BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

HOLLAND ENERGY, LLC	)	
	)	
Petitioner,	)	
	)	
v.	)	
	)	**PCB 11-_____**
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY,	)	**(NPDES Permit Appeal – Water)**
	)	
	)	
Respondent.	)	
	)	

NOTICE OF ELECTRONIC FILING

TO:

John Therriault, Clerk
Illinois Pollution Control Board
James R. Thompson Center
100 West Randolph Street, Suite 11-500
Chicago, IL 60601

PLEASE TAKE NOTICE that I have today electronically filed with the Office of the Clerk of the Illinois Pollution Control Board, Petitioner's **NOTICE OF ELECTRONIC FILING, NOTICE OF APPEARANCE, PETITION FOR REVIEW OF AGENCY NPDES PERMIT DECISION AND MOTION TO CONFIRM AUTOMATIC STAY OF NPDES PERMIT** and **CERTIFICATE OF SERVICE**, copies of which are attached herewith served upon you.

Respectfully submitted,

ICE MILLER, LLP

By: /s/ Susan Charles
One of its Attorneys

Date: May 18, 2011

Susan Charles
ICE MILLER LLP
200 West Madison Street
Suite 3500
Chicago, Illinois 60606
Phone: 312-726-1567
Facsimile: 312-726-7102
Susan.Charles@IceMiller.com

BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

HOLLAND ENERGY, LLC	)	
	)	
Petitioner,	)	
	)	
v.	)	
	)	**PCB 11-_____**
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY,	)	**(NPDES Permit Appeal – Water)**
	)	
	)	
Respondent.	)	
	)	

NOTICE OF APPEARANCE

PLEASE TAKE NOTICE THAT, pursuant to 35 Ill. Adm. Code Section 101.400(a)(4), Susan Charles, with the law firm of ICE MILLER, LLP, hereby files her Appearance in this proceeding on behalf of petitioner, HOLLAND ENERGY, LLC.

Respectfully submitted,

ICE MILLER, LLP

By: /s/ Susan Charles
One of its Attorneys

Date: May 18, 2011

Susan Charles
ICE MILLER LLP
200 West Madison Street
Suite 3500
Chicago, Illinois 60606
Phone: 312-726-1567
Facsimile: 312-726-7102
Susan.Charles@IceMiller.com

CERTIFICATE OF SERVICE

I, the undersigned, certify that on this 18th day of May, 2011, I have served electronically the attached NOTICE OF ELECTRONIC FILING, NOTICE OF APPEARANCE and PETITION FOR REVIEW OF AGENCY NPDES PERMIT DECISION AND MOTION TO CONFIRM AUTOMATIC STAY OF NPDES PERMIT upon the following person:

John Therriault, Clerk
Illinois Pollution Control Board
James R. Thompson Center
100 West Randolph Street, Suite 11-500
Chicago, IL 60601

and by U.S. Mail, first class postage prepaid, to the following persons:

Division of Legal Counsel
Illinois Environmental Protection Agency
1021 North Grand Avenue East
P.O. Box 19276
Springfield IL 62794-9276

/s/ Susan Charles
Susan Charles

BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

HOLLAND ENERGY, LLC	)	
	)	
Petitioner,	)	
	)	
v.	)	
	)	**PCB 11-____**
ILLINOIS ENVIRONMENTAL PROTECTION AGENCY,	)	**(NPDES Permit Appeal – Water)**
	)	
	)	
Respondent.	)	
	)	

PETITION FOR REVIEW OF AGENCY NPDES PERMIT DECISION
AND
MOTION TO CONFIRM AUTOMATIC STAY OF NPDES PERMIT

HOLLAND ENERGY, LLC ("Holland"), through its counsel and pursuant to Section 5/40(a)(1) of the Illinois Environmental Protection Act, 415 ILCS 5/1, *et seq.* ("Act"), and 35 Ill. Adm. Code §§ 105.200, *et seq.*, respectfully submits this Petition for Review of Agency NPDES Permit Decision ("Petition") and Motion to Confirm Automatic Stay of NPDES Permit ("Motion") to the Illinois Pollution Control Board ("Board"). Holland requests a hearing to contest final decisions included in Special Condition Numbers 13 and 14 of National Pollutant Discharge Elimination System ("NDPES") Permit Number IL0074268 ("Permit") issued on April 18, 2011, by the Illinois Environmental Protection Agency ("IEPA") to the Holland Energy Facility. Holland also seeks confirmation that the effectiveness of the Permit is automatically stayed pending final resolution of this appeal. A copy of the Permit is attached as Exhibit 1.

I. BACKGROUND

A. The Holland Energy Facility

1. The Holland Energy Facility is an electric generating station built on a 240-acre site near Beecher City in Shelby County, Illinois. Construction began on the 640-megawatt natural gas combined cycle facility in June 2000 and was completed in September 2002.

2. The Permit authorizes Holland to discharge to the Kaskaskia River from the Holland Energy Facility, located in Section 16, Township 9 North, Range 4 East, in Holland Township, Illinois.

3. The Permit includes three authorized outfalls – Outfalls 001, 002 and 003. Treated plant effluent is transported to Outfall 001 through a 10-inch buried HPDE pipe and discharges directly to the Kaskaskia River through a multi-port diffuser pipe mounted on the River's bottom. The effluent from Outfall 001 consists primarily of cooling tower blowdown, evaporative cooler blowdown, demineralizer regenerate, filter backwash and turbine wash. Outfall 001 has a daily average flow ("DAF") of 1.42 million gallons per day ("MGD").

4. Outfall 002 has a DAF of 0.105 MGD and discharges from the north stormwater basin to an unnamed tributary to Bush Creek. Effluent from Outfall 002 consists primarily of hydrostatic test water, water line clean out and stormwater. Outfall 003 has a DAF of 0.1 MGD and discharges from the south stormwater basin to an unnamed tributary to Bush Creek. Effluent from Outfall 003 consists primarily of hydrostatic test water and stormwater.

B. Holland's Prior NPDES Permit.

5. On July 21, 2000, the Agency issued NPDES Permit No. IL0074268 to Holland Energy, LLC ("Prior NPDES Permit"). *See* Exhibit 2. The Prior NPDES Permit has an effective date of July 21, 2000. *Id.* The Agency issued a modification to the Prior NPDES Permit on July 25, 2000. *Id.* The Agency issued a second modification to the Prior NPDES Permit on December 31, 2001. *Id.* The Prior NPDES Permit (as modified on December 31, 2001) has an expiration date of June 30, 2005. *Id.* Holland timely filed an application to renew the Prior NPDES Permit on December 29, 2004. *See* Exhibit 3. Pursuant to 35 Ill. Adm. Code § 309.104, the Prior NPDES Permit remained in effect pending the Agency's final decision on Holland's renewal application.

6. The Agency drafted a renewal NPDES Permit for the Holland Energy Facility and placed it for public notice on December 7, 2007. The public notice period closed January 7, 2008. Hoosier Energy Rural Electric Cooperative, Inc. ("Hoosier Energy") and Wabash Valley Power Association, Inc. ("WVPA") acquired Holland and the Holland Energy Facility on January 7, 2009. Pursuant to 35 Ill. Adm. Code § 325.435, Holland submitted a notification of a change in ownership to the Agency on January 8, 2009.

7. Hoosier Energy and WVPA submitted written comments on the draft renewal Permit on January 19, 2009. The comments addressed, among other Permit conditions, Special Condition Nos. 13 and 14. The Agency issued the Permit on April 18, 2011. The transmittal letter attached to the Permit included specific responses to the comments submitted by Hoosier Energy and WVPA on January 19, 2009. *See* Exhibit 4, at p. 1. However, the Agency did not

incorporate any of Hoosier Energy's and WVPA's comments with respect to Special Condition Nos. 13 and 14. *Id.*

8. The Permit states that it is effective May 1, 2011. Pursuant to 415 ILC 5/40(a)(1) and 35 Ill. Adm. Code § 105.206(a), Holland has 35 days from the date it is served with the Permit to petition the Board for review of the Agency's decision regarding the Permit. Holland received the Permit on approximately April 19, 2011. Holland filed this Petition with the Board on May 18, 2011. This Petition is timely filed with the Board.

II. ISSUES ON APPEAL

9. Holland appeals two conditions of its Permit: (a) Special Condition 13, Biomonitoring Plan; and (b) Special Condition 14, Monitoring Plan. The bases for its appeal are set forth in detail below.

A. Special Condition 13 – Biomonitoring Plan

10. Holland challenges Special Condition 13 of the Permit as internally inconsistent, impossible to comply with and, therefore, arbitrary, capricious, an abuse of discretion and otherwise not in accordance with law. Special Condition 13 provides, in relevant part:

> The Permittee shall prepare a preliminary biomonitoring plan and submit the plan to IEPA for review and approval within ninety (90) days of the effective date of this Permit. *The Permittee shall begin biomonitoring effluent from Outfall 001 the first summer after plan approval.*
>
> Biomonitoring
>
> 1. Toxicity Test – Acute (4-d) and short-term (14-d) toxicity tests shall be run on juveniles of mussel species representative of the aquatic community of the receiving stream. Procurement and testing of organisms must be consistent with Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater

> Mussels (ASTM E2455-06). Guidelines for measuring effluent toxicity must be consistent with Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (Fifth Ed.) EPA/821-R-02-012. Unless substitute tests are pre-approved, the following test is required.
>
> > An acute (4-d) and short-term (14-d) static-renewal toxicity test using newly-transformed juvenile fatmucket (*Lampsilis siliquoidea*) or another IEPA pre-approved native species.
>
> 2. Testing Frequency - * * * *Testing must be conducted once per year for two year [sic] beginning the first Summer after permit issuance.*

See Exhibit 1, Special Condition 13, at p. 9 (emphasis added).

11. Special Condition 13 is inconsistent on its face and should be revised so that compliance is possible. Special Condition 13 requires Holland to conduct 4-day acute and 14-day short-term toxicity testing. However, the Permit also requires that Holland's procurement and testing methods be consistent with ASTM E2455-06.[1] ASTM E2455-06 contemplates a 4-day acute and 21 - 28-day chronic testing period. *See* Exhibit 7, M E2455-06, Table A1.4, p. 46 at Column Titled, "Recommended Test Conditions." ASTM E2455-06 does not reference any parameters for "short-term" toxicity testing. *Id.* The 14-day short term test required by the Permit is therefore inconsistent with ASTM E2455-06.

12. Special Condition 13 should be revised so that its requirements are internally consistent. Holland requests that the requirement in Special Condition 13 to conduct a "short-term (14-d) toxicity test[]" be removed and replaced with the requirement to conduct a 21 – 28-day chronic test, as set forth in ASTM E2455-06. *Cf. People v. Holloway*, 177 Ill. 2d 1, 8, 682

[1] The toxicity testing procedures required under Special Condition 13 is not included as an approved test method by the United States Environmental Protection Agency ("USEPA") in 40 CFR § 136.3. If a testing method is not included in 40 CFR § 136.3, the Agency is required to obtain USEPA's approval of that alternative testing method pursuant to the requirements of 40 CFR § 136.5 prior to including the alternative testing method in a NPDES permit. By letter dated September 23, 2010, the Agency requested approval of the test method included in Special Condition 13 from USEPA as an alternative test method pursuant to 40 CFR § 136.5. *See* Exhibit 5. By letter dated September 23, 2010, USEPA approved the testing protocol included in Special Condition 13. *See* Exhibit 6.

N.E.2d 59, 63 (1997) ("A term is considered ambiguous if more than one interpretation of it is reasonable."); *See, e.g., Caterpillar Tractor Co. v. Illinois EPA*, 1981 Ill. ENV LEXIS 113 (Ill. ENV 1981) (holding that permit should state with certainty the permittee's duty and that, when a provision is subject to multiple interpretations, the provision or term is ambiguous).

13. The requirement to conduct a 14-day short-term test with newly-transformed juvenile fatmucket (less than five (5) days old) also is inconsistent with ASTM E2455-06. Juveniles are appropriate for a 4-day acute test (*see* Exhibit 7,Table A1.4, at p. 46, comparing rows 3 and 11) but are inappropriate for evaluating chronic toxicity results – the freshwater mussels will not have grown enough in 14 days to develop a measurable sublethal growth endpoint. ASTM E2455-06 reports that tests with durations greater than 4 days typically use 60 to 120 day old mussels. *Id.* The Permit's requirement to utilize juvenile fatmuckets in the 14-day test is inappropriate and inconsistent with ASTM E2455-06.

14. Special Condition 13 should be revised so that its requirements are internally consistent and capable of compliance. Holland requests that the requirement in Special Condition 13 to conduct a 14-day short-term test with newly-transformed juvenile fatmucket (less than five (5) days old) be removed and replaced with the requirement to conduct a 21 – 28-day chronic test using 60 to 120 day old mussels, as set forth in ASTM E2455-06. *Cf. People v. Holloway*, 177 Ill. 2d 1, 8, 682 N.E.2d 59, 63 (1997) ("A term is considered ambiguous if more than one interpretation of it is reasonable."); *Caterpillar Tractor Co. v. Illinois EPA*, 1981 Ill. ENV LEXIS 113 (Ill. ENV 1981) (holding that permit should state with certainty the permittee's duty and that, when a provision is subject to multiple interpretations, the provision or term is ambiguous).

15. Special Condition 13 also is arbitrary, capricious and an abuse of discretion because it's timing requirements are inherently inconsistent and render compliance impossible. Special Condition 13 requires that the biomonitoring toxicity testing be conducted "once per year for two year [sic] *beginning the first Summer after permit issuance.*" *See* Exhibit 1 at Special Condition 13, ¶ 2 (emphasis added). The permit's effective date is May 1, 2011, and the first summer after the Permit's issuance will begin in June 2011. Notwithstanding this requirement, Special Condition 13 also contemplates ninety (90) days for Holland to submit a preliminary biomonitoring plan to the Agency (*e.g.*, not later than July 29, 2011). *Id.* The Agency then has an unspecified time period to approve the preliminary biomonitoring plan. *Id.* Considering the timing associated with submission and approval of a biomonitoring plan, it will be impossible for Holland to complete biomonitoring toxicity testing during the summer of 2011.

16. Special Condition 13 should be revised so that compliance with the biomonitoring plan required under Special Condition 13 it is not required until the first summer after the Agency approves the monitoring plan. *See, e.g., Caterpillar Tractor Co. v. Illinois EPA*, 1981 Ill. ENV LEXIS 113 (Ill. ENV 1981) (noting that a permit provision or term is ambiguous if it does not state with certainty the permittee's duty or is subject to multiple interpretations); *Village of Sauget v. Illinois EPA*, 1988 Ill. Env. Lexis 516 (Ill. Env. 1988) (noting that it was impossible for the permittee to comply with a permit when the permit did not state with certainty the discharger's duty due to the permit's broad definition of "contaminant" that did not clearly identify to permittee which contaminants to monitor or what permittee should do); *see also Browning Ferries Industries of Illinois, Inc. v. Lake County Board of Supervisors*, PCB No. 82-101, 1982 Ill. ENV LEXIS 255, 31-32 (Ill. ENV 1982) (holding grant conditions requiring the

testing of unspecified "pollutants before pumping begins" and testing of private wells were vague and unenforceable because no clear test, or parameters for action, was specified).

B. Special Condition 14 – Monitoring Plan

17. Holland challenges Special Condition 14 of the Permit as internally inconsistent, impossible to comply with and, therefore, arbitrary, capricious, an abuse of discretion and otherwise not in accordance with law. Special Condition 14 provides, in relevant part:

> The Permittee shall prepare a monitoring plan for the following biological parameters in the Kaskaskia River and submit the plan to IEPA for review and approval within 90 days of the effective date of the Permit. The Permittee shall *implement the biological monitoring plan during the first defined low flow conditions between July and September after approval of the plan.*
>
> > The monitoring plan shall include mussel surveys that repeat previous surveys conducted prior to and throughout the initial permit issuance. The surveys must be conducted during defined low flow conditions between July and September. *Mussel surveys shall be conducted the first summer after permit issuance and annually thereafter.*

See Exhibit 1, Special Condition 14, at p. 10 (emphasis added).

18. Special Condition 14 is inconsistent on its face and should be revised so that compliance is possible. Special Condition 14 first requires that the monitoring of biological parameters be implemented "during the first defined low flow conditions between July and September after approval of the plan." *Id.* Special Condition 14 then goes on to require that the monitoring plan be conducted "the first summer after permit issuance." *Id.* The permit's effective date is May 1, 2011, and the first summer after the Permit's issuance will begin in June 2011. Notwithstanding this requirement, Special Condition 14 also contemplates ninety (90) days for Holland to submit a preliminary biological monitoring plan to the Agency (*e.g.*, not later

than July 29, 2011). *Id.* The Agency then has an unspecified time period to approve the preliminary biological monitoring plan. Considering the timing associated with submission and approval of a biological monitoring plan, it will be impossible for Holland to complete biological monitoring testing during the summer of 2011.

19. Special Condition 14 should be revised so that compliance with the biological monitoring plan required under Special Condition 14 it is not required until the first summer after the Agency approves the biological monitoring plan. *See, e.g., Caterpillar Tractor Co. v. Illinois EPA*, 1981 Ill. ENV LEXIS 113 (Ill. ENV 1981) (noting that a permit provision or term is ambiguous if it does not state with certainty the permittee's duty or is subject to multiple interpretations); *Village of Sauget v. Illinois EPA*, 1988 Ill. Env. Lexis 516 (Ill. Env. 1988) (noting that it was impossible for the permittee to comply with a permit when the permit did not state with certainty the discharger's duty due to the permit's broad definition of "contaminant" that did not clearly identify to permittee which contaminants to monitor or what permittee should do); *see also Browning Ferries Industries of Illinois, Inc. v. Lake County Board of Supervisors*, PCB No. 82-101, 1982 Ill. ENV LEXIS 255, 31-32 (Ill. ENV 1982) (holding grant conditions requiring the testing of unspecified "pollutants before pumping begins" and testing of private wells were vague and unenforceable because no clear test, or parameters for action, was specified).

III. MOTION TO CONFIRM AUTOMATIC STAY OF PERMIT.

21. Under the Administrative Procedures Act, 5 ILCS 100/1-1 *et seq.* ("APA"), Holland's Permit is automatically stayed and the Prior NPDES Permit remains in full force and effect pending resolution of this Petition. Section 10-65(b) of the APA provides:

> When a licensee has made timely and sufficient application for the renewal of a license or a new license with reference to any activity of a continuing nature, the existing license shall continue in full force and effect until the final agency decision on the application has been made unless a later date is fixed by order of a reviewing court.

5 ILCS 100/10-65(b)(2008). Under Section 1-35, a "license" is defined to include the Permit. 5 ILCS 100/1-35 ("'License' includes the whole or part of any agency permit, certificate, approval, registration, charter, or similar form of permission required by law, but it does not include a license required solely for revenue purposes.").

22. The Board recently confirmed that, under Section 10-65(b) of the APA, a permit issued under 415 ILCS 5/39.5 is automatically stayed pending resolution of a timely filed appeal. *See KCBX Terminals Company v. Illinois Environmental Protection Agency,* PCB 11-43 (Ill. P. Control Bd. April 21, 2011). This result also has been specifically applied to an appeal of a NPDES permit. *Id., citing* Borg-*Warner Corp. v. Mauzy,* 100 Ill. App.3d 862, 427 N.E.2d 415 (3rd Dist. 1981); *see also Citgo Petroleum Corporation v. Illinois Environmental Protection Agency,* PCB 07-10 (Ill. P. Control Bd. Sept. 21, 2006).

23. The Agency issued the Permit on April 18, 2011 and Holland received the Permit on approximately April 19, 2011. Pursuant to 415 ILC 5/40(a)(1) and 35 Ill. Adm. Code § 105.206(a), Holland has 35 days from the date it is served with the Permit to petition the Board for review of the Agency's decision regarding the Permit. Holland filed this Petition with the Board on May 18, 2011. This Petition is timely filed with the Board.

24. Interpreting Section 10-65(b) of the APA in the context of an appealed NPDES permit, the appellate court held in *Borg-Warner*:

> Borg-Warner made the application for renewal of its NPDES permit, that application was timely and sufficient on the record before us, and therefore its original permit continues in effect until final action on the application by the administrative bodies charged with making the determination. A final decision, in the sense of a final and binding decision coming out of the administrative process before the administrative agencies with the decision making power, will not be forthcoming in the instant case until the [Board] rules on the permit application, after Borg-Warner has been given its adjudicatory hearing before the [Board]. Thus, until that time, under [the APA automatic stay], the effectiveness of the renewed permit issued by the EPA is stayed.

Borg-Warner, 100 Ill. App. 3d at 870-71, 427 N.E.2d at 421.

22. Here, the Permit is a "license." Holland timely filed with the Agency an application to renew the Prior NPDES Permit. The Agency issued the renewed Permit on April 18, 2011, conditions of which are the subject of this appeal. This appeal was timely filed. Accordingly, under the APA and Board precedent, Holland's Prior NPDES Permit should continue in full force and effect until final resolution of this Petition.

WHEREFORE, for the reasons set forth above, Holland appeals Special Condition Numbers 13 and 14 included in the Permit. Additionally, Holland requests that the Board confirm that the automatic stay authorized under the Section 10-65(b) of the Administrative Procedures Act applies and Holland's Prior NPDES Permit continues in full force and effect until final resolution of this Petition.

Respectfully submitted,

HOLLAND ENERGY, LLC

By:

/s/ Susan Charles
One of its Attorneys

Dated: May 18, 2011

ICE MILLER LLP
Susan Charles
300 West Wacker Drive, Suite 3500
Chicago, Illinois 60606
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Susan.Charles@IceMiller.com

NPDES Permit No. IL0074268

Illinois Environmental Protection Agency

Division of Water Pollution Control

1021 North Grand Avenue East

Post Office Box 19276

Springfield, Illinois 62794-9276

NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM

Reissued (NPDES) Permit

Expiration Date: April 30, 2016

Issue Date: April 18, 2011
Effective Date: May 1, 2011

Name and Address of Permittee:

Holland Energy, LLC
722 North High School Road
Indianapolis, IN 46214

Facility Name and Address:

Holland Energy Facility
RR2, 270-A
P.O. Box 65
Beecher City, Illinois 62414-0065
(Shelby County)

Discharge Number and Name:	Receiving Waters:
001 Cooling Tower Blowdown, Evaporative Cooler Blowdown, Demineralizer Regenerate, Filter Backwash, and Turbine Wash	Kaskaskia River
002 Hydrostatic Test water, Water Line Clean Out, and Stormwater	Unnamed Tributary to Brush Creek
003 Hydrostatic Test water and Stormwater	Unnamed Tributary to Brush Creek

In compliance with the provisions of the Illinois Environmental Protection Act, Title 35 of Ill. Adm. Code, Subtitle C and/or Subtitle D, Chapter 1, and the Clean Water Act (CWA), the above-named permittee is hereby authorized to discharge at the above location to the above-named receiving stream in accordance with the standard conditions and attachments herein.

Permittee is not authorized to discharge after the above expiration date. In order to receive authorization to discharge beyond the expiration date, the permittee shall submit the proper application as required by the Illinois Environmental Protection Agency (IEPA) not later than 180 days prior to the expiration date.

Alan Keller

Alan Keller, P.E.
Manager, Permit Section
Division of Water Pollution Control

SAK:DEL:LRL:06100601.daa

EXHIBIT 1

NPDES Permit No. IL0074268

Effluent Limitations and Monitoring

1. From the effective date of this permit until the expiration date, the effluent of the following discharge(s) shall be monitored and limited at all times as follows:

	LOAD LIMITS lbs/day DAF (DMF)		CONCENTRATION LIMITS mg/l			
PARAMETER	30 DAY AVERAGE	DAILY MAXIMUM	30 DAY AVERAGE	DAILY MAXIMUM	SAMPLE FREQUENCY	SAMPLE TYPE
Outfall 001 - Cooling Tower Blowdown, Evaporative Cooler Blowdown, Demineralizer Regenerate, Filter Backwash, and Turbine Wash (Daily Average Flow (DAF) = 1.42 MGD)						
Flow	See Special Condition 1.				Continuous While Discharging	
pH	See Special Condition 3.				1/Month	Grab
Total Suspended Solids			15	30	1/Month	Grab
Oil and Grease			15	20	1/Month	Grab
Chloride	See Special Condition 17.			Monitor Only	1/Month	Grab
Temperature	See Special Condition 6.				Continuous While Discharging	
Total Residual Chlorine				0.05	1/Month	Grab
Zinc (Total)*			1.0	1.0	1/Quarter	Grab
Phosphorus					1/Month	Grab
Chromium (Total)*			0.2	0.2	1/Quarter	Grab
Ammonia Nitrogen	See Special Condition 16.			Monitor Only	1/Month	Grab

* - Quarterly sampling results for Chromium (Total) and Zinc (Total) shall be submitted during the months of April, July, October and January for the preceding three month period.

NPDES Permit No. IL0074268

Effluent Limitations and Monitoring

1. From the effective date of this permit until the expiration date, the effluent of the following discharge(s) shall be monitored and limited at all times as follows:

	LOAD LIMITS lbs/day DAF (DMF)		CONCENTRATION LIMITS mg/l			
PARAMETER	30 DAY AVERAGE	DAILY MAXIMUM	30 DAY AVERAGE	DAILY MAXIMUM	SAMPLE FREQUENCY	SAMPLE TYPE
Outfall 002 - North Stormwater Basin* (DAF = 0.105 MGD)						
Flow	See Special Condition 1.				Measure When Monitoring	
pH	See Special Condition 3.				Daily While Discharging	Grab
Total Suspended Solids			15	30	Daily While Discharging	Grab
Iron (total)			2	4	Daily While Discharging	Grab
Oil and Grease			15	30	Daily While Discharging	Grab

* - See Special Condition 12. Monitoring requirements and limitations apply only when discharging hydrostatic test water and/or water supply pipeline cleaning water.

Outfall 003 - South Stormwater Basin* (DAF = 0.1 MGD)						
Flow	See Special Condition 1.				Measure When Monitoring	
pH	See Special Condition 3.				Daily While Discharging	Grab
Total Suspended Solids			15	30	Daily While Discharging	Grab
Iron (total)			2	4	Daily While Discharging	Grab
Oil and Grease			15	30	Daily While Discharging	Grab

* - See Special Condition 12. Monitoring requirements and limitations apply only when discharging hydrostatic test water and/or water supply pipeline cleaning water.

NPDES Permit No. IL0074268

Special Conditions

SPECIAL CONDITION 1. The discharge flow shall be measured on a continuous basis and in units of Million Gallons per Day (MGD) and reported as a monthly average and a daily maximum on the monthly discharge Monitoring Report.

SPECIAL CONDITION 2. This permit is written with the expressed understanding that there will be no discharge from this facility during extreme low river flow conditions. Extreme low river flow is defined as those times when flow in the Kaskaskia River drops below 10 cubic feet per second immediately upstream of the outfall.

SPECIAL CONDITION 3. The pH shall be in the range 6.0 to 9.0. The monthly minimum and monthly maximum values shall be reported on the DMR form.

SPECIAL CONDITION 4. Samples taken in compliance with the effluent monitoring requirements shall be taken at a point representative of the discharge, but prior to entry into the receiving stream.

SPECIAL CONDITION 5. If an applicable effluent standard or limitation is promulgated under Sections 301(b)(2)(C) and (D), 304(b)(2), and 307(a)(2) of the Clean Water Act and that effluent standard or limitation is more stringent than any effluent limitation in the permit or controls a pollutant not limited in the NPDES Permit, the Agency shall revise or modify the permit in accordance with the more stringent standard or prohibition and shall so notify the permittee.

SPECIAL CONDITION 6. A thermal mixing zone is recognized from Outfall 001 downstream for 22 feet in the Kaskaskia River and for 25% of the river width. Continuous temperature readings must be collected at the point 22 feet downstream of the outfall at a point midway in the 25% of stream width beginning at the east bank. The following limits must be met at this point:

A. The following maximum temperature limits must not be exceeded at the edge of the mixing zone during more than one percent of the hours in the 12 month period ending with any month. Moreover, at no time will the water temperature at the edge of the mixing zone exceed these limits by more than three degrees F.

	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
°F	60	60	60	90	90	90	90	90	90	90	90	60
°C	16	16	16	32	32	32	32	32	32	32	32	16

B. In addition, the discharge shall not cause abnormal temperature changes that may adversely affect aquatic life unless caused by natural conditions.

C. The rise in temperature at the edge of the mixing zone may not exceed the river temperature measured at the river water intake point by more than five degrees F.

D. The monthly maximum temperature at the edge of the mixing zone must be reported on the DMR form along with the number of hours temperatures exceeded the values in the above table and the accumulated time that the temperature at the edge of the mixing zone exceeded the river temperature at the intake by more than five degrees F.

E. In the event that the facilities thermocouple used to measure the mixed stream temperature should fail, the following equation may be used to determine the mixed stream temperature:

TMR = TU + [(QC(TC-TU))/Stream Flow]

TMR = mixed river temperature (°F)
TU = upstream river temperature (°F)
TC = effluent temperature (°F)
QC = effluent flow (MGD)
Stream Flow = one half the daily flow value of the receiving stream in MGD

The permittee shall notify the Agency when they discover a failure in the thermocouple that would result in the use of this equation. The permittee shall repair the thermocouple in a timely fashion and use of this equation may be suspended should the Agency determine that the facility has not repaired the thermocouple in a reasonable amount of time.

NPDES Permit No. IL0074268

Special Conditions

SPECIAL CONDITION 7. The Permittee shall record monitoring results on Discharge Monitoring Report (DMR) Forms using one such form for each outfall each month.

In the event that an outfall does not discharge during a monthly reporting period, the DMR Form shall be submitted with no discharge indicated.

The Permittee may choose to submit electronic DMRs (eDMRs) instead of mailing paper DMRs to the IEPA. More information, including registration information for the eDMR program, can be obtained on the IEPA website, http://www.epa.state.il.us/water/edmr/index.html.

The completed Discharge Monitoring Report forms shall be submitted to IEPA no later than the 15th day of the following month, unless otherwise specified by the permitting authority.

Permittees not using eDMRs shall mail Discharge Monitoring Reports with an original signature to the IEPA at the following address:

Illinois Environmental Protection Agency
Division of Water Pollution Control
1021 North Grand Avenue East
Post Office Box 19276
Springfield, Illinois 62794-9276

Attention: Compliance Assurance Section, Mail Code # 19

SPECIAL CONDITION 8. There shall be no discharge of the 126 priority pollutants, except chromium (total) and zinc (total) from outfall 001. The discharge from Outfall 001 shall be monitored once per year for the metals and phenols, as found at 35 Ill. Adm. Code Section 304.124, as well as the 126 priority pollutants listed in 40 CFR 423 Appendix A. All analysis shall be completed using an appropriate method contained in 40 CFR 136 on other USEPA approved methods. The results of this yearly monitoring shall be submitted with the December Discharge Monitoring Report.

SPECIAL CONDITION 9. All samples for total residual chlorine shall be analyzed by an applicable method contained in 40 CFR 136, equivalent in accuracy to low-level amperometric titration. Any analytical variability of the method used shall be considered when determining the accuracy and precision of the results obtained.

SPECIAL CONDITION 10. In the event that the permittee shall require the use of water treatment chemicals, other than those proposed in the application for this permit, the permittee shall notify the Agency in writing in accordance with the Standard Conditions, Attachment H. The permit may then be modified or revised following public notice and opportunity for hearing.

SPECIAL CONDITION 11. There shall be no discharge of polychlorinated biphenyl (PCB) compounds such as those commonly used for transformer fluids.

SPECIAL CONDITION 12.

STORM WATER POLLUTION PREVENTION PLAN (SWPPP)

A. A storm water pollution prevention plan shall be maintained by the permittee for the storm water associated with industrial activity at this facility. The plan shall identify potential sources of pollution which may be expected to affect the quality of storm water discharges associated with the industrial activity at the facility. In addition, the plan shall describe and ensure the implementation of practices which are to be used to reduce the pollutants in storm water discharges associated with industrial activity at the facility and to assure compliance with the terms and conditions of this permit.

B. The owner or operator of the facility shall make a copy of the plan available to the Agency at any reasonable time upon request.

C. The permittee may be notified by the Agency at any time that the plan does not meet the requirements of this condition. After such notification, the permittee shall make changes to the plan and shall submit a written certification that the requested changes have been made. Unless otherwise provided, the permittee shall have 30 days after such notification to make the changes.

D. The discharger shall amend the plan whenever there is a change in construction, operation, or maintenance which may affect the discharge of significant quantities of pollutants to the waters of the State or if a facility inspection required by paragraph G of this

condition indicates that an amendment is needed. The plan should also be amended if the discharger is in violation of any conditions of this permit, or has not achieved the general objective of controlling pollutants in storm water discharges. Amendments to the plan shall be made within the shortest reasonable period of time, and shall be provided to the Agency for review upon request.

E. The plan shall provide a description of potential sources which may be expected to add significant quantities of pollutants to storm water discharges, or which may result in non-storm water discharges from storm water outfalls at the facility. The plan shall include, at a minimum, the following items:

1. A topographic map extending one-quarter mile beyond the property boundaries of the facility, showing: the facility, surface water bodies, wells (including injection wells), seepage pits, infiltration ponds, and the discharge points where the facility's storm water discharges to a municipal storm drain system or other water body. The requirements of this paragraph may be included on the site map if appropriate.

2. A site map showing:

   i. The storm water conveyance and discharge structures;

   ii. An outline of the storm water drainage areas for each storm water discharge point;

   iii. Paved areas and buildings;

   iv. Areas used for outdoor manufacturing, storage, or disposal of significant materials, including activities that generate significant quantities of dust or particulates.

   v. Location of existing storm water structural control measures (dikes, coverings, detention facilities, etc.);

   vi. Surface water locations and/or municipal storm drain locations

   vii. Areas of existing and potential soil erosion;

   viii. Vehicle service areas;

   ix. Material loading, unloading, and access areas.

3. A narrative description of the following:

   i. The nature of the industrial activities conducted at the site, including a description of significant materials that are treated, stored or disposed of in a manner to allow exposure to storm water;

   ii. Materials, equipment, and vehicle management practices employed to minimize contact of significant materials with storm water discharges;

   iii. Existing structural and non-structural control measures to reduce pollutants in storm water discharges;

   iv. Industrial storm water discharge treatment facilities;

   v. Methods of onsite storage and disposal of significant materials;

4. A list of the types of pollutants that have a reasonable potential to be present in storm water discharges in significant quantities.

5. An estimate of the size of the facility in acres or square feet, and the percent of the facility that has impervious areas such as pavement or buildings.

6. A summary of existing sampling data describing pollutants in storm water discharges.

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F. The plan shall describe the storm water management controls which will be implemented by the facility. The appropriate controls shall reflect identified existing and potential sources of pollutants at the facility. The description of the storm water management controls shall include:

1. Storm Water Pollution Prevention Personnel - Identification by job titles of the individuals who are responsible for developing, implementing, and revising the plan.

2. Preventive Maintenance - Procedures for inspection and maintenance of storm water conveyance system devices such as oil/water separators, catch basins, etc., and inspection and testing of plant equipment and systems that could fail and result in discharges of pollutants to storm water.

3. Good Housekeeping - Good housekeeping requires the maintenance of clean, orderly facility areas that discharge storm water. Material handling areas shall be inspected and cleaned to reduce the potential for pollutants to enter the storm water conveyance system.

4. Spill Prevention and Response - Identification of areas where significant materials can spill into or otherwise enter the storm water conveyance systems and their accompanying drainage points. Specific material handling procedures, storage requirements, spill clean up equipment and procedures should be identified, as appropriate. Internal notification procedures for spills of significant materials should be established.

5. Storm Water Management Practices - Storm water management practices are practices other than those which control the source of pollutants. They include measures such as installing oil and grit separators, diverting storm water into retention basins, etc. Based on assessment of the potential of various sources to contribute pollutants, measures to remove pollutants from storm water discharge shall be implemented. In developing the plan, the following management practices shall be considered:

   i. Containment - Storage within berms or other secondary containment devices to prevent leaks and spills from entering storm water runoff;

   ii. Oil & Grease Separation - Oil/water separators, booms, skimmers or other methods to minimize oil contaminated storm water discharges;

   iii. Debris & Sediment Control - Screens, booms, sediment ponds or other methods to reduce debris and sediment in storm water discharges;

   iv. Waste Chemical Disposal - Waste chemicals such as antifreeze, degreasers and used oils shall be recycled or disposed of in an approved manner and in a way which prevents them from entering storm water discharges.

   v. Storm Water Diversion - Storm water diversion away from materials manufacturing, storage and other areas of potential storm water contamination;

   vi. Covered Storage or Manufacturing Areas - Covered fueling operations, materials manufacturing and storage areas to prevent contact with storm water.

6. Sediment and Erosion Prevention - The plan shall identify areas which due to topography, activities, or other factors, have a high potential for significant soil erosion and describe measures to limit erosion.

7. Employee Training - Employee training programs shall inform personnel at all levels of responsibility of the components and goals of the storm water pollution control plan. Training should address topics such as spill response, good housekeeping and material management practices. The plan shall identify periodic dates for such training.

8. Inspection Procedures - Qualified plant personnel shall be identified to inspect designated equipment and plant areas. A tracking or follow-up procedure shall be used to ensure appropriate response has been taken in response to an inspection. Inspections and maintenance activities shall be documented and recorded.

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G. The permittee shall conduct an annual facility inspection to verify that all elements of the plan, including the site map, potential pollutant sources, and structural and non-structural controls to reduce pollutants in industrial storm water discharges are accurate. Observations that require a response and the appropriate response to the observation shall be retained as part of the plan. Records documenting significant observations made during the site inspection shall be submitted to the Agency in accordance with the reporting requirements of this permit.

