 1. Examples of a test board and randomizing template:
1) test board with positions for six columns of ten replicate test chambers with each position numbered for recording results on data sheets,
2) cardboard randomizing template prepared by randomly drawing numbers (1-6) for each position in a row across the board, and
3) test board with random locations showing from template.

In practice: test board 1 is placed on top of the 2) the randomizing template, and the test organisms are assigned from one brood cup to each treatment within a given block. Following placement of test chambers, test organisms are allocated using blocking by known parentage.

Laboratory Counting 4th Brood of Neonates in The *Ceriodaphnia dubia* Chronic Test (Method 1002.0)

- 13.10.9.1 – "Tests should be terminated when 60% or more of the surviving control females have produced their 3rd brood, or at the end of 8 days, whichever comes first".
- 4th and higher broods should not be counted and not included in the total number of neonates produced during the test.
- Laboratory should be able, based on their cultures, to determine individual broods in the test.

Data Sheet Showing Brood Tracking



Conc	Day	A B/N	B	C	D	E	F	G	H	I	J	Number of Live Adults
0%	1	0										10
	2	0										10
	3	0	0	0	0	0	0	1/4	1/3	0	0	10
	4	1/4	1/6	1/5	1/3	1/2	1/5	1/1	1/1	1/3	1/3	10
	5	2/7	2/9	2/8	2/6	2/7	2/7	2/8	2/7	2/7	2/10	10
	6	2.3/13	2.3/17	3/15	3/10	2.3/13	2.3/15	3/12	3/14	2.3/16	3/13	10
	7											
	8											
32%	1	0										10
	2	0										10
	3	0	0	0	0	0	0	0	1/2	0	1/5	10
	4	1/3	1/4	1/5	1.3/5	1/3	1/4	1/5	0	1/3	0	10
	5	1.2/9	2/6	2/8	2/8	X 0	2/9	2/12	2/9	2/11	2/11	9
	6	3/15	3/11	3/12	3/11		3/11	3/13	3/7	2.3/17	3/15	9
	7											
	8											
42%	1	0										10
	2	0										10
	3	0										10
	4	1/3	1/4	1/4	1/3	1/3	1/4	1/3	1/2	1/5	1/4	10
	5	2/8	2/7	2/6	2/9	2/8	2/10	2/12	2/9	2/12	2/11	10
	6	3/11	3/9	2.3/14	3/13	3/11	3/12	3/15	3/12	3/11	3/8	10
	7											
	8											
56%	1	0										10
	2	0										10
	3	0	0	0	0	0	0	1/3	1/3	0	0	10
	4	1/4	1/2	1/4	1/3	1/3	1/4	0	0	1/4	1/3	10
	5	2/9	2/6	2/9	2/8	3/11	2/17	2/10	2/11	2/11	2/12	10
	6	3/10	3/9	3/11	3/11	3/15	3/7	2/1	3/11	3/11	3/12	10
	7											
	8											
75%	1	0										10
	2	0										10
	3	0	0	0	0	0	0	1/3	1/3	0	0	10
	4	1/3	1/4	1/4	0	1/1	1/2	0	1/2	1/4	1/4	10
	5	1.2/11	1.2/12	2/8	1/6	1.2/13	2/11	2/9	2/8	2/9	3/X9	9
	6	3/11	3/8	3/12	1.2/11	3/8	3/9	3/11	3/11	3/7		9
	7											
	8											
100%	1	0										10
	2	0										10
	3	0	0	0	0	0	0	1/3	1/2	0	1/5	10
	4	1/4	1/2	0	1/3	1/2	1/2	0	2	1/1	0	10
	5	2/6	2/5	1/5	2/6	2/4	2/9	2/8	2/9	1.2/9	2/7	10
	6	3/9	3/7	2/8	2.3/12	3/3	2.3/15	3/12	0	2.3/9	3/7	10
	7											
	8											

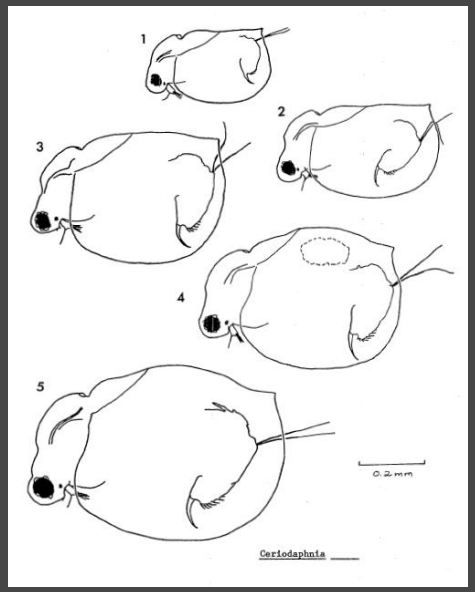
Key: X=dead adult, X"=adult had n neonates before death, M=male.
B/N = Brood count/#neonates