H. This plan should briefly describe the appropriate elements of other program requirements, including Spill Prevention Control and Countermeasures (SPCC) plans required under Section 311 of the CWA and the regulations promulgated thereunder, and Best Management Programs under 40 CFR 125.100.

I. The plan is considered a report that shall be available to the public under Section 308(b) of the CWA. The permittee may claim portions of the plan as confidential business information, including any portion describing facility security measures.

J. The plan shall include the signature and title of the person responsible for preparation of the plan and include the date of initial preparation and each amendment thereto.

Construction Authorization

K. Authorization is hereby granted to construct treatment works and related equipment that may be required by the Storm Water Pollution Prevention Plan developed pursuant to this permit.

This Authorization is issued subject to the following condition(s).

1. If any statement or representation is found to be incorrect, this authorization may be revoked and the permittee there upon waives all rights thereunder.

2. The issuance of this authorization (a) does not release the permittee from any liability for damage to persons or property caused by or resulting from the installation, maintenance or operation of the proposed facilities; (b) does not take into consideration the structural stability of any units or part of this project; and (c) does not release the permittee from compliance with other applicable statutes of the State of Illinois, or other applicable local law, regulations or ordinances.

3. Plans and specifications of all treatment equipment being included as part of the stormwater management practice shall be included in the SWPPP.

4. Construction activities which result from treatment equipment installation, including clearing, grading and excavation activities which result in the disturbance of one acre or more of land area, are not covered by this authorization. The permittee shall contact the IEPA regarding the required permit(s).

REPORTING

L. The facility shall submit an annual inspection report to the Illinois Environmental Protection Agency. The report shall include results of the annual facility inspection which is required by Part G of the Storm Water Pollution Prevention Plan of this permit. The report shall also include documentation of any event (spill, treatment unit malfunction, etc.) which would require an inspection, results of the inspection, and any subsequent corrective maintenance activity. The report shall be completed and signed by the authorized facility employee(s) who conducted the inspection(s).

M. The first report shall contain information gathered during the one year time period beginning with the effective date of coverage under this permit and shall be submitted no later than 60 days after this one year period has expired. Each subsequent report shall contain the previous year's information and shall be submitted no later than one year after the previous year's report was due.

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N. Annual Inspection reports shall be mailed to the following address:

Illinois Environmental Protection Agency
Bureau of Water
Compliance Assurance Section
Annual Inspection Report
1021 North Grand Avenue East
Post Office Box 19276
Springfield, Illinois 62794-9276

O. If the facility performs inspections more frequently than required by this permit, the results shall be included as additional information in the annual report.

SPECIAL CONDITION 13. The Permittee shall prepare a preliminary biomonitoring plan and submit the plan to the IEPA for review and approval within ninety (90) days of the effective date of this Permit. The Permittee shall begin biomonitoring of effluent from Outfall 001 the first summer after plan approval.

Biomonitoring

1. Toxicity Test - Acute (4-d) and short-term (14-d) toxicity tests shall be run on juveniles of mussel species representative of the aquatic community of the receiving stream. Procurement and testing of organisms must be consistent with Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM E2455-06). Guidelines for measuring effluent toxicity must be consistent with Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (Fifth Ed.) EPA/821-R-02-012. Unless substitute tests are pre-approved; the following test is required.

   An acute (4-d) and short-term (14-d) static-renewal toxicity test using newly-transformed juvenile fatmucket (*Lampsilis siliquoidea*) or another IEPA pre-approved native species.

2. Testing Frequency - The above test shall be conducted using 8-hour composite effluent samples (one initial sample and sufficient renewal samples to be determined in biomonitoring plan) discharged under normal operating conditions unless otherwise authorized by the IEPA. Upstream water of the Kaskaskia River is to be supplied to conduct serial dilutions. Testing must be conducted once per year for two year beginning the first summer after permit issuance.

3. Reporting - Results shall be reported according to EPA/821-R-02-012, Section 12, Report Preparation and shall be submitted to IEPA, Bureau of Water, Compliance Assurance Section within one week of receipt from the laboratory. Reports are due to the IEPA no later than 3 months following the test date.

4. Toxicity Assessment - Should the review of the results of the biomonitoring program identify toxicity, the IEPA may require that the Permittee prepare a plan for toxicity reduction evaluation and identification. This plan shall be developed in accordance with Toxicity Reduction Evaluation Guidance for Municipal Wastewater Treatment Plants, EPA/833B-99/002, and shall include an evaluation to determine which chemicals have a potential for being discharged in the plant wastewater, a monitoring program to determine their presence or absence and to identify other compounds which are not being removed by treatment, and other measures as appropriate. The Permittee shall submit to the IEPA its plan for toxicity reduction evaluation within ninety (90) days following notification by the IEPA. The Permittee shall implement the plan within ninety (90) days or other such date as contained in a notification letter received from the IEPA.

   The IEPA may modify this Permit during its term to incorporate additional requirements or limitations based on the results of the biomonitoring. In addition, after review of the monitoring results, the IEPA may modify this Permit to include numerical limitations for specific toxic pollutants. Modifications under this condition shall follow public notice and opportunity for hearing.

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SPECIAL CONDITION 14. The Permittee shall prepare a monitoring plan for the following biological parameters in the Kaskaskia River and submit the plan to IEPA for review and approval within 90 days of the effective date of the Permit. The Permittee shall implement the biological monitoring plan during the first defined low flow conditions between July and September after approval of the plan.

The monitoring plan shall include mussel surveys that repeat previous surveys conducted prior to and throughout the initial permit issuance. The surveys must be conducted during defined low flow conditions between July and September. Mussel surveys shall be conducted the first summer after permit issuance and annually thereafter.

SPECIAL CONDITION 15. The water supply necessary for the operation of this facility is to be obtained from Lake Shelbyville via the Kaskaskia River, through a water supply agreement between the permittee and the Illinois Department of Natural Resources. While an alternate water supply is not prohibited by this special condition, it may require the modification of this permit. The Agency must be notified in writing prior to use of an alternate water supply. The Agency will modify the permit following public notice and opportunity for hearing.

SPECIAL CONDITION 16. The permittee shall monitor Ammonia as N and report the concentration in mg/L being discharged. The sample frequency shall be once a month. The results of the monthly sampling shall be submitted with the monthly Discharge Monitoring Report. After two years the permittee may request a modification to the permit to remove the ammonia sampling if justified by the sampling results.

SPECIAL CONDITION 17. Chloride shall be monitored on a monthly basis for a year. Upon collection of the 12 monthly samples, and upon written notification to the Agency the sampling may cease. The Agency may modify the permit based on the results of the sampling data to include further monitoring or limitations following public notice and opportunity for comment.

Standard Conditions

Definitions

Act means the Illinois Environmental Protection Act, 415 ILCS 5 as Amended.

Agency means the Illinois Environmental Protection Agency.

Board means the Illinois Pollution Control Board.

Clean Water Act (formerly referred to as the Federal Water Pollution Control Act) means Pub. L. 92-500, as amended. 33 U.S.C. 1251 et seq.

NPDES (National Pollutant Discharge Elimination System) means the national program for issuing, modifying, revoking and reissuing, terminating, monitoring and enforcing permits, and imposing and enforcing pretreatment requirements, under Sections 307, 402, 318 and 405 of the Clean Water Act.

USEPA means the United States Environmental Protection Agency.

Daily Discharge means the discharge of a pollutant measured during a calendar day or any 24-hour period that reasonably represents the calendar day for purposes of sampling. For pollutants with limitations expressed in units of mass, the "daily discharge" is calculated as the total mass of the pollutant discharged over the day. For pollutants with limitations expressed in other units of measurements, the "daily discharge" is calculated as the average measurement of the pollutant over the day.

Maximum Daily Discharge Limitation (daily maximum) means the highest allowable daily discharge.

Average Monthly Discharge Limitation (30 day average) means the highest allowable average of daily discharges over a calendar month, calculated as the sum of all daily discharges measured during a calendar month divided by the number of daily discharges measured during that month.

Average Weekly Discharge Limitation (7 day average) means the highest allowable average of daily discharges over a calendar week, calculated as the sum of all daily discharges measured during a calendar week divided by the number of daily discharges measured during that week.

Best Management Practices (BMPs) means schedules of activities, prohibitions of practices, maintenance procedures, and other management practices to prevent or reduce the pollution of waters of the State. BMPs also include treatment requirements, operating procedures, and practices to control plant site runoff, spillage or leaks, sludge or waste disposal, or drainage from raw material storage.

Aliquot means a sample of specified volume used to make up a total composite sample.

Grab Sample means an individual sample of at least 100 milliliters collected at a randomly-selected time over a period not exceeding 15 minutes.

24-Hour Composite Sample means a combination of at least 8 sample aliquots of at least 100 milliliters, collected at periodic intervals during the operating hours of a facility over a 24-hour period.

8-Hour Composite Sample means a combination of at least 3 sample aliquots of at least 100 milliliters, collected at periodic intervals during the operating hours of a facility over an 8-hour period.

Flow Proportional Composite Sample means a combination of sample aliquots of at least 100 milliliters collected at periodic intervals such that either the time interval between each aliquot or the volume of each aliquot is proportional to either the stream flow at the time of sampling or the total stream flow since the collection of the previous aliquot.

(1) **Duty to comply.** The permittee must comply with all conditions of this permit. Any permit noncompliance constitutes a violation of the Act and is grounds for enforcement action, permit termination, revocation and reissuance, modification, or for denial of a permit renewal application. The permittee shall comply with effluent standards or prohibitions established under Section 307(a) of the Clean Water Act for toxic pollutants within the time provided in the regulations that establish these standards or prohibitions, even if the permit has not yet been modified to incorporate the requirements.

(2) **Duty to reapply.** If the permittee wishes to continue an activity regulated by this permit after the expiration date of this permit, the permittee must apply for and obtain a new permit. If the permittee submits a proper application as required by the Agency no later than 180 days prior to the expiration date, this permit shall continue in full force and effect until the final Agency decision on the application has been made.

(3) **Need to halt or reduce activity not a defense.** It shall not be a defense for a permittee in an enforcement action that it would have been necessary to halt or reduce the permitted activity in order to maintain compliance with the conditions of this permit.

(4) **Duty to mitigate.** The permittee shall take all reasonable steps to minimize or prevent any discharge in violation of this permit which has a reasonable likelihood of adversely affecting human health or the environment.

(5) **Proper operation and maintenance.** The permittee shall at all times properly operate and maintain all facilities and systems of treatment and control (and related appurtenances) which are installed or used by the permittee to achieve compliance with conditions of this permit. Proper operation and maintenance includes effective performance, adequate funding, adequate operator staffing and training, and adequate laboratory and process controls, including appropriate quality assurance procedures. This provision requires the operation of back-up, or auxiliary facilities, or similar systems only when necessary to achieve compliance with the conditions of the permit.

(6) **Permit actions.** This permit may be modified, revoked and reissued, or terminated for cause by the Agency pursuant to 40 CFR 122.62 and 40 CFR 122.63. The filing of a request by the permittee for a permit modification, revocation and reissuance, or termination, or a notification of planned changes or anticipated noncompliance, does not stay any permit condition.

(7) **Property rights.** This permit does not convey any property rights of any sort, or any exclusive privilege.

(8) **Duty to provide information.** The permittee shall furnish to the Agency within a reasonable time, any information which the Agency may request to determine whether cause exists for modifying, revoking and reissuing, or terminating this permit, or to determine compliance with the permit. The permittee shall also furnish to the Agency upon request, copies of records required to be kept by this permit.

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(9) **Inspection and entry.** The permittee shall allow authorized representative of the Agency or USEPA (including an authorized contractor acting as a representative of the Agency or USEPA), upon the presentation of credentials and other documents as may be required by law, to:
  (a) Enter upon the permittee's premises where a regulated facility or activity is located or conducted, or where records must be kept under the conditions of this permit;
  (b) Have access to and copy, at reasonable times, any records that must be kept under the conditions of this permit;
  (c) Inspect at reasonable times any facilities, equipment (including monitoring and control equipment), practices, or operations regulated or required under this permit; and
  (d) Sample or monitor at reasonable times, for the purpose of assuring permit compliance, or as otherwise authorized by the Act, any substances or parameters at any location.

(10) **Monitoring and records.**
  (a) Samples and measurements taken for the purpose of monitoring shall be representative of the monitored activity.
  (b) The permittee shall retain records of all monitoring information, including all calibration and maintenance records, and all original strip chart recordings for continuous monitoring instrumentation, copies of all reports required by this permit, and records of all data used to complete the application for this permit, for a period of at least 3 years from the date of this permit, measurement, report or application. Records related to the permittee's sewage sludge use and disposal activities shall be retained for a period of at least five years (or longer as required by 40 CFR Part 503). This period may be extended by request of the Agency or USEPA at any time.
  (c) Records of monitoring information shall include:
    (1) The date, exact place, and time of sampling or measurements;
    (2) The individual(s) who performed the sampling or measurements;
    (3) The date(s) analyses were performed;
    (4) The individual(s) who performed the analyses;
    (5) The analytical techniques or methods used; and
    (6) The results of such analyses.
  (d) Monitoring must be conducted according to test procedures approved under 40 CFR Part 136, unless other test procedures have been specified in this permit. Where no test procedure under 40 CFR Part 136 has been approved, the permittee must submit to the Agency a test method for approval. The permittee shall calibrate and perform maintenance procedures on all monitoring and analytical instrumentation at intervals to ensure accuracy of measurements.

(11) **Signatory requirement.** All applications, reports or information submitted to the Agency shall be signed and certified.
  (a) **Application.** All permit applications shall be signed as follows:
    (1) For a corporation: by a principal executive officer of at least the level of vice president or a person or position having overall responsibility for environmental matters for the corporation;
    (2) For a partnership or sole proprietorship: by a general partner or the proprietor, respectively; or
    (3) For a municipality, State, Federal, or other public agency: by either a principal executive officer or ranking elected official.
  (b) **Reports.** All reports required by permits, or other information requested by the Agency shall be signed by a person described in paragraph (a) or by a duly authorized representative of that person. A person is a duly authorized representative only if:
    (1) The authorization is made in writing by a perso described in paragraph (a); and
    (2) The authorization specifies either an individual or position responsible for the overall operation of th facility, from which the discharge originates, such a a plant manager, superintendent or person c equivalent responsibility; and
    (3) The written authorization is submitted to the Agency
  (c) **Changes of Authorization.** If an authorization under (b Is no longer accurate because a different individual c position has responsibility for the overall operation of th facility, a new authorization satisfying the requirements c (b) must be submitted to the Agency prior to or togethe with any reports, information, or applications to be signe by an authorized representative.
  (d) **Certification.** Any person signing a document unde paragraph (a) or (b) of this section shall make th following certification:

  I certify under penalty of law that this document and a attachments were prepared under my direction o supervision in accordance with a system designed t assure that qualified personnel properly gather an evaluate the information submitted. Based on my inquir of the person or persons who manage the system, o those persons directly responsible for gathering th information, the information submitted is, to the best o my knowledge and belief, true, accurate, and complete. am aware that there are significant penalties fo submitting false information, including the possibility o fine and imprisonment for knowing violations.

(12) **Reporting requirements.**
  (a) **Planned changes.** The permittee shall give notice to the Agency as soon as possible of any planned physica alterations or additions to the permitted facility.
  Notice is required when:
    (1) The alteration or addition to a permitted facility may meet one of the criteria for determining whether a facility is a new source pursuant to 40 CFR 122.29 (b); or
    (2) The alteration or addition could significantly change the nature or increase the quantity of pollutants discharged. This notification applies to pollutants which are subject neither to effluent limitations in the permit, nor to notification requirements pursuant to 40 CFR 122.42 (a)(1).
    (3) The alteration or addition results in a significant change in the permittee's sludge use or disposa practices, and such alteration, addition, or change may justify the application of permit conditions that are different from or absent in the existing permit, including notification of additional use or disposal sites not reported during the permit application process or not reported pursuant to an approved land application plan.
  (b) **Anticipated noncompliance.** The permittee shall give advance notice to the Agency of any planned changes in the permitted facility or activity which may result in noncompliance with permit requirements.
  (c) **Transfers.** This permit is not transferable to any person except after notice to the Agency.
  (d) **Compliance schedules.** Reports of compliance or noncompliance with, or any progress reports on, interim and final requirements contained in any compliance schedule of this permit shall be submitted no later than 14 days following each schedule date.
  (e) **Monitoring reports.** Monitoring results shall be reported at the intervals specified elsewhere in this permit.
    (1) Monitoring results must be reported on a Discharge Monitoring Report (DMR).

(2) If the permittee monitors any pollutant more frequently than required by the permit, using test procedures approved under 40 CFR 136 or as specified in the permit, the results of this monitoring shall be included in the calculation and reporting of the data submitted in the DMR.

(3) Calculations for all limitations which require averaging of measurements shall utilize an arithmetic mean unless otherwise specified by the Agency in the permit.

(f) **Twenty-four hour reporting.** The permittee shall report any noncompliance which may endanger health or the environment. Any information shall be provided orally within 24-hours from the time the permittee becomes aware of the circumstances. A written submission shall also be provided within 5 days of the time the permittee becomes aware of the circumstances. The written submission shall contain a description of the noncompliance and its cause; the period of noncompliance, including exact dates and time; and if the noncompliance has not been corrected, the anticipated time it is expected to continue; and steps taken or planned to reduce, eliminate, and prevent reoccurrence of the noncompliance. The following shall be included as information which must be reported within 24-hours:

(1) Any unanticipated bypass which exceeds any effluent limitation in the permit.

(2) Any upset which exceeds any effluent limitation in the permit.

(3) Violation of a maximum daily discharge limitation for any of the pollutants listed by the Agency in the permit or any pollutent which may endanger health or the environment.

The Agency may waive the written report on a case-by-case basis if the oral report has been received within 24-hours.

(g) **Other noncompliance.** The permittee shall report all instances of noncompliance not reported under paragraphs (12) (d), (e), or (f), at the time monitoring reports are submitted. The reports shall contain the information listed in paragraph (12) (f).

(h) **Other information.** Where the permittee becomes aware that it failed to submit any relevant facts in a permit application, or submitted incorrect information in a permit application, or in any report to the Agency, it shall promptly submit such facts or information.

(13) **Bypass.**

(a) Definitions.

(1) Bypass means the intentional diversion of waste streams from any portion of a treatment facility.

(2) Severe property damage means substantial physical damage to property, damage to the treatment facilities which causes them to become inoperable, or substantial and permanent loss of natural resources which can reasonably be expected to occur in the absence of a bypass. Severe property damage does not mean economic loss caused by delays in production.

(b) Bypass not exceeding limitations. The permittee may allow any bypass to occur which does not cause effluent limitations to be exceeded, but only if it also is for essential maintenance to assure efficient operation. These bypasses are not subject to the provisions of paragraphs (13)(c) and (13)(d).

(c) Notice.

(1) Anticipated bypass. If the permittee knows in advance of the need for a bypass, it shall submit prior notice, if possible at least ten days before the date of the bypass.

(2) Unanticipated bypass. The permittee shall submit notice of an unanticipated bypass as required in paragraph (12)(f) (24-hour notice).

(d) Prohibition of bypass.

(1) Bypass is prohibited, and the Agency may take enforcement action against a permittee for bypass, unless:

(i) Bypass was unavoidable to prevent loss of life, personal injury, or severe property damage;

(ii) There were no feasible alternatives to the bypass, such as the use of auxiliary treatment facilities, retention of untraated wastes, or maintenance during normal periods of equipment downtime. This condition is not satisfied if adequate back-up equipment should have been installed in the exercise of reasonable engineering judgment to prevent a bypass which occurred during normal periods of equipment downtime or preventive maintenance; and

(iii) The permittee submitted notices as required under paragraph (13)(c).

(2) The Agency may approve an anticipated bypass, after considering its adverse effects, if the Agency determines that it will meet the three conditions listed above in paragraph (13)(d)(1).

(14) **Upset.**

(a) Definition. Upset means an exceptional incident in which there is unintentional and temporary noncompliance with technology based permit effluent limitations because of factors beyond the reasonable control of the permittee. An upset does not include noncompliance to the extent caused by operational error, improperly designed treatment facilities, inadequate treatment facilities, lack of preventive maintenance, or careless or improper operation.

(b) Effect of an upset. An upset constitutes an affirmative defense to an action brought for noncompliance with such technology based permit effluent limitations if the requirements of paragraph (14)(c) are met. No determination made during administrative review of claims that noncompliance was caused by upset, and before an action for noncompliance, is final administrative action subject to judicial review.

(c) Conditions necessary for a demonstration of upset. A permittee who wishes to establish the affirmative defense of upset shall demonstrate, through properly signed, contemporaneous operating logs, or other relevant evidence that:

(1) An upset occurred and that the permittee can identify the cause(s) of the upset;

(2) The permitted facility was at the time being properly operated; and

(3) The permittee submitted notice of the upset as required in paragraph (12)(f)(2) (24-hour notice).

(4) The permittee complied with any remedial measures required under paragraph (4).

(d) Burden of proof. In any enforcement proceeding the permittee seeking to establish the occurrence of an upset has the burden of proof.

(15) **Transfer of permits.** Permits may be transferred by modification or automatic transfer as described below:

(a) Transfers by modification. Except as provided in paragraph (b), a permit may be transferred by the permittee to a new owner or operator only if the permit has been modified or revoked and reissued pursuant to 40 CFR 122.62 (b) (2), or a minor modification made pursuant to 40 CFR 122.63 (d), to identify the new permittee and incorporate such other requirements as may be necessary under the Clean Water Act.

(b) Automatic transfers. As an alternative to transfers under paragraph (a), any NPDES permit may be automatically transferred to a new permittee if:

(1) The current permittee notifies the Agency at least 30 days in advance of the proposed transfer date;

(2) The notice includes a written agreement between the existing and new permittees containing a specified date for transfer of permit responsibility, coverage and liability between the existing and new permittees; and

(3) The Agency does not notify the existing permittee and the proposed new permittee of its intent to modify or revoke and reissue the permit. If this notice is not received, the transfer is effective on the date specified in the agreement.

(16) All manufacturing, commercial, mining, and silvicultural dischargers must notify the Agency as soon as they know or have reason to believe:

(a) That any activity has occurred or will occur which would result in the discharge of any toxic pollutant identified under Section 307 of the Clean Water Act which is not limited in the permit, if that discharge will exceed the highest of the following notification levels:

(1) One hundred micrograms per liter (100 ug/l);

(2) Two hundred micrograms per liter (200 ug/l) for acrolein and acrylonitrile; five hundred micrograms per liter (500 ug/l) for 2,4-dinitrophenol and for 2-methyl-4,6 dinitrophenol; and one milligram per liter (1 mg/l) for antimony.

(3) Five (5) times the maximum concentration value reported for that pollutant in the NPDES permit application; or.

(4) The level established by the Agency in this permit.

(b) That they have begun or expect to begin to use or manufacture as an intermediate or final product or byproduct any toxic pollutant which was not reported in the NPDES permit application.

(17) All Publicly Owned Treatment Works (POTWs) must provide adequate notice to the Agency of the following:

(a) Any new introduction of pollutants into that POTW from an indirect discharge which would be subject to Sections 301 or 306 of the Clean Water Act if it were directly discharging those pollutants; and

(b) Any substantial change in the volume or character of pollutants being introduced into that POTW by a source introducing pollutants into the POTW at the time of issuance of the permit.

(c) For purposes of this paragraph, adequate notice shall include information on (i) the quality and quantity of effluent introduced into the POTW, and (ii) any anticipated impact of the change on the quantity or quality of effluent to be discharged from the POTW.

(18) If the permit is issued to a publicly owned or publicly regulated treatment works, the permittee shall require any industrial user of such treatment works to comply with federal requirements concerning:

(a) User charges pursuant to Section 204 (b) of the Clean Water Act, and applicable regulations appearing in 40 CFR 35;

(b) Toxic pollutant effluent standards and pretreatment standards pursuant to Section 307 of the Clean Water Act; and

(c) Inspection, monitoring and entry pursuant to Section 308 of the Clean Water Act.

(19) If an applicable standard or limitation is promulgated under Section 301(b)(2)(C) and (D), 304(b)(2), or 307(a)(2) and that effluent standard or limitation is more stringent than any effluent limitation in the permit, or controls a pollutant not limited in the permit, the permit shall be promptly modified or revoked, and reissued to conform to that effluent standard or limitation.

(20) Any authorization to construct issued to the permittee pursuant to 35 Ill. Adm. Code 309.154 is hereby incorporated by reference as a condition of this permit.

(21) The permittee shall not make any false statement, representation or certification in any application, record, report, plan or other document submitted to the Agency or the USEPA, or required to be maintained under this permit.

(22) The Clean Water Act provides that any person who violates a permit condition implementing Sections 301, 302, 306, 307, 308, 318, or 405 of the Clean Water Act is subject to a civil penalty not to exceed $25,000 per day of such violation. Any person who willfully or negligently violates permit conditions implementing Sections 301, 302, 306, 307, 308, 318 or 405 of the Clean Water Act is subject to a fine of not less than $2,500 nor more than $25,000 per day of violation, or by imprisonment for not more than one year, or both. Additional penalties for violating these sections of the Clean Water Act are identified in 40 CFR 122.41 (a)(2) and (3).

(23) The Clean Water Act provides that any person who falsifies, tampers with, or knowingly renders inaccurate any monitoring device or method required to be maintained under this permit shall, upon conviction, be punished by a fine of not more than $10,000, or by imprisonment for not more than 2 years, or both. If a conviction of a person is for a violation committed after a first conviction of such person under this paragraph, punishment is a fine of not more than $20,000 per day of violation, or by imprisonment of not more than 4 years, or both.

(24) The Clean Water Act provides that any person who knowingly makes any false statement, representation, or certification in any record or other document submitted or required to be maintained under this permit, including monitoring reports or reports of compliance or non-compliance shall, upon conviction, be punished by a fine of not more than $10,000 per violation, or by imprisonment for not more than 6 months per violation, or by both.

(25) Collected screening, slurries, sludges, and other solids shall be disposed of in such a manner as to prevent entry of those wastes (or runoff from the wastes) into waters of the State. The proper authorization for such disposal shall be obtained from the Agency and is incorporated as part hereof by reference.

(26) In case of conflict between these standard conditions and any other condition(s) included in this permit, the other condition(s) shall govern.

(27) The permittee shall comply with, in addition to the requirements of the permit, all applicable provisions of 35 Ill. Adm. Code, Subtitle C, Subtitle D, Subtitle E, and all applicable orders of the Board or any court with jurisdiction.

(28) The provisions of this permit are severable, and if any provision of this permit, or the application of any provision of this permit is held invalid, the remaining provisions of this permit shall continue in full force and effect.

(Rev. 7-9-2010 bah)

NPDES Permit No. IL0074268

Illinois Environmental Protection Agency

Division of Water Pollution Control

1021 North Grand Avenue East

Post Office Box 19276

Springfield, Illinois 62794-9276

NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM

Modified (NPDES) Permit

Expiration Date: June 30, 2005

Issue Date: July 21, 2000
Effective Date: July 21, 2000
Modification Date: July 25, 2000
Modification Date: December 31, 2001

Name and Address of Permittee:

Holland Energy, LLC
c/o Constellation Power Development, Inc.
111 Market Place, Suite 200
Baltimore, Maryland 21201-7110

Facility Name and Address:

Holland Energy Facility
Section 16, T 9 N, R 4 E
Holland Township
(Shelby County)

Discharge Number and Name:		Receiving Waters:
001	Cooling Tower Blowdown Evaporative Cooler Blowdown, Clarifier Sludge Dewatering, Plant Sumps and Drains	Kaskaskia River
002	North Stormwater Basin - Stormwater, Hydrostatic Test Water, Water Supply Pipeline Cleanout Water	Unnamed Tributary to Brush Creek
003	South Stormwate Basin - Stormwater, Hydrostatic Test Water	Unnamed Tributary to Brush Creek

In compliance with the provisions of the Illinois Environmental Protection Act, Title 35 of Ill. Adm. Code, Subtitle C and/or Subtitle D, Chapter 1, and the Clean Water Act (CWA), the above-named permittee is hereby authorized to discharge at the above location to the above-named receiving stream in accordance with the standard conditions and attachments herein.

Permittee is not authorized to discharge after the above expiration date. In order to receive authorization to discharge beyond the expiration date, the permittee shall submit the proper application as required by the Illinois Environmental Protection Agency (IEPA) not later than 180 days prior to the expiration date.

Thomas G. McSwiggin, P.E.
Manager, Permit Section
Division of Water Pollution Control

TGM:DEL:00040601.daa

EXHIBIT 2

 Modification Date: December 31, 2001

NPDES Permit No. IL0074268

Effluent Limitations and Monitoring

PARAMETER	LOAD LIMITS lbs/day DAF (DMF) 30 DAY AVERAGE	LOAD LIMITS lbs/day DAF (DMF) DAILY MAXIMUM	CONCENTRATION LIMITS mg/l 30 DAY AVERAGE	CONCENTRATION LIMITS mg/l DAILY MAXIMUM	SAMPLE FREQUENCY	SAMPLE TYPE
1. From the effective date of this permit until the expiration date, the effluent of the following discharge(s) shall be monitored and limited at all times as follows:						
Outfall(s): 001						
Flow (MGD)	See Special Condition 1				Continuous	
pH	See Special Condition 3				2/Month	Grab
Total Suspended Solids			15	30	2/Month	Grab
Oil and Grease			15	20	2/Month	Grab
Total Dissolved Solids				1,800	2/Month	Grab
Sulfates				700	2/Month	Grab
Temperature	See Special Condition 4				Continuous	
Total Residual Chlorine				0.05	2/Month	Grab
Zinc (total)			1.0	1.0	1/Month	Grab
Phosphorous					1/Month	Grab
Chromium (total)*			0.2	0.2	1/Quarter	Grab
*Quarterly sampling results for Chromium (total) shall be submitted during the months of April, July, October and January for the preceeding three month period.						
Outfalls: 002 and 003, North and South Stormwater Basins**						
Flow (MGD)	See Special Condition 1				Measure When Monitoring	
pH	See Special Condition 3				2/Month	Grab
Total Suspended Solids			15	30	2/Month	Grab
Iron (total)			2	4	2/Month	Grab
Oil and Grease			15	30	2/Month	Grab

**See Special Condition 16. Monitoring requirements and limitations apply only when discharging hydrostatic test water or water supply pipeline cleaning water.

NPDES Permit No. IL0074268

Special Conditions

SPECIAL CONDITION 1. The discharge flow from outfall 001 shall be measured on a continuous basis, and shall be reported as a monthly average and a daily maximum. The flow in the receiving stream immediately upstream of the outfall shall also be monitored, and shall be reported as a daily minimum, monthly average and daily maximum. Within 90 days of the effective date of this permit, the permittee shall submit a stream monitoring plan outlining the methods and procedures which will be used to monitor and record stream flow. The plan should include the type and location of the stream flow monitoring device, as well as expected performance of the device when measuring lower river flows. The stream flow monitoring plan required under this special condition may be included with, and submitted as part of, the Monitoring Plan required in Special Condition 12.

SPECIAL CONDITION 2. This permit is written with the expressed understanding that there will be no discharge from this facility during extreme low river flow conditions. Extreme low river flow is defined as those times when flow in the Kaskaskia River drops below 10 cubic feet per second immediately upstream of the outfall.

SPECIAL CONDITION 3. The pH shall be in the range 6.0 to 9.0. The monthly minimum and monthly maximum values shall be reported on the DMR form.

SPECIAL CONDITION 4.

1. Water temperature measured at the end-of-pipe for Outfall 001 must not exceed the limits in the following table during more than one percent of the hours in the 12 month period ending with any month. Moreover, at no time will the water temperature at such location exceed the limits in the following table by more than 3° F (1.7°C).

	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
°F	60	60	60	90	90	90	90	90	90	90	90	60
°C	16	16	16	32	32	32	32	32	32	32	32	16

2. Compliance with the water temperature limits above will be determined through evaluation of the following monitoring requirements. A continuous monitoring device will be operated at the end of the discharge pipe and will measure temperature in the effluent before discharge to the Kaskaskia River. Monthly Discharge Monitoring Reports will provide the instantaneous daily maximum water temperature recorded for each day. In addition, the number of hours that the hourly average effluent temperature exceeds the appropriate value from the above table must be reported, along with the individual hourly average temperature for the hours when such excursions occur.

3. Additionally, the water temperature below the mixing zone must not exceed by 5° F or more the temperature of the river water removed from the Kaskaskia River in the intake pipe.

4. Compliance with provision #3 will be assessed on an hourly basis. Intake water temperature will be measured by a continuous monitoring device and the average hourly temperature will be compared with the average hourly temperature reading from a continuous monitoring device installed in the river immediately below the mixing zone. The monthly Discharge Monitoring Reports must list all hours in which the river temperature at the edge of the mixing zone exceeded the intake temperature by more that 5° F as well as the intake and river temperatures during these events.

SPECIAL CONDITION 5. Samples taken in compliance with the effluent monitoring requirements shall be taken at a point representative of the discharge, but prior to entry into the receiving stream.

SPECIAL CONDITION 6. If an applicable effluent standard or limitation is promulgated under Sections 301(b)(2)(C) and (D), 304(b)(2), and 307(a)(2) of the Clean Water Act and that effluent standard or limitation is more stringent than any effluent limitation in the permit or controls a pollutant not limited in the NPDES Permit, the Agency shall revise or modify the permit in accordance with the more stringent standard or prohibition and shall so notify the permittee.

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Special Conditions

SPECIAL CONDITION 7. The permittee shall record monitoring results on Discharge Monitoring Report forms using one such form for each discharge each month. The completed Discharge Monitoring Report form shall be submitted monthly to IEPA, no later than the 15th of the following month, unless otherwise specified by the Agency, to the following address:

Illinois Environmental Protection Agency
Bureau of Water
Compliance Assurance Section
1021 North Grand Avenue East
Post Office Box 19276
Springfield, Illinois 62794-9276

SPECIAL CONDITION 8. There shall be no discharge of the 126 priority pollutants, except chromium (total) and zinc (total), in detectable amounts from outfall 001. The discharge from Outfall 001 shall be monitored once per year for the metals and phenols, as found at 35 Ill. Adm. Code Section 304.124, as well as the 126 priority pollutants listed in 40 CFR 423 Appendix A. All analysis shall be completed using an appropriate method contained in 40 CFR 136 on other USEPA approved methods. The results of this yearly monitoring shall be submitted with the December Discharge Monitoring Report.

SPECIAL CONDITION 9. All samples for total residual chlorine shall be analyzed by an applicable method contained in 40 CFR 136, equivalent in accuracy to low-level amperometric titration. Any analytical variability of the method used shall be considered when determining the accuracy and precision of the results obtained.

SPECIAL CONDITION 10. In the event that the permittee shall require the use of water treatment chemicals, other than those proposed in the application for this permit, the permittee shall notify the Agency in writing in accordance with the Standard Conditions, Attachment H. The permit may then be modified or revised following public notice and opportunity for hearing.

SPECIAL CONDITION 11.

The permittee shall conduct biomonitoring of the effluent from Outfall 001. Quarterly biomonitoring of the effluent discharge shall be performed on a normal operating day. Annual biomonitoring shall be performed on a normal operating day during July, August or September.

Biomonitoring

1. Acute Toxicity

   A. Acute Toxicity - Standard definitive acute toxicity tests shall be run on at least two trophic levels of aquatic species (fish, invertebrate) representative of the aquatic community of the receiving stream. Except as noted here and in the IEPA document "Effluent Biomonitoring and Toxicity Assessment", testing must be consistent with Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms EPS 600/4-90-027F. Unless substitute tests are pre-approved, the following tests are required:

      a. Fish - 96 hour static LC50 Bioassay using one to two week old fathead minnows (Pimephales promelas).

      b. Invertebrate 48 - hour static LC50 Bioassay using Ceriodaphnia.

   B. Testing Frequency - The above tests shall be conducted during the first four quarters of facility operation for which there is a discharge and on an annual basis for the duration of this permit, using 24 - hour composite effluent samples unless otherwise authorized by the Agency. Results shall be reported according to EPS 600/4-90-027F, Section 12, Report Preparation, and shall be submitted to IEPA within 45 days of receipt of results.

2. Chronic Toxicity

   A. Chronic Toxicity - Chronic toxicity tests shall be conducted on at least two trophic levels of aquatic species (fish, invertebrate) representative of the aquatic community of the receiving stream. Testing must be consistent with USEPA's Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms (Third Edition) (EPA 600-4-91-002). Chronic biomonitoring should require the following tests:

      (a.) Fish - Fathead minnow (Pimephales promelas) larval survival and growth test (method 1000.0)

      (b.) Invertebrate - Daphnid (Ceriodaphnia dubia) survival and reproduction test (method 1000.0)

Special Conditions

B. Test Frequency - The above tests shall be conducted during the first four quarters of facility operation for which there is a discharge, using 24 - hour composite effluent samples unless otherwise authorized by the Agency. Results shall be in reported according to EPA 600-4-91-002, Section 10, Report Preparation, and shall be submitted to IEPA within 45 days of receipt of results.

3. Toxicity Assessment - Should the review of the results of the biomonitoring program identify toxicity, the Agency shall require that the permittee prepare a plan for toxicity reduction evaluation and identification. This plan shall include an evaluation to determine which chemicals have a potential for being discharged in the plant wastewater, a monitoring program to determine their presence or absence and to identify other compounds which are not being removed by treatment and/or other measures as appropriate.

The Agency may modify this permit during its term to incorporate additional requirements or limitations based on the results of any biomonitoring. In addition, after review of the monitoring results, the Agency may modify this permit to include numerical limitations for specific toxic pollutants. Modifications under this condition shall follow public notice and opportunity for hearing.

SPECIAL CONDITION 12. The Permittee shall prepare a monitoring plan for the following chemical, physical and biological parameters in the Kaskaskia River and submit the plan to IEPA for review and approval within 90 days of the effective date of the permit. The plan shall address phosphorus/algae productivity and aquatic species. The Permittee shall implement the biological monitoring plan during the first defined low flow conditions between July and September after approval of the plan.

1. Phosphorus/Algae Productivity

The Monitoring Plan will include sampling of selected water quality parameters and indicators of biological productivity related to discharge of phosphorus at a maximum of four locations upstream and downstream of the facility discharge during defined low flow conditions between July and September. Locations of the monitoring locations, selection of water quality parameters, definition of low flow conditions, and methods of measurement of biological productivity, will be identified in the Plan.

The phosphorus monitoring shall be conducted each year prior to the initiation of the facility operational discharge to establish baseline conditions and annually thereafter.

2. Aquatic Species

The Monitoring Plan shall include surveys for mussels and the western sand darter that repeat the surveys conducted for mixing zone and nondegradation evaluations prior to permit issuance. The surveys will be conducted during defined low flow conditions between July and September.

Aquatic species monitoring shall be conducted each year prior to the initiation of the facility operational discharge, in the first year of operation, and biennially thereafter for the first term on the permit.

3. Temperature

The monitoring plan shall include a description of the methods to implement Special Condition 4, including documentation and delineation of the mixing zone.

4. Reporting

An annual report of the results of the stream monitoring program will be submitted to the Agency by December 31st of each year.

SPECIAL CONDITION 13. Prior to commencing operation, the permittee shall apply for and receive an NPDES permit modification for the discharge of stormwater associated with industrial activity. The modification request should include a site map indicating the location of each stormwater outfall and the area drained by each outfall, an estimate of stormwater characteristics for each outfall. The name of the receiving stream, and the latitude and longitude of each outfall. A completed Form 2F including sampling, shall be submitted for each stormwater outfall within 180 days of the start of operation.

SPECIAL CONDITION 14. The water supply necessary for the operation of this facility is to be obtained from Lake Shelbyville via the Kaskaskia River, through a water supply agreement between the permittee and the Illinois Department of Natural Resources. A copy of the final water supply agreement must be submitted to the IEPA prior to commencing operation. While an alternate water supply is not prohibited by this special condition, it may require the modification of this permit. The Agency must be notified in writing prior to use of an alternate water supply. The Agency will modify the permit following public notice and opportunity for hearing.

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Special Conditions

SPECIAL CONDITION 15. There shall be no discharge of polychlorinated biphenyl (PCB) counpounds such as those commonly used for transformer fluids.

SPECIAL CONDITION 16.

STORM WATER POLLUTION PREVENTION PLAN (SWPPP)

A. A storm water pollution prevention plan shall be developed by the permittee for the storm water associated with industrial activity at this facility. The plan shall identify potential sources of pollution which may be expected to affect the quality of storm water discharges associated with the industrial activity at the facility. In addition, the plan shall describe and ensure the implementation of practices which are to be used to reduce the pollutants in storm water discharges associated with industrial activity at the facility and to assure compliance with the terms and conditions of this permit.

B. The plan shall be completed within 180 days of the effective date of this permit. Plans shall provide for compliance with the terms of the plan within 365 days of the effective date of this permit. The owner or operator of the facility shall make a copy of the plan available to the Agency at any reasonable time upon request. [Note: If the plan has already been developed and implemented it shall be maintained in accordance with all requirements of this special condition.]

C. The permittee may be notified by the Agency at any time that the plan does not meet the requirements of this condition. After such notification, the permittee shall make changes to the plan and shall submit a written certification that the requested changes have been made. Unless otherwise provided, the permittee shall have 30 days after such notification to make the changes.

D. The discharger shall amend the plan whenever there is a change in construction, operation, or maintenance which may affect the discharge of significant quantities of pollutants to the waters of the State or if a facility inspection required by paragraph G of this condition indicates that an amendment is needed. The plan should also be amended if the discharger is in violation of any conditions of this permit, or has not achieved the general objective of controlling pollutants in storm water discharges. Amendments to the plan shall be made within the shortest reasonable period of time, and shall be provided to the Agency for review upon request.

E. The plan shall provide a description of potential sources which may be expected to add significant quantities of pollutants to storm water discharges, or which may result in non-storm water discharges from storm water outfalls at the facility. The plan shall include, at a minimum, the following items:

1. A topographic map extending one-quarter mile beyond the property boundaries of the facility, showing: the facility, surface water bodies, wells (including injection wells), seepage pits, infiltration ponds, and the discharge points where the facility's storm water discharges to a municipal storm drain system or other water body. The requirements of this paragraph may be included on the site map if appropriate.

2. A site map showing:

   i. The storm water conveyance and discharge structures;

   ii. An outline of the storm water drainage areas for each storm water discharge point;

   iii. Paved areas and buildings;

   iv. Areas used for outdoor manufacturing, storage, or disposal of significant materials, including activities that generate significant quantities of dust or particulates.

   v. Location of existing storm water structural control measures (dikes, coverings, detention facilities, etc.);

   vi. Surface water locations and/or municipal storm drain locations

   vii. Areas of existing and potential soil erosion;

   viii. Vehicle service areas;

   ix. Material loading, unloading, and access areas.

3. A narrative description of the following:

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Special Conditions

i. The nature of the industrial activities conducted at the site, including a description of significant materials that are treated, stored or disposed of in a manner to allow exposure to storm water;

ii. Materials, equipment, and vehicle management practices employed to minimize contact of significant materials with storm water discharges;

iii. Existing structural and non-structural control measures to reduce pollutants in storm water discharges;

iv. Industrial storm water discharge treatment facilities;

v. Methods of onsite storage and disposal of significant materials;

4. A list of the types of pollutants that have a reasonable potential to be present in storm water discharges in significant quantities.

5. An estimate of the size of the facility in acres or square feet, and the percent of the facility that has impervious areas such as pavement or buildings.

6. A summary of existing sampling data describing pollutants in storm water discharges.

F. The plan shall describe the storm water management controls which will be implemented by the facility. The appropriate controls shall reflect identified existing and potential sources of pollutants at the facility. The description of the storm water management controls shall include:

1. Storm Water Pollution Prevention Personnel - Identification by job titles of the individuals who are responsible for developing, implementing, and revising the plan.

2. Preventive Maintenance - Procedures for inspection and maintenance of storm water conveyance system devices such as oil/water separators, catch basins, etc., and inspection and testing of plant equipment and systems that could fail and result in discharges of pollutants to storm water.

3. Good Housekeeping - Good housekeeping requires the maintenance of clean, orderly facility areas that discharge storm water. Material handling areas shall be inspected and cleaned to reduce the potential for pollutants to enter the storm water conveyance system.

4. Spill Prevention and Response - Identification of areas where significant materials can spill into or otherwise enter the storm water conveyance systems and their accompanying drainage points. Specific material handling procedures, storage requirements, spill clean up equipment and procedures should be identified, as appropriate. Internal notification procedures for spills of significant materials should be established.

5. Storm Water Management Practices - Storm water management practices are practices other than those which control the source of pollutants. They include measures such as installing oil and grit separators, diverting storm water into retention basins, etc. Based on assessment of the potential of various sources to contribute pollutants, measures to remove pollutants from storm water discharge shall be implemented. In developing the plan, the following management practices shall be considered:

i. Containment - Storage within berms or other secondary containment devices to prevent leaks and spills from entering storm water runoff;

ii. Oil & Grease Separation - Oil/water separators, booms, skimmers or other methods to minimize oil contaminated storm water discharges;

iii. Debris & Sediment Control - Screens, booms, sediment ponds or other methods to reduce debris and sediment in storm water discharges;

iv. Waste Chemical Disposal - Waste chemicals such as antifreeze, degreasers and used oils shall be recycled or disposed of in an approved manner and in a way which prevents them from entering storm water discharges.

v. Storm Water Diversion - Storm water diversion away from materials manufacturing, storage and other areas of potential storm water contamination;

vi. Covered Storage or Manufacturing Areas - Covered fueling operations, materials manufacturing and storage areas to prevent contact with storm water.

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Special Conditions

6. Sediment and Erosion Prevention - The plan shall identify areas which due to topography, activities, or other factors, have a high potential for significant soil erosion and describe measures to limit erosion.

7. Employee Training - Employee training programs shall inform personnel at all levels of responsibility of the components and goals of the storm water pollution control plan. Training should address topics such as spill response, good housekeeping and material management practices. The plan shall identify periodic dates for such training.

8. Inspection Procedures - Qualified plant personnel shall be identified to inspect designated equipment and plant areas. A tracking or follow-up procedure shall be used to ensure appropriate response has been taken in response to an inspection. Inspections and maintenance activities shall be documented and recorded.

G. The permittee shall conduct an annual facility inspection to verify that all elements of the plan, including the site map, potential pollutant sources, and structural and non-structural controls to reduce pollutants in industrial storm water discharges are accurate. Observations that require a response and the appropriate response to the observation shall be retained as part of the plan. Records documenting significant observations made during the site inspection shall be submitted to the Agency in accordance with the reporting requirements of this permit.

H. This plan should briefly describe the appropriate elements of other program requirements, including Spill Prevention Control and Countermeasures (SPCC) plans required under Section 311 of the CWA and the regulations promulgated thereunder, and Best Management Programs under 40 CFR 125.100.

I. The plan is considered a report that shall be available to the public under Section 308(b) of the CWA. The permittee may claim portions of the plan as confidential business information, including any portion describing facility security measures.

J. The plan shall include the signature and title of the person responsible for preparation of the plan and include the date of initial preparation and each amendment thereto.

Construction Authorization

K. Authorization is hereby granted to construct treatment works and related equipment that may be required by the Storm Water Pollution Prevention developed pursuant to this permit.

This Authorization is issued subject to the following condition(s).

1. If any statement or representation is found to be incorrect, this authorization may be revoked and the permittee there upon waives all rights thereunder.

2. The issuance of this authorization (a) does not release the permittee from any liability for damage to persons or property caused by or resulting from the installation, maintenance or operation of the proposed facilities; (b) does not take into consideration the structural stability of any units or part of this project; and ©does not release the permittee from compliance with other applicable statutes of the State of Illinois, or other applicable local law, regulations or ordinances.

3. Plans and specifications of all treatment equipment being included as part of the stormwater management practice shall be included in the SWPPP.

4. Construction activities which result from treatment equipment installation, including cleaning, grading and excavation activities which result in the disturbance of five acres or more of land area, are not covered by this authorization. The permittee shall contact the IEPA regarding the required permit(s).

REPORTING

L. The facility shall submit an annual inspection report to the Illinois Environmental Protection Agency. The report shall include results of the annual facility inspection which is required by Part G of the Storm Water Pollution Prevention Plan of this permit. The report shall also include documentation of any event (spill, treatment unit malfunction, etc.) Which would require an inspection, results of the inspection, and any subsequent corrective maintenance activity. The report shall be completed and signed by the authorized facility employee(s) who conducted the inspection(s).

M. The first report shall contain information gathered during the one year time period beginning with the effective date of coverage under this permit and shall be submitted no later than 60 days after this one year period has expired. Each subsequent report shall contain the previous year's information and shall be submitted no later than one year after the previous year s report was due.

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Special Conditions

N. Annual inspection reports shall be mailed to the following address:

Illinois Environmental Protection Agency
Bureau of Water
Compliance Assurance Section
Annual Inspection Report
1021 North Grand Avenue East
Post Office Box 19276
Springfield, Illinois 62794-9276

O. If the facility performs inspections more frequently than required by this permit, the results shall be included as additional information in the annual report.

Attachment H

Standard Conditions

Definitions

Act means the Illinois Environmental Protection Act, 415 ILCS 5 as Amended.

Agency means the Illinois Environmental Protection Agency.

Board means the Illinois Pollution Control Board.

Clean Water Act (formerly referred to as the Federal Water Pollution Control Act) means Pub. L. 92-500, as amended. 33 U.S.C. 1251 et seq.

NPDES (National Pollutant Discharge Elimination System) means the national program for issuing, modifying, revoking and reissuing, terminating, monitoring and enforcing permits, and imposing and enforcing pretreatment requirements, under Sections 307, 402, 318 and 405 of the Clean Water Act.

USEPA means the United States Environmental Protection Agency.

Daily Discharge means the discharge of a pollutant measured during a calendar day or any 24-hour period that reasonably represents the calendar day for purposes of sampling. For pollutants with limitations expressed in units of mass, the "daily discharge" is calculated as the total mass of the pollutant discharged over the day. For pollutants with limitations expressed in other units of measurements, the "daily discharge" is calculated as the average measurement of the pollutant over the day.

Maximum Daily Discharge Limitation (daily maximum) means the highest allowable daily discharge.

Average Monthly Discharge Limitation (30 day average) means the highest allowable average of daily discharges over a calendar month, calculated as the sum of all daily discharges measured during a calendar month divided by the number of daily discharges measured during that month.

Average Weekly Discharge Limitation (7 day average) means the highest allowable average of daily discharges over a calendar week, calculated as the sum of all daily discharges measured during a calendar week divided by the number of daily discharges measured during that week.

Best Management Practices (BMPs) means schedules of activities, prohibitions of practices, maintenance procedures, and other management practices to prevent or reduce the pollution of waters of the State. BMPs also include treatment requirements, operating procedures, and practices to control plant site runoff, spillage or leaks, sludge or waste disposal, or drainage from raw material storage.

Aliquot means a sample of specified volume used to make up a total composite sample.

Grab Sample means an individual sample of at least 100 milliliters collected at a randomly-selected time over a period not exceeding 15 minutes.

24 Hour Composite Sample means a combination of at least 8 sample aliquots of at least 100 milliliters, collected at periodic intervals during the operating hours of a facility over a 24-hour period.

8 Hour Composite Sample means a combination of at least 3 sample aliquots of at least 100 milliliters, collected at periodic intervals during the operating hours of a facility over an 8-hour period.

Flow Proportional Composite Sample means a combination of sample aliquots of at least 100 milliliters collected at periodic intervals such that either the time interval between each aliquot or the volume of each aliquot is proportional to either the stream flow at the time of sampling or the total stream flow since the collection of the previous aliquot.

(1) Duty to comply. The permittee must comply with all conditions of this permit. Any permit noncompliance constitutes a violation of the Act and is grounds for enforcement action, permit termination, revocation and reissuance, modification, or for denial of a permit renewal application. The permittee shall comply with effluent standards or prohibitions established under Section 307(a) of the Clean Water Act for toxic pollutants within the time provided in the regulations that establish these standards or prohibitions, even if the permit has not yet been modified to incorporate the requirement.

(2) Duty to reapply. If the permittee wishes to continue an activity regulated by this permit after the expiration date of this permit, the permittee must apply for and obtain a new permit. If the permittee submits a proper application as required by the Agency no later than 180 days prior to the expiration date, this permit shall continue in full force and effect until the final Agency decision on the application has been made.

(3) Need to halt or reduce activity not a defense. It shall not be a defense for a permittee in an enforcement action that it would have been necessary to halt or reduce the permitted activity in order to maintain compliance with the conditions of this permit.

(4) Duty to mitigate. The permittee shall take all reasonable steps to minimize or prevent any discharge in violation of this permit which has a reasonable likelihood of adversely affecting human health or the environment.

(5) Proper operation and maintenance. The permittee shall at all times properly operate and maintain all facilities and systems of treatment and control (and related appurtenances) which are installed or used by the permittee to achieve compliance with conditions of this permit. Proper operation and maintenance includes effective performance, adequate funding, adequate operator staffing and training, and adequate laboratory and process controls, including appropriate quality assurance procedures. This provision requires the operation of back-up, or auxiliary facilities, or similar systems only when necessary to achieve compliance with the conditions of the permit.

(6) Permit actions. This permit may be modified, revoked and reissued, or terminated for cause by the Agency pursuant to 40 CFR 122.62. The filing of a request by the permittee for a permit modification, revocation and reissuance, or termination, or a notification of planned changes or anticipated noncompliance, does not stay any permit condition.

(7) Property rights. This permit does not convey any property rights of any sort, or any exclusive privilege.

(8) Duty to provide information. The permittee shall furnish to the Agency within a reasonable time, any information which the Agency may request to determine whether cause exists for modifying, revoking and reissuing, or terminating this permit, or to determine compliance with the permit. The permittee shall also furnish to the Agency, upon request, copies of records required to be kept by this permit.

(9) Inspection and entry. The permittee shall allow an authorized representative of the Agency, upon the presentation of credentials and other documents as may be required by law, to:

(a) Enter upon the permittee's premises where a regulated facility or activity is located or conducted, or where records must be kept under the conditions of this permit;

(b) Have access to and copy, at reasonable times, any records that must be kept under the conditions of this permit;

(c) Inspect at reasonable times any facilities, equipment (including monitoring and control equipment), practices, or operations regulated or required under this permit; and

(d) Sample or monitor at reasonable times, for the purpose of assuring permit compliance, or as otherwise authorized by the Act, any substances or parameters at any location.

(10) Monitoring and records.

(a) Samples and measurements taken for the purpose of monitoring shall be representative of the monitored activity.

(b) The permittee shall retain records of all monitoring information, including all calibration and maintenance records, and all original strip chart recordings for continuous monitoring instrumentation, copies of all reports required by this permit, and records of all data used to complete the application for this permit, for a period of at least 3 years from the date of this permit, measurement, report or application. This period may be extended by request of the Agency at any time.

(c) Records of monitoring information shall include:

(1) The date, exact place, and time of sampling or measurements;

(2) The individual(s) who performed the sampling or measurements;

(3) The date(s) analyses were performed;

(4) The individual(s) who performed the analyses;

(5) The analytical techniques or methods used; and

(6) The results of such analyses.

(d) Monitoring must be conducted according to test procedures approved under 40 CFR Part 136, unless other test procedures have been specified in this permit. Where no test procedure under 40 CFR Part 136 has been approved, the permittee must submit to the Agency a test method for approval. The permittee shall calibrate and perform maintenance procedures on all monitoring and analytical instrumentation at intervals to ensure accuracy of measurements.

(11) Signatory requirement. All applications, reports or information submitted to the Agency shall be signed and certified.

(a) Application. All permit applications shall be signed as follows:

(1) For a corporation: by a principal executive officer of at least the level of vice president or a person or position having overall responsibility for environmental matters for the corporation;

(2) For a partnership or sole proprietorship: by a general partner or the proprietor, respectively; or

(3) For a municipality, State, Federal, or other public agency: by either a principal executive officer or ranking elected official.

(b) Reports. All reports required by permits, or other information requested by the Agency shall be signed by a person described in paragraph (a) or by a duly authorized representative of that person. A person is a duly authorized representative only if:

(1) The authorization is made in writing by a person described in paragraph (a); and

(2) The authorization specifies either an individual or a position responsible for the overall operation of the facility, from which the discharge originates, such as a plant manager, superintendent or person of equivalent responsibility; and

(3) The written authorization is submitted to the Agency.

(c) Changes of Authorization. If an authorization under (b) is no longer accurate because a different individual or position has responsibility for the overall operation of the facility, a new authorization satisfying the requirements of (b) must be submitted to the Agency prior to or together with any reports, information, or applications to be signed by an authorized representative.

(12) Reporting requirements.

(a) Planned changes. The permittee shall give notice to the Agency as soon as possible of any planned physical alterations or additions to the permitted facility.

(b) Anticipated noncompliance. The permittee shall give advance notice to the Agency of any planned changes in the permitted facility or activity which may result in noncompliance with permit requirements.

(c) Compliance schedules. Reports of compliance or noncompliance with, or any progress reports on, interim and final requirements contained in any compliance schedule of this permit shall be submitted no later than 14 days following each schedule date.

(d) Monitoring reports. Monitoring results shall be reported at the intervals specified elsewhere in this permit.

(1) Monitoring results must be reported on a Discharge Monitoring Report (DMR).

(2) If the permittee monitors any pollutant more frequently than required by the permit, using test procedures approved under 40 CFR 136 or as specified in the permit, the results of this monitoring shall be included in the calculation and reporting of the data submitted in the DMR.

(3) Calculations for all limitations which require averaging of measurements shall utilize an arithmetic mean unless otherwise specified by the Agency in the permit.

(e) Twenty-four hour reporting. The permittee shall report any noncompliance which may endanger health or the environment. Any information shall be provided orally within 24 hours from the time the permittee becomes aware of the circumstances. A written submission shall also be provided within 5 days of the time the permittee becomes aware of the circumstances. The written submission shall contain a description of the noncompliance and its cause; the period of noncompliance, including exact dates and time; and if the noncompliance has not been corrected, the anticipated time it is expected to continue; and steps taken or planned to reduce, eliminate, and prevent reoccurrence of the noncompliance. The following shall be included as information which must be reported within 24 hours:

(1) Any unanticipated bypass which exceeds any effluent limitation in the permit;

(2) Violation of a maximum daily discharge limitation for any of the pollutants listed by the Agency in the permit to be reported within 24 hours.

The Agency may waive the written report on a case-by-case basis if the oral report has been received within 24 hours.

(f) Other noncompliance. The permittee shall report all instances of noncompliance not reported under paragraphs (12)(c), (d), or (e), at the time monitoring reports are submitted. The reports shall contain the information listed in paragraph (12)(e).

(g) Other information. Where the permittee becomes aware that it failed to submit any relevant facts in a permit application, or submitted incorrect information in a permit application, or in any report to the Agency, it shall promptly submit such facts or information.

(13) Transfer of permits. A permit may be automatically transferred to a new permittee if:

(a) The current permittee notifies the Agency at least 30 days in advance of the proposed transfer date;

(b) The notice includes a written agreement between the existing and new permittees containing a specific date for transfer of permit responsibility, coverage and liability between the current and new permittees; and

(c) The Agency does not notify the existing permittee and the proposed new permittee of its intent to modify or revoke and reissue the permit. If this notice is not received, the transfer is effective on the date specified in the agreement.

(14) All manufacturing, commercial, mining, and silvicultural dischargers must notify the Agency as soon as they know or have reason to believe:

(a) That any activity has occurred or will occur which would result in the discharge of any toxic pollutant identified under Section 307 of the Clean Water Act which is not limited in the permit, if that discharge will exceed the highest of the following notification levels:

(1) One hundred micrograms per liter (100 ug/l);

(2) Two hundred micrograms per liter (200 ug/l) for acrolein and acrylonitrile; five hundred micrograms per liter (500 ug/l) for 2,4-dinitrophenol and for 2-methyl-4,6 dinitrophenol; and one milligram per liter (1 mg/l) for antimony.

(3) Five (5) times the maximum concentration value reported for that pollutant in the NPDES permit application; or

(4) The level established by the Agency in this permit.

(b) That they have begun or expect to begin to use or manufacture as an intermediate or final product or byproduct any toxic pollutant which was not reported in the NPDES permit application.

(15) All Publicly Owned Treatment Works (POTWs) must provide adequate notice to the Agency of the following:

(a) Any new introduction of pollutants into that POTW from an indirect discharge which would be subject to Sections 301 or 306 of the Clean Water Act if it were directly discharging those pollutants; and

(b) Any substantial change in the volume or character of pollutants being introduced into that POTW by a source introducing pollutants into the POTW at the time of issuance of the permit.

(c) For purposes of this paragraph, adequate notice shall include information on (i) the quality and quantity of effluent introduced into the POTW, and (ii) any anticipated impact of the change on the quantity or quality of effluent to be discharged from the POTW.

(16) If the permit is issued to a publicly owned or publicly regulated treatment works, the permittee shall require any industrial user of such treatment works to comply with federal requirements concerning:

(a) User charges pursuant to Section 204(b) of the Clean Water Act, and applicable regulations appearing in 40 CFR 35;

(b) Toxic pollutant effluent standards and pretreatment standards pursuant to Section 307 of the Clean Water Act; and

(c) Inspection, monitoring and entry pursuant to Section 308 of the Clean Water Act.

(17) If an applicable standard or limitation is promulgated under Section 301(b)(2)(C) and (D), 304(b)(2), or 307(a)(2) and that effluent standard or limitation is more stringent than any effluent limitation in the permit, or controls a pollutant not limited in the permit, the permit shall be promptly modified or revoked, and reissued to conform to that effluent standard or limitation.

(18) Any authorization to construct issued to the permittee pursuant to 35 Ill. Adm. Code 309.154 is hereby incorporated by reference as a condition of this permit.

(19) The permittee shall not make any false statement, representation or certification in any application, record, report, plan or other document submitted to the Agency or the USEPA, or required to be maintained under this permit.

(20) The Clean Water Act provides that any person who violates a permit condition implementing Sections 301, 302, 306, 307, 308, 318, or 405 of the Clean Water Act is subject to a civil penalty not to exceed $10,000 per day of such violation. Any person who willfully or negligently violates permit conditions implementing Sections 301, 302, 306, 307, or 308 of the Clean Water Act is subject to a fine of not less than $2,500 nor more than $25,000 per day of violation, or by imprisonment for not more than one year, or both.

(21) The Clean Water Act provides that any person who falsifies, tampers with, or knowingly renders inaccurate any monitoring device or method required to be maintained under permit shall, upon conviction, be punished by a fine of not more than $10,000 per violation, or by imprisonment for not more than 6 months per violation, or by both.

(22) The Clean Water Act provides that any person who knowingly makes any false statement, representation, or certification in any record or other document submitted or required to be maintained under this permit shall, including monitoring reports or reports of compliance or non-compliance shall, upon conviction, be punished by a fine of not more than $10,000 per violation, or by imprisonment for not more than 6 months per violation, or by both.

(23) Collected screening, slurries, sludges, and other solids shall be disposed of in such a manner as to prevent entry of those wastes (or runoff from the wastes) into waters of the State. The proper authorization for such disposal shall be obtained from the Agency and is incorporated as part hereof by reference.

(24) In case of conflict between these standard conditions and any other condition(s) included in this permit, the other condition(s) shall govern.

(25) The permittee shall comply with, in addition to the requirements of the permit, all applicable provisions of 35 Ill. Adm. Code, Subtitle C, Subtitle D, Subtitle E, and all applicable orders of the Board.

(26) The provisions of this permit are severable, and if any provision of this permit, or the application of any provision of this permit is held invalid, the remaining provisions of this permit shall continue in full force and effect.

(Rev. 3-13-86)

HOLLAND ENERGY

A Member of the Constellation Energy Group

December 29, 2004

Illinois Environmental Protection Agency
Bureau of Water
Compliance Assurance Section
1021 N. Grand Avenue East
P.O. Box 19276
Springfield, Illinois 62794-9276

RE: NPDES Permit # IL0074268 5-year renewal

Please find enclosed the renewal application forms from Holland Energy for the NPDES Permit # IL0074268 5-year renewal.

Holland is asking for the following modifications to be made during the renewal process.

Special Conditions:

11) Change the requirement of 24-hour composite sample for Bio-monitoring to a composite sample during at least an 8-hour day during times of operation. (This is due to the facility not continuously operating in 24-hour periods).

Effluent Limitations and Monitoring:
Outfall 001

Specify Monitoring Point for outfall 001 at the lift pump at the facility and piped to the lab in the water treatment building.

Change frequency of sampling to once per month while operating as limited operation of facility suggests and previous samples do not show any upward trends of monitored parameters.

RR2, Box 270-A, Beecher City, IL 62414-0065 • Phone: 618-487-5190 • Fax: 618-487-5192

HOLLAND ENERGY

A Member of the Constellation Energy Group

Change Limit Concentration for Daily Maximum to 30 mg/l for Oil and Grease.

Change frequency from "continuous" to "daily" for flow and temperature readings.

Eliminate Chromium, Zinc, Oil and Grease, and Chlorine from list of parameters.

Outfalls 002 and 003

Change the sampling frequency from twice per month to once per discharge.

If you have any questions, please contact Steve Dobbs or myself at 618-487-9120.

Respectfully yours,

Barry Hatfield
General Manager
Holland Energy, LLC.

Enclosures

Cc: Dale Linaweaver
Edward F. Tracey

File: Water/NPDES Permit

RR2, Box 270-A, Beecher City, IL 62414-0065 • Phone: 618-487-5190 • Fax: 618-487-5192

ILLINOIS ENVIRONMENTAL PROTECTION AGENCY
1021 North Grand Avenue East, P.O. Box 19276, Springfield, Illinois 62794-9276 • (217) 782-2829
James R. Thompson Center, 100 West Randolph, Suite 11-300, Chicago, IL 60601 • (312) 814-6026
PAT QUINN, GOVERNOR DOUGLAS P. SCOTT, DIRECTOR

217/782-0610

April 18, 2011

Holland Energy, LLC
722 North High School Road
Indianapolis, IN 46214

Re: Holland Energy, LLC
NPDES Permit No. IL0074268
Final Permit

Gentlemen:

Attached is the final NPDES Permit for your discharge. The Permit as issued covers discharge limitations, monitoring, and reporting requirements. Failure to meet any portion of the Permit could result in civil and/or criminal penalties. The Illinois Environmental Protection Agency is ready and willing to assist you in interpreting any of the conditions of the Permit as they relate specifically to your discharge.

The Agency received your letters dated January 8, 2009 and January 19, 2009 regarding the draft NPDES permit. Based on the information provided the following changes were made to the permit.

1. The mailing address for the facility was changed as requested.

2. Special Condition 6 will remain in the permit. The facility was granted a mixing zone for temperature and the limits will remain in the permit to ensure that the facility is in compliance with the thermal standard.

3. ASTM Method E2455-06 will remain in Special Condition 13. The Agency received a letter dated February 23, 2011 from USEPA Region 5 supporting the use of this test method.

4. Special Condition 14 will remain in the permit. Prior to approval of the initial permit, IEPA raised concerns over the potential impacts that the high volume wastestream would have on aquatic life within the Kaskaskia River. The pre and post-operational mussel surveys conducted under the previous permit were required in order to assure that the large wastestream would not adversely impact aquatic life within the receiving water. It is inherent that biosurveys of this nature are subject to variability from year to year due to sampling inconsistencies as well as variable conditions of the receiving water. However, based on a comparison of pre and post-operational surveys there is concern that mussel recruitment may have declined in post-operational years. Given that these biosurveys were initially required in order to assure that biology of the Kaskaskia River would be preserved, the Agency has determined that additional mussel surveys are necessary when it appears that mussel recruitment may be declining downstream of the facility. If additional mussel surveys conducted pursuant to the attached permit suggest that mussel recruitment is not impacted by the facility, the Agency will review the information and determine if this special condition could be removed in the next permit renewal.

EXHIBIT 4

The Agency also received a letter dated January 7, 2008 from Prairie Rivers Network regarding the draft NPDES permit. Based on the information provided the following changes were made to the permit.

1. Chloride monitoring was added to outfall 001. Special Condition 17 was added which outlines the monitoring requirement for chloride.

2. The Total Dissolved Solids (TDS) limit was removed from the permit. The Illinois Pollution Control Board eliminated the TDS water quality standard on September 4, 2008.

3. The sulfate limit was removed from the permit. Based on existing data, there is no reasonable potential for the facility to exceed the water quality standard for sulfate.

The Agency has begun a program allowing the submittal of electronic Discharge Monitoring Reports (eDMRs) instead of paper Discharge Monitoring Reports (DMRs). If you are interested in eDMRs, more information can be found on the Agency website, http://epa.state.il.us/water/edmr/index.html. If your facility is not registered in the eDMR program, a supply of preprinted paper DMR Forms for your facility will be sent to you prior to the initiation of DMR reporting under the reissued permit. Additional information and instructions will accompany the preprinted DMRs upon their arrival.

The attached Permit is effective as of the date indicated on the first page of the Permit. Until the effective date of any re-issued Permit, the limitations and conditions of the previously-issued Permit remain in full effect. You have the right to appeal any condition of the Permit to the Illinois Pollution Control Board within a 35 day period following the issuance date.

Should you have questions concerning the Permit, please contact Leslie Lowry at 217/782-0610.

Sincerely,

Alan Keller

Alan Keller, P.E.
Manager, Permit Section
Division of Water Pollution Control

SAK:DEL:LRL:06100601.daa

Attachment: Final Permit

cc: Records
Compliance Assurance Section
Champaign Region
Billing

ILLINOIS ENVIRONMENTAL PROTECTION AGENCY
1021 North Grand Avenue East, P.O. Box 19276, Springfield, Illinois 62794-9276 • (217) 782-2829
James R. Thompson Center, 100 West Randolph, Suite 11-300, Chicago, IL 60601 • (312) 814-6026
PAT QUINN, GOVERNOR DOUGLAS P. SCOTT, DIRECTOR

217/558-2012

September 23, 2010

Ms. Susan Hedman
USEPA Region 5, R-19J
77 West Jackson
Chicago, IL 60604

RE: Request for Approval of Alternate Test Method Per 40 CFR 136.5

Dear Ms. Hedman:

Our office has been in contact with Rob Pepin of USEPA Region 5 Permit Section regarding the use of a unionid mussel toxicity test. Illinois EPA desires to issue a permit to an intermittent electric power generation plant (peaker plant) with a condition requiring mussel whole effluent toxicity testing using an ASTM Method (E2455-06). We may also find that this test is necessary at other facilities where unionid mussels are of interest. We are hereby requesting the approval of the Regional Administrator pursuant to 40 CFR 136.5 regarding our use of this test method.

ASTM Method E2455-06 is designed similarly to existing USEPA approved methods such as 2002.0 and 1002.0, acute and chronic effluent toxicity test methods for daphnids. Mussels are increasingly being tested due to their sensitivity to pollutants and declining populations in river habitats. USEPA's recent publication of National Water Quality Criteria for Ammonia (December, 2009) uses similar if not identical mussel toxicity tests as justification for the ammonia criteria. The approval of this test under 40 CFR 136.5 by the Regional Administrator is vital to our use and defense of the mussel test in permits. I have enclosed a page from the draft permit that represents our intended use of the mussel test as a permit condition. Future uses in permits would be similar or identical.

Please consider approval of this request at your earliest convenience. Rob Pepin is familiar with some of the details and I may be contacted at the above phone number.

Sincerely,

Robert Mosher
Manager, Water Quality Standards Unit
Division of Water Pollution Control

RGM:djp/hollandmusselltet

cc: Rob Pepin, USEPA Region 5, WN-16J
√Leslie Lowry

EXHIBIT 5

NPDES Permit No. IL0074268

Special Conditions

N. Annual inspection reports shall be mailed to the following address:

Illinois Environmental Protection Agency
Bureau of Water
Compliance Assurance Section
Annual Inspection Report
1021 North Grand Avenue East
Post Office Box 19276
Springfield, Illinois 62794-9276

O. If the facility performs inspections more frequently than required by this permit, the results shall be included as additional information in the annual report.

SPECIAL CONDITION 13. The Permittee shall prepare a preliminary biomonitoring plan and submit the plan to the IEPA for review and approval within ninety (90) days of the effective date of this Permit. The Permittee shall begin biomonitoring of effluent from Outfall 001 the first summer after plan approval.

Biomonitoring

1. Toxicity Test - Acute (4-d) and short-term (14-d) toxicity tests shall be run on juveniles of mussel species representative of the aquatic community of the receiving stream. Procurement and testing of organisms must be consistent with Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM E2455-06). Guidelines for measuring effluent toxicity must be consistent with Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (Fifth Ed.) EPA/821-R-02-012. Unless substitute tests are pre-approved; the following test is required.

   An acute (4-d) and short-term (14-d) static-renewal toxicity test using newly-transformed juvenile fatmucket (*Lampsilis siliquoidea*) or another IEPA pre-approved native species.

2. Testing Frequency - The above test shall be conducted using 8-hour composite effluent samples (one initial sample and sufficient renewal samples to be determined in biomonitoring plan) discharged under normal operating conditions unless otherwise authorized by the IEPA. Upstream water of the Kaskaskia River is to be supplied to conduct serial dilutions. Testing must be conducted once per year for two year beginning the first summer after permit issuance.

3. Reporting - Results shall be reported according to EPA/821-R-02-012, Section 12, Report Preparation and shall be submitted to IEPA, Bureau of Water, Compliance Assurance Section within one week of receipt from the laboratory. Reports are due to the IEPA no later than 3 months following the test date.

4. Toxicity Assessment - Should the review of the results of the biomonitoring program identify toxicity, the IEPA may require that the Permittee prepare a plan for toxicity reduction evaluation and identification. This plan shall be developed in accordance with Toxicity Reduction Evaluation Guidance for Municipal Wastewater Treatment Plants, EPA/833B-99/002, and shall include an evaluation to determine which chemicals have a potential for being discharged in the plant wastewater, a monitoring program to determine their presence or absence and to identify other compounds which are not being removed by treatment, and other measures as appropriate. The Permittee shall submit to the IEPA its plan for toxicity reduction evaluation within ninety (90) days following notification by the IEPA. The Permittee shall implement the plan within ninety (90) days or other such date as contained in a notification letter received from the IEPA.

   The IEPA may modify this Permit during its term to incorporate additional requirements or limitations based on the results of the biomonitoring. In addition, after review of the monitoring results, the IEPA may modify this Permit to include numerical limitations for specific toxic pollutants. Modifications under this condition shall follow public notice and opportunity for hearing.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
REGION 5
77 WEST JACKSON BOULEVARD
CHICAGO, IL 60604-3590

FEB 23 2011

REPLY TO THE ATTENTION OF: WC-15J

Mr. Robert Mosher
Manager, Water Quality Standards Unit
Division of Water Pollution Control
Illinois Environmental Protection Agency
P.O. Box 19276
Springfield, Illinois 62794-9276

Dear Mr. Mosher:

I am responding to your Alternate Test Procedure (ATP) Application for *Limited Use* of ASTM Method E2455-06. Method E2455-06 is the "Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels." We have received and reviewed the corresponding Illinois Environment Protection Agency request and supplemental materials. We are pleased to inform you that based on our review and the recommendation from our technical staff, the proposed procedures are approved for *Limited Use*.

This approval is provided under the authority granted to me in 40 CFR Section 136.5. and may be used in relation to the Clean Water Act National Pollutant Discharge Elimination System (NPDES) and Pretreatment Program Monitoring. This approval is for the analysis of Acute and Chronic Whole Effluent Toxicity using freshwater mussels as a test species and applies exclusively to the Holland Energy Facility, Illinois NPDES IL0074268.