CERIO2 Rev.3.0

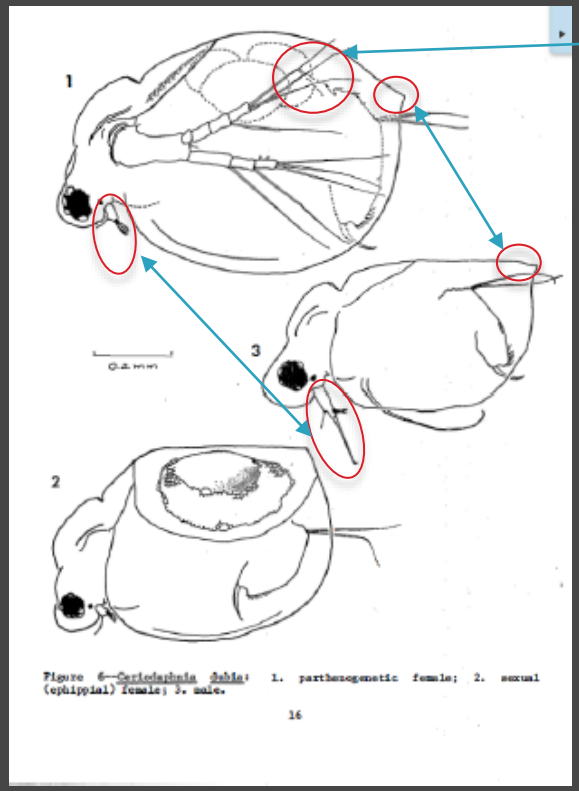
Laboratory Did Not Identify Males: Chronic *Ceriodaphnia dubia* Test (Method 1002.0)

- 13.10.9.3- "Any animal not producing young should be examined to determine if it is a male (Berner, 1986). In most cases, the animal will need to be placed on a microscope slide before examining (see Subsection 13.6.16.4).
  - Even though the above statement says "should", it is one of those "shoulds" that will affect the test results.

Taxonomy of *Ceriodaphnia dubia*

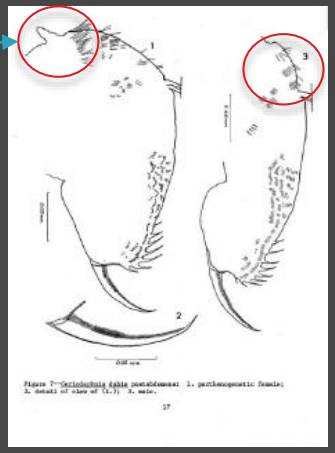


Shape changes during growth of *Ceriodaphnia dubia* parthenogenic females.
Figures 1-3 are juveniles; Figures 4 and 5 are adults.



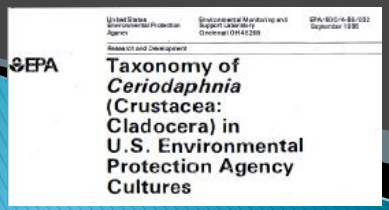
Gamoetogenetic female: Length, about 0.73mm, Height, about 0.76 times length. Shape rounded, flattened dorsally along top of ephippium Lower borders of ephippium forming a rounded curve or a broad V. Ephippium exhibits three distinct regions:

- a flattened border region lacking cellular outliers,
- a raised, semicircular region of deep polygonal cells having slightly domed surfaces
- the dorsal locule, which is covered with small, circular bumps. These become more prominent as ecdysis (moulting) approaches.