Thank you for your participation in the ATP process. If you have questions regarding this letter, please contact Kenneth Gunter, ATP Coordinator, Water Division, at (312) 353-9076.

Sincerely,

Tinka G. Hyde
Director, Water Division

RECEIVED
MAR - 3 2011
Surface Water Section
BUREAU OF WATER

Enclosures

cc: Barbara Conner, IEPA

EXHIBIT 6

ALTERNATE TEST PROCEDURE TITLE: WHOLE EFFLUENT TOXICITY TEST USING FRESH WATER MUSSELS

I	ASPECT: Codification In Federal Regulations	PERSON ASSIGNED RESPONSIBILITY: US EPA
ISSUES: The WET test methods are incorporated by reference		
I(a)	COMPONENT: Three test methods are specified	SOURCE: 40 CFR part 136
SUMMARY The WET test methods and their procedures are described in EPA manuals (EPA-821-R02-012,013, and 014). These manuals identify the selection of the test species as well as practices where flexibility is allowed.		

I (b)	COMPONENT: USEPA. October 2002. Testing the Toxicity of Surface Water on Fish and Other Aquatic Species Fourth Edition.	SOURCE: EPA 821-R-02-012, EPA 821-R-02-013, and EPA 821-R-02-014.
SUMMARY: . The use of any test species or test conditions other than those described in the methods summary tables in this manual shall be subject to application and approval of alternate test procedures under 40 CFR 136.4 and 40 CFR 136.5.		

II	ASPECT: **HOLLAND ENERGY LLC** RR 2 BOX 270A BEECHER CITY, IL 62444	PERSON ASSIGNED RESPONSIBILITY: Illinois EPA
ISSUE: KASKASKIA RIVER		
II(a)	COMPONENT: WATER QUALITY LIMITED SEGMENT	SOURCE: IL0074268 Outfall 001
SUMMARY: The stream segment receiving the discharge from outfall(s) 001 is on the 303 (d) list of impaired waters.		

II(b)	COMPONENT: Special Condition 18	SOURCE: IL0074268 Outfall 001
SUMMARY: The Permittee shall prepare a preliminary biomonitoring plan and submit the plan to the IEPA for review and approval within ninety (90) days of the effective date of this Permit. The Permittee		

shall begin biomonitoring of effluent from Outfall 001 the first summer after plan approval

II(c)	COMPONENT: Biomonitoring	SOURCE: IL0074268 Outfall 001
SUMMARY: Toxicity tests shall be run on juveniles of mussel species representative of the aquatic community of the receiving stream		

II(d)	COMPONENT: Test Methods	SOURCE: IL0074268 Outfall 001
SUMMARY: Acute (4-d) and short-term (14-d) toxicity tests consistent with Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM E2455-06) and Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (Fifth Ed.) EPA/821-R-02-012.		

III	ASPECT: **Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels**	PERSON ASSIGNED RESPONSIBILITY:
ISSUE: Protectiveness of WQC		
III (a)	COMPONENT: Water Quality Criteria for ammonia	SOURCE: : ASTM E2455
SUMMARY: Results of acute toxicity tests (24 to 96 h) for 10 species in 8 genera were used to calculate genus mean acute values (GMAVs) ranging from 2.56 to 8.97 mg/L (total ammonia as N at pH 8 at 25°C).		

III(b)	COMPONENT: Acute sensitivity of freshwater mussels	SOURCE: ASTM E2455
SUMMARY: Recalculation of the criteria maximum concentration (CMC) including these mussel data resulted in a CMC 75 % lower than the CMC of 5.62 mg/L total ammonia as N at pH 8 at 25°C		

III (c)	COMPONENT CHRONIC Sensitivity of freshwater mussels	SOURCE: ASTM E2455
SUMMARY: The range of acute to chronic ratios were used to estimate a criteria continuous concentration (CCC), the estimated CCC for mussels was 20 to 75 % less than the CCC of 1.24 mg/L total ammonia as N at pH 8 and 25°		

III (d)	COMPONENT: COMPONENT	SOURCE: ASTM E2455
SUMMARY: Keller et al (2005) () concluded that U.S. Environmental Protection Agency (USEPA) water quality criteria (WQC) for some metals and ammonia may not be protective of freshwater mussels.		

IV	ASPECT: PROTOCOL APPROVAL OF ATPs	PERSON ASSIGNED RESPONSIBILITY: US EPA Office of Water (EAD)
ISSUE: Scope of guidance		
IV (a)	COMPONENT, Applicability	SOURCE: EPA 821-B-98-002
SUMMARY: Subject protocol does not establish or affect legal obligations under federal regulations.		

IV(b)	COMPONENT: : Definition	SOURCE: EPA 821-B-98-002
SUMMARY: A proposed procedure is considered a "modified" procedure if it involves only minor changes in established test conditions for an approved species/method, , or if it employs a "new" species to be used as a substitute for a related, Agency-approved species and can be performed with essentially the same test conditions and methods of data analysis used for current Agency-approved species/methods		

IV(c)	COMPONENT:	SOURCE: EPA 821-B-98-002
SUMMARY: The sensitivity of the proposed test species/method must be demonstrated to be equal to or greater than the sensitivity of current Agency-approved species /methods, using reference toxicants or effluents		

V	ASPECT: REQUEST FOR APPROVAL	PERSON ASSIGNED RESPONSIBILITY: IL EPA
ISSUES: Test Procedure		
V (a)	COMPONENT: Comparability	SOURCE: 9/23/2010 LETTER
SUMMARY ASTM Method E2455-06 is designed similarly to existing USEPA approved methods i.e., 2002.0 and 1002.0		
V (b)	COMPONENT: Species selection	SOURCE: 9/23/2010 LETTER
SUMMARY: Mussels are increasingly being tested due to their sensitivity to pollutants and declining populations in river habitats.		

V(c)	COMPONENT: Criteria	SOURCE: 9/23/2010 LETTER
SUMMARY: Recent US EPA publication of National Water Quality Criteria for Ammonia use similar mussel toxicity test as justification for the ammonia criteria.		

Electronic Code of Federal Regulations
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e-CFR Data is current as of March 7, 2011

Title 40: Protection of Environment

PART 136—GUIDELINES ESTABLISHING TEST PROCEDURES FOR THE ANALYSIS OF POLLUTANTS

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§ 136.5 Approval of alternate test procedures.

(a) The Regional Administrator of the region in which the discharge will occur has final responsibility for approval of any alternate test procedure proposed by the responsible person or firm making the discharge.

(b) Within thirty days of receipt of an application, the Director will forward such application proposed by the responsible person or firm making the discharge, together with his recommendations, to the Regional Administrator. Where the Director recommends rejection of the application for scientific and technical reasons which he provides, the Regional Administrator shall deny the application and shall forward this decision to the Director of the State Permit Program and to the Alternate Test Procedure Program Coordinator, Office of Science and Technology (4303), Office of Water, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460.

(c) Before approving any application for an alternate test procedure proposed by the responsible person or firm making the discharge, the Regional Administrator shall forward a copy of the application to the Alternate Test Procedure Program Coordinator, Office of Science and Technology (4303), Office of Water, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460.

(d) Within ninety days of receipt by the Regional Administrator of an application for an alternate test procedure, proposed by the responsible person or firm making the discharge, the Regional Administrator shall notify the applicant and the appropriate State agency of approval or rejection, or shall specify the additional information which is required to determine whether to approve the proposed test procedure. Prior to the expiration of such ninety day period, a recommendation providing the scientific and other technical basis for acceptance or rejection will be forwarded to the Regional Administrator by the Alternate Test Procedure Program Coordinator, Washington, DC. A copy of all approval and rejection notifications will be forwarded to the Alternate Test Procedure Program Coordinator, Office of Science and Technology (4303), Office of Water, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460, for the purposes of national coordination.

(e) *Approval for nationwide use.* (1) As expeditiously as is practicable after receipt by the Alternate Test Procedure Program Coordinator, Washington, DC, of an application for an alternate test procedure for nationwide use, the Alternate Test Procedure Program Coordinator, Washington, DC, shall notify the applicant in writing whether the application is complete. If the application is incomplete, the applicant shall be informed of the information necessary to make the application complete.

(2) As expeditiously as is practicable after receipt of a complete package, the Alternate Test Procedure Program Coordinator shall perform any analysis necessary to determine whether the alternate test procedure satisfies the applicable requirements of this part, and the Alternate Test Procedure Program Coordinator shall recommend to the Administrator that he/she approve or reject the application and shall also notify the application of the recommendation.

(3) As expeditiously as practicable, an alternate method determined by the Administrator to satisfy the applicable requirements of this part shall be proposed by EPA for incorporation in subsection 136.3 of 40 CFR part 136. EPA shall make available for review all the factual bases for its proposal, including any performance data submitted by the applicant and any available EPA analysis of those data.

(4) Following a period of public comment, EPA shall, as expeditiously as practicable, publish in theFederal Registera final decision to approve or reject the alternate method.

[38 FR 28760, Oct. 16, 1973, as amended at 41 FR 52785, Dec. 1, 1976; 55 FR 33440, Aug. 15, 1990; 62 FR 30763, June 5, 1997; 72 FR 11239, Mar. 12, 2007]

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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, disease, predation and pollution are considered causal or contributing factors in many areas of the United States (Neves et al 1997) (**4**). Over the past decade, there have been over 75 published studies conducted that have evaluated the role of contaminants in the decline of populations of freshwater mussels (Kernaghan et al 2005) (**5**). In these studies, early life stages of mussels of several species are highly sensitive to some metals and ammonia in water exposures when compared to many of the most sensitive species of other invertebrates, fish, or amphibians that are commonly used to establish U.S. Environmental Protection Agency Water Quality Criteria (WQC; Augspurger et al 2003 (**6**), Keller et al 2005 (**7**), Kernaghan et al 2005 (**5**); USGS (2005a,b) (**8, 9**) section 1.5). Importantly, results of these previous studies indicate WQC for individual chemicals established for the protection of aquatic organisms may not be adequately protective of sensitive stages of freshwater mussels.

1.3 *Summary of Life History of Freshwater Mussels*:

1.3.1 Freshwater mussels are bivalve mollusks belonging to the family Unionidae or Margaritiferidae (section 10.1). Adults 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 appropriate fish host and encyst to complete development. Although glochidia may survive for months during brooding in the female mussel, glochidia typically survive for only a few days after release unless the glochidia reach a compatible host. Encystment on the host occurs by overgrowth of host tissue. Metamorphosis of juvenile mussels on the fish host occurs within days or weeks, depending on species and temperature. Host fish specificity varies among mussels. While some mussel species appear to require a single host organism, other species can transform their glochidia into juvenile mussels on several species of host fish. Following proper host infestation, glochidia transform into microscopic juveniles and excyst (drop off) and settle into suitable habitat to survive. The transformation of glochidia to juveniles results in the development of internal organs necessary for self-sustained existence as a benthic organism.

1.3.3 Newly-transformed juvenile mussels have a life style different from adult mussels. Transformed juvenile mussels may be at the sediment-water interface or may burrow several centimeters into sediment and rely on water percolating between substrate particles of sediment for food and oxygen. Newly-transformed juvenile mussels feed using ciliary currents on the foot and mantle. Older juvenile and adult mussels likely use different food types when living in different microenvironments. Given that glochidia and juvenile mussels are ecologically and physiologically different from adult mussels, protection of habitat quality of adult life stages may not be protective

[1] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.03 on Sediment Assessment and Toxicology.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

EXHIBIT 7

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. Efforts to assess effects of contaminants on mussels need to evaluate potential exposure to host fish in addition to exposure to each unique life stage of freshwater mussels.

1.4 *Summary of Toxicity Testing Conditions*:

1.4.1 Section 4 provides a summary of conditions for conducting toxicity tests with glochidia and juvenile mussels. Annex A1 provides guidance for conducting water-only toxicity tests with glochidia and juvenile mussels. Recommended test conditions for conducting these toxicity tests are based on various published methods outlined in Table A1.1 and Table A1.4 in Annex A1 and are based on the conditions used to conduct an inter-laboratory toxicity test with glochidia and juvenile mussels (section 16.5). Glochidia and juvenile mussels are only available on a seasonal basis. Section 10 describes procedures for collecting adult female mussels from the field to obtain glochidia for conducting toxicity tests or for obtaining glochidia to propagate juvenile mussels using a host organism.

1.4.2 In the field, mussels may be exposed to contaminants in water, sediment, or food. This standard only addresses effects associated with exposure of mussels to contaminants in water.

1.4.3 Guide E 724 describes procedures for conducting acute 48-h toxicity tests with embryos or larvae of saltwater bivalve mollusks. Endpoints measured in Guide E 724 include survival or shell deposition. Procedures outlined in Guide E 724 may be useful in helping to design studies for conducting toxicity tests with freshwater mussels as outlined in Annex A1.

1.4.4 Results of tests, even those with the same species, using procedures different from those described in Annex A1 may not be comparable. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting toxicity tests with aquatic organisms. If tests are conducted with procedures different from those described in this standard, uddditional tests are required to determine comparability of results. General procedures described in this standard might be useful for conducting tests with other aquatic organisms; however, modifications may be necessary.

1.5 *Summary of Results of Toxicity Tests Conducted with Freshwater Mussels*:

1.5.1 Keller et al (2005) (7) summarized results of acute laboratory toxicity tests conducted with glochidia and juvenile mussels described in 16 published studies. Freshwater mussels tended to be less sensitive in exposures to some pesticides and other organic compounds compared to other commonly-tested aquatic organisms. In contrast, Keller et al (2005) (7) concluded that U.S. Environmental Protection Agency (USEPA) water quality criteria (WQC) for some metals and ammonia may not be protective of freshwater mussels.

1.5.2 Augspurger et al (2003) (6) evuluated ammonia toxicity data generated for glochidia and juvenile of freshwater mussels in laboratory toxicity tests. Specifically, these toxicity data were used to estimate concentrations that would not likely be harmful to mussels in acute and chronic exposures and were used to evaluate the protectiveness of the WQC for ammonia. Results of acute toxicity tests (24 to 96 h) for 10 species in 8 genera were used to calculate genus mean acute values (GMAVs) ranging from 2.56 to 8.97 mg/L (total ammonia as N at pH 8 at 25°C). The freshwater mussels are at the sensitive end of the range when added to the GMAVs from the database used to derive the acute WQC for ammonia. Recalculation of the criteria maximum concentration (CMC) including these mussel data resulted in a CMC 75 % lower than the CMC of 5.62 mg/L total ammonia as N at pH 8 at 25°C (for application when salmonids absent). No chronic ammonia toxicity data (for example, 21 to 28-d exposures) were available for freshwater mussels; however, when a range of acute to chronic ratios were used to estimate a criteria continuous concentration (CCC), the estimated CCC for mussels was 20 to 75 % less than the CCC of 1.24 mg/L total ammonia as N at pH 8 and 25°C. Hence, Augspurger et al (2003) (6) concluded that the acute and chronic WQC for ammonia may not be protective of freshwater mussels.

1.5.3 Milam et al (2005) (**10**) conducted a series of 24-h acute toxicity tcsts with glochidia of six freshwater mussel species, *Leptodea fragilis*, *Utterbackia imbecillis*, *Lampsilis cardium*, *Lampsilis siliquoidea*, *Megalonaias nervosa*, and *Ligumia subrostrata*, and with two commonly-tested organisms, *Ceriodaphnia dubia* and *Daphnia magna*. Chemicals selected for testing (carbaryl, copper, 4-nonylphenol, pentachlorophenol, permethrin, and 2,4-dichlorophenoxyacetic acid [2,4-D]) represented different chemical classes and different toxie modes of action (Dwycr et al 2005a,b) (**11, 12**). No single chemical elieited consistently high or low toxicity; however, carbaryl and 2,4-D were generally the least toxic to the species tested. Milam et al (2005) (**10**) concluded that the toxicity data generated with *C. dubia* and *D. magna* were relatively protective of the range of sensitivities exhibited by glochidia of the mussels species tested. However, toxicity data generated with the commonly-tested *U. imbecillis* were not always protective of the range of sensitivities exhibited by the other mussel speeies tested.

1.6 This standard is arranged as follows:

1.7 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.

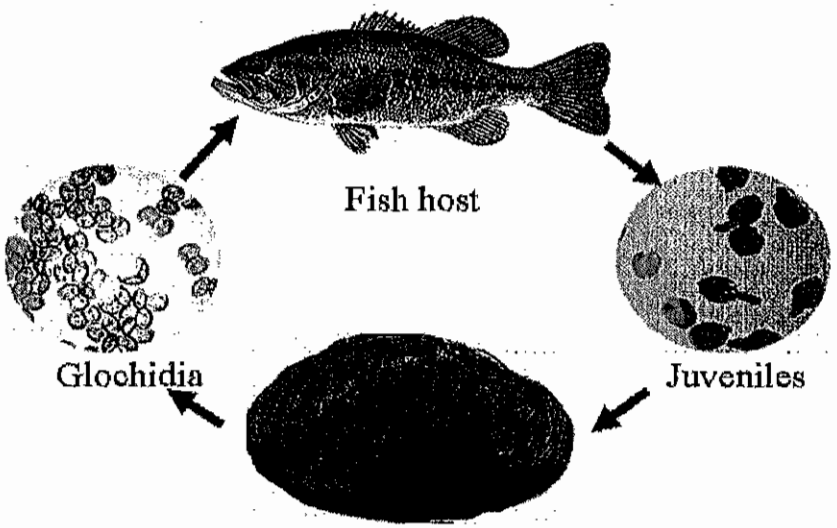


FIG. 1 Life Cycle of a Freshwater Mussel (Chris Barnhart, Missouri State University, Springfield, MO)

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:* [3]

D 4447 Guide for Disposal of Laboratory Chemicals and Samples

E 177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E 691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E 724 Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs

E 729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, und Amphibians

E 943 Terminology Relating to Biological Effects and Environmental Fate

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E 1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes

E 1367 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates

E 1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

E 1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

E 1850 Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests

IEEE/ASTM SI 10 Standard for Use of the International System of Units (SI) (the Modernized Metric System)

3. Terminology

3.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this standard. "Must" is used to express an absolute requirement, that is, to state that a test ought to be designed to satisfy the specified conditions, unless the purpose of the test requires a different design. "Must" is used only in connection with the factors that relate directly to the aeceptability of a test. "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although the violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions*—For definitions of other terms used in this standard, refer to Guides E 729 and E 1241 and Terminology E 943 and D 1129. For an explanation of units and symbols, refer to Practice E 380. A listing of the common and scientific names of freshwater mussels in North America can be found in AFS (1998) (13).

3.3 *Definitions of Terms Specific to This Standard:*

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

3.3.1 *acute test*—a comparative study in which organisms, that are subjected to different treatments, are observed for a short period usually not constituting a substantial portion of their life span (for example, 24- to 96-h exposures).

3.3.2 *chronic test*—a comparative study in which organism, that are subjected to different treatments, are observed for a long period or a substantial portion of their life span (for example, 21- to 28-d exposures). There is no test duration that represents a distinct boundary between acute and chronic test durations for any species. Although acute or chronic test procedures may specify standard duration(s), these durations have not been intended to define an acute:chronic boundary. Acute tests often utilize mortality as the only measure of effect; chronic tests usually include additional measures of effect such as growth or reproduction.

3.3.3 *EC50*—a statistically or graphically estimated concentration that is expected to cause one or more specified effects in 50 % of a group of organisms under specified conditions.

3.3.4 *IC50*—a point estimate of the toxicant concentration that would cause a 50 % reduction in a non-quantal measurement such as fecundity or growth.

3.3.5 *LC50*—a statistically or graphically estimated concentration that is expected to be lethal to 50 % of a group of organisms under specified conditions.

3.3.6 *lowest-observed-effect concentration (LOEC)—in a toxicity test*, the tested concentration of one or more chemicals immediately above the highest tested concentration that did not result in a statistically significant change in the particular toxicological variable compared to that value in the control.

3.3.7 *no-observed-effect concentration (NOEC)—in a toxicity test*, the test concentration of one or more chemicals immediately below the lowest tested concentration that resulted in a statistically significant change in a particular toxicological variable compared to the control.

3.3.8 *reconstituted water*—a dilution water that is prepared by adding appropriate amounts of selected chemicals to water, which is usually prepared using deionization or reverse osmosis, so that the concentrations and ratios of the major ions in the dilution water are similar to those in comparable natural surface waters.

3.3.9 *surrogate species*—a species that is tested to estimate responses of another species, for which direct testing is impractical.

3.3.10 *toxicity test*—an experiment used to study the adverse effect(s) of one or more chemicals on whole organisms, tissues, or cells.

3.3.11 *Unionoidea*—the super family of freshwater bivalves that includes the North American families Unionidae and Margaritiferidae. The family Unionidae includes three subfamilies (Unioninae, Anodontinae, and Lampsliniae).

3.3.12 *unionoid*—any mussel species in the super family Unionoidea.

3.3.13 *unionid*—any mussel species in the family Unionidae.

3.3.14 *margaritiferid*—any mussel species in the family Margaritiferidae.

3.3.15 *bradytictic*—a mussel species spawning its gametes in late summer and the female broods the glochidia over winter for release the following spring (also called long-term brooders).

3.3.16 *tachytictic*—a mussel species spawning its gametes in spring and the female releases the glochidia in late spring or summer of that year (also called short-term brooders).

3.3.17 *glochidia*—bivalve larvae of unionid mussels which are generally parasitic on the gills of fish.

3.3.18 *marsupium*—a brood pouch for developing eggs and glochidia in unionid mussels, formed by a restricted portion of the outer gill, the complete outer gill, or all gills.

4. Summary of Guide

4.1 Annex A1 provides guidance for conducting water-only toxicity tests with glochidia and juvenile mussels. Recommended test conditions for conducting these toxicity tests are based on various published methods outlined in Table A1.1 and Table A1.4 in Annex A1 and are based on the conditions used to conduct an inter-laboratory toxicity test with glochidia and juvenile mussels (section 16.5). Glochidia and juvenile mussels are only available for a limited time on a seasonal basis. Section 10 describes procedures for collecting adult female mussels from the field to obtain glochidia for conducting toxicity tests or for obtaining glochidia to propagate juvenile mussels using a host organisim.

4.1.1 Toxicity tests with glochidia and juvenile mussels should be conducted at 20°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lux. Toxicity tests with glochidia are typically started within 2 h after glochidia are isolated from the gills of the female mussels; however, some toxicity tests have been started with glochidia isolated from female mussels for about 24 h before the start of a test. The endpoint measured in toxicity tests with glochidia is survival (viability) as determined by the response of organisms to the addition of a solution of NaCl. Glochidia that close their valves with the addition of a salt solution are classified as alive (viable) in a toxicity test. For most species, the duration of a toxicity test conducted with glochidia should be up to 24 h with survival measured at 6 and 24 h. Control survival is typically >90 % at the end of 24-h toxicity tests conducted with glochidia. Longer duration toxicity tests with glochidia (for example, 48 h) can be conducted as long as control survival >90 % is achieved. For example, toxicity tests conducted for 48 h with glochidia might be used for species for which juvenile mussels are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates).Effect concentrations are typically calculated based on the percentage of viable glochidia in the control at a particular sampling time. Glochidia are not fed during the toxicity test. Survival can be determined throughout the toxicity test by subsampling each replicate.

4.1.2 Toxicity tests with juvenile mussels are typically started with organisms <5 d after release from the host; however, some toxicity tests have been started with 2- to 4-month-old juvenile mussels. Acute toxicity tests with juvenile mussels are typically conducted for 96 h with survival

measured at 48 and 96 h. Chronic toxicity tests started with 2- to 4-month-old juvenile mussels have been conducted for 21 to 28 d with measures of survival (based on movement of the foot) and growth (based on shell length). Control survival is typically >90 % at the end of 96-h toxicity tests conducted with juvenile mussels and is typically >80 % at the end of toxicity tests conducted for 10 to 28 d with juvenile mussels. Juvenile mussels are not typically fed during toxicity tests conducted for up to 10 d. Algae have been used as a food source in toxicity tests conducted for 10 to 28 d.

5. Significance and Use

5.1 Protection of a species requires prevention of unacceptable effects on the number, weight, health, and uses of the individuals of that species. Toxicity tests can be used provide information about the toxicity of a test material to a specific life stage of a particular species of mussel. The primary adverse effects studied are reduced survival or growth.

5.2 Results of toxicity tests might bc used to predict effects likely to occur on mussels in field situations as a result of an exposure under comparable conditions.

5.3 Results of toxicity tests might be used to compare the sensitivities of different mussel species and the toxicity of different test materials, and to study the effects of various environmental factors on results of such tests.

5.4 Results of toxicity tests conducted with mussels might be an important consideration when assessing the risks of test materials to aquatic organisms or when deriving USEPA Water Quality Criteria for aquatic organisms (Guide E 1241).

5.5 Results of acute toxicity tests (for example, 24- to 96-h tests) might be useful for predicting the results of chronic tests on the same test material with the same species in another water or with another species in the same or a different water. Most predictions take into account the results of acute toxicity tests, and so the usefulness of the results of a chronic toxicity test is greatly increased by reporting also the results of an acute toxicity test conducted with a similar life stage of the same species under the same conditions (Guide E 729).

5.6 Results of toxicity tests might be useful for studying the biological availability of, and structure-activity relationships between, test materials.

5.7 Results of toxicity tcsts will depend on temperature, composition of the dilution water, condition of the test organisms, and other factors.

5.8 *Interferences*—A number of factors can impede or prevent selection and use of freshwater mussels for toxicity testing (Guide E 1850). The following should be considered when selecting a test species and measuring the sensitivity of the test species during toxicity tests.

5.8.1 Handling of field-collected adult mussels resulting from collection or transport to the laboratory might cause excessive mortality or sublethal effects.

5.8.2 The age, health, and physical condition of adult mussels (for example, the presence of parasites, bacteria, and disease) collected from a resident population might not be adequately known.

5.8.3 The physical eharactcristics of the testing environment (such as water quality, temperature, water flow, light) and food requirements might affect thc ability of the test organisms to acclimate, recover from handling, or adapt to the laboratory environment conditions.

5.8.4 The degree of eontamination and the history of contamination at the collection of the adult mussels might not be adequately known.

5.8.5 In the field, mussels may be exposed to contaminants in water, sediment, or food. This standard only addresses effects associated with exposure of mussels to contaminants in water. 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.

5.8.6 There are insufficient data available to determine if juvenile mussels are able to avoid exposure to chemicals by valve closure. If it is suspected that juvenile mussels are avoiding exposure to a chemical in a toxicity test, it may be desirable to place the suspected live test organisms into dilution water that does not contain any added test material for 1 to 2 d after the end of the toxicity test to determine whether these test organisms are alive or dead (section A1.4.7; Guide E 729).

6. Apparatns

6.1 *Facilities*—Although some small organisms can be held and acclimated in static or renewal (for example, static renewal) systems, most organisms are held, acclimated, and cultured in flow-through systems. Test chambers should be in a constant-temperature room, incubator, or recirculating water bath. For static and renewal tests a dilution-water tank, which may be used to prepare reconstituted water, is often elevated so that dilution water can be delivered by gravity into holding and acclimation tanks and test chambers. For flow-through tests an elevated head box is often desirable so that dilution water can be delivered by gravity into holding and acclimation tanks and into the metering system (6.4), which prepares the test solutions and delivers them to the test chambers. Strainers and air traps should be included in the water-supply system. Head boxes and holding, acclimation, culture, and dilution-water tanks should be equipped for temperature control and aeration. Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Filtration of air through a 0.22-μm bacterial filter might be desirable (Guide E 729). The facility should be well-ventilated and free of fumes. To further reduce the possibility of contamination by test materials and other substances, especially volatile ones, holding, acclimation, and culture tanks should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned. A timing device should be used to provide a controlled photopcriod. A 15 to 30-min transition period when the lights go on might be desirable to reduce the possibility of organisms being stressed by large, sudden increases in light intensity. A transition period when the lights go off might also be desirable (Guide E 729).

6.2 *Special Requirements*—Some organisms may require special conditions during holding, acclimation, and testing. For example, adult mussels should be provided a substrate suitable for burrowing.

6.3 *Construction Materials*—Equipment and facilities that contact stock solutions, tcst solutions, or any water into which

test organisms will be placed should not contain substanees that ean be leaehed or dissolved by aqueous solutions in amounts that adversely affeet test organisms. In addition, equipment and facilities that contaet stock solutions or test solutions should be ehosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastics should be used whenever possible to minimize dissolution or leaching. Concrete and rigid plastics may be used for holding, acclimation, and culture tanks in the water-supply system, but these materials should be soaked, preferably in flowing dilution water, for a week or more before use (Guide E 729). Cast iron pipe should not be used for water-supply systems because colloidal iron may be added to the dilution water, and strainers will be needed to remove rust particles. Brass, copper, lead, galvanized metal, and natural rubber should not contact dilution water, stock solutions, or test solutions before or during the test. Items made of neoprene rubber or other materials not previously mentioned should not be used unless it has been shown that either *(1)* unfed individuals of a sensitive aquatic species (for example, *Daphnia magna*) do not show more signs of stress, unusual behavior, or death, when held for at least 48 h in static dilution water in which the item is soaking than when held in static dilution water that does not eontain the item or *(2)* their use will not adversely affeet survival, growth, or reproduction of a sensitive species (Section 8 and Guide E 729).

6.4 *Metering System:*

6.4.1 For flow-through tests, the metering system should be designed to accommodate the type and concentration(s) of the test material and the necessary flow rates of test solutions. The system should permit the mixing of test material with dilution water immediately before entrance to the test chambers and permit the supply of the selected concentration(s) of test material (section 9.3) in a reproducible fashion. Various metering systems, using different combinations of such as syringes, siphons, pumps, saturators, solenoids, valves have been used successfully to control the concentrations of test material in, and the flow rates of, test solutions. Proportional diluters use an intermittent flow design and various devices for metering the test material. Continuous-flow metering systems are also available, as are systems that prepare the different test solutions independently of eaeh other. See Guide E 729, E 1241 and Test Method E 1706 for additional detail on metering systems.

6.4.2 The metering system should be calibrated before and after the test by determining the flow rate through each test ehamber and by measuring either the concentration of test material in each test chamber or the volume of solution used in each portion of the metering system. The general operation of the metering system should be visually checked daily in the morning and afternoon throughout the test. The metering system should be adjusted during the test if necessary. It is usually desirable to construct the metering system so that it can provide at least ten-volume additions per 24 h, if desired, in case *(1)* the loading is high or *(2)* there is rapid loss of test material due to microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, or volatilization. At uny partieular time during the test, the flow rates through any two test chambers should not differ by more than 10 %.

6.4.3 The frequency of water addition to the eaeh test chamber should be based on the duration of the exposure and on the stability of the exposure eoncentrations (for example, based on degradation, hydrolysis, oxidation, photolysis, reduction, sorption, or volatilization). Ideally, preliminary tests should be conducted to determine how frequently water should be added to maintain water quality and exposure concentrations of the test material. For exaiiple, in 96-h exposures with ammonia and juvenile mussels, water was renewed every two days to maintain relatively consistent exposure concentrations (USGS 2005a (8)). In 28-d exposures starting with 2-month-old juvenile mussels, about 4 volume additions/d were delivered to each test ehamber in copper and ammonia toxicity tests (USGS 2005b (9)).

6.4.4 Speciation of some metals (for example, lead or eopper) and perhaps other test materials is not instantaneous and may change over a period of time (perhaps hours or days), even in test solutions that do not contain test organisms. Water-renewal systems have been designed with "equilibration chambers" that provide a residence time for test solution before the test solution is delivered to the exposure chambers (Kim et al. 1999, Besser et al. 2005(14, 15)).

6.5 *Test Chambers:*

6.5.1 In a toxicity test with aquatie organisms, test chambers are defined as the smallest physical units between which no water connections exist. However, screens, cups may be used to create two or more compartments within eaeh chamber. Therefore, the test solution can flow from one compartment to another within a test chamber, but, by definition, cannot flow from one chamber to another. Because the solution can flow from one compartment to another in the same test chamber, the temperature, concentration of test material, and levels of pathogens and extraneous contaminants are likely to be more similur between compartments in the same test chamber than between compartments in different test chambers in the same treatment. All chambers (and compartments) in a test must be identical.

6.5.2 Test chambers may be constructed by welding, but not soldering, stainless steel or by gluing double-strength or stronger window glass with clear silicone adhesive. Stoppers and silicone adhesive sorb some organic chemicals, which are then difficult to remove. Therefore, as few stoppers and as little adhesive as possible should be in contact with test solution. If extra beads of adhesive are needed for strength, the extra adhesive should be on the outside of chambers rather than on the inside. Especially in static and renewal tests, the size and shape of the test chamber might affect the results of tests on materials that volatilize or sorb onto the chambers in substantial quantities.

6.5.3 The dimensions of test chambers and volume of water to test depends on the age and number of the organisms being tested (Annex A1).

6.6 *Cleaning*—The metering system, test chambers, and equipment used to prepare and store dilution water, stock solutions, and test solutions should be cleaned before use. New items should be washed with detergent and rinsed with water,

a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid (HCl)), and rinsed at least twice with deionized or dilution water. Reagent grade solvents are recommended. If lesser grades are used, possible contaminants should be considered with respect to the purpose of the test (some lots of some organic solvents might leave a film that is insoluble in water). A dichromate-sulfuric acid cleaning solution may be used in place of both the organic solvent and the acid, but it might attack silicone adhesive. At the end of the test, all items that are to be used again should be immediately *(1)* emptied, *(2)* rinsed with water, *(3)* cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases, detergent, organic solvent, or activated carbon to remove organic chemicals), and *(4)* rinsed at least twice with deionized or dilution water. Acid can be used to remove mineral deposits, and 200 mg of hypochlorite (ClO^-)/L can be used to remove organic matter and for disinfection. A solution containing about 200 mg of ClO^-/L may be prepared by adding 6 mL of liquid household chlorine bleach to 1 L of water. However, ClO^- is quite toxic to many aquatic animals and is difficult to remove from some construction materials. It can be removed by soaking in a sodium thiosulfate, sodium sulfite, or sodium bisulfite solution, by autoclaving in deionized water for 20 min, or by drying the item and letting it sit for at least 24 h before use. An item cleaned or disinfected with hypochlorite should not be used unless it has been demonstrated at least once that unfed individuals of a sensitive aquatic species do not show more signs of stress, such as discoloration, unusual behavior, or death, when held for at least 48 h in static dilution water in which the item is soaking than when held in static dilution water containing a similar item that was not treated with ClO^- (Guide E 729). The metering system and test chambers should be rinsed with dilution water just before use.

6.7 *Acceptability*—Before a toxicity test is conducted in new test facilities, it is desirable to conduct a "non-toxicant" test, in which all test chambers contain dilution water without added test material. Determine before the first test: *(a)* whether test organisms will meet test acceptability requirements outlined in Annex A1, *(b)* whether the food, water, or handling procedures are acceptable, *(c)* whether there are any location effects on either survival or growth of organisms, and *(d)* the magnitudes of the within-chamber and between-chamber variances.

7. Hazards

7.1 *General Precautions*:

7.1.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management and includes: *(1)* the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, *(2)* the preparation of a formal, written health and safety plan, which is provided to each laboratory staff member, *(3)* an ongoing training program on laboratory safety, and *(4)* regular safety inspections.

7.1.2 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and glasses, and by using dip nets, forceps, or tubes to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans, recommended handling procedures, and biological, chemical, and physical properties of the test material should be studied before a test is begun (section Appendixes X2, X3, and X4 in Guide E 1023). **Warning**—Special procedures might be necessary with radiolabeled test materials and with test materials that are, or are suspected of being, carcinogenic (Guide E 729).

7.1.3 Collection and use of environmental samples (for example, sediments, effluents) may involve substantial risks to personal safety and health. Chemicals in field-collected samples may include carcinogens, mutagens, and other potentially toxic compounds. Inasmuch as testing is often started before chemical analyses can be completed, worker contact with field-collected samples needs to be minimized by *(1)* using personal safety gear, *(2)* manipulating samples under a ventilated hood or in an enclosed glove box, and *(3)* enclosing and ventilating the exposure system. Personnel collecting samples and conducting tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation because of lack of oxygen or presence of noxious gases.

7.2 *Safety Equipment*:

7.2.1 Before beginning sample collection or laboratory work, personnel should determine that all required safety equipment and materials have been obtained and are in good condition.

7.2.2 *Personal Safety Gear*—Personnel should use safety equipment, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, face shields, hard hats, and safety shoes.

7.2.3 *Laboratory Safety Equipment*—Laboratories should be provided with safety equipment such as first-aid kits, fire extinguishers, fire blankets, emergency showers, and eye wash stations. Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

7.3 *General Laboratory and Field Operations*:

7.3.1 Special handling and precautionary guidance in Material Safety Data Sheets (MSDS) should be followed for reagents and other chemicals purchased from supply houses.

7.3.2 It is advisable to wash exposed parts of the body with bactericidal soap and water immediately after collecting or manipulating field-collected samples.

7.3.3 Strong acids and volatile organic solvents should be used in a fume hood or under an exhaust canopy over the work area.

7.3.4 **Warning**—An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

7.3.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only under a fume hood.

7.3.6 Although disposal of stoek solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

7.3.7 Use of ground-fault systems and leak detectors is strongly recommended to help prevent electrical shocks. Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories should not be used. Ground-fault interrupters should be installed in all "wet" laboratories where electrical equipment is used.

7.3.8 All containers should be adequately labeled to indicate their contents.

7.3.9 A clean and well-organized work place contributes to safety and reliable results.

7.4 *Disease Prevention*—Personnel handling samples which are known or suspected to contain human wastes should be immunized against hepatitis B, tetanus, typhoid fever, and polio. Thorough washing of exposed skin with bacterieidal soap should follow handling of samples collected from the field.

7.5 *Safety Manuals*—For further guidance on safe practices when handling field-collected samples and conducting toxieity tests, check with the permittee and consult general industrial safety manuals (Test Method E 1706).

7.6 *Pollution Prevention, Waste Management, and Sample Disposal*—Work with some field-collected samples may require compliance with rules pertaining to the handling of hazardous materials. Guidelines for the handling and disposal of hazardous materials should be strictly followed (Guide D 4447). The Federal Governmcnt has published regulations for the management of hazardous waste and has given the States the option of either adopting those regulations or developing their own. If States develop their own regulations, these regulations are required to be at least as stringent as the Federal regulations. As a handler of hazardous materials, it is your responsibility to know and comply with the pertinent regulations applicable in the State in which you are operating (Test Method E 1706).

8. Dilution Water

8.1 *Requirements*—The dilution water should *(a)* be available in adequate supply, *(b)* be acceptable to the test organisms, *(c)* be of uniform quality, and *(d)* except as stated in 8.1.4, not unnecessarily affect results of the test. Additional details on dilution water for use in culture or toxicity testing can be found in Guide E 729.

8.1.1 The minimal requirement for an acceptable dilution water for toxicity tests is that healthy test organisms survive in it through acclimation and testing without showing signs of stress, such as discoloration, unusual behavior, or death. A better eriterion for an acceptable dilution water is that at least one species of aquatic animal (preferably of the one being tested or one taxonomically similar) will survive, grow, or reproduce satisfactorily in the water. Because daphnids are more sensitive to some test materials than many other aquatic animal species, water in which daphnids (less than 24-h old) will survive for 48 h without showing signs of stress is probably acceptable for toxicity tests with most freshwater animal species. Water in which daphnids will survive, grow, and reproduce satisfactorily in a life-cycle test is probably an acceptable dilution water for tests with most freshwater animal speeies.

8.1.2 The quality of the dilution water should be uniform so that the test organisms are cultured or acclimated and the test conducted in water of the same quality. The range of hardness should be within 10 % of the average.

8.1.3 The dilution water should not unnecessarily affect the results of a toxicity test because of sueh things as sorption or complexation of test material. Except as in accordance with section 8.1.4, it is desirable for the purpose of reducing inter-laboratory variability that the concentrations of both total organie carbon (TOC) and partieulate matter should be less than 5 mg/L.

8.1.4 If it is desired to study the effect of an environmental factor such as TOC, particulate matter, or dissolved oxygen on the results of a toxicity test, it will be necessary to use a water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such a water is used, it is important that adequate analyses be performed to characterize the water and that a comparable test be available or be conducted in a more usnal dilution water to facilitate interpretation of the results in the special water.

8.2 *Source*:

8.2.1 *Reconstituted Water*:

8.2.1.1 Tables 1 and 2 in Guide E 729 provide recipes for preparing a variety of reconstituted waters that have been used successfully to conduct toxicity tests. Reconstituted water is prepared by adding specified amounts of reagent grade chemicals to high-quality water with *(a)* resistivity greater than 1 MΩ water and *(b)* either total organic carbon (TOC) less than 2 mg/L or chemical oxygen demand (COD) less than 5 mg/L. Acceptable water can usually be prepared using properly operated deionization or reverse osmosis units. Conductivity should be measured on each batch und TOC or COD should be measured at least twice a year and whenever substantial changes might be expected. If the water is prepared from surface water, TOC or COD should be measured on each batch. The reconstituted watcr should be aerated before use. Problems have been encountered with some species in reconstituted waters, but sometimes these problems have been overcome by aging the reconstituted water for one or more weeks.

8.2.2 *Natural Dilution Water*:

8.2.2.1 If natural dilution water is used, it should be obtained from an uncontaminated, uniform quality source. The quality of water from a well or spring is usually more uniform than that of watcr from a surface water. If a surface water is used as a source of water, the intake should be positioned (for example, about one meter below the surface) to minimize fluctuations in quality and the possibility of contamination, and to maximize the concentration of dissolved oxygen to help ensure that the concentrations of sulfide and iron are not high.

8.2.2.2 Water quality characteristics (such as hardness, conductivity, pH) may be adjusted, if desired, by addition of appropriate reagent grade chemicals, acid, base, or deionized water if desired (Guide E 729). Chlorinated water should not be used as, or in the preparation of, dilution water because residual chlorine and chlorine-produced oxidants are toxic to many aquatic animals (Guide E 729). Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Sodium bisulfite is probably better for dechlorinating water than sodium sulfite and both are more reliable than carbon filters, especially for removing chloramines. Some organic chloramines, however, react slowly with sodium bisulfite. In addition to residual chlorine, municipal drinking water often contains high concentrations of copper, lead, zinc, and fluoride, and quality is often rather variable. The concentrations of most metals can usually be reduced with a chelating resin, but use of different dilution water might be preferable. If dechlorinated water is used as dilution water or in its preparation, during the test it should be demonstrated that a sensitive aquatic species (for example, daphnids less than 24-h old) do not show more signs of stress, such as discoloration, unusual behavior, or death, when held in the water for at least 48 h without food than when similarly held in a water that was not chlorinated and dechlorinated).

8.3 *Treatment*:

8.3.1 Dilution water should be aerated intensively by such means as air stones, surface aerators, or column aerators before adding test material. Adequate aeration will bring the pH and the concentrations of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be between 90 and 100 % of saturation to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Super-saturation by dissolved gases, which might be caused by heating the dilution water, should be avoided (Guide E 729).

8.3.2 Filtration through bag, sand, sock, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low and as a pretreatment before ultraviolet sterilization or filtration through a finer filter.

8.3.3 Dilution water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 µm or less (Guide E 729).

8.4 *Characterization*—The following items should be measured at least twice each year, or more often *(a)* if such measurements have not been made semiannually for at least two years, or *(b)* if a surface water is used: pH, particulate matter, TOC, organo-phosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc, hardness, alkalinity, conductivity, sodium, and chloride. For each analytical method used the detection limit should be below either *(a)* the concentration in the dilution water or *(b)* the lowest concentration that has been shown to unacceptably affect the test species (Guide E 729).

9. Test Material

9.1 *General*—The test material should be reagent grade or better, unless a test on a formulation, commercial product, or technical-grade or use-grade material is specifically needed (Guide E 729). Before a test is begun, the following should be known about the test material: *(1)* Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 1 % of the material, *(2)* Solubility and stability in the dilution water, *(3)* Measured or estimated acute or chronic toxicity to the test species, *(4)* Precision and bias of the analytical method at the planned concentration(s) of the test material, if the test concentrations are to be measured, *(5)* Estimate of toxicity to humans, and *(6)* Recommended handling procedures (Section 7).

9.2 *Stock Solution*:

9.2.1 In some cases the test material can be added directly to the dilution water, but usually it is dissolved in a solvent to form a stock solution that is then added to the dilution water. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

9.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is dilution water, although filtration or sterilization, or both, of the water might be necessary. If the hardness of the dilution water will not be affected, deionized water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials (Guide E 729). The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than use of the minimum necessary amount of a strong acid or base.

9.2.3 If a solvent other than dilution water is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect the test species. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals, low volatility, and high ability to dissolve many organic chemicals (Guide E 729). Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but these materials might stimulate undesirable growths of microorganisms (Guide E 729; **Warning**—Acetone is also quite volatile). If an organic solvent is used, it should be reagent grade or better and its concentration in any test solution must not exceed 0.5 mL/L in 96-h tests (Guide E 729) or 0.1 mL/L in longer-term tests (Guide E 1241). A surfactant must not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions (these limitations do not apply to any ingredient in a mixture, formulation, or commercial product unless an extra

amount of solvent is used in the preparation of the stock solution or if the test is on a solvent or surfactant).

9.2.4 If a solvent other than dilution water is used, at least one solvent control using solvent from the same batch used to make the stock solution must be included in the test. If no solvent other than water is used, a dilution-water control must be included in the test and the survival and growth of test organisms in the dilution-water control must meet test acceptability requirements in order for the test to be considered acceptable (Annex A1). Using no solvent other than dilution water is the most desirable option because using any other solvent means that antagonism, synergism, and confounding are possible (Guide E 1241). Using different concentrations of a solvent at the different concentrations of the test material should be avoided because both the concentration of the solvent and the concentration of the test material vary across the treatments, potentially resulting in confounding. Therefore, it is desirable to test the same concentration of solvent in all of the test solutions.

9.2.4.1 If the concentration of solvent is the same in all test solutions that contain test material, the solvent control must contain the same concentration of solvent.

9.2.4.2 If the concentration of solvent is not the same in all test solutions that contain test material, either *(a)* a toxicity test must be conducted to determine whether survival or growth of the test organisms is related to the concentration of the solvent over the range used in the toxicity test, or *(b)* such a toxicity test must have been conducted on the solvent using the same dilution water and test species. If survival or growth are related to the concentration of solvent, a toxicity test with that species in that water is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival nor growth are related to the concentration of solvent, a toxicity test with that same species in that same water may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments (Guide E 1241).

9.2.4.3 There may be instances when a toxicity test is to be conducted with a species that is not routinely available for testing (for example, such as with an endangered species.) In these instances, the toxicity test used to evaluate potential effects of a solvent outlined in 9.2.4.2 may be conducted with species in the same family (preferably the same genus) as long as the concentrations of solvent are at least double the concentration of solvent used in the toxicity test on the test material. Testing at least double the concentration of solvent used in the toxicity test would provide some margin of safety in extrapolating results of toxicity tests between species in the same family. For example, Dwyer et al (2005a,b) (**11, 12**) and Besser et al (2005) (**16**) reported the sensitivity of endangered species of fish was within a factor of about 2 of commonly-tested surrogate fish species for a variety of organic and inorganic chemicals in acute or chronic toxicity tests. Similarly, USEPA (2003) (**17**) reported similar sensitivity of aquatic species to a variety of organic or inorganic chemicals in toxicity tests conducted within a family.

9.2.4.4 If the test contains both a dilution-water control and a solvent control, the survival and growth of the organisms in the two controls should be compared. If a statistically significant difference in survival or growth is detected between the two controls, only the solvent control may be used for meeting the requirements of outlined in Table A1.3 or Table A1.5 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be pooled for meeting the requirements outlined in Table A1.3 or Table A1.5 and as the basis for calculation of results.

9.2.5 If a solvent other than water is used to prepare a stock solution, it might be desirable to conduct simultaneous tests on the test material using two chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of solvent on the toxicity of the test material or the sensitivity of the test species.

9.3 *Test Concentration(s):*

9.3.1 If the test is intended to allow calculation of an LC50, EC50, or IC50, the test concentrations should bracket the predicted concentration. The prediction might be based on the results of a test on the same or a similar test material with the same or a similar species. In acute toxicity tests, if a useful prediction is not available, it is usually desirable to conduct a range-finding toxicity test in which groups of five or more organisms are exposed for 24 to 96 h to a control and three to five concentrations of the test material that differ by a factor of ten. Replicate chambers are not typically evaluated in range-finding toxicity tests. The greater the similarity between the range-finding test and the definitive test, the more useful the range-finding test will be. If necessary, concentrations above solubility should be used because organisms in the real world are sometimes exposed to concentrations above solubility and because solubility in dilution water is often not well known. The use of concentrations that are more than ten times greater than solubility are probably not worthwhile. With some test materials it might be found that concentrations above solubility do not kill or affect a greater percentage of test organisms than does the concentration that is the solubility limit; such information is certainly worth knowing.

9.3.2 In chronic toxicity tests, the test concentrations should bracket the best prediction of that concentration. Such a prediction can be based on the results of an acute toxicity test using the same dilution water, test material, and species (Guide E 729). If an acute-chronic ratio has been determined for the test material with a species of comparable sensitivity, the result of the acute test can be divided by the acute-chronic ratio. Except for a few materials, acute-chronic ratios with sensitive species are often less than five. Thus, if no other useful information is available, the highest concentration of test material in an early life-stage test is often selected to be equal to the lowest concentration that caused adverse effects in a comparable acute test (Guide E 1241).

9.3.3 In some (usually regulatory) situations, it is necessary only to determine *(a)* whether a specific concentration of test material is acutely toxic to the test species, or *(b)* whether the LC50, EC50, or IC50 is above or below a specific concentration. For example, the specific concentration might be the concentration occurring in surface water, the concentration resulting from the direct application of the material to a body of water, or the solubility limit of the material in water. When

there is interest only in a specific concentration, it is often necessary only to test that concentration, and it is not necessary to actually determine the LC50, EC50, or IC50.

10. Test Organisms

10.1 *Life History of Freshwater Mussels*:

10.1.1 Freshwater mussels are bivalve mollusks belonging to the family Unionidae or Margaritiferidae. Adults 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 (Murray and Leonard 1962) (18), such as detritus, phytoplankton, zooplankton, diatoms, bacteria, and other microorganisms (Fuller 1974 (19), Strayer et al 2004 (20)). The extent of selectivity exhibited by mussels feeding on each of these food groups is poorly understood and is likely to vary by species (Beck and Neves 2003) (21). Recent evidence suggests that detritus, bacteria, and zooplankton may be important food sources (Silverman et al 1997 (22), Nichols and Garling 2000 (23)). The early juvenile stage use 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 (Yeager et al 1994 (24), Silverman et al 1997 (22)) in addition to unicellular algae (Gatenby et al 1997 (25), O'Beirn et al 1998 (26), Parker et al 1998 (27), Beck and Neves 2003 (21)). Pedal feeding in juvenile mussels is accomplished by movements of microscopic cilia lining the foot that carry food particles into the mantle cavity and into the mouth. Juvenile mussels also use the foot in a sweeping motion to draw particles toward the mantle cavity (Reid et al 1992) (28).

10.1.2 Unionid mussels have an unusual and complex mode of reproduction, which for most species includes a brief, obligatory parasitic stage on fish (Fig. 1). Freshwater mussels are typically dioecious, but some species may be hermaphroditic (for example, *Toxolasma parvus*, *Lasmigona compressa*, *Utterbackia imbecillis*; Watters 2005). During the breeding season, males release sperm into the water column and females draw the sperm in through the incurrent aperture. The eggs are fertilized in the suprabranchial chambers in the gills and are moved to the marsupial region of the gill until released as mature glochidia by the thousands to millions (Fig. 2)

10.1.3 Spawning takes place in the spring for most amblemines and in the summer for most anodontines and lampsilines

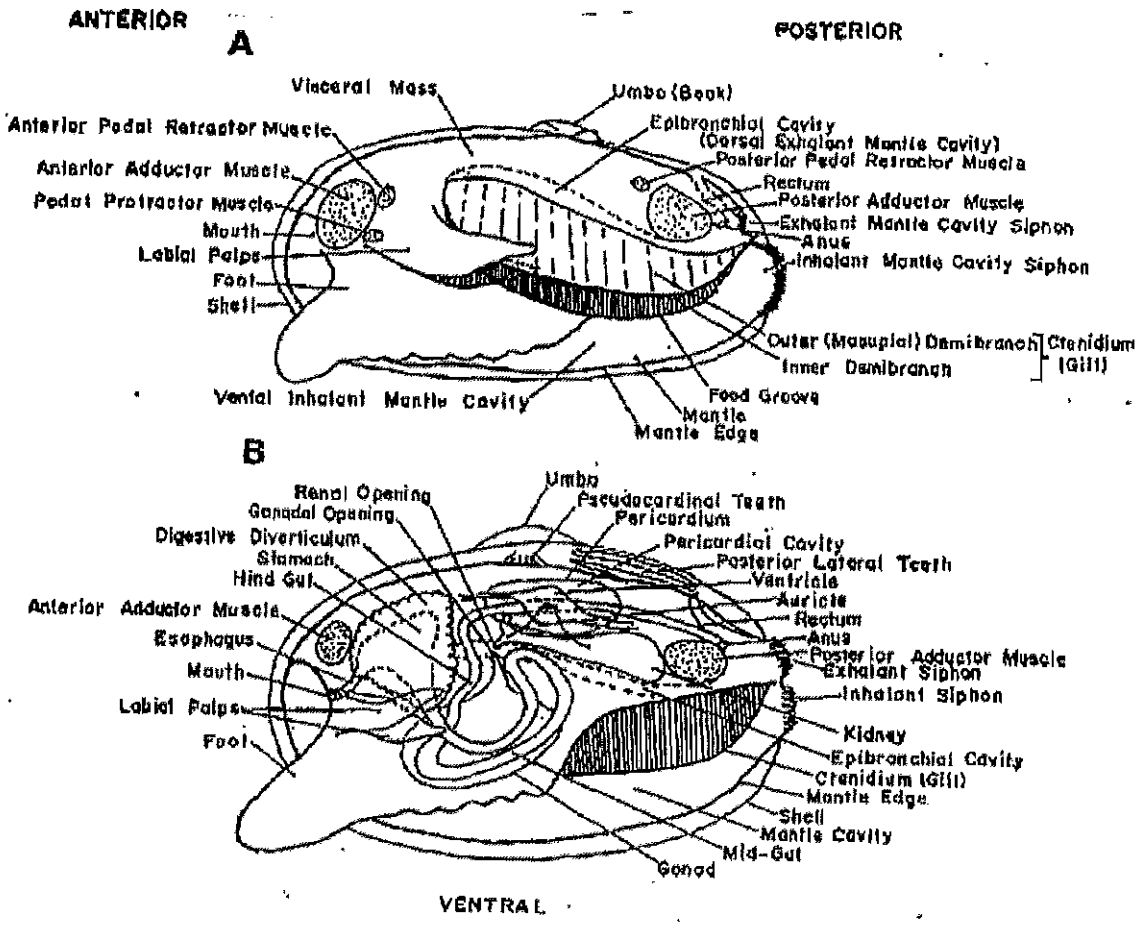


FIG. 2 General External Anatomy of the Soft Tissues (A), and Internal Anatomy, Organs, and Organ Systems of Soft Tissues of a Unionid Mussel (B); adapted from McMahon and Bogan, Academic Press, 2001, (29) Copyright Academic Press

(Watters 2005) (30). Depending on the species, mature glochidia may be brooded for several months or may be released shortly after maturation. Winter-brooding mussels produce glochidia in the late summer or fall, but do not release the glochidia until the following spring or summer (bradytictic or long-term brooders). Summer-brooding mussels produce glochidia in the late spring or early summer and release them in the summer (tachytictic or short-term brooders). Some mussels release glochidia in the fall or winter and after attaching to a host, the glochidia remain dormant over winter until a threshold temperature is reached in the spring, at which time the glochidia metamorphose and excyst as juvenile mussels (for example, *Pyganodon grandis* and *Leptodea fragilis*; Watters 2005) (30).

10.1.4 The successful transfer of mature glochidia to a suitable host constitutes a critical event in the life cycle of most freshwater mussels. Various adaptations have evolved to facilitate this process. High levels of mortality occur during the passage of glochidia from the female mussel to the host fish due to low incidence of fish host contact. Once encysted in the gill, glochidia may be relatively protected from *in situ* exposure contaminants in water (Jacobson et al 1997) (31). The method of host infestation greatly varies among species. While some species simply broadcast glochidia into the surrounding water to haphazardly come into contact with the appropriate host, the process is more intricate and direct for other species. For example, females in the genus *Lampsilis* have an extension of the mantle tissue that resembles a small fish or invertebrate complete with eye spots and appendages. This lure is displayed outside the shell between the valves and is twitched repetitively to attract a predaceous fish host. The host is infested while attempting to eat the lure when the marsupial gills of the female are ruptured (Kraemer 1970 (32), Barnhart and Roberts 1997 (33)). Some species release conglutinates (small structures containing glochidia) freely into the water. In many conglutinate-produeing species (for example, *Elliptio*, *Fusconaia*, *Pleurobema*, *Plethobasus*, *Cyprogenia*, and *Quadrula*), conglutinates are released as cohesive masses made up of unfertilized eggs that hold together mature glochidia. Conglutinates of some species (for example, *Ptychobranchus*) are made up of gelatinous material that enclose large numbers of glochidia (Hartfield and Hartfield 1996) (34). Conglutinates may resemble prey items of the host fish; the host fish are infested with glochidia when fish attempt to eat conglutinates (Chamberlain 1934 (35), Barnhart and Roberts 1997 (33), Jones et al 2004 (36)).

10.1.5 Glochidia range in size from about 50 to 400 µm (Hoggarth 1999 (37), McMahon and Bogan 2001 (29), Wachtler et al 2001 (38)). Tbe only visible behavior of which glochidia are capable is closure of the valves, which is accomplished by a single adductor muscle. The valves close in response to a variety of artificial tactile and chemical stimuli such as insertion of objects placed between valves, hypo-osmotic solutions, saturated NaCl or KCl solutions, or the blood of vertebrates (LeFevre and Curtis 1912 (39), Arey 1921 (40)). In nature, glochidia will attach to the gills or the fins of a host fish upon contact. The sharp valves cut into the epithelium of the host, enclosing and compressing the tissue (LeFevre and Curtis 1912 (39), Arey 1932 (41)). After glochidia are released from the female, glochidia need to attach to the gills or the fins of an appropriate fish host and encyst to complete development. Although glochidia may survive for months during brooding in the femule mussel, glochidia typically survive for only a few days after release unless the glochidia reach a compatible host. Encystment on the host occurs from encapsulation by host tissue (Zimmerman and Neves 2002) (42).

10.1.6 Metamorphosis of juvenile mussels on the fish host occurs within days or weeks, depending on species and temperature. Host fish specificity varies among mussels. While some mussel species appear to require a single host organism, other species can transform their glochidia into juvenile mussels on many species of host fish. Following proper host infestation, glochidia transform into microscopic juveniles and excyst (drop off) and settle into suitable habitat to survive. The transformation of glochidia to juveniles results in the development of internal organs necessary for self-sustained existence as a benthic organism. Newly-transformed juvenile mussels have a life style different from adult mussels. Transformed juvenile mussels may be at the sediment-water interface or may burrow several centimeters into sediment and rely on water percolating between substrate particles of sediment for food and oxygen (Neves and Widlak 1987) (43).

10.1.7 Newly-transformed juvenile mussels feed using ciliary currents on the foot and mantle. Older juvenile and adult mussels likely use different food types when living in different micro-environments. Given that 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 (Watters 2005) (30). Distributions of adult mussels are dependent both on the presence of host fish and on microhabitat conditions. Efforts to assess effects of contaminants on mussels need to evaluatc potential exposure to host fish in addition to exposure to each unique life stage of freshwater mussels (Watters 2005) (30).

10.1.8 Photographs of lures and conglutinates that mimic prey items of the host fish can be found at the following websites: *(1)* http://unionid.smsu.edu/default.htm and *(2)* http://courses.smsu.edu/mcb095f/gallery/. Additional information on the life history or propagation techniques for freshwater mussels can be found in Gordon and Layzer (1989) (44), Parmalee and Bogan (1998) (46), Bishop et al (2005) (47), and Watters (1995, 2005) (45, 30).

10.1.9 *Anatomy of Adult Mussels*—Fig. 2 illustrates the *(a)* general external anatomy of the soft tissues and *(b)* internal anatomy, organs, and organ systems of soft tissues of a unionid mussel. McMahon and Bogan (2001) (29) provide an overview of the basis anatomy and physiology of freshwater mussels. Information is also provided in McMahon and Bogan (2001) (29) on the ecology and evolution and on the collection, identification and rearing freshwater mussels. Unlike most epibenthic marine bivalves, North American freshwater mussels lack true siphons or tubes for water intake and release. Because of this, freshwater mussels frequently burrow only to the posterior edge of the shell (Watters 2005) (30). However,

anecdotal observations suggest that certain freshwater speeies are routinely found near the sediment-water interface (that is, *Amblema plicata*), while other species maybe be found well below the sediment-water interface (for example, *Obliquaria reflexa*). In temperate locations, mussels may burrow deeper into the substrate during the winter.

10.1.10 *Tolerance Limits of Mussels*:

10.1.10.1 Dimock and Wright (1993) (**48**) reported oxygen, pH and temperature requirements for juvenile *Utterbackia imbecillis* and *Pyganodon cataracta* and found that 7- to 10-d old juvenile mussels could not survive 24 h in an anoxic condition. Temperatures above 30°C were lethal (for example, 96-h median lethal effect at 31.5°C for *Utterbackia imbecillis* and 33°C for *Pyganodon cataracta*). Slight acidity was tolerated with >70 % survival in all groups above a pH value of 5.0 with LC50s of pH 4.5 for both species. Chen et al (2001a) (**49**) summarizes oxygen consumption by 9 species of freshwater mussels. Sparks and Strayer (1998) (**50**) reported that juvenile *Elliptio complanata* were sensitive to low concentrations of dissolved oxygen with survival significantly reduced at 1.3 mg/L and behavior affected at 2 to 4 mg/L.

10.2 *Test Species and Life Stage*:

10.2.1 Table A1.1 and Table A1.4 lists examples of species that have been used to conduct toxicity tests with glochidia or juvenile mussels. These species were selected for testing based on availability, past successful testing, and ease of handling in the laboratory. Selection of the test species or the life stage to be tested depends on the purpose and scope of the study and should be appropriate to the overall objective of the study (Guide E 1850). For example, early life stages of a species might be sensitive to a certain toxicant and readily acclimate to the laboratory environment. These organisms may be used in an acute toxicity test or sublethal test designed to assess toxicity using a growth endpoint (Annex A1), but would not provide information on reproduction.

10.2.2 Before mussels are collected from the field, appropriate federal or state permits for collection of mussels are mandatory. In addition, permission is needed to collect mussels from private landowners. Specific guidance on collection of adult mussels in the field can be obtained from Strayer and Smith (2003) (**51**).

10.2.3 When selecting the appropriate test species, the following selection criteria should be considered in order of importance (Guide E 1850):

10.2.3.1 *Ease of Organism Procurement and Laboratory Culture and Handling*—Species should be screened for ease of handling, ease of collection, and resistance to shock and handling. Preference might be given to those species that can be successfully cultured in the laboratory and are amenable to laboratory testing (Table A1.1 and Table A1.4). Organisms for use in testing should not have had prior exposure to contaminants or other known sources of stress. Potential criteria to determine whether a given batch of field-collected organisms is suitable for laboratory testing should include the following:

(1) Adult mussels collected from the field should not have signs of obvious physical abnormalities such as broken shells or lesions. High survival of adult mussels several days after placement in the laboratory environment should indicate that the organisms have adapted to the new environment.

(2) Organisms should exhibit normal behavior (for example, feeding or locomotory, if appropriate).

(3) Reference-toxicant tests should be performed with subsamples of each batch of glochidia or juvenile mussels used in toxicity tests (following the recommended conditions for conducting toxicity tests in Table A1.1 and Table A1.4). Results of these reference-toxicant tests can be used to compare test organism sensitivity over time either with previously reported results of toxicity tests or with laboratory data being developed for that species and life stage (section 16.3).

10.2.3.2 *Ease of Method Development*—Test procedures might exist for the species of interest or an ecologically similar species (Table A1.1 and Table A1.4). Alternatively, preliminary tests should be conducted with the species and life stage of interest to determine how well the selected species will respond in laboratory conditions.

10.2.3.3 *Potential Sensitivity to Contaminants*—A variety of references are available that categorize species in terms of general sensitivity to organic enrichment and other contaminants (Guide E 1850). It is desirable to use species for whicb data are available, indicating their relative sensitivity to a given test material or class of test materials (for example, Keller et al 2005) (7).

10.2.3.4 *Test Performance Characterization*—To document the quality of the data produced from a given test organism (and surrogate species as well) and to determine the comparability of the selected test organism with other species data for the same test material, method performance characteristics should be determined, preferably before definitive toxicity testing of the test material of interest (Guide E 1850). The degree to which a toxicity test with selected test organisms yields meaningful data will depend on how well the test performance characteristics meet the data quality objectives of the study (for example, Table A1.3 and Table A1.5). Test performance characterization should include the following steps:

(1) Different batches of the same species and the same life stage should be collected and tested over time in order to obtain a measure of the variability associated with testing the particular species. The relative sensitivity and quality of test organisms can then be determined through an assessment of test organism response to a known toxicant or, preferably, different classes of toxicants (for example, NaCl, metals, chlorinated organic compounds, or polycyclic aromatic hydrocarbons) in which the toxicity effect is theoretically constant across tests. Repeated tests using standard or reference materials could be used to compare the sensitivity of the selected test organism with existing data for surrogate test species, through the development of a reference-toxicant control chart for the species and the test material being used (Section 16.3).

(2) The appropriate exposure time required for testing should be determined. Different life stages of the same species (for example, glochidia versus juvenile mussels) might require different exposure durations in order to obtain meaningful test endpoints (section 10.3, Annex A1). As a general rule, acute toxicity tests should conducted for at least 24 h with glochidia

and for 96 h with juvenile mussels. However, shorter time periods for glochidia toxicity tests might be needed for a particular species depending on the survival time of the glochidia (Table A1.2, section A1.5.2). A 48-h toxicity test with glochidia might be used for species for which juvenile mussels are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates). Longer exposure periods may be required for older life stages of mussels that are capable of avoiding exposure for short periods of time (older juvenile mussels and adult mussels; Guide E 729 and 5.8.6).

(3) If a hypothesis test is used, the statistical power of a particular toxicity testing method (Guide E 1850, Section 14). This information will provide a measure of test reliability, given the method and test species used. For regression, probit, or logit-based endpoints such as LC50 or IC25, test reliability and data quality of objectives are best stated in terms of the range of the 95 % confidence limit around the endpoint; the tighter the confidence intervals of the endpoint, the more reliable the test.

(4) The method precision (degree to which independent tests using the same concentration of test material elicits a similar response or test endpoint) should be determined and compared in relation to the decision criteria or data quality objectives to the study (for example, Section 16). For certain applications, it might be desirable or necessary to determine test precision before conducting the definitive testing of a particular test material.

(5) Appendix X3 in Guide E 1850 provides a flow chart that summarizes the factors described above that should be considered when selecting a test species.

10.3 *Age*:

10.3.1 Annex A1, Table A1.1, and Table A1.4 describe the age of test organisms to be used and recommended to start a toxicity test.

10.4 *Source*:

10.4.1 Adult mussels collected from the field should be representative of the organisms that could occur at the study site based on habitat features available and historic species records for the region and should not have been previously exposed to contaminants or pathogens (Guide E 1850). Therefore, adult mussels should be obtained from reference areas (Test Method E 1706), outside of the direct influence of point- or non-point sources of contamination. Adult mussels collected to produce either glochidia or juvenile mussels should be obtained from the same location. Priority pollutant analyses of the site water, sediment, or organism tissues might be used to determine whether organisms have had exposure to source-related contaminants at the collection site. The taxonomic identity of test species should be determined by appropriate keys and verified by an appropriate expert (section 11.5).

10.4.2 Table 1 provides a summary of facilities that have cultured juvenile mussels as of May 2005. Table 2 and section 10.5 provide a summary of techniques that have been used to transform juvenile mussels. Transformation of juvenile mussels has been reported for many species using either fish hosts (*in vivo*) or artificial media (*in vitro*; Bishop et al 2005 (47); section 10.5). Additionally, Watters (1994) (52) reported over 150 species of fish hosts for 95 species of freshwater mussels. While the main focus of the culture facilities listed in Table 1 is propagation of juvenile mussels for release into the environment, these facilities may also be a source of either glochidia or juveniles for use in toxicity tests. Individuals at these facilities will be able to provide additional guidance on handling and culturing of freshwater mussels. The following sections briefly summarize activities at each of the facilities listed in Table 1.

10.4.2.1 *Mammoth Spring National Fish Hatchery, AR*—Over 2500 individuals comprising 28 species of native mussels from the White and Ouachita Rivers in Arkansas have been held in refugia at the Mammoth Spring National Fish Hatchery since 1995. This facility was designed to hold adult mussels in response to a zebra mussel infestation predicted by personnel at the state game and fish commission. Species were held and monitored for survival and physiological condition (cellulolytic enzyme activity), using surrogate species, for four years (some species are still surviving in the hatchery raceways nearly seven years after initial collection). Survival from year one (90 %) to year four (60 %) was measured and indicated that the hatchery provided suitable conditions (high water quality, adequate food source, and continuous water temperatures throughout the year) for short- and long-term holding of native mussels. Since 1994, this hatchery has supported freshwater mussel propagation for recovery and restoration projects in Arkansas and Ohio. Six species (including two federally-endangered species), have been propagated using a combination of host fish and artificial media for the production of juvenile mussels (*L. streckeri, Arkansia wheeleri, P. grandis, L. siliquoidea, L. ventricosa, Fusconaia flava,* and *U. imbecillis*). About 10 000 juvenile mussels of these species were maintained in recirculating streams for several weeks and reintroduced into watersheds to support restoration goals of the U.S. Fish and Wildlife Service.

10.4.2.2 *Lost Valley State Fish Hatchery, MO*—Since 2002, personnel at the Lost Valley State Fish Hatchery have propagated, via host fish, about 5000 *Epioblasma triquetra* and 40 000 *Lampsilis teres* juvenile mussels. *Epioblasma triquetra* is considered rare by the state of Missouri and is currently listed as a candidate species by U.S. Fish and Wildlife Service.

10.4.2.3 *Warm Springs National Fish Hatchery, GA*—Due to drought conditions that were occurring in a small tributary of the Flint River, Georgia, 1500 individual mussels were transported to the Warms Springs National Fish Hatchery in the late 1990s. Two species federally listed as endangered (*Lampsilis subangulata* and *Pleurobema pyriform*) have been propagated at the hatchery. Most of the mussels recovered from the dry tributary were maintained at the hatchery in recireulating tanks for about one year. Propagation efforts at the hatchery began in 2000 using a variety of host fish. Hatchery managers reported the successful transformation juvenile *Villosa vibex, V. lienosa,* and *L. subangulata. Lampsilis subangulata* is listed as endangered by the federal government and consideration of this listing has prompted hatchery personnel to focus efforts on propagating this and other species in the region. From these

TABLE 1 Facilities Currently Conducting Freshwater Mussel (Unionidae) Propagation and Refugium Efforts

Facility	State or Province	Species	Contact
Mammoth Spring National Fish Hatchery	AR	*Lampsilis streckeri* FE[A] *Arkansia wheeleri* FE *Pyganodon grandis* *L. siliquoidea* *L. ventricosa* *Fusconaia flava* *Utterbackia imbecillis*	Richard Shelton (Hatchery Manager) 870/625-3912 mammothspring@fws.gov http://mammothspring.fws.gov
Lost Valley State Fish Hatchery	MO	*Epioblasma triquetra* SR[B] *L. teres*	Ken Neubrand (Hatchery Manager) 660/438-4465 Ken.neubrand@mdc.mo.gov http:// www.conservation.state.mo.us/areas/hatchery/lostvalley/
Warm Springs National Fish Hatchery	GA	*Villosa vibex* *V. lienosa* *L. subanbulata* FE	Curtis Echevarria (Hatchery Manager) 706/655-3382 warmsprings@fws.gov http://warmspringshatchery.fws.gov
White Sulphur Springs National Fish Hatchery	WV	*L. fasciola* *V. iris* *E. rangiana* FE *P. clava* FE *V. fabalis* CD[C] *Amblema plicata* *Cyclonaias turberculata* *A. ligamentina* *E. dilatata* *Epioblasma* spp.	Catherine Gatenby (Project Leader) 304/536-1361 catherine_gatenby@fws.gov http://northeast.fws.gov/wv/wssnfh.html
Genoa National Fish Hatchery	WI	*L. higginsi* FE *O. Fragosa* FE *L recta* SR *O. olivaria* SR *L. cardium* *L. siloquoidea* SR *L. teres* SR	Tony Brady (Mussel Biologist) Doug Aloisi (Hatchery Manager) Roger Gordon (Mussel Program Supervisor) 608/869-2605 Doug_Aloisi@fws.gov http://midwest.fws.gov/Genoa
Aquatic Wildlife Conservation Center at Buller Fish Culture Station	VA	*Actinonaias ligamentina* *A. pectorosa* *Epioblasma capsaeformis* FE *E. brevidens* FE *E. f. walkeri* FE *Epioblasma triquetra* SR *Lampsilis fasciola* *L. ovata* *Polamilus alatus* *Villosa iris* *V. perpurpurea* FE	Nathan Eckert (SW VA Mussel Recovery Coordinator) Joe Ferraro (Mussel Propagation Specialist) 276/783-2136 Nathan.Ekert@DGIF.virginia.gov http:// www.dgif.virginia.gov/wildlife/freshwater_mussels.html
Tennessee Aquarium Research Institute	GA	*Lampsilis altilis* *L. virescens* *Lasmigona holstonia (etowehensis)* *Medionidus acutissimus* *Pleurobema decisum* *P. georgianum* *Ptychobranchus greenii* *Villosa nebulosa* *V. umbrans*	Paul Johnson (Director) 706/694-4419 pdj@tnari.org http://www.tennis.org/get_involved/research_tnari.asp
Kentucky Department of Fish and Wildlife Resources	KY	59 species including 7 federally-listed species	Monte McGregor (Aquatic Scientist) 502/564-7109 monte.mcgregor@ky.gov www.kdfwr.state.kv.us
Missouri State University	MO	Various species	Chris Barnhart 417/836-5166 chrisbarnhart@smsu.edu http://biology.smsu.edu/aquatic/smsuwebs.htm
Cooperative Fish and Wildlife Research Unit, Virginia Polytech and State University	VA	Various species	Richard Neves 540/231-5927 mussel@vt.edu http://www.fw.vt.edu/fisheries/neves.htm
Arkansas State University	AR	Various species	Jerry Farris 501/972-3082 jlfarris@astate.edu
Department of Fisheries and Wildlife, University of Minnesota	MN	Various species	Mark Hove 612/624-3019 mark_hove@umn.edu http:// www.fw.umn.edu/Personnel/staff/Hove/Personal.Page

TABLE 1 *Continued*

Facility	State or Province	Species	Contact
Cooperative Fisheries Research Unit, Tennessee Tech University	TN	Various species	Jim Layzer 931/372-3032 Jlm-layzer@tntech.edu
Department of Zoology, University of Guelph	ONT	Various species	Gerald Mackie 519/767-6684 http://www.uoguelph.ca/cbs/

[A]FE: Federally endangered
[B]SR: State rare
[C]CD: Candidate for listing

three species, nearly 8000 juvenile mussels were released into Spring Creek, GA. An additional 20 000 juvenile mussels have been maintained in laboratory conditions and are being monitored for growth and survival of viable juvenile mussels in these hatchery conditions.