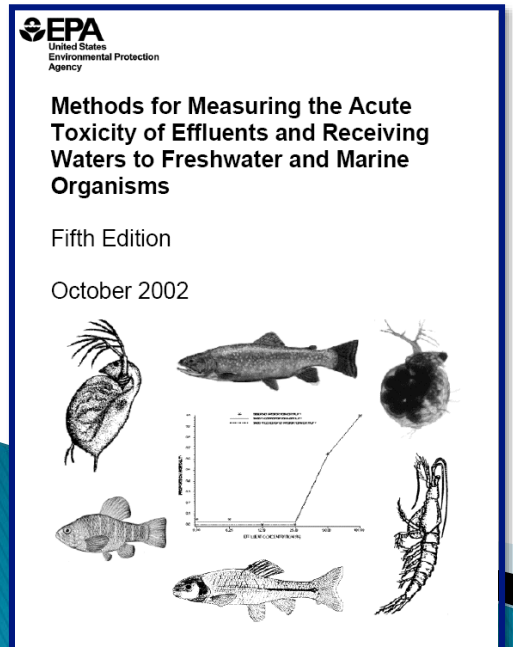
Male: Length, about 0.58 mm Height, about 0.55 times length .Scope, elongate oval, flattened dorsally and ventrally.
Head, larger in proportion to body to a female, and not as fully depressed, with a distinct dorsal fenestra and supraocular depression. Antennule only slightly longer than that of female, with short aesthetascs and a very short, straight terminal male seta, equal or shorter in length than that of the body of the antennule.

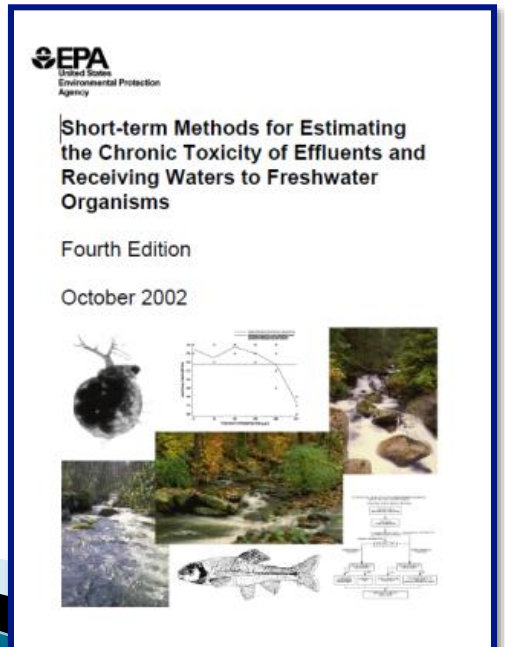
Source:

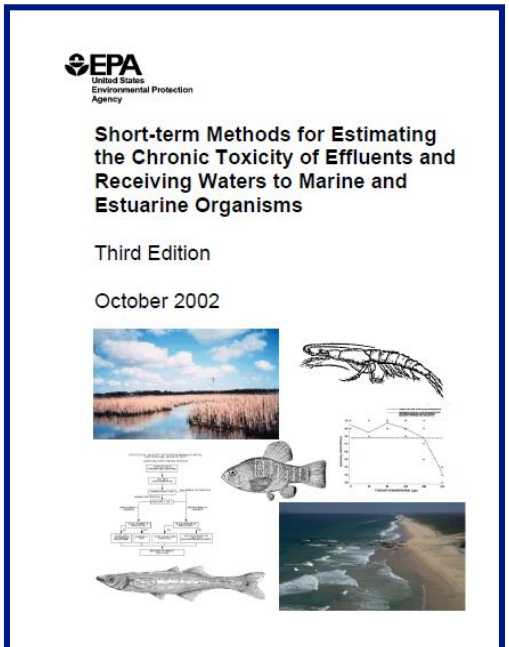


Laboratory's Reference Toxicant Test Data Did Not Exhibit Two or More Partial Mortalities

- This is a "should" not a "must".
- 4.14 – "….A reference toxicant concentration series (0.5 or higher) **SHOULD** be selected that will consistently provide partial mortalities at two or more concentrations."