10.4.2.4 *White Sulphur Springs National Fish Hatchery, WV*—In response to an emergency salvage order, White Sulphur Springs was involved in the collection and holding of various mussels species from the Ohio River in 1995. While high mortality occurred in mussels held in <5 cm of substrate during winter months, the following years yielded a much high survival of mussels held in containers with at least 20 cm of substrate. The propagation of two common mussels, *Lampsilis fasciola* and *V. iris* indicated that conditions at the hatchery may be limiting for the successful transformation of other species. While juvenile mussels were successfully propagated using fish host techniques, mean survival of *V. iris* and *L. fasciola* juvenile mussels following three months was 50 % and 6 %, respectively.

10.4.2.5 *Genoa National Fish Hatchery, WI*—The Genoa hatchery is focusing its recovery efforts on the propagation and reintroduction of federally endangered juvenile *Lampsilis higginsi*, and *Quadrula fragosa*. Various propagation techniques are being implemented including hatchery propagation (using host fish) and holding of juvenile mussels for survival and growth. Over 4 years, about 1 500 000 juvenile mussels were released into watersheds known to maintain existing or historic populations of *L. higginsi*. The majority of juvenile mussels produced are by cage propagation in river systems using host fish. Other propagation techniques include the free release of infested host fish. Nearly 20 500 host fish were released in 2003 and 2004 and results indicate that for cage releases, over 7000 sub-adults are living and growing from these 2 year classes. Other mussel work includes host fish studies and propagation for the native mussel species. In 2004, channel catfish were infested and held with *Q. fragosa* glochidia and held until releases are favorable in the spring.

10.4.2.6 *Aquatic Wildlife Conservation (AWCC), Buller Fish Cultural Station, VA*—AWCC was established in 1998 to recover mussels within the Upper Tennessee River Drainage of Virginia. The facility has held over 30 species of adult mussels with a survival rate of 95 %. Additionally, at least 16 species have spawned at the AWCC including both state and federally listed species. These mussels are held in 1 meter round diameter tanks fed with natural river water. Propagation and release has been successful for *Actinonaias ligamentina*, *A. pectorosa*, *Epioblasma brevidens*, *E. capsaeformis*, *E. florentina walkeri*, *Lampsilis fasciola*, *L. ovata*, *Villosa iris* and *V. perpurpurea*. Over 70 000 individuals, ranging from 1 week to 6 years of age, have been released into the Powell and Clinch Rivers. Grow-out of propagated juvenile mussels past one year has been attempted and successful for 4 species (*E. brevidens*, *E. capsaeformis*, *L. fasciola* and *V. iris*). Due to concerns over impacts in Indian Creek, Tazewell County, VA, an Ark population of 2 federally endangered species, *E. florentina walkeri* and *V. perpurpurea*, was established at AWCC. Both species have spawned providing a number of females on hand for propagation during the upcoming season.

10.4.2.7 *Tennessee Aquarium Research Institute (TNARI), GA*—To stem the tide of extinction in southeastern rivers and streams, TNARI surveys and monitors mollusks within the region and to propagate mussels and snails in captivity for reintroduction into the wild. TNARI scientists have successfully bred in captivity the Georgia rocksnail, the plicate rocksnail and the spiny riversnail—snails selected for propagation because habitat destruction has resulted in the loss of these species from over 85 percent of their historical range. In 2002, TNARI researchers produced about 12 000 snails in captivity. More than 2700 spiny riversnails were released into the Tennessee River in 2002. The TNARI has propagated the following species since 2000: *Io fluvialis*, *Lampsilis altilis*, *L. virescens*, *Lasmigona holstonia*, *Leptoxis foremani*, *Leptoxis plicata*, *Medionidus acutissimus*, *Pleurobema decisum*, *P. georgianum*, *Ptychobranchus greenii*, *Villosa nebulosa* and *V. umbrans*.

10.4.3 Bishop et al (2005) (**47**) reported both successful and unsuecessful shipment of gravid mussels of various species based on numerous personal communications with facilities involved in mussel transport. Shipping gravid mussels is often necessary because mussels are not in the area where the propagation laboratory is located.

10.4.3.1 Long-term brooders (Lampsilinae and Anodontinae) tend to hold their embryos or glochidia during shipping and handling. Adult mussels can be transported to the laboratory at about 4 to 10°C using ice bags or ice packs placed in a cooler. The ice bags or ice packs should not be in direct contact with the mussels or with the water containing the mussels (if mussels are shipped with water). Specifically, there should be some insulation around the ice bags or ice packs. Cope et al (2004) (**76**) recommends shipping adult mussels in moist burlap in coolers with ice in plastic bags for transport duration <12 h at a temperature within 2°C of the collection water (if possible). Alternatively, Chen et al (2001b) (**77**) and Gordon (2001) (**78**) recommend shipping adult mussels in well-aerated

TABLE 2 Summary of Techniques Used to Transform Juvenile Mussels (adapted from Bishop et al, 2005) (47) (reprinted with permission)

Species	Technique	Purpose	Reference
Alasmidonta raveneliana	Fish host	Toxicity testing	Keller and Augspurger 2005 (53)
Amblema plicata	Fish host	Reintroduction	Hubbs 2000 (54)
	Media	Culture development	B Hudson and M Barfield (personal communication)
Anodonta suborbiculata	Fish host	Host suitability	Barnhart and Roberts 1997 (33)
Anodontoides ferussacianus	Fish host	Host suitability	Hove et al 1997 (55)
Toxolasma cylindrellus	Fish host	Unknown	Hudson and Isom 1984 (56)
Cyclonaias tuberculata	Fish host	Host suitability	Hove et al 1997 (55)
Elliptio angustata	Media	Toxicity testing	Hudson et al 1996 (57)
E. complanata	Media	Culture development	B Hudson and M Barfield (personal communication)
E. crassidens	Media	Unknown	D Simbeck (personal communication)
E. icenterina	Fish host	Toxicity testing	Keller and Ruessler 1997 (58)
Fusconaia ebena	Media	Culture development	Isom and Hudson 1982 (59)
Fusconaia flava	Media	Reintroduction	Milam et al 2000 (60)
Lampsilis cardium	Fish host	Toxicity testing	Keller and Ruessler 1997 (58)
	Media	Reintroduction	Milam et al 2000 (60)
	Fish host	Toxicity testing	Newton et al 2003 (61)
L. fasciola	Fish host	Reintroduction	Morgan et al 1997 (62)
	Media		D Simbeck (personal communication)
L. ovata	Fish host	Culture development	Isom and Hudson 1982 (59)
L. rafinesqueana	Fish host	Host suitability	Barnhart and Roberts 1997 (33)
			Shiver 2002 (63)
L. reevelana	Fish host	Host suitability	Barnhart and Roberts 1997 (33)
L. siliquoidea	Media	Reintroduction	Milam et al 2000 (60)
	Media	Survival and growth	Myers-Kinzie 2000 (64)
L. streckeri	Fish host	Host suitability and reintroduction	Winterringer 2003 (65)
L. subangulata	Fish host	Host suitability	C Echevarria (personal communication)
L. teres	Media	Unknown	Keller and Zam 1990 (66)
Ligumia recta	Media	Culture development	Isom and Hudson 1982 (59)
			Milam et al 2000 (60)
Medionidus conradicus	Fish host	Reintroduction	Morgan et al 1997 (62)
Megalonaias gigantia	Media	Unknown	B Isom, D Simbeck (personal communication)
M. nervosa	Fish host	Reintroduction	Hubbs 2000 (54)
Pleurobema coccineum	Fish host	Host suitability	Hove et al 1997 (55)
P. cordatum	Media	Culture development	Hudson and Isom 1984 (56)
Ptychobranchus occidentalis	Fish host	Host suitability	Barnhart and Roberts 1997 (33)
Pyganodon cataracta	Media	Unknown	Dimock and Wright 1993 (48)
P. grandis	Fish host	Toxicity testing	Keller and Ruessler 1997 (58)
	Fish host	Reintroduction	Milam et al 2000 (60)
	Media		B Isom (personal communication)
Strophitus undulatus	Fish host	Host suitability	Hove et al 1997 (55)
Utterbackia imbecillis	Fish host	Toxicity testing	Keller and Zam 1991 (67)
			Warren 1996 (68)
			Clem 1998 (69)
	Media	Culture development	Isom and Hudson 1982 (59)
			Barfield et al 1997 (70)
		Toxicity testing	Hudson and Shelbourne 1990 (71)
			Wade et al 1989 (72)
	Fish host	Physiological effects	Dimock and Wright 1993 (48)
	Fish host	Viability	Fisher and Dimock 2000 (73)
	Media	Unknown	Keller and Zam 1990 (66)
Venustaconcha ellipsiformis	Fish host	Host suitability	Riusech and Barnhart 2000 (74)
V. pleasii	Fish host	Host suitability	Riusech and Barnhart 2000 (74)
Villosa iris	Fish host	Toxicity testing	Jacobson et al 1993 (75)
	Fish host	Behavior	Yeager et al 1994 (24)
	Media	Unknown	D Simbeck (personal communication)
V. lienosa	Fish host	Toxicity testing	Keller and Ruessler 1997 (58)
	Fish host	Host suitability	C Echevarria (personal communication)
	Media	Unknown	Keller and Zam 1990 (66)
V. taeniata	Fish host	Reintroduction	Morgan et al 1997 (62)
V. vibex	Fish host	Host suitability	C Echevarria (personal communication)

water. The approach used may be dependent on the species of mussel being shipped. For species that are relatively tolerant of low of oxygen, it may not matter which approach is used for short intervals of time (Chen et al 2001b) (77). Lampsilinae and Anodontinae mussels will not likely abort glochidia during transport, but can abort glochidia after been warmed and placed into culture systems. Once received in the laboratory, the temperature of the water containing the mussels should be gradually adjusted to the test temperature (for example, increase by no more than about 3°C/h). Some culture facilities have had better success when adult mussels are held for a day or two before the glochidia are extracted for propagation of juvenile mussels (Bishop et al 2005) (47).

10.4.3.2 Short-term brooders (Unioninae) tend to abort embryos or glochidia during shipping or following shipping (although less than 5 % may abort, resulting in partial demibranch release during transportation). Adult amblemine mussels transported in wet towels in an ice chest often abort when returned to water. *Quadrula* species seem to be especially prone to aborting glochidia when disturbed (Bishop et al 2005b) (47).

10.4.4 Glochidia have been shipped free from the marsupia in cool, well oxygenated natural or reconstituted water (Gordon 2001 (78); section 10.4.4). Excised gravid marsupia have also been shipped for use in propagation efforts. However, the most appropriate way to ship glochidia is free from the marsupia because the female mussel is not killed (section 10.5.3). Alternatively, cold storage at about 4°C of inflated marsupia for up to 4 d has been shown to be effective in maintaining the condition of encapsulated glochidia for toxicity testing (Bishop et al 2005) (47). Glochidia of *Lampsilis higginsi* were held at 8 to 12°C for 24 h without a substantial reduction in viability (Gordon 2001) (78). Zimmerman and Neves (2002) (42) compared glochidia from two species over time in different temperature regimes and found that glochidia in the cooler temperatures (0 and 10°C) remained viable longer than those at 25°C (75 % survival at 7.5 days for *Villosa iris* and at 14.4 days for *Actinonaias pectorosa*) and were able to be transformed on fish following this time period (Table A1.2).

10.4.5 *Shipping Glochidia or Juvenile Mussels:*:

10.4.5.1 Section 10.5.3 describes procedures for isolation of glochidia from female mussels and section 10.5.4 describes procedures for culturing juvenile mussels. It may be desirable to ship adult mussels containing glochidia rather than ship glochidia isolated from female mussels. Once glochidia have been isolated, the female mussel can be returned to the collection site (Keller and Augspurger 2005) (53).

10.4.5.2 Young juvenile mussels or glochidia isolated from female mussels are fragile and should be shipped with care. The glochidia or juvenile mussels should be shipped from the source to the laboratory in as short of a period of time as possible using an over night delivery service. Check to determine that the vendor accepts live organisms for shipment. Before shipping, empty shells or detritus should be separated from the glochidia or juvenile mussels. The mussels should then be placed into clean culture water or acclimated to the dilution water before shipment (section A1.4.2.2). It is not necessary to feed the juvenile mussels during shipping. In fact, food may adversely affect the water quality during transit.

10.4.5.3 Either plastic bags or square, wide mouth polyethylene bottles (for example, 250 to 1000 mL) work well for contain mussels when placed into strong-walled containers for shipping. Square bottles, when properly sealed, can be laid on their sides; the square form may help prevent piling or bunching of mussels during shipment. Teflon tape can be wound around the threads of the bottle to help seal the cap of the bottle. Flat (or square) bottom fish-shipping bags also work well for containing mussels. Use of the pleated bag (flat bottom) provides a larger surface area for the mussels to lie on during shipping. For added security, the shipping bag should be doubled bagged. Each bag should be sealed with rubber bands. Zip-lock bags should not be used because these bags may open during shipment. Pure oxygen can be added to the water containing the mussels before sealing the bags or bottles for shipment.

10.4.5.4 Shipping containers should be durable and water tight. Six-pack beverage coolers are well insulated, durable, and work well for shipping bottles or bags containing glochidia or juvenile mussels. The addition of bubble wrap, newspaper or foam peanuts will reduce jostling and keep the bottles or bags more secure in the container. These materials also add an additional layer of insulation. Coolers containing test organisms should be firmly taped shut before shipment.

10.4.5.5 Care should be taken in shipping mussels when outdoor temperatures are reduced or elevated. Insulated shipping containers will help protect from temperature fluctuations during shipping. Ice packs can be used to stabilize the temperature of the shipping container. Small temperature recorders can be used to monitor temperature of the container during shipment. Once received in the laboratory, the temperature of the water and the water quality characteristics of the water containing the mussels should be gradually adjusted (for example, a temperature increase of no more than about 3°C/h). See section A1.4.2.2 for additional guidance on acclimation of test organisms before the start of a toxicity test.

10.5 *Care and Handling of Organisms in the Laboratory*:

10.5.1 Information in the following sections and in section 10.6 summarizes procedures for the culture of mussels.

10.5.2 *Adult Mussels*:

10.5.2.1 In the laboratory, adult mussels can be maintained in aquaria with a substrate of sediment or gravel. Maintaining the physiological condition of adult mussels in the laboratory is difficult because the diet and nutritional requirements for mussels are poorly understood (Cope et al 2004) (76). Adult mussels held for up to one month without feeding can produce viable glochidia; however feeding adult mussels algae enhanced survival of adult mussels (Johnson et al 1993 (79), Patterson et al 1999 (80), Gatenby et al 2000 (81)). Holding and maintaining adult mussels in laboratory conditions is necessary to allow for transport acclimation, glochidia development, and in some cases, for reproduction to occur. *Villosa* spp. and *Lampsilis* spp. are particularly easy to maintain in the laboratory when given adequate food quantity and quality (Bishop et al 2005) (47). Maintenance of these species results in relatively low mortality and measurable growth, indicating

that these individuals are in reasonably good condition. Females of *Villosa*, *Pyganodon*, *Utterbackia*, *Tritogonia*, *Elliptio*, and *Pleurobema* have repeatedly become gravid in holding conditions (Bishop et al 2005) (**47**).

10.5.2.2 Adult mussels should be observed daily for signs of stress or mortality. Gaping mussels that do not close when touched with a probe should be discarded. Mussels that never open or do not deposit feces should be discarded. Waste and feces should be siphoned out of the culture systems as needed. Concentrations of glycogen in the adult mussels should also be monitored during the time that the organisms are held in the laboratory (Patterson et al 1999 (**80**), Naimo et al 1998 (**82**), Naimo and Monroe 1999 (**83**), Cope et al 2004 (**76**)).

10.5.2.3 Cope et al (2004) summarizes conditions for holding adult mussels in the laboratory or in ponds and recommends feeding adult mussels 1×10^5 algal cells/mL or 4.0 mg/L dry weight of algae twice daily or 2 to 5×10^4 algal cells/mL or 1.9 mg/L dry weight of algae on a continuous bases (Gatenby et al 2000 (**81**) and Gatenby 2002 (84)). The amount of algae required is dependent on the biomass of adult mussels in a particular culture location.

10.5.2.4 Adult *Lampsilis cardium* have been held in the laboratory in aerated 100 to 150-L flow-through aquaria receiving about 20 to 30 L/h containing sand and aerated well water at 10 to 15°C. Adults were fed a commercial shellfish diet[4] at a ration of 1.2 mL/individual/day. To deliver feed, about 80 % of the water was siphoned from the aquaria and the shellfish diet was added (mixed with about 500 mL of well water) and then the tank was filled with water back to volume. Adults were usually fed three times a week and the ration was adjusted accordingly (for example, to get a 7-d supply of food delivered in 3 feedings). Adult *L. cardium* have been held in this manner with few to no mortality for up to one year (Newton et al 2003) (**61**).

10.5.2.5 USGS (2004) held adult mussels containing glochidia in an indoor laboratory setting. Well water (hardness 280 mg/L as $CaCO_3$ at 10 to 17°C) was provided at a rate of about 1 volume addition/h. Mussels were held in 250 to 600-L tanks. Plastic containers (35 by 24 by 23 cm) were placed in the fiberglass tanks and a 10-cm layer of creek gravel (about 0.5 to 2 cm diameter) was used as a substrate in each container. About 10 adult mussels were placed in each container. About 15 mL of two instant algae mixtures (prepared from non-viable microalgae concentrates of *Nannochloropsis* and from a commercial shellfish diet)[4] were added every other day to each container (section A1.4.5 for a description of the process used to prepare these two instant algae mixtures).

10.5.2.6 Adult mussels have been held in a 0.1 hectare pond for more than 1 year in suspended pocket nets or in sediment-filled containers placed on the bottom of the pond (Dick Neves, USGS, Blacksburg, VA; personal communication).

10.5.3 *Glochidia*:

10.5.3.1 During early development, glochidia are carried in the gills of the female mussel. The maturity of the glochidia can be determined by the color of the gills of the female. Gills containing mature glochidia are enlarged and brown in color whereas enlarged beige or white gills may contain immature glochidia (Johnson et al 1993) (**79**). Many short-term brooders have conglutinates that change in color from red to pink as the glochidia mature (Jones et al 2004) (**36**). Visual examination of gill of a female mussel can be done by carefully prying the sides of the shell open.

10.5.3.2 Mature glochidia can be gently flushed from the marsupium of a female mussel into a basin or shallow container using a sterile hypodermic syringe filled with dilution water in which the female mussels are held. The gage of the needle used should be based on the size of marsupium of the mussel (for example, needle about 3.8-mm long, 16 to 20 gauge). Care should be taken not to damage the gill structure within the marsupium. The valves of the adult mussel should be slowly opened with reverse pliers (Gordon 2001) (**78**) or with a small nasal speculum. Opening mussels too quickly or too wide can crack the valves or rip the adductor mussels. The valves can be propped open with a silicon stopper or similar object. Caution should be taken not to damage internal organs, labial palps, or gill structure (Gordon 2001) (**78**). Glochidia have also been isolated by cutting a section of gill from the female mussel and then teasing out the glochidia in water. This latter technique is destructive to the gills of the adult female and should be avoided if possible. No studies were identified where glochidia were isolated for toxicity testing from conglutinates released into the water by female mussels (Kernaghan et al 2005) (**5**).

10.5.3.3 Isolated glochidia can be held in glass chambers before the start of a toxicity test or before the glochidia are used to produce juvenile mussels (section 10.5.4). Glochidia of anodontines may stick together due to byssal thread adhesion. These aggregates of glochidia can be separated by carefully aspirating the aggregates in and out of a pipette. The maturity of glochidia can be determined through microscopic examination. Mature glochidia will be free of embryonic membranes and the shell valves of viable glochidia will open and close sporadically in anodontinc species. Viability of glochidia isolated from a female mussel should be evaluated before the start of a toxicity test using a solution of NaCl (section A1.4.8.4).

10.5.3.4 Gravid female mussels are usually collected from the field and held in the laboratory before isolating glochidia to start a toxicity test. Alternatively, Zimmerman and Neves (2002) (**42**) suggested glochidia of some species (including *Villosa iris* and *Actinonaias pectorosa*) could be extracted in the field from a female and transported back to the laboratory in cool water where the glochidia can remain viable for several days without a reduction in ability to successfully attach on a host fish. This procedure may be particularly useful when glochidia of endangered species are extracted in the field, and the female mussels should be immediately returned to their habitat.

[4] The sole source of supply of the materials known to the committee at this time is Instant Algae 520 McGlincy Lane #9, Campbell, CA 95008. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

10.5.3.5 Before starting an exposure, the viability of gloehidia should be evaluated by the response of the gloehidia to the addition of a solution of NaCl (seetion A1.4.8.4). Mature and healthy glochidia will snap shut in response to the addition of a salt solution. Immature glochidia isolated from the marsupium of a female will often be enclosed in an egg membrane and will be fragile and tend to fracture. Toxicity tests are usually started if >90 % viability of the glochidia is observed (Annex A1). If an abundance of immature glochidia are isolated from a female mussel, progeny of this female should not be used to conduct a toxieity test.

10.5.3.6 Exposures are usually started the same day that glochidia are isolated from female mussels without an extended acclimation period in the dilution water before the start of a toxicity test (Table A1.1 and Table A1.3). However, Wang et al (2003) (85) observed that the sensitivity of *Lampsilis siliquoidea* glochidia held for 24 h after isolation from a female was similar to newly-released glochidia in exposures to copper. The viability of glochidia isolated from each female should be evaluated before glochidia are pooled together (section A1.4.8.4). Toxicity tests should be conducted by pooling glochidia from at least three female mussels. Toxicity tests can be conducted with glochidia obtained from one female mussel (for example, when a limited number of organisms of an endangered species is available for testing); however, the results of tests conducted with a limited number of mussels should be interpreted with caution. Additional research is needed to determine the minimum number of females that should be sampled to obtain glochidia to start a toxicity test. This research might include an evaluation of the variability in sensitivity of glochidia obtained from individual females using a variety of toxicants (seetion A1.6).

10.5.4 *Juvenile Mussels*:

10.5.4.1 Toxicity tests with juvenile mussels are typically started within about 5 d after juvenile mussels are released from a fish host (Table A1.4; for example, *in vivo* propagation; Lefevre and Curtis 1912) (39). Alternatively, artificial media has also been used to transform juvenile mussels for use in toxicity testing (for example, *in vitro* propagation; Johnson et al 1993 (79), Clem 1998 (69), Isom and Hudson 1982 (59), Summers 1998 (86), Hudson et al 2003 (87)).

10.5.4.2 Bishop et al (2005) (47) provides an overview of *in vitro* and *in vivo* methods used to culture juvenile mussels. 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*. Comparisons of physiological conditions of juvenile mussels transformed *in vitro* and *in vivo* indicate that individuals that transform on a fish host tend to be healthier than individuals that transform in artificial culture media. Juvenile mussels transformed with fish exhibited several features that were not present in juvenile mussels transformed *in vitro* (Fisher and Dimock 2002) (73). There was little evidence of lipids and glycogen in the larval mantle cells of the juvenile mussels transformed *in vitro*, whereas the juvenile mussels transformed with fish had numerous lipid droplets and glycogen granules in the basal portions of the cells (Fisher 2002 (88), Hudson et al 2003 (87)). Juvenile mussels transformed *in vivo* on fish hosts were less sensitive to thermal and hypoxie stresses eompared to juvenile mussels transformed *in vitro* (Fisher 2002) (88). Juvenile *U. imbecillis* transformed *in vitro* were less sensitive compared to juvenile mussels transformed *in vivo* in 24-h exposures to sodium dodecylsulfate; however, sensitivity to cadmium or ammonia was similar between the two groups of juvenile mussels (Summers 1998) (86). Comparisons of toxicity tests conducted with *in vitro*- and *in vivo*-transformed juvenile mussels indieated that juvenile mussels transformed in an artificial medium were more sensitive to copper than the juvenile mussels transformed on a fish host (Warren and Klaine 1994) (89).

10.5.4.3 Table 2 provides a summary of techniques that have been used to transform juvenile mussels (Bishop et al 2005) (47). Most freshwater mussels require a host fish for reproductive success. Freshwater mussels are identified as either generalists, where glochidia can transform on a variety of fish species, or specialists, where only one or two host fish have been identified that successfully metamorphose glochidia to the juvenile life stage. Techniques for determination of fish hosts for a particular species have been reported and used by many researchers for decades, while some unconventional hosts (for example, amphibians) have also been used to transform juvenile mussels. Some freshwater mussels can transform from glochidia directly to juvenile mussels inside the marsupial pouch of the mussel (for example, *Strophitus undulatus*, *Utterbackia imbecillis*, *Obliquaria* spp.; Bishop et al 2005) (47).

10.5.4.4 Common species as well as state and federally listed species are often difficult to transform due to the lack of knowledge of life history complexities and requirements (section 10.1). Glochidial attachment can range from several days to several months depending on the mussel species, fish health, water temperature, and other unknown variables (Bishop et al 2005b) (47). Alternatively, fish survival can be jeopardized by excessive glochidial infestation, limiting gas exchange across the gill lamellae. Maintenance of healthy host fish before and during encystment is critical to the success of transforming juvenile mussels. While 50 to 100 gloehidia/gill for fish 15 to 25 cm in length have been reported as adequate, others investigators have directly infested host fish with several thousand and achieved successful transformation and still maintained fish viability (Bishop et al 2005) (47). Transformation of glochidia to juveniles on the fish gill (or in artificial media) may range from 7 to >110 d, depending on mussel species, water temperature, and host fish condition (Bishop et al 2005) (47).

10.5.4.5 Host fish should not be fed for several days before the release of the transformed juvenile mussels. The bottom of the chamber holding the host fish should be kept clean of debris before the release of the newly-transformed juvenile mussels. Bottom-feeding minnows and catostomids may feed on newly-transformed juvenile mussels; therefore these fish should be separated from the bottom of the chamber with fine mesh (Bishop et al 2005) (47). The newly-transformed juvenile mussels can be siphoned from bottom of the chamber holding the host fish and collected using a sieve of appropriate size (for

example, 130 µm). A polarized lens attached to the objective lens of a dissecting microscope can be used to reflect, through under stage lighting, only prismatic objects and block out sediment or feces that can make juvenile identification and counting difficult (Watters 1996) (90).

10.5.4.6 Section 10.4 provides guidance on obtaining and shipping juvenile mussels from facilities that culture mussels. The following sections provide examples of approaches used by culture facilities to transform juvenile mussels. Laboratories interested in transforming juvenile mussels in their own facilities are encouraged to obtain the publications cited in the sections below for additional detail. Laboratories interested in transforming juvenile mussels at their own facilities may also want to contact facilities listed in Table 2 for guidance.

10.5.4.7 Techniques for determining fish host suitability include the use of aeration tanks, direct gill placement, and the use of anesthetics to reduce handling stress on the fish (Zale and Neves 1982) (91). Aeration tanks have been used when there are viable glochidia with several fish species and cohorts. However, if glochidia are limited or the fish are small, direct gill placement using pipettes is a viable alternative to aeration techniques for attachment onto the gill (Bishop et al 2005b) (47). Host suitability trials should include multiple attempts using several individuals of the same host organism with glochidia from different females to assure that metamorphosis occurs in at least two different test trials (Bishop et al 2005) (47).

10.5.4.8 The U.S. Fish and Wildlife Service Genoa National Fish Hatchery in Genoa, WI uses the following procedure to encyst glochidia of federally-endangered *Lampsilis higginsi* using largemouth (*Micropterus salmoides*) or smallmouth (*M. dolomieu*) bass as the fish hosts (Tony Brady, Genoa, WI, personal communication; Gordon 2001) (78). Glochidia are flushed from the gills of 1 to 3 adult mussels. About 2 mL of glochidia are added to 1 to 2 L of water, and 10 fish are then placed into this solution for about 3 minutes. Host fish should be introduced after the addition of the glochidia to minimize fouling of the chamber with excess feces or mucus. A smaller volume of water allows for more concentrated glochidia when infesting fish. Aeration with an air stone is used to keep the glochidia in suspension. The target infestation is 250 glochidia per fish. Light levels should be reduced as much as feasible to minimize activity of the infested fish.

10.5.4.9 Barnhart (2003) (92) described a system used to transform juvenile mussels of three species of freshwater mussels: *Lampsilis rafinesqueana*, *L. abrupta*, and *Leptodea leptodon*. A large-scale recirculating system for mussel propagation was developed and used to produce large numbers (14 000 to 375 000) of juvenile mussels. Barnhart (2003) (92) also provides a description of procedures that can be used to encyst the glochidia on the fish hosts and maintain the host fish during the transformation of the juvenile mussels. Host fish containing encysted glochidia were held in flow-through raceways and then transferred into low-flow or recirculating tanks during the drop-off period to avoid losing the juvenile mussels. Water supplies at hatcheries often contain a wide variety of zooplankton that are the same size as glochidia or juvenile mussels. Some invertebrates such as flatworms and hydra are predators on juvenile mussels. Other species are the same size range of glochidia or juvenile mussels and are very difficult to separate (for example, cladocerans, ostracods, bryozoans). Efforts to remove invertebrates by pre-filtering water supplies were unsatisfactory. Vacuuming the tanks holding the host fish to remove transformed juvenile mussels was labor intensive and missed a large proportion of the juvenile mussels that dropped from the fish host. The recirculating propagation system (RPS) developed by Barnhart (2003) (92) was designed to hold several hundred host fish and recover glochidia or juvenile mussels continuously from the recirculating flow of water (Figure 1 to 7 in Barnhart 2003 (92)). The RPS consists of: *(1)* 2 conical-bottom 1000-L tanks each with a double stand pipe to contain the host fish, *(2)* a sump containing a biological filter to maintain water quality, *(3)* recovery filters to recover juvenile mussels from each tank, and *(4)* a pump to recirculate water. Host fish can be held in the RPS during the entire encystment period or the fish can be moved to the RPS shortly before drop-off of the juvenile mussels. Host fish are not fed for several days in advance of the drop-off of the juvenile mussels. The RPS system eliminates most problems with zooplankton because these organisms do not enter the system. Vacuuming debris from the bottom of the tank is also eliminated because recirculation of water is used to recover the juvenile mussels by moving them to a filtration system. The juvenile mussels can be removed from the filters to facilitate counts and expedite handling for use in culture or toxicity testing.

10.5.4.10 Newton et al (2003) (61) used *in vivo* infestation to obtain about 2000 juvenile *Lampsilis cardium* from largemouth bass (*Micropterus salmoides*). Glochidia were combined from at least three female mussels and used to infest four, 8 to 15-cm long largemouth bass. Glochidia were isolated from a female mussel by flushing the gill with about 30 mL of well water (delivered three times via a 10 mL syringe). The water containing isolated glochidia was placed into a glass dish and glochidia viability was determined on a subsample and then glochidia isolated from all of the female mussels were composited into one dish. Four fish were placed into a 19-L bucket with about 9.5 L of vigorously aerated well water followed by the addition of the glochidial solution. After 10 min, one fish was randomly removed and placed into a separate 19 L bucket with 9.5 L of well water and 1.0 g MS-222. Once the fish became lethargic, the gills were checked for level of glochidial infestation (the target was about 400 to 500 glochidia/fish). If the infestation was low, the fish was put back into the bucket containing glochidia for about 2 to 5 min and re-checked to evaluate infestation. Once the encystment was complete, the fish were transferred into 38-L flow-through aquaria (about 500 mL/min) containing dechlorinated well water at 22°C. Temperature, dissolved oxygen, and flow rate were measured daily and tank bottoms were siphoned daily. At this temperature, juvenile mussels began to excyst in about 17 to 19 d. Encysted fish were fed rainbow trout, *Oncorhynchus mykiss*, until about 7 to 10 d before the expected release of juvenile mussels. About 3 d before the expected release of juvenile mussels, fish were consolidated into 2 aquaria using a plastic baffle to separate the fish. To determine post-excystment age, water was

siphoned from the aquaria bottoms daily through a 153-µm sieve and the contents was examined under a microscope. Juvenile mussels from a given day were transferred into 4.4-em inner diameter glass cylinders fitted with a 153-µm mesh bottom and suspended in 38-L flow-through aquaria at 22°C until use in toxicity testing. This procedure has been used to produce juveniles for conducting more than 10 toxicity tests and has resulted in acceptable survival of both host fish and juvenile mussels.

10.6 *Feeding*:

10.6.1 *Adult Mussels*—See 10.5 for a description of procedures for feeding adult mussels held in the laboratory.

10.6.2 *Glochidia*—Glochidia isolated from female mussels are not fed in culture or in toxicity tests.

10.6.3 *Juvenile Mussels*:

10.6.3.1 The following sections summarize information on general feeding requirements of juvenile mussels. Examples of procedures used by facilities to culture newly-transformed juvenile mussels are also presented. Bishop et al (2005) (47) also describes procedures for rearing juvenile mussels caged in rivers and describes case studies where facilities have propagated and reintroduced juvenile mussels into the environment.

10.6.3.2 Little is known about the survival, growth, and reproduction of naturally produced mussels once the juvenile mussels excyst from the host organisms. Growth of juvenile mussels during the first year is variable among species and consequently, collection from the wild and assessment of these young individuals is difficult. Certain species of juvenile mussels may only grow a few millimeters to centimeters in a typical year. Percentage of juvenile survival that results in reproductively-viable adults for most species is unknown (Bishop et al 2005) (47); however, some information is available for some European species of freshwater mussels (Bauer and Wachtler 2000) (93).

10.6.3.3 The addition of sediment fines as a substrate has been shown to inerease growth rates of juvenile mussels of some species in the laboratory (Hudson and Isom 1984 (56), Gatenby et al 1997 (25), O'Beirn et al 1998 (26)). Juvenile mussels can use the organie matter that coats small sediment particles. While some juvenile mussels do well in fine sediment, juvenile mussels of other spccies (typically riffle-dwelling species) do poorly in fine sediment (Neves 2004) (3). Sediment used to culture juvenile mussels is typically sieved to remove larger particles and autoclaved to remove invertebrate predators and fungal growth that may kill juvenile mussels. Hudson et al (2003) (87) report that sediment pretreated with low concentrations of bentonite clay or EZ mud[5] clears the suspension of the finest clay particles, resulting in better survival of juvenile mussels. This indicates that finer particles may impair gill function of juvenile mussels (Bishop et al 2005) (47).

10.6.3.4 Nutrition in juvenile and adult mussels is important for the survival, growth, and reproduction of mussel populations. However, little is known about the quantity or quality of food source that provides conditions for sustaining populations in the wild or in the laboratory (Gatenby et al 2003 (94), Christian et al 2004 (95)). A diversity of algae reportedly improves growth of juvenile mussels (Hudson and Isom 1984 (56); Gatenby et al 1997 (25), 1999a (96); Beck and Neves 2003 (21)). Algae containing higher levels of lipids (for example, *Neochloris oleoabundans*) promoted the best growth of juvenile mussels (Gatenby et al 1997, 2003) (25, 94).

10.6.3.5 Barnhart (2005) (97) described a eompact recireulating system for rearing newly-transformed juvenile freshwater mussels. The system consisted of nested buekets that partition a volume of 18 L of culture water into an upper and lower compartment. A small submersible pump is used to move water from the lower compartment to the upper compartment, and the water then returns to the lower compartments through cylindrical sereen-capped ehambers that contain juvenile mussels. The design minimizes space requirements and facilitates the isolation, containment, and handling of juvenile mussels. Newly-transformed juvenile mussels of 8 species were held in these systems for several months and fed continuously by drip with a monoculture of algae (*Neochloris oleoabundans*). River water filtered to remove particles >30 µm was used to culture juvenile mussels to provide a natural community of microorganisms which may aid in digestion. Survival rates were higher than most previous reports for captive juvenile mussels. Survival of newly-transformed *Lampsilis siliquoidea* and *L. reeveiana* exceeded 95 % over 2 months. Changes in shell length in these two species were about linear ranging from 4.2 to 12.5 µm/day at 22°C. These growth rates are similar to or higher than previous reports of growth of juvenile mussels in recirculating systems. The bucket rearing system may be particularly useful for conducting studies feeding studies with juvenile mussels. This recirculating system might also be adapted for conducting chronic toxicity tests with juvenile mussels.

10.6.3.6 Henley et al (2001) (98) described two air-driven recirculating water systems for eulturing juvenile mussels. An 8-L system was used to hold newly-transformed juvenile mussels for about 10 weeks. Juvenile mussels were then transferred to polyvinyl chloride (PVC) trays place into a larger 350-L system for grow out. The 8-L system consisted of two interconnected polypropylene containers. One container served as the juvenile rearing tank and the other as a reservoir. Water entered the rearing tank via an airlift through silicone tubing from the reservoir and exited the rearing tank through a stand pipe. The rearing tank was designed to have some algal settling for juvenile mussels at a pedal-feeding stage of development. Juvenile mussels were fed periodically to maintain an algal cell density of about 30 000 cells/mL in the water column (Neves 2004) (3). *Scenedesmus*, *Nannochloropsis*, and *Neochloris* were genera of algae that are suitable for the diet of juvenile mussels (Neves 2004) (3). The 350-L system consisted of an interconnected polyethylene feed trough, a polyethylene drum and a polyvinyl chloride airlift and return tubes. A series of air stones were used to suspend algae in the trough containing the trays with juvenile mussels and in the drum and were used to recirculate water from the drum to the trough. Juvenile mussels

[5] The sole source of supply of the apparatus kuown to the committee at this time is BAROID Industrial Drilling Prodnets. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will reeeive eareful cousideration at a meetiag of the responsible technical committee,[1] which you may attend.

were placed in PVC trays (0.2 m by 1.2 m by 20 mm; bottom area about 0.25 m^2) containing about 10 mm of course sand and silt substrate. Algal rations were added to the trough through an algal recirculating system. Similar types of juvenile mussel systems using electrical pumps to recirculate water were described by O'Beirn et al (1998) (**26**), Jones and Neves (2002) (**99**).