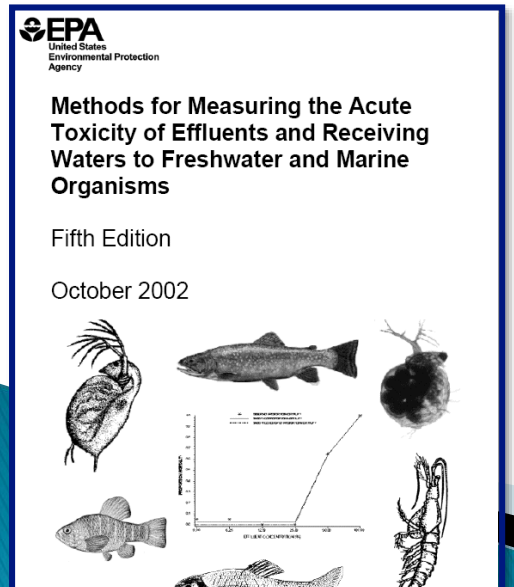


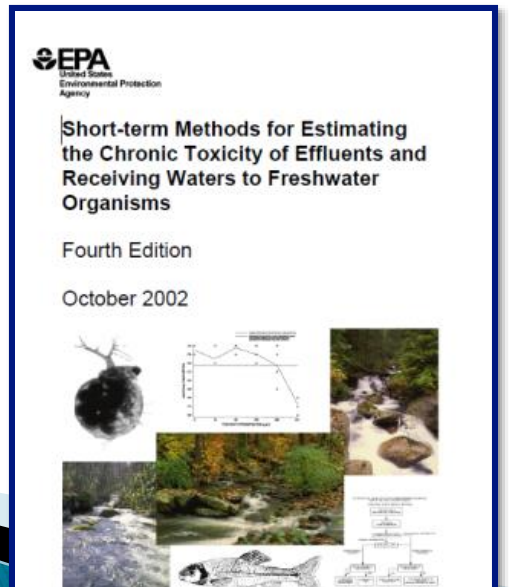
Batch or Lot Information Not Provided from Marine Salt Manufacturer

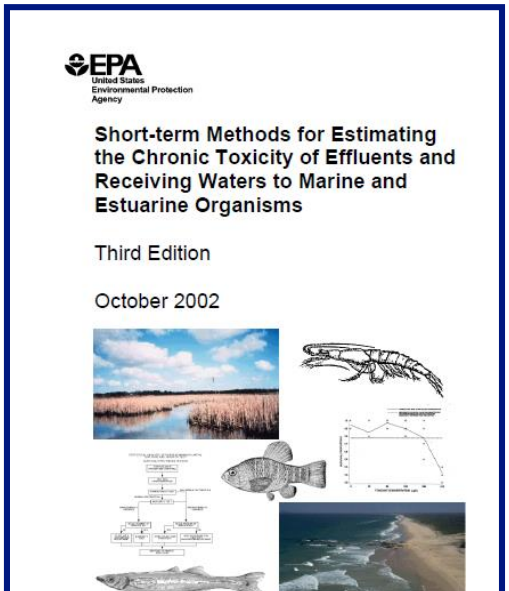
- WET laboratories buy products that, sometimes, do not have easily obtainable certificate of analysis documents. When this occurs, laboratories should develop a system for receiving and using these products, as well as ensuring that the products are not affecting organism health or test results. Examples are:
  - Marine sea salt
  - Food

Taxonomy of Test Organisms Not Positively Identified to Species

- **Must** be positively identified annually.
  - Use a taxonomic reference, citing the page and name of the taxonomic expert(s) and author.
  - Certification must be kept on file at the laboratory for every organism cultured in-house or purchased.
  - In all references and in 1.7.1.6d of V1M7 of TNI Standard.







No Documentation On Light Cycle and Intensity Levels

- 1.7.1.6p of V1 M7 of the TNI 2009 Standard: "Light intensity shall be maintained as specified in the methods. Measurements shall be made a recorded on a yearly basis. Photoperiod shall be maintained as specified in the methods and shall be documented at least quarterly. For algal and plant tests, the light intensity shall be measured and recorded at the start of each test."

Test Containers Used for Different Tests

- The same containers used to make dilutions for one test were not properly cleaned before using for another test.
- Section 5 of the Method Manual. MUST!

Closing Remarks

- EPA now has recorded webinars for Whole Effluent Toxicity Testing (2016) available at https://www.epa.gov/npdes/npdes-training#wettraining

Module 1: Overview of the NPDES WET Permitting Program
Module 2: NPDES Testing Methods for Whole Effluent Toxicity
Module 3: NPDES Reviewing WET Tests and WET QA/QC
Module 4: NPDES WET Statistical Analysis and Data Interpretation
Module 5: Determining WET Reasonable Potential for NPDES Permitting
Module 6: NPDES WET Permit Development
Module 7: NPDES WET Testing Decision-Making and WET Permit Language Review
Module 8: NPDES WET Compliance and Enforcement
Module 9: NPDES Toxicity Reduction Evaluations and Toxicity Identification Evaluations

Closing Remarks

EPA Freshwater Series Videos	EPA Saltwater Series Videos
Ceriodaphnia Survival and Reproduction Toxicity Test Training Video	Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests Training Video Culturing Mysids (*Americamysis bahia*) Training Video
Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Test Training Video	Sperm Cell Toxicity Tests Using the Sea Urchin (*Arbacia punctulata)* Training Video
Culturing Fathead Minnows (*Pimephales promelas*) Training Video	Red Algal (*Champia parvula*) Sexual Reproduction Toxicity Tests Training Video
	Sheepshead Minnow (*Cyprinodon variegatus*) and Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Toxicity Tests Training Video

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