10.6.3.7 Jones and Neves (2002) (**99**) also described a static system for culturing juvenile mussels in 6 cm square and 5 cm deep plastic containers. Juvenile mussels were placed in containers containing 50 mL of water, 50 mL of an algal suspension, and about 0.5 mL of a fine sediment (particle size <105 µm). The sediment was autoclaved to kill predators such as flatworms and diptera larvae before placement into the containers (Jones et al 2004) (**36**). The water, algae, and sediment were exchanged every 2 d. Better survival of juvenile *Cyprogenia stegaria* was observed in the static system compared to a recirculating system; however, the density of algae in the static system was higher than the algae in the reeirculating system (Jones and Neves 2002) (**99**).

10.6.3.8 Beaty and Neves (2004) (**100**) described a flow-through culture system using natural river water to maintain newly-transformed juvenile *Villosa iris* for about 90 days. Juvenile mussels were placed in containers partially filled with sieved river sediment, providing both a food source and some protection from physical disturbance. Most of the juvenile mussels were found in a loose, flocculent layer of sediment brought into the containers by the river water. Survival and growth of juvenile mussels was best when cultures were started in June eompared to cultures started in August or September, perhaps due to warmer temperatures earlier in the summer.

10.6.3.9 USGS (2005a,b) (**8, 9**) conducted a 28-d feeding study with 2-month-old juvenile *Lampsilis siliquoidea* that compared the influence of various sources of algae, concentrations of algae, and the presence of sediment on survival or growth of juvenile mussels. Juvenile mussels were fed three species of live algae (*Neochloris oleoabundans, Pseudokirchneriella subcapitata (formerly Selenastrum capricornutum)*, or *Nannochloropsis oculata*) at three feeding concentrations or two combinations of commercial Instant Algae[4] brand nonviable microalgae concentrates [*Nannochloropsis* or a combination of *Nannochloropsis* and Shellfish Diet; Reed Mariculture, Campbell, CA]) at three feeding concentrations: *(1)* amount recommended by the food providers, *(2)* two times the recommended amount, and *(3)* three times the recommended amount. The feeding study was conducted in a flow-through system with about 60-mL additional water added to each chamber once every 4 h. Juvenile mussels were fed twice a day right after the addition of the new water. By the end of 28-d experiment, the mean survival (n=2) of controls (no-food or sediment-only) ranged 25 to 35 %. Survival of juvenile mussels fed with various foods at the recommended feeding rates ranged from 70 to 90 %. Higher feeding rates generally did not increase the survival of juvenile mussels. The better survival rates (≥85 %) were observed in feeding treatments with the two microalgae concentrates. The results of this feeding study indicate that 28-d chronic toxicity tests starting with 2-month-old juvenile *L. siliquoidea* might be conducted with a control survival of over 80 % using a diluter system and Instant Algae[4] brand microalgae concentrates. Survival of *Villosa iris* was ≥85 % in a subsequent 28-d feeding study using this combined diet of Instant Algae[4] brand microalgae concentrates (USGS 2005b) (**9**).

10.6.3.10 Water hardness concentrations ranging from 250 to 350 mg/L (as $CaCO_3$) have been shown to support the long-term maintenance of juvenile mussels (Bishop et al 2005) (**47**). Others have found that water hardness concentrations as low as 180 mg/L provide adequate levels of calcium and magnesium to support juvenile and adult survival (Farris et al 1998) (**101**). A daily ration of about 30 000 cells/mL of *Neochloris oleoabundans* or *Nannochloropsis oculata* (small-eelled species with high lipids) provided adequate nutrition for survival and growth of juvenile mussels for several weeks or months (Henley et al 2001 (**98**), Bishop et al 2005 (**47**)). Holding juvenile mussels in recirculating water system provides a continuous assortment of fine sediments used as a food source and provides more consistent water quality compared to static systems. Juvenile mussels held in recirculating systems for several weeks increased in size by 7 to 12-fold from the newly-transformed juvenile mussels (Milam et al 2000) (**60**).

10.6.3.11 Hudson and Isom (1984) (**56**) observed an 18-fold increase in growth of juvenile *Utterbackia imbecillis* mussels held in raceways over a 74-d period using river water supplemented with sediment and plankton under static conditions. Mussels cultured at 30°C exhibited a slight increase in growth compared to mussels cultured at 23°C. Hudson and McKissick (1999) (**102**) raised artificially-transformed juvenile mussels in a static system for 93 d and observed a 10-fold inerease in growth in sediment from the Conasauga River, TN. Although juvenile mussels can survive and grow in static systems, water should be renewed to reduce waste products or the build up of bacteria or fungus (Michaelson and Neves 1995 (**103**), Layzer et al (1993) (**104**). Hanlon (2000) (**105**) reported 82 % survival of juvenile *Lampsilis fasciola* held in concrete raceways for 90 d using recirculating water with sediment fines added as a substrate.

10.6.3.12 Most investigators have observed high mortality of juvenile mussels about 4 to 6 weeks after transformation (as reviewed by Kernaghan et al 2005 (**5**)). As a result of this problem, the duration of toxicity tests started with newly-transformed juvenile mussels is less than 14 d, with survival or growth measured at the end of the exposures (Table A1.4). Food (mixtures of different species of algae) and sediment have been added to test chambers. Some investigators have found that newly-transformed juvenile mussels will survivc for at least 14 d without the addition of food (Table A1.4). Thc high mortality of newly-transformed juvenile mussels in toxicity tests conducted for >14 d is likely related to a lack of an understanding of the nutritional requirements of mussels at this life stage (section 10.5.4).

10.6.3.13 Newly-transformed juvenile mussels depend on pedal-feeding to obtain food (cilia on the foot are used to move food into the juvenile mussel; see 10.1). Juvenile mussels gradually begin to use a combination of pedal- and suspension-feeding to obtain food until the mussels eventually depend on suspension-feeding to obtain food by abont 6 months in

laboratory cultures supplied with a silt-clay sediment substrate. However, in the field, juvenile mussels probably depend on a combination of suspension-, deposit- and pedal-feeding in coarser substrates. Research is ongoing to improve culturing methods for propagation, holding, and feeding of newly-transformed juvenile mussels (Keller and Zam 1990 (**66**); Gatenby et al 1996 (**106**), 1997 (**25**); Henley et al 2001 (**98**); Jones and Neves 2002 (**99**); Jones et al 2004 (**36**); Bishop et al 2005 (**47**)). Once developed, these culturing methods should help to refine methods for conducting chronic exposures with juvenile mussels.

10.6.3.14 Valenti et al (2005) (**107**) conducted toxicity tests starting with 2-month-old juvenile mussels of *Villosa iris* and observed control survival >90 % in 21-d exposures. Juvenile mussels were held in a small amount of sediment and were fed algae (*Neochloris*) and survival and growth were the endpoints. USGS (2005a,b) (**8, 9**) and Bringolf et al (2005) (**108**) conducted toxicity tests starting with 2- to 4-month old juvenile *Actinonaias ligamentina, Lampsilis siliquoidea* or *Villosa iris* and observed control survival >88 % in 21- to 28-d exposures when algae was used as a food source (Table A1.4). The size of the algal cells used to feed the juvenile mussels should meet the dietary requirements of the species (for example, usually <10 µm; Gatenby et al 2003 (**94**)), but can be species specific. The algae should be high in polyunsaturated fats (Gatenby et al 2003) (**94**). Addition of a small amount of sediment substrate improves survival and growth of some species of newly-transformed juvenile mussels (Neves 2004) (**3**).

10.7 *Disease Treatment*:

10.7.1 Whenever adult mussels are brought into a facility, these organisms should be quarantined until use for 14 d or until these organisms appear free of disease and a record of the general health of the mussels should be made at least weekly. If a group of mussels is severely diseased, it is often best to destroy the entire group immediately. Although little is known about diseases of freshwater mussels inhabiting North America, there is a potential for pathogen transmission among mussels and fish (Cope et al 2004) (**76**). Disease transmission between mussels and fish may be particularly problematic when mussel culturing facilities are co-located with fish hatcheries. Cope et al (2004) (**76**) recommend establishing a pathogen and disease monitoring plan for adult mussels similar to approaches used for hatchery-reared fish. For example, Newton et al (2001) (**109**) certified that adult mussels collected from the upper Mississippi River were free of bacterial and viral agents based on inspections conducted by the U.S. Fish and Wildlife Service Fish Disease Control Center in Onalaska, WI.

10.7.2 Zimmerman et al (2003) (**110**) described a procedure for control of predatory flatworms in culturing juvenile mussels. Newly-transformed juvenile mussels did not survive in concentrations of formalin required to kill flatworms. Therefore, Zimmerman et al (2003) (**110**) recommend treatment of host fish with formalin before these fish are used to transform mussels.

10.7.3 Adult mussels collected from the field should be inspected for the presence of zebra mussels (*Dreissena polymorpha*). Soft brushes should be used to remove attached zebra mussels. The adult mussels should be held in a quarantined area for at least one month to determine whether additional zebra mussels are present (Gatenby et al 2000 (**94**), Newton et al 2001 (**109**), Cope et al 2004 (**76**)). The equipment used in mussel cultures suspected to be infested with *D. polymorpha* should be treated with 25 to 250 mg/L hypochlorite and effluent water from the mussel cultures should treated to a concentration of at least 5 mg/L hypochlorite. Additional guidance on handling or control of zebra mussels is describe in:

(1) Gatenby et al (1999b, 2000) (**111, 81**), Newton et al 2001 (**109**) and Cope et al (2004) (**76**)

(2) http://sgnis.org/publicat/papers/zmr_2_06.pdf

(3) http://nas.er.usgs.gov/zebra.mussel/

(4) http://www.clo2.com/reading/Subject_Papers/zebra-mussel-control.htm

(5) http://ag.ansc.purdue.edu/EXOTICSP/732_articles_related_to_ZM.htm

10.8 *Acclimation*—Section A1.4.2.2 provides information of acclimation of test organisms before the start of a toxicity test.

10.9 *Quality*—Section 11 provides information on quality assurance and quality control for the culture and testing of test organisms.

11. Quality Assurance and Quality Control

11.1 *Introduction*:

11.1.1 Developing and maintaining a laboratory Quality Assurance (QA) program requires an ongoing commitment by laboratory management and also includes the following: *(1)* appointment of a laboratory quality assurance officer with the responsibility and authority to develop and maintain a QA program, *(2)* preparation of a Quality Assurance Project Plan with Data Quality Objectives, *(3)* preparation of written descriptions of laboratory Standard Operating Procedures (SOPs) for test organism culturing, testing, instrument calibration, sample chain-of-custody, laboratory sample tracking system, and *(4)* provision of adequate, qualified technical staff and suitable space and equipment to ensure reliable data (Guide E 1391).

11.1.2 Quality Assurance (QA) practices within a testing laboratory should address all activities that affect the quality of the final data, such as: *(1)* sample sampling and handling, *(2)* the source and condition of the test organisms, *(3)* condition and operation of equipment, *(4)* test conditions, *(5)* instrument calibration, *(6)* replication, *(7)* use of reference toxicants, *(8)* record keeping, and *(9)* data evaluation.

11.1.3 Quality Control (QC) practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance, and general guidance on good laboratory practices related to testing, see Guide E 1391 and Test Method E 1706).

11.2 *Performance-based Criteria*:

11.2.1 The USEPA Environmental Monitoring Management Council (EMMC) recommended the use of performance-based methods in developing standards for chemical analytical methods (Test Method E 1706). Performance-based methods were defined by EMMC as a monitoring approach which permits the

use of appropriate methods that meet preestablished demonstrated performance standards. Minimum required elements of performance, such as precision, reproducibility, bias, sensitivity, and detection limits should be specified and the method should be demonstrated to meet the performance standards.

11.2.2 No single method is required for collection or culture of mussels used conduct a toxicity test. Success of a test relies on the health of the culture from which organisms are taken for testing. Having healthy organisms of known quality and age for testing is the key consideration relative to culture methods. Therefore, a performance-based criteria approach is the preferred method through which individual laboratories can evaluate culture health rather than requiring all laboratories to use the same culturing procedure. Performance-based criteria are used in ASTM standards dealing with toxicity testing to allow eaeh laboratory to optimize culture methods while providing organisms that produce reliable and comparable test results (for example, Test Methods E 1367 and E 1706). See Table A1.3 and Table A1.5 in Annex A1 for a listing of performance criteria for culturing and testing of organisms.

11.3 *Facilities, Equipment, and Test Chambers*:

11.3.1 Separate areas must be maintained for eulturing and testing organisms to avoid loss of cultures because of cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemieal analysis laboratories or sample storage and preparation areas into organism culturing or toxicity testing areas, and from toxicity testing laboratories and sample preparation areas into culture areas.

11.3.2 Equipment for temperature eontrol should be adequate to maintain recommended test-water temperatures. Recommended materials should be used in the fabrication of the test equipment whieh comes in contaet with the dilution water (that is, water or sediment).

11.3.3 Before a toxicity test is conducted in a new facility, a "non-contaminant" test should be conducted in which all test chambers contain control water. This information is used to demonstrate that the facility, control water, and handling procedures provide acceptable responses of test organisms.

11.3.4 *Water*—Quality of water ased for organism culturing and testing is extremely important. Water used to conduct toxicity tests and water ased to culture organisms should be uniform in quality. Acceptable water should allow satisfactory survival or growth of the test organisms. Organisms should not show signs of disease or apparent stress (for example, diseoloration, unusual behavior). See Section 8 for additional details.

11.4 *Test Conditions*—Temperatures should be maintained within the limits speeified for each test. Dissolved oxygen, alkalinity, water hardness, conductivity, ammonia, and pH in toxicity tests should be checked in accordance with Annex A1.

11.5 *Quality of Test Organisms*:

11.5.1 Test organisms should appear healthy, behave normally, and have low mortality in cultures, during holding, and in test eontrols (for example, <20 % for 48 h before the start of a javenile mussel toxicity test).

11.5.2 Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CaSO_4$, see 16.4). Data from these reference-toxicant tests can be used to assess genetic strain or life-stage sensitivity of test organisms to select chemicals.

11.5.3 All organisms in a test must be from the same source. The supplier of organisms should also certify the species identification of the organisms, and provide the taxonomic references, or name(s) of the taxonomic expert(s) consulted.

11.6 *Quality of Food*—Problems with the nutritional suitability of the food will be reflected in the survival or growth of the test organisms in cultures or in toxicity tests.

11.7 *Test Acceptability*—Table A1.3 and Table A1.5 in Annex A1 outline requirements for aceeptability of tests. An individual test may be conditionally aceeptable if temperature, dissolved oxygen, and other specified eonditions fall outside specifications, depending on the degree of the departure and the objectives of the toxicity test (see test condition summaries in Table A1.1 and Table A1.4). The acceptability of a test will depend on the experienee and professional judgment of the laboratory analyst and the reviewing staff of the regulatory authority. Any deviation from test specifications should be noted when reporting data from a test.

11.8 *Analytical Methods*:

11.8.1 All routine chemical and physical analyses for culture and testing water, food, and sediment should include established quality assurance practices (Guide E 1391).

11.8.2 Reagent containers should be dated when received from the supplier and the shelf life of the reagent should not be exceeded. Working solutions should be dated when prepared and the recommended shelf life should not be exceeded.

11.9 *Calibration and Standardization*:

11.9.1 Instruments used for routine measurements of chemical and physical characteristics such as pH, dissolved oxygen, temperature, and conductivity should be calibrated before use each day aecording to the instrument manufacturer's procedures as indieated in the general section on quality assurance (see Test Method E 1706 for a listing of USEPA Methods) Calibration data should be recorded in a permanent log.

11.9.2 Known-quality water should be included in the analyses of each batch of water samples (for example, water hardness, alkalinity, conductivity). It is desirable to inelude eertified standards in the analysis of water samples.

11.10 *Replication and Test Sensitivity*—Sensitivity of toxicity tests will depend in part on the number of replicates/treatment, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of a test will inerease as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data (Section 14).

11.11 *Demonstrating Acceptable Performance*:

11.11.1 Before conducting tests with chemicals of interest, it is strongly recommended that the laboratory condact the toxicity test with control water alone. Results of these preliminary studies should be used to determine if the use of the eontrol water and other test conditions result in acceptable performance in the toxicity test as outlined in Annex A1.

11.11.2 Section 16.4 provides a summary of techniques to evaluate acceptable laboratory performance (for example,

reference-toxicity tests, variance associated with intra-laboratory toxicity tests, variance associated with inter-laboratory toxicity tests). Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CuSO_4$, see 16.4).

11.12 *Record Keeping*—Section 14.1 outlines recommendations for recorded keeping (that is, data files, chain-of custody).

12. Experimental Design

12.1 Decisions concerning such aspects of experimental design as the dilution factor, number of treatments, and numbers of test chambers and organisms per treatment should be based on the purpose of the test and the type of procedure that is to be used to calculate results (Section 14). One of the following two types of experimental design will probably be appropriate in most cases.

12.1.1 A toxicity test intended to allow calculation of an LC50, EC50, IC50, NOEC, or LOEC usually consists of one or more control treatments and a geometric series of at least five concentrations of test material. In the dilution-water or solvent control(s), or both (section 9.3), organisms are exposed to dilution water to which no test material has been added. Except for the control(s) and the highest concentration, each concentration should be at least 50 to 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.5 to 0.6, five properly chosen concentrations will often provide LC50s, EC50s, IC50s, NOECs, and LOECs for several durations (Annex A1) and are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of toxicity is particularly uncertain (section 9.3), six or seven concentrations might be desirable. If it is desirable to provide extensive information concerning the dependence of adverse effects on time or concentration, or both, seven or more appropriately spaced concentrations might be desirable to cover the range from effects on almost all organisms at quite short times to effects on few organisms at quite long time.

12.1.2 If it is only necessary to determine *(a)* whether a specific concentration is acutely toxic to the test species or *(b)* whether the LC50, EC50, or IC50 is above or below a specific concentration (section 9.3), only that concentration and the control(s) are necessary. Two additional concentrations at about one half and two times the specific concentration of concern are desirable to increase confidence in the results.

12.1.3 If an endpoint near the extremes of toxicity, such as an LC5 or LC95, is to be calculated, at least one concentration of test material should have killed or affected a percentage of test organisms, other than 0 or 100 %, near the percentage for which the LC, EC, or IC is to be calculated. This requirement might be met in a test to determine an LC50, EC50, or IC50, but a special test with appropriate test concentrations and more test organisms per treatment will usually be necessary.

12.2 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned. Because test solution can flow from one compartment to another, but not from one test chamber to another (section 6.5), the test chamber is the experimental unit. As the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom increases and, therefore, the width of the confidence interval on a point estimate decreases and the power of a hypothesis test increases. With respect to factors that might affect results within the test chambers and the results of the test, all chambers in the test should be treated as similarly as practical. For example, the temperature in all test chambers should be as similar as practical unless the purpose of the test is to study the effect of temperature. Test chambers are usually arranged in one or more rows. Treatments must be randomly assigned to individual test chamber locations and may be randomly reassigned during the test. A randomized block design (with each treatment being present in each block, which may be a row or a rectangle) is preferable to a completely randomized design.

12.3 The minimum desirable number of test chambers and organisms per treatment should be calculated from *(a)* the expected variance within test chambers, *(b)* the expected variance between test chambers within a treatment, and *(c)* the maximum acceptable width of the confidence interval on the LC50, EC50, or IC50 (Guide E 729). Organisms in each treatment should be divided between two or more test chambers in order to allow estimation of experimental variation. If the controls are important in the calculation of results, such as because of correction for spontaneous mortality using Abbott's formula or because the results are calculated as a percent reduction from the controls, it might be desirable to use more test chambers and test organisms for the control treatment(s) than for each of the other treatments (Guide E 729).

12.4 The shape of the concentration-effect curve is critical for the determination of time-independent toxicity levels, and observations of dead and affected organisms should be with sufficient frequency to facilitate the estimation of a time-independent value, either directly or mathematically. Depending on the objectives of the test, a design should be selected that includes sufficient observations to determine the desired endpoint. If regulatory or cost factors are a consideration, observations may be made in acute toxicity tests at 24, 48, and 96 h or as stipulated by the regulatory guideline. Depending on the shape of the toxicity curve, more observations will typically be desirable (for example, 3, 6, 12, and 24 h and twice daily thereafter) to provide a sound measurement of a time-independent toxicity value. For chronic toxicity tests, ideally, survival should be measured weekly during the exposures. It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results.

13. Analytical Methodology

13.1 If samples of dilution water, stock solutions, or test solutions cannot be analyzed immediately, the samples should be handled and stored to minimize loss of test material by microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

13.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other

reliable sourees (Guide E 729). The concentration of unionized ammonia may be calculated from the pH, temperature, and coneentration of total ammonia (Guide E 729).

13.3 Methods used to analyze food or test organisms for chemicals of interest should be obtained from appropriate sources (Guide E 729).

13.4 The precision and bias of each analytical method used should be determined in an appropriate matrix, for example, in water samples from a control test chamber or brood-stock tank, in food, and in test organisms. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

14. Calculation of Results

14.1 *Data Recording*—Quality assurance project plans with data quality objectives and standard operating procedures should be developed before starting a test. Procedures should be developed by each laboratory to verify and archive data (Guide E 1391). A file should be maintained for each toxicity test or group of tests on closely related samples. This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the toxicity test(s); chemical analysis data on the sample(s); control data sheets for reference toxieants; detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the culibration of equipment and instruments; test eonditions used; and results of reference-toxicant tests. Original data sheets should be signed and dated by the laboratory personnel performing the toxicity tests and archived. Electronic copies of data should also be archived.

14.2 *Data Analysis*:

14.2.1 *Introduction*—The goals of statistical analysis are to summarize, display, quantify, and provide objective yardsticks for assessing the structure, relations, and anomalies in data (Guide E 1241). The data display and statistical techniques most commonly used to achieve these goals are *(a)* preliminary and diagnostic graphical displays, *(b)* pairwise comparison techniques such as t-tests and 2 by 2 contingency table tests, *(c)* analysis of variance (ANOVA) and corresponding contingency table tests, *(d)* multiple comparison techniques for simultaneous pairwise comparison of other treatment groups with control groups, *(e)* concentration-effect curve analyses, and *(f)* multiple regression. If used correctly, each of these techniques can provide useful information about the results of an acceptable toxicity test. The three kinds of data that can be obtained from toxicity tests are dichotomous or categorical (for example, mortality), and continuous (for example, length or weight). Statistical methods for analyzing dichotomous and other categorical data are directly analogous to those for analyzing count and continuous data. However, for technical reasons and because they arose from different application areas, different terminologies and computing tools were developed for analyzing the three kinds of data.

14.2.2 *Endpoint*—The endpoint determined in toxicity tests generally has been defined in terms of whether differences from control organisms are statistically significant at the 5 % level (that is, analysis of variance followed by mean separation; Guide E 1241). One of the main coneeptual problems with sueh a definition of the endpoint is that the notions of biological importanee and statistical significance are logically distinct. Effeets of considerable biological importance might not be statistically significant if sample sizes are small or if effects are extremely variable or both. Conversely, biologically trivial effects might be highly statistically significant if sample sizes are large or effects are very reproducible. An endpoint based solely on statistical significance might depend as much or more on sample sizes as on the magnitudes of the effeets. An alternative is to define the endpoint in terms of a specified absolute or relative amount of difference in a biological attribute from the control treatment(s). A regression-type model would be fitted to the data and the eoncentration associated with a specified amount of difference from the control treatment(s) would be estimated using the model. For example, the coneentration resulting in a speeified percent decrease in survival or shell length might be estimated along with confidence limits on the estimated concentration. The result of a toxicity test would then be reported as a point estimate, preferably with confidence limits, of the concentration expected to cause an amount of effect that had been pre-selected as being biologically unacceptable.

14.2.2.1 In general, an endpoint defined in terms of a statistically significant difference is calculated using analysis of variance, contingency tables, or other hypothesis testing procedures. An endpoint defined in terms of a specified amount of effect is calculated using regression analysis, concentration-effect curve analysis, and other point estimation procedures. Regardless of the procedure used, sufficient data should be presented in reports to permit calculation of endpoints other than those chosen by the investigator and to ullow other uses, such as modeling.

14.2.3 For each set of data the LC50, EC50, IC50 and its 95 % confidence limits or NOEC and LOEC should be calculated on the basis of *(a)* the measured initial concentrations of test material, if available, or the calculated initial concentrations for static tests, and *(b)* the average measured concentrations of test material, if available, or the calculated average concentrations for flow-through tests. If other LCs, ECs, or ICs are calculated, their 95 % confidence limits should also be calculated (Guide E 729, Guide E 1241).

14.2.4 Most acute toxicity tests produce quantal or dichotomous data, that is, counts of the number of organisms in two mutually exclusive categories, such as alive or dead. A variety of methods summarized in Guide E 729 and Test Method E 1706 can be used to calculate an LC50 or EC50 and its 95 % confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent dead or affected is between 0 and 100. The method used should appropriately take into account the number of test chambers per treatment and the number of test organisms per chamber. When fewer than two concentrations kill or affect between 0 and 100 %, the binomial test can usually be used to obtain statistically sound information about the LC50 or EC50. The binomial test does not provide a point estimate of the LC50 or EC50, but it does provide a range within which the

LC50 or EC50 should lie. If desired, an interpolation procedure may be used to obtain an approximate LC50 or EC50.

14.2.5 Although they generally require more effort to obtain, quantitative data on individual organisms, such as time-to-death or shell length, contain more information per organism than do quantal data. Quantitative data can usually be analyzed to calculate an IC50. For each test chamber in each treatment other than the control treatment(s), the percent inhibition (*%I*) should usually be calculated as follows:

$$\%I = 100(M - X) / M \quad (1)$$

where:

M = average value for the control test chambers, and
X = value for a test chamber in any other treatment.

14.2.5.1 The *%I* for each test chamber should be plotted against the corresponding concentration of test material after transformation of *%I* or concentration, or both, if appropriate. The IC50 ean then be obtained from a line of best fit by determining the concentration eorresponding to $\%I = 50$. If possible, the 95 % confidence limits on the IC50 should be calculated, appropriately taking into account the number of test chambers per treatment, the number of test organisms exposed in each chamber, the range of concentrations tested, and the variance within each treatment, especially in the controls. Alternatively, an appropriate linear or nonlinear inverse regression technique can be used to calculate the IC50 and its 95 % confidence limits (Guide E 729). If the percent inhibition covers an appropriate range, such as at least 37 to 63 %, a variety of regression models will usually give nearly the same IC50 from a set of data. However, only the correct model, which is not known to be available at this time, will appropriately take into account the variance between the test chambers in the control treatment(s) and give the eorrect confidenee limits.

14.2.6 The values for X may be plotted against the corresponding concentrations of test material, after transformation of X or concentration, or both, if appropriate, and the IC50 determined by graphical or statistical interpolation to the concentration of test material at which a line of best fit = $M/2$.

14.2.7 An endpoint near an extreme of toxicity, such as an LC5 or LC95, should not be calculated unless at least one concentration of test material killed or affected a percentage of test organisms, other than 0 or 100 %, near the percentage for whieh the LC, EC, or IC is to be ealculated. Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of test material that actually killed or affected no greater a percentage of the test organisms than did the control treatment(s) or to report the lowest concentration of test material that actually killed or affected all test organisms exposed to it. These alternatives are usually more reliable than reporting a ealculated result such as an LC5 or LC95 unless several percent killed or affected were obtained close to 5 or 95 %.

14.2.8 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material killed or affected a statistically significant number of the exposed organisms. If a hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures and tests of heterogeneity. Then a pair wise eomparison teehnique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. Presentation of results of each hypothesis test should inelude the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test. See Guide E 1241, Practice E 1847, and Test Method E 1706 for additional detail on hypothesis testing.

15. Report

15.1 The record of the results of an acceptable toxicity test should include the following information either directly or by referencing available documents:

15.1.1 Name of test and investigator(s), name and location of laboratory, and dates of start and end of test.

15.1.2 For sediment testing, source of control or test sediment, method for collection, handling, shipping, storage, and disposal of sediment.

15.1.3 Source of test material, lot number if applicable, composition (identities and concentrations of major ingredients and impurities if known), known chemical and physical properties, and the identity and concentration(s) of any solvent used.

15.1.4 Source and characteristics of dilution water, description of any pretreatment, and results of any demonstration of the ability of an organism to survive or grow in the water.

15.1.5 Source, history, and age of test organisms; culture procedures; and source and date of collection of organisms from the field, scientific name, name of person who identified the organisms and the taxonomic key used, age or life stage, means and ranges of shell length, observed diseases or unusual appearance, treatments, holding, and acclimation procedures.

15.1.6 Source and composition of food, concentrations of test material and other contaminants, procedure used to prepare food, feeding methods, frequency, and ration.

15.1.7 Description of the experimental design and test chambers, volume water in the chambers, lighting, number of test chambers and number of test organisms/treatment, date and time test starts and ends, temperature measurements, dissolved oxygen concentration (as percent saturation), and any aeration used before starting a test and during the conduct of a test.

15.1.8 Methods used for physical and ehemical characterization of water or sediment samples.

15.1.9 Definition(s) of the effects used to calculate LC50 or EC50s, biologieal endpoints for tests, and a summary of general observations of other effects.

15.1.10 Methods used for statistical analyses of data: *(a)* summary statistics of the transformed or raw data as applicable (for example, mean, standard deviation, coefficient of variation, precision and bias); *(b)* hypothesis testing (raw data, transformed data, null hypothesis, alternate hypothesis, target Type I and II error rates, statistics used (including calculation of test statistic)), decision rule used (for example, approach used to establish the rejection of the null hypothesis), calculated test statistic and decision rule result, achieved Type I and II error rates (for some discrete tests, achieved error rates only approximate the target rates); *(c)* results of regression analyses

(parameters of regression fit, uncertainty limits on the regression parameters, correlation coefficient).

15.1.11 Summary of general observations on other effects or symptoms.

15.1.12 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

15.2 Published reports should contain enough information to clearly identify the methodology used and the quality of the results.

16. Precision and Bias

16.1 *Determining Precision and Bias*:

16.1.1 Precision is a term that describes the degree to which data generated from replicate measurements differ and reflects the closeness of agreement between randomly selected test results. Bias is the difference between the value of the measured data and the true value and is the closeness of agreement between an observed value and an accepted reference value (Practices E 177 and E 691). Quantitative determination of precision and bias in toxicity testing of aquatic organisms is difficult or may be impossible in some cases, as compared to analytical (chemical) determinations. This is due, in part, to the many unknown variables which affect organism response. Determining the bias of a toxicity test using field samples is not possible since the true values are not known. Since there is no acceptable reference material suitable for determining the bias of toxicity tests, bias of the procedures described in this standard has not been determined (section 16.2).

16.1.2 Toxicity tests exhibit variability due to several factors. Test variability can be described in terms of two types of precision, either single laboratory (intra-laboratory or repeatability; see 16.5.1) precision or multi-laboratory (inter-laboratory or reproducibility; see 16.5.2) precision (also referred to as round-robin or ring tests). Intra-laboratory precision reflects the ability of trained laboratory personnel to obtain consistent results repeatedly when performing the same test on the same organism using the same toxicant. Inter-laboratory precision is a measure of how reproducible a method is when conducted by a large number of laboratories using the same method, organism, and toxic sample. Generally, intra-laboratory results are less variable than inter-laboratory results (Test Method E 1706).

16.1.3 A measure of precision can be calculated using the mean and relative standard deviation, or percent coefficient of variation (CV % = standard deviation/mean × 100) of the calculated endpoints from the replicated endpoints of a test. However, precision reported as the CV should not be the only approach used for evaluating precision of tests and should not be used for the no-observed-effect concentrations (NOECs) derived from statistical analyses of hypothesis testing. The CVs may be very high when testing extremely toxic or nontoxic samples. For example, if there are multiple replicates with no survival and one with low survival the CV may exceed 100 %, yet the range of response is actually quite consistent. Therefore, additional estimates of precision should be used, such as range of responses and minimum detectable differences (MDD) compared to control survival or growth (Test Method E 1706). Several factors can affect the precision of the test, including test organism age, condition, sensitivity, handling, and feeding of the test organisms, overlying water quality, and the experience in conducting tests. For these reasons, it is recommended that trained laboratory personnel conduct the toxicity tests in accordance with the procedures outlined in Annex A1. Quality assurance practices should include: *(a)* single laboratory precision determinations that are used to evaluate the ability of the laboratory personnel to obtain precise results using reference toxicants for each of the test organisms and *(b)* preparation of control charts (Figure 16 in Test Method E 1706) for each reference toxicant and test organism. The single laboratory precision determinations should be made before conducting routine toxicity tests.

16.1.4 Intra-laboratory precision data are routinely calculated for test organisms using water-only acute exposures to a reference toxicant such as NaCl or $CuSO_4$. Intra-laboratory precision data should be tracked using a control chart. Each laboratory's reference-toxicant data will reflect conditions unique to that facility, including dilution water, culturing, and other variables (Section 11). However, each laboratory's reference toxicant CVs should reflect good repeatability.

16.1.5 Results of one intra-laboratory toxicity study and one inter-laboratory (round-robin) study using 24 and 48-h toxicity tests with glochidia and 48 and 96-h toxicity tests with juvenile mussels are reported in section 16.5.

16.2 *Bias*—Bias of toxicity tests cannot be determined since there is no acceptable reference material. The bias of the reference-toxicant tests can only be evaluated by comparing test responses to control charts.

16.3 *Replication and Test Sensitivity*—Sensitivity of toxicity tests will depend in part on the number of replicates per concentration, the probability levels (alpha and beta), and the type of statistical analysis. For a specific level of variability, the sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data (Section 14).

16.4 *Demonstrating Acceptable Laboratory Performance*:

16.4.1 Subsamples of each batch of test organisms used in toxicity tests should be evaluated using a reference toxicant (for example, NaCl or $CuSO_4$). Bringolf et al (2005) (**108**) reported 24-h EC50s ranging from 0.55 to 3.3 g NaCl/L for glochidia of five species of mussels and 96-h EC50s ranging from 4.0 to 6.3 g NaCl/L for 5 species of juvenile mussels in reference-toxicity tests. USGS (2005b) reported 24-h EC50s ranging from 10 to >100 µg Cu/L for glochidia of 11 species of mussels and 96-h EC50s ranging from 6.8 to 60 µg Cu /L for 7 species of juvenile mussels in reference-toxicity tests (hardness 170 mg/L as $CaCO_3$). Test conditions for conducting reference toxicity tests should follow the recommended conditions for conducting toxicity tests with glochidia outlined in Table A1.1 and with juvenile mussels outlined in Table A1.4.

16.4.2 Intra-laboratory precision, expressed as a coefficient of variation (CV), of the range for each type of test to be used in a laboratory can be determined by performing multiple toxicity tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (for example, the same test duration, type

of water, age of test organisms, feeding), and same data analysis methods. A reference-toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations of the test chemical.

16.4.3 A control chart can be prepared for each combination of reference toxicant and test organism. Each control ehart should include the most current data. Endpoints from five tests are adequate for establishing the control charts. In this technique, a running plot is maintained for the values (X_i) from successive tests with a given reference toxicant (Figure 16 in Test Method E 1706), and the endpoint (LC50, NOEC, ICp) are examined to determine if these endpoints are within prescribed limits. Control charts as described in Test Method E 1706 are used to evaluate the cumulative trend of results from a series of samples. The mean and upper and lower control limits (±2 SD) are recalculated with each successive test result.

16.4.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified using control charts. With an alpha of 0.05, one in 20 tests would be expected to fall outside of the control limits by chance nlone. If 2 of 20 reference-toxicant tests fall outside the control limits, the toxicity tests conducted during the time in which the second reference-toxicant test failed are suspect, and should be considered as provisional and subject to careful review.

16.4.5 A toxicity test may be acceptable if specified conditions of a reference-toxicant test fall outside the expected ranges. Specifically, a toxicity test should not be judged unacceptable if the LC50 for a given reference-toxicant test falls outside the expected range or if control survival in the reference-toxicant test is less that the acceptability requirement outlined in Annex A1. All the performance criteria outlined in Annex A1 should be considered when determining the acceptability of a toxicity test. The acceptability of the toxicity test would depend on the experience and judgment of the investigator and the regulatory authority.

16.4.6 If the value from a given test with the reference toxicant falls more than two standard deviation (SD) outside the expected range, the sensitivity of the organisms and the overall credibility of the test system may be suspect (Test Method E 1706). In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

16.4.7 Performance should improve with experience, and the control limits for point estimates should gradually narrow. However, control limits of ±2 SD, by definition, will be exceeded 5 % of the time, regardless of how well a laboratory performs. Highly proficient laboratories which develop a very narrow control limit may be unfairly penalized if a test which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not an outlier is to be rejected. This determination should be made by the regulatory authority evaluating the data.

16.4.8 The recommended reference-toxicant test consists of a control and five or more concentrations in which the endpoint is an estimate of the toxicant concentration which is lethal to 50 % of the test organisms in the time period prescribed by the test. The LC50 is determined by an appropriate procedure, such as the trimmed Spearman-Karber Method, Probit Method, Graphical Method, or the Linear Interpolation Method (Section 14 and Test Method E 1706).

16.4.9 The point estimation unalysis methods recommended in this standard have been chosen primarily because point estimates are well-tested, well-documented, and are applicable to most types of test data. Many other methods were considered in the selection process, and it is recognized that the methods selected are not the only possible methods of analysis for toxicity data.

16.5 *Precision of Toxicity Tests Conducted with Glochidia or Juvenile Mussels*:

16.5.1 *Intra-laboratory Precision*—Table 3 summarizes the results of intra-laboratory toxicity tests condueted with gloehidia of *Actinonaias ligamentina* and *Lampsilis siliquoidea* (USGS 2004) (**112**) and juvenile mussels of *L. siliquoidea* (USGS 2005b (**9**)). Test conditions for conducting the toxicity tests with glochidia were in aceordance with the recommended test conditions outlined in Table A1.1 and all of the toxieity tests met the test acceptability requirements outlined in Table A1.3 (**112**). The dilution water was reconstituted hard water (160-180 mg/L us $CaCO_3$; Guide E 729). Survival of glochidia (based on valve closure in response to a solution of NaCl) was measured at 24 and 48 h. Survival of juvenile mussels (based on movement of the foot) was measured at 48 and 96 h. The variability of EC50s for glochidia toxicity tests conducted with copper, ammonia, or chlorine over two exposure periods, expressed as the coefficient of variation (CV), ranged between 13 and 36 % for toxicity tests conducted with glochidia of *A. ligamentina* and between 15 and 38 % for toxicity tests conducted with glochidia of *L. siliquoidea* (Table 3). The variability of EC50s for toxicity tests conducted with juvenile mussels and copper at 48 and 96 h, expressed as the

TABLE 3 Intra-laboratory Precision of EC50s (expressed as the Coefficient of Variation; CV) from Toxicity Tests with Glochidia or Juveniles of *Actinonaias ligamentina* or *Lampsilis siliquoidea* (USGS 2004, 2005b) (9)(112)

Test Organism	Life Stage	Exposure Duration	Copper (µg/L)			Ammonia (mg NIL)[A]			Chlorine (µg/L)		
			N	EC50	CV(%)	N	EC50	CV(%)	N	EC50	CV(%)
A. ligamentina	Glochidia	24 h	4	53	25	4	8	25	3	91	17
A. ligamentina	Glochidia	48 h	4	26	22	4	5	36	3	47	13
L. siliquoidea	Glochidia	24 h	6	35	15	5	13	20	5	77	27
L. siliquoidea	Glochidia	48 h	6	23	25	5	11	20	5	66	38
L. siliquoidea	Juvenile	48 h	4	40	26						
L. siliquoidea	Juvenile	96 h	4	22	13						

[A] At about pH 8.3

TABLE 4 Inter-laboratory Precision of EC50s in Copper Toxicity Tests (µg Cu/L and 95 % Confidence Intervals) with Glochidia and Juveniles of *Lampsilis siliquoidee* (USGS 2004) (112)

Lab	Glochidia		Juvenile	
	24-h EC50	48-h EC50	48-h EC50	96-h EC50
1. CERC	29 (28-31)	13 (12-14)	29 (23-36)	18 (15-22)
2. NCSU	33 (32-35)	24 (22-25)	48 (40-59)	18 (16-20)
3. OSU	27 (25-29)	26 (24-28)	47 (40-54)	41 (35-47)
4. UMESC	38 (35-41)	21 (20-23)	34 (26-45)	21 (17-25)
5. WSLH	32 (31-34)	20 (19-21)	36 (24-54)	19 (12-30)
Mean EC50 (µg/L)	32	21	39	23
SD	4.2	5.0	8.3	9.9
Coefficent of variation (%)	13	24	22	42
H/L EC50	1.4	2.0	1.7	2.3

CV, ranged from 13 to 26% (Table 3). These measures of intra-laboratory precision were similar to previous measures of intra-laboratory precision for tests conducted using commonly-tested species and reference toxicants (i.e., Lewis and Weber 1985, USEPA 1993,**113,114**).

16.5.2 *Inter-laboratory Precision*—Table 4 summarizes the results of an inter-laboratory toxieity test conducted with glochidia and juvenile mussels of *Lampsilis siliquoidea* (USGS 2004) (**112**). Test conditions for conducting the toxicity tests with glochidia were in accordance with the recommended test conditions outlined in Table A1.1 and test conditions for conducting the toxicity tests with juvenile mussels were in accordance with the recommended test conditions outlined in Table A1.4. Survival of glochidia (based on valve closure in response to a solution of NaCl) was measured at 24 and 48 h. Survival of juvenile mussels (based on movement of the foot) was measured at 48 and 96 h. The dilution water was reconstituted hard water (160-180 mg/L as $CaCO_3$; Guide E 729). One laboratory prepared the dilution water, the high concentration of test water, and supplied each laboratory with the testing equipment. A separate facility produced the glochidia (about <24-h old at the start of the toxicity tests) and juvenile mussels (about 4-d old at the start of the toxicity tests). Test organisms were shipped overnight at about 10°C to five laboratories participating in the inter-laboratory toxicity test. The testing laboratories included 2 federal faeilities and 3 university facilities. All of the laboratories met the test acceptability requirements outlined in Table A1.3 for glochidia toxicity tests and met the test acceptability requirements outlined in Table A1.5 for juvenile mussel toxicity tests (USGS 2004) (**112**). Control survival across all of the testing laboratories was >92 % at 24 and 48 h in the glochidia toxicity tests and was >95 % at 48 and 96 h in the juvenile toxicity tests. The variability of EC50s for glochidia, expressed as the CV, was 13 % for the 24-h EC50s and was 24 % for the 48-h EC50s (Table 4). The variability of EC50s for juvenile mussels, expressed as the CV, was 22 % for the 48-h EC50s and was 42 % for the 96-h EC50s (Table 4). The ratio of the high to low EC50 was less than 2.3 for all of the toxicity tests conducted. These measures of inter-laboratory precision in glochidia or juvenile mussel toxicity tests were similar to the variation reported for previous inter-laboratory studies in water-only exposures (for example, Lewis and Weber 1985, USEPA 1993 (**113,114**)) or in sediment exposures, (for example USEPA 2000 (**115**), Test Method E 1706) using eommonly-tested organisms.

17. Keywords

17.1 acute toxicity test; bivalve; chronic toxicity test; freshwater; gloehidia; juvenile mussels; Margaritiferidae; Margaritiferid mussels; mollusc; mollusk; mussels; sediment; Unionidae; Unionid mussels; Unionoidea

ANNEX

(Mandatory Information)

A1. GUIDANCE FOR CONDUCTING WATER-ONLY TOXICITY TESTS WITH EARLY LIFE STAGES OF FRESHWATER MUSSELS

A1.1 *Significance*

A1.1.1 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)). Habitat alteration, introduction of exotic species, over-utilization, disease, predation and pollution are considered causal or contributing factors in many areas of the United States (Neves et al 1997) (4). Numerous laboratory toxicity studies have been conducted with freshwater mussels in an attempt to understand the role of contaminants in the decline of mussel populations in the field. Kernaghan et al (2005) (5) provides a review of over 75 toxicity studies conducted with a variety of freshwater species of mussels and contaminants in laboratories worldwide. Three critical life stages (glochidia, juvenile mussels, and adults) have been used in these toxicity assessments. Toxicity studies are separated according to the medium of exposure (water, sediment, and host fish; Kernaghan et al 2005 (5)). In these studies, early life stages of mussels of several species are highly sensitive to some metals and ammonia in water exposures when compared to many of the most sensitive species of other invertebrates, fish, or amphibians that are commonly used to establish U.S. Environmental Protection Agency Water Quality Criteria (WQC; Augspurger et al 2003 (6), Keller et al 2005 (7); USGS (2005a,b)(8, 9) section 1.5). Importantly, results of these previous studies indicate WQC for individual chemicals established for the protection of aquatic organisms may not be adequately protective of sensitive stages of freshwater mussels.

A1.1.2 Short-term 24-h exposures with glochidia may be useful for screening of chemicals, but response of juvenile mussels may be more ecologically relevant (A1.4.2,A1.5.2, and A1.5.3). Use of glochidia to screen the relative sensitivity of a particular mussel species to chemicals would be particularly useful when evaluating species where only a limited number of adult mussels are available for methods development or for generating juvenile mussels for toxicity testing. Moreover, the host fish for some species of mussels or techniques for transforming juvenile mussels in the laboratory may be unknown for some species.

A1.1.3 In the field, mussels may be exposed to contaminants in water, sediment, or food. Annex A1 only addresses effects associated with exposure of mussels to contaminants in water.

A1.1.4 Sections 12 and A1.5 provide guidance on experimental design of toxicity tests with glochidia or juvenile mussels. Section A1.2 provides guidance for conducting water-only toxicity tests with glochidia isolated from adult mussels. Section A1.3 provides guidance for conducting water-only toxicity tests with juvenile mussels. Refinement of these methods may be described in future versions of this standard after additional laboratories have used these methods (section A1.5). Results of tests using procedures different from the procedures described in section A1.2 or A1.3 may not be comparable. Comparisons of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting toxicity tests with aquatic organisms. If tests are conducted with procedures different from the procedures described in this standard, additional tests are required to determine comparability of results (section 1.4).

A1.2 *Test Conditions for Conducting Water-only Toxicity Tests with Glochidia of Freshwater Mussels*

A1.2.1 Test conditions used by investigators to conduct toxicity tests with glochidia are summarized in Table A1.1. Selection of specific test conditions and decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water-quality characteristics should be based on the purpose of the test and the methods of data analysis (Sections 12 and 16). When variability remains constant, the statistical sensitivity of a test increases as the number of replicates increase.

A1.2.2 Table A1.1 also provides a list of recommended test conditions for conducting toxicity tests with glochidia. The list of recommended test conditions is based on the various methods outlined in Table A1.1 and is based on the conditions used to conduct an inter-laboratory toxicity test with glochidia (section 16.5). Toxicity tests with glochidia should be conducted at 20°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lux (Table A1.1). Toxicity tests are typically started within 2 h after glochidia are isolated from the gills of the female mussels; however, some toxicity tests have been started with glochidia isolated from female mussels for about 24 h before the start of a toxicity test. The endpoint measured in toxicity tests with glochidia is survival (viability) as determined by the response of organisms to the addition of a solution of NaCl (KCl has also been previously been used, but this standard recommends use of NaCl in order to have more consistency between laboratories). Glochidia that close their valves with the addition of a salt solution are classified as alive (viable) in a toxicity test. For most species, the duration of a toxicity test conducted with glochidia should be up to 24 h with survival measured at 6 and 24 h. Control survival is typically

>90 % at the end of 24-h toxicity tests conducted with glochidia. Longer duration toxicity tests with glochidia (for example, 48 h) can be conducted as long as control survival >90 % is achieved. Toxicity tests conducted for >24 h with glochidia might be used for species for which juvenile mussels are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates).

A1.2.3 Glass test chambers should be used to conduct toxicity tests with glochidia. Test chambers should be a minimum of volume of 100 mL containing a minimum of 75 mL of dilution water. Static, renewal, or flow-through conditions can be used depending on the chemical being tested. Glochidia are not fed during the toxicity test and aeration of dilution water is not necessary unless dissolved oxygen is below acceptable concentrations (section A1.4.9.3). Dilution water should be a source of water that has been demonstrated to support survival of glochidia for the duration of the toxicity test. For site-specific evaluations, the characteristics of the dilution water should be as similar as possible to the site of interest.

A1.2.4 The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. A minimum of 3 replicates should be tested, each replicate containing about at least 500 glochidia (preferably 1000 glochidia/replicate if survival is to be evaluated in subsamples of glochidia collected during the toxicity test). Survival can be determined throughout the toxicity test by subsampling each replicate (for example, by subsampling about 100 glochidia at 6 and 24 h and then placing these organisms into one well of a multi-well plate to determine survival with the addition of a salt solution; Wang et al 2003 (85) and A1.4.8.4). Water-quality characteristics of the dilution water (dissolved oxygen, pH, ammonia, hardness, alkalinity, and conductivity) should be measured at the start and end of the exposures in at minimum the high and medium test concentrations and in the control. Requirements for test acceptability for toxicity tests conducted with glochidia are summarized in Table A1.3.

A1.2.5 Toxicity tests with glochidia have been conducted for up to 144 h, but 24 and 48-h exposures are most often used (Table A1.1). The relatively short duration of toxicity tests with glochidia is based on the relatively short duration between release of glochidia into the water column and encystment on the host and is based on the relatively short survival time of glochidia after isolation from the female mussel (Table A1.2). If the life history of a particular species is not known (for example, the host required for encystment or how long glochidia released from a female mussel can remain in the water column before encysting on a host), it might be appropriate to conduct toxicity tests with glochidia for longer than 24 h as long as 90 % control survival can be achieved at the end of the test.

A1.2.6 The time between the release of glochidia from the marsupium of the female mussel to attachment of these glochidia on a host may only take a few seconds for some species (10.1.4), but hours are required for the gill tissue of a fish to migrate to form a cyst around the glochidia. During that time, the glochidia may be exposed to water-borne toxicants. Many anodontinae species release glochidia into water column that remain viable for days before infesting a host fish. Therefore, a prolonged glochidial test would have ecological relevance for these species. Other species release glochidia in mucus strands that coat the bottom or remain suspended on vegetation, waiting for their hosts to swim by and still other species release glochidia packaged in conglutinates that serve as a lure to host fish. Hence, glochidia of these species may also be in water for extended periods of time; however, it is not known how exposure to water-borne contaminants would be influenced by the mucus or conglutinate surrounding the glochidia. Toxicity tests conducted for 24 h with glochidia may not be as ecologically relevant in some cases as toxicity tests conducted with juvenile mussels, but may be useful for some purposes such as deriving concentrations of a chemical that may be protective of the species. Use of glochidia to evaluate the relative sensitivity of a particular mussel species to chemicals would be particularly useful when evaluating species where only a limited number of adult mussels are available for methods development or a limited number of adults are available for producing juvenile mussels for toxicity testing. Moreover, the host fish for some species of mussels or techniques for transforming juvenile mussels in the laboratory may be unknown.

A1.3 *Test Conditions for Conducting Water-only Toxicity Tests with Juvenile Freshwater Mussels*

A1.3.1 Test conditions used by investigators to conduct toxicity tests with juvenile mussels are summarized in Table A1.4. Selection of specific test conditions and decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water-quality characteristics should be based on the purpose of the test and the methods of data analysis (Sections 12 and 14). When variability remains constant, the statistical sensitivity of a test increases as the number of replicates increase.

A1.3.2 Table A1.4 also provides a list of recommended test conditions for conducting toxicity tests with juvenile mussels. The list of recommended test conditions is based on the various methods outlined in Table A1.4 and is based on the conditions used to conduct an inter-laboratory toxicity test with juvenile mussels (section 16.5). Toxicity tests with juvenile mussels should be conducted at 20°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lux (Table A1.4). Toxicity tests are typically started with newly-transformed juvenile mussels <5 d after release from the host; however, some toxicity tests have been started with 2- to 4-month-old juvenile mussels. Acute toxicity tests with juvenile mussels are typically conducted for 96 h with survival measured at 48 and 96 h. Chronic toxicity tests started with 2- to 4-month-old juvenile mussels have been conducted for 21 to 28 d with measures of survival (based on movement of the foot) and growth (based on shell length). Control survival is typically >90 % at the end of 96-h toxicity tests conducted with juvenile mussels and is

typically >80 % at the end of toxicity tests conducted for 10 to 28 d with juvenile mussels (Table A1.4).

A1.3.3 In acute static tests, glass test chambers should be a minimum of volume of 50 mL containing a minimum of 30 mL of dilution water. In chronic tests or in flow-through tests, glass chambers should be a minimum volume of 300 mL containing a minimum volume of 200 mL of dilution water. Static, renewal, or flow through conditions can be used depending on the chemical being tested. Juvenile mussels are not typically fed during acute toxicity tests. Algae have been used as a food source in toxicity tests conducted for 10 to 28 d. Aeration of dilution water is not necessary unless dissolved oxygen is below acceptable concentrations (section A1.4.9.3). Dilution water should be a source of water that has been demonstrated to support survival of juvenile mussels for the duration of the toxicity test. For site-specific evaluations, the characteristics of the dilution water should be as similar as possible to the site of interest.

A1.3.4 The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. In 96-h toxicity tests, a minimum of 20 organisms should be exposed to each concentration (for example, 4 replicates each containing a minimum of 5 juvenile mussels). It may be desirable to test only 5 juvenile mussels in each replicate when a limited number of test organisms are available or when test organisms are relatively small (for example, when juvenile mussels are small, it may be difficult to observe more than about 5 test organisms simultaneously in a replicate test chamber under the microscope). However, some investigators have tested 10 to 20 juvenile mussels in each replicate. In chronic toxicity tests, a minimum of 3 replicates should be tested, each replicate containing a minimum of 10 juvenile mussels. Water-quality characteristics of the dilution water (dissolved oxygen, pH, ammonia, hardness, alkalinity, and conductivity) should be measured at the start and end of the acute exposures and at least weekly in chronic exposures in the high and medium test concentrations and in the control as live organisms are present. Requirements for test acceptability for toxicity tests conducted with juvenile mussels are summarized in Table A1.5.

A1.4 *Conducting a Toxicity Test*

A1.4.1 Procedures for constructing and maintaining exposure systems are outlined in Section 6 and in section A1.4.3. Hazards associated with conducting the toxicity tests are outlined in Section 7. Procedures for preparing dilution water are outlined in Section 8. Procedures for preparation and delivery of the test material to test chambers are outlined in Section 9 and in section A1.4.3. Procedures for obtaining test organisms are outlined in Section 10. Procedures for addressing quality assurance and quality control associated with a toxicity test are outlined in Section 11 and in Section 16. Considerations of experimental design for a toxicity test are outlined in Section 12 and in A1.5. Procedures for analysis of test materials are outlined in Section 13. Procedures for analyzing data generated from a toxicity test are outlined in Section 14. Reporting requirements for a toxicity test are outlined in Section 15.

A1.4.2 *Beginning the Test:*

A1.4.2.1 Section 10.5 provides information on obtaining glochidia or juvenile mussels to start a toxicity test.

A1.4.2.2 *Acclimation*—Glochidia should be acclimated to a 50 to 50 mixture of culture to dilution water for about 2 h before the start of a toxicity test. Juvenile mussels should be acclimated to the dilution water for at least 24 h before the start of a toxicity test (for example, by holding juvenile mussels for 2 h in a 50 to 50 mixture of culture water to dilution water, then for 2 h in a 25 to 75 mixture of culture water to dilution water, followed by a transfer into 100 % dilution water until the start of the toxicity test). The temperature of the water used to acclimate test organisms and the water quality characteristics of the water should be gradually adjusted over the acclimation period (for example, increase by no more than about 3°C /h). Glochidia and newly-transformed juvenile mussels are not fed during the acclimation period; however, older juvenile mussels should be fed during the acclimation period (A1.4.5).

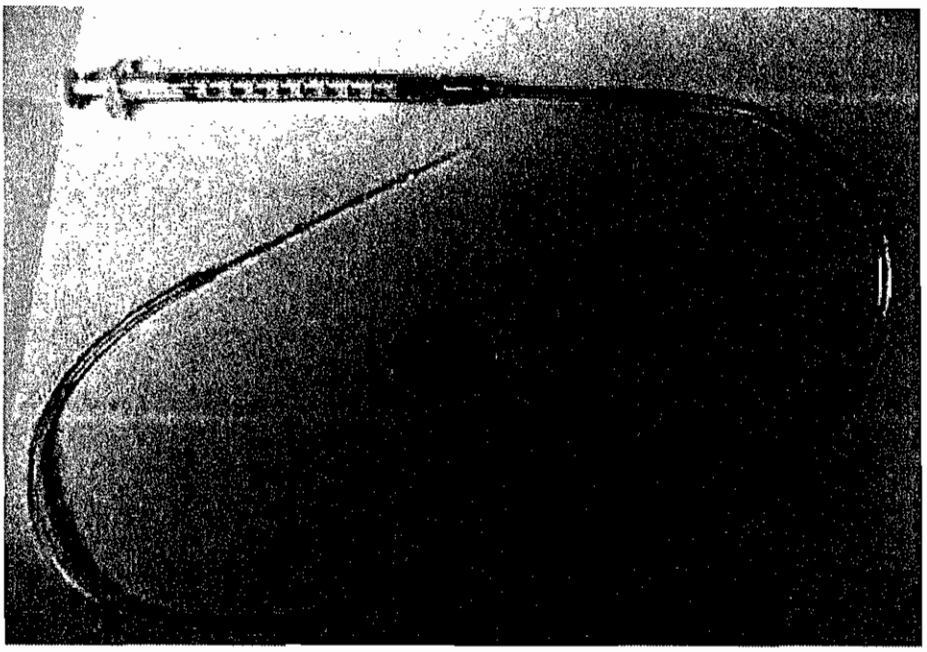
A1.4.2.3 *Placing Test Organisms in Test Chambers*—The test begins when the test organisms are first placed in dilution water containing test material. Section A1.4.8.4 provides information on establishing the viability of glochidia at the start of a toxicity test. Only active juvenile mussels should be used to start a toxicity test (that is, with foot movement).

A1.4.2.4 A representative sample of the test organisms must be impartially distributed among the test chambers. Caution should be exercised to minimize the transfer of dilution water with the test organism to the chambers. Test organisms should be handled as little as possible. Test organisms should be introduced into the test water below the air-water interface. A pipette or syringe can be used to place organisms directly into the test water. Fig. A1.1 illustrates a syringe system used to transfer newly-transformed juvenile mussels into test water (Wang et al 2003) (85). This syringe system consists of a glass capillary tube (1.17-mm inner diameter), connected to vinyl tubing (1.0-mm inner diameter), connected to a 2.5-cm, 16-gauge needle; that is connected to a 1-mL syringe. For 2- to 4-month old juveniles, a larger system should be used (for example, 2.2-mm inner diameter glass capillary tube connected to a 2.3 mm inner diameter vinyl tube, connected to a 5-mL syringe). If the shell of a juvenile mussel is broken, this organism should not be used in a toxicity test. A subsample of about 30 juvenile mussels should be archived at the start of chronic toxicity tests for subsequent length measurements (section A1.4.8.3). This information can be used to determine consistency in the size of the juvenile mussels used to start a test.

A1.4.3 *Static, Renewal, and Flow-through Exposure Systems*:

A1.4.3.1 Section 6 provides a description of procedures for constructing exposure systems.

A1.4.3.2 Static and renewal tests should begin by placing test organisms in the chambers within 30 min after the test material was added to the dilution water. Flow-through tests should begin by either *(a)* placing test organisms in the chambers after the test solutions have been flowing through the chambers long enough for the concentrations of test material to have reached steady state or *(b)* activating the metering device in the metering system several days after organisms were



NOTE—The tubing is secured to the needle with a small piece of tape.
FIG. A1.1 Syringe Used to Transfer Juvenile Mussels (Wang et al, 2003) (85)

placed in test chambers that had dilution water flowing through them. This second alternative requires the addition of a "spike" that is, an aliquot of test material sufficient to establish the desired test concentration in the test chamber at the time of activation of the metering device. The first alternative *(a)* allows the investigator to study the properties of the test material and the operation of the metering system immediately before the test, whereas the second alternative *(b)* allows the organisms to partially adjust to the chambers before the beginning of the test.

A1.4.3.3 In flow-through tests with glochidia or juvenile mussels, where there may be turbulence with each addition of dilution water, it is desirable to place a stainless-steel baffle in the test chamber to reduce turbulence. Specifically, Wang et al (2003) (**85**) placed stainless-steel mesh screen (4 by 15 cm; 300-µm opening) bent over the surface of the water in a 300-mL beaker used in flow-through tests to reduce the turbulence of water. Each of these beakers contained 200 mL of test water and had a 2.5-cm hole in the side covered with stainless-steel mesh screen (300-µm opening; Wang et al 2003 (**85**)). A description of the flow-through exposure system used by Wang et al (2003) (**85**) to conduct toxicity tests with glochidia and juvenile mussels can be found in USEPA (2000) (**115**),Figure A.5. Survival of glochidia in 48-h toxicity tests and survival of juvenile mussels in 10-d toxicity tests with copper and ammonia were similar in static or renewal exposure systems compared to flow-through exposure systems (Wang et al 2003) (**85**).

A1.4.3.4 Alternative test chambers that have been used to conduct toxicity tests with glochidia are multi-well (6 or 12 well) polystyrene (or other types of plastic) tissue-culture plates containing about 4 to 12 mL of water and a specific number of glochidia/chamber (Table A1.1). Larger glass test chambers have also been used to conduct toxicity tests with glochidia (for example, 250- to 400-mL beakers). A difficulty in using small multi-well plates is that there is a limited volume of water available for conducting water quality or chemical analyses. Jacobson (1990) (**116**) suggested that subsampling of glochidia from a smaller test chamber (for example, 12-well plates) may result in a biased sampling of glochidia. Wang et al (2003) (**85**) exposed groups of about 1000 glochidia in 200-mL glass chambers in about 100 to 150 mL of exposure water. Survival was then evaluated with the addition of a solution of NaCl at 6, 24, and 48 h to subsamples of glochidia (that is, about 100 glochidia in about 2 mL of exposure water placed into one well of a multi-well plate; see A1.4.8.4). Use of larger test chambers permits easier sampling of water quality and chemical concentrations during the exposures (Wang et al 2003) (**85**). In addition, exposures in larger chambers can be conducted using water-renewal systems (for example, Zumwalt et al 1994 (**117**), Brunson et al 1998 (**118**)). Similar survival of glochidia from several species was observed when glochidia were held under control conditions in multi-well plates or in larger chambers under static, renewal, or flow-through conditions (Wang et al 2003) (**85**). Wang et al (2003) (**85**) also observed that concentrations of copper in the multi-well plates substantially decreased during 48-h exposures; whereas, the concentration of copper in larger glass chambers remained relatively consistent over this time period.

A1.4.3.5 Alternative test chambers used to conduct toxicity tests with juvenile mussels have included multi-well tissue-culture plates for short-term exposures or larger chambers for longer exposures (Table A1.4). Investigators have also exposed juvenile mussels in glass cylinders with a mesh bottom placed inside larger test chambers (Dimock and Wright 1993 (**48**);

Wade et al 1993 (**119**); McKinney and Wade 1996 (**120**); Farris et al 1994, 1995 (**121, 122**)).

A1.4.4 *Loading*—Table A1.1 outlines the number of glochidia added to each replicate test chamber and Table A1.4 outlines the number of juvenile mussels added to each test chamber. Loading should be limited to ensure that *(a)* the concentrations of dissolved oxygen and test material do not fall below acceptable levels, *(b)* concentrations of metabolic products do not exceed acceptable levels, and *(c)* the test organisms are not stressed because of crowding. Guides E 729 and E 1241 provide additional guidance on loading of organisms used in acute or chronic toxicity tests.

A1.4.5 *Feeding*:

A1.4.5.1 Glochidia are not fed during toxicity tests.

A1.4.5.2 Juvenile mussels are not typically fed during an acute toxicity test (for example, ≤ 96 h) or for a time before the test because fecal matter and uneaten food can decrease the dissolved oxygen concentration and can influence the bioavailability of some test materials. Toxicity tests with juvenile mussels have been conducted for 10 d without feeding juvenile mussels (USGS 2004) (**112**). The acute toxicity of copper was determined in 48-h tests with juvenile *Lampsilis siliquoidea* and *L. rafinesqueana* that had been held for 10 d under control conditions (for example, with the replacement of dilution water, but without the addition of food; USGS 2004 (**112**)). Similar 48-h EC50s were observed in tests conducted with juvenile mussels held for 10 d before testing compared to tests started with newly-transformed juvenile mussels. Results of these tests indicate that the sensitivity of juvenile mussels did not change over the 10-d exposure without feeding.

A1.4.5.3 In 10 to 28-d toxicity tests, algae have been used as a source of food (Table A1.4). USGS (2005b) (**9**) described a procedure for conducting 28-d toxicity tests starting with 2-month-old juvenile *Villosa iris*. In this 28-d toxicity test, juvenile mussels were fed 4-mL of an instant algae mixture twice daily. The instant algae mixture was prepared from commercial Instant Algae[4] brand non-viable microalgae concentrates (Reed Mariculture, Campbell, CA) by adding 1 mL of a *Nannochloropsis* concentrate and 2 mL of a Shellfish Diet (a mix of four marine microalgae [*Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira weissflogii*]) to 1.8 L of well water. Control survival of the juvenile mussels was 88 % in a 28-d copper toxicity test and was 100 % in a 28-d ammonia toxicity test (USGS 2005b (**9**).) Additional information on feeding of juvenile mussels in culture or in toxicity tests is included in 10.6.3

A1.4.6 *Monitoring a Test*—Operation of the exposure system should be monitored daily. A microscope is needed to determine survival of test organisms. Therefore, survival of juvenile mussels typically monitored only periodically during a toxicity test (for example, at 48 and 96 h in an acute test and at 4, 7, 10, 14, 21, or 28 d in a chronic test).

A1.4.7 *Duration of Test*—Toxicity tests with glochidia are typically conducted for at least 24 h (Table A1.1; section A1.2.5), A 48-h toxicity test with glochidia might be used for species for which juvenile mussels are not readily available for testing or for species with a life history where glochidia are released into the water column and remain viable for days before attaching to a host (in contrast to species that release glochidia in mucus strands or in conglutinates). Acute toxicity test with juvenile mussels are typically conducted for 96 h, and chronic toxicity tests starting with 2- to 4-month-old juvenile mussels have been conducted for 21 to 28 d (Table A1.4). The duration of an acute toxicity test should be no more than half of the length of time that 90% of the organisms survive in the dilution water under test conditions. Specifically, survival of control organisms in control water might be evaluated for an additional time period after the end of an acute test to further evaluate the quality of the test organisms (for example, control survival should be >90% for 24 h after the end of a 24-h glochidia toxicity test and control survival should be >90% for 96 h after the end of a 96-h juvenile toxicity test). At the end of the test it may be desirable to place the live test organisms for 1 to 2 d in dilution water that does not contain any added test material to determine whether delayed effects occur (Guide E 729). It may also be desirable to maintain all test chambers with surviving organisms until at least 10% mortality occurs in each chamber.

A1.4.8 *Biological Data*:

A1.4.8.1 Endpoints measured in the toxicity tests with glochidia include survival (that is, measured as viability of glochidia at 6 and 24 h; Table A1.1). Endpoints measured in toxicity tests with juvenile mussels include survival (measured at 48 and 96 h in acute tests and about weekly in chronic tests) and growth (measured at the end of a chronic test; Table A1.4). Newton et al (2003) (**61**) observed small reductions in growth of juvenile mussels in 96-h toxicity tests.

A1.4.8.2 *Measurement of Juvenile Survival*—The endpoint typically measured in juvenile mussel toxicity tests is survival based on movement of the foot. However, ciliary activity on the foot, heartbeat, or vital staining has also been used to establish survival of juvenile mussels at the end of a toxicity test (Table A1.4). Survival of juvenile mussels in each replicate should be determined using a microscope to observe movement of the foot of each juvenile mussel within a 5-min period. Laboratories may also want to evaluate other measures of survival such as heart beat or cilia movement on the foot. In order to observe the juvenile mussels under the microscope during a test, it may be necessary to remove some of the water from the test chamber (for example, it is easier to observe foot movement of a juvenile mussel with a microscope if there is less than about 1 cm of water in the test chamber). Gently swirling the test chamber will create a slight vortex in the water, concentrating the juvenile mussels in a small area in the chamber, making it easier to see all of the organisms simultaneously in the field of view under the microscope.

A1.4.8.3 *Measurement of Juvenile Growth*—Growth of juvenile mussels has been measured at the maximum shell length parallel to the hinge or at the maximum shell height perpendicular to the hinge. These measurement provide comparable results, but the maximum shell height is somewhat easier to measure; (Teresa Newton, USGS, LaCrosse, WS, personal communication). Subsamples of about 30 juvenile mussels at the start of a toxicity test and juvenile mussels surviving at the end of the toxicity test can be preserved for subsequent growth measurements. Juvenile mussels can be placed in a small glass

vial and preserved in 70 % ethanol until growth is measured. Alternatively, juveniles can be placed in neutral buffered formalin for 24 h and then transferred to 70 % ethanol until growth is measured (Newton et al 2003) (**61**). Growth can be measured using a microscope interfaced with a digitizing system (for example, Newton et al 2003 (**61**)).

A1.4.8.4 *Evaluation of Viability of Glochidia*—Percent survival (viability) of glochidia should be calculated from the proportion of glochidia that close with the addition of a saturated salt solution (NaCl). Specifically, survival of glochidia should be calculated as: Survival (%) = 100 (# of closed glochidia after adding salt solution – # of closed glochidia before adding salt solution) / (# of open and closed glochidia after adding salt solution). A subsample of 100 to 200 glochidia isolated from each female mussel should be evaluated at the beginning of a toxicity test to confirm the viability of the glochidia from that female using a saturated salt solution. Readings of percent viable glochidia should be made about 1 min after the addition of the saturated salt solution. The saturated solution of NaCl can be prepared by adding about 12 g of reagent-grade NaCl to 50 mL of deionized water. About 1 drop of this saturated salt solution should be added to about 2 mL of a water sample containing glochidia. If viability is >80 % (preferably >90 %), the rest of glochidia collected from that female can be used for toxicity testing. Glochidia with >80 % (preferably >90 %) viability from at least three female mussels should be composited into in a large chamber before the start of a toxicity test or before a host is infested with glochidia to produce juvenile mussels. The recommendation to record the response of the glochidia 1 min after addition of a specific amount of NaCl is based on the observations that after addition of a saturated salt solution, glochidia sometimes closed slowly (USGS 2004) (**112**) or initially close then reopen after several minutes (for example, *Utterbackia imbecillis*; Bringolf et al 2005 (**108**)).

A1.4.9 *Other Measurements*:

A1.4.9.1 *Water Quality*—Water-quality characteristics (dissolved oxygen, pH, ammonia, hardness, alkalinity, and conductivity) should be measured at the start and end of an acute toxicity test and at least weekly in chronic toxicity tests in a minimum of the high and medium test concentrations and in the control (as long as live organisms are present). Measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable in the dilution water. It may be necessary to composite water samples from individual replicates. The pipette used to collect water samples should be checked to make sure no organisms are removed during sampling of water. Water quality should be measured for each new batch of water prepared for the test.

A1.4.9.2 *Temperature*—Toxicity tests should be conducted at 20°C. In static and renewal tests, either *(a)* in at least one test chamber temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily or *(b)* if the test chambers are in a water bath or a constant-temperature room or incubator, the temperature of the water or air must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured at least daily. In addition, temperature must be measured concurrently near both the beginning and end of the test in all test chambers or in various parts of the water bath, room, or incubator. In flow-through tests, in at least one chamber either temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily. In addition, near both the beginning and end of the test, temperature must be measured concurrently in all test chambers. Uniform temperature is important to maintain in a test because survival or growth of test organisms can be influenced by temperature. The stated requirements are necessary to prevent confounding and unnecessary large variance in temperature. Table A1.3 and Table A1.5 summarize acceptable variation in temperature during a toxicity test.

A1.4.9.3 *Dissolved Oxygen*—Dissolved oxygen (and pH and conductivity) can be measured directly in the overlying water with a probe. If a probe is used to measure dissolved oxygen, it should be rinsed between samples to minimize cross contamination. Concentrations of dissolved oxygen should be maintained above 4 mg/L during the test. Sparks and Strayer (1998) (**50**) observed effects on behavior of juvenile *Elliptio complanata* at dissolved oxygen concentrations of 2 to 4 mg/L. Gentle aeration can be used if dissolved oxygen in the test water is below 4 mg/L (that is, about 1 bubble/second from a glass pipette in the test water). Turbulence should be avoided because it might stress test organisms or increase volatilization of the test material. Aeration should be the same in all test chambers, including the control(s), throughout the test.

A1.4.10 *Test Material*:

A1.4.10.1 If the test material is uniformly dispersed throughout the test chamber, water samples should be taken by using a pipette or by siphoning water through glass or fluorocarbon plastic tubing from a point midway between the top, bottom, and sides of the test chamber and should not include any surface scum or material stirred up from the bottom or sides (Guide E 729). If test material might be lost due to sorption onto the walls of the sample container, the container and the siphon or pipette should be rinsed with test solution before collecting the sample. Water samples should be collected into appropriate-sized containers from which the test material can be extracted or analyzed directly. If the test material is not uniformly dispersed in the test chamber in static and renewal tests, the whole volume of solution in the test chamber should be *(a)* used as the sample or *(b)* treated appropriately (for example, by adding acid, base, or surfactant and mixing thoroughly) to uniformly distribute the test material before a sample is taken. If the test material is not uniformly dispersed in the test chamber in flow-through tests, a large volume of the solution flowing into the test chambers should be collected and used as the sample or treated appropriately to uniformly distribute the test material in the sample before a subsample is taken.

A1.4.10.2 If some of the test material is not dissolved, measurement of the concentration of dissolved test material in each treatment might be desirable.

A1.4.10.3 In acute tests, the concentration of test material in the exposure chambers should be measured in the control and high, medium and low concentrations of test material at least at beginning and end of a test. In chronic tests, concentration of

test material in the exposure chambers should be measured at the beginning and weekly in the control and high, medium and low concentrations of test material. It is desirable to measure the concentration of test material in all of the test concentrations. Measurement of degradation products might be desirable. Whenever a serious malfunction is detected in the metering system, the test material in the test chambers should be measured. Guides E 729 and E 1241 provide additional guidance on calibration of flow-through systems before the start of a toxicity test and on monitoring concentrations during a toxicity test.

A1.5 *Additional Information on Experimental Design and Interpretation of Data Generated in Toxicity Tests Conducted with Glochidia or Juvenile Mussels*

A1.5.1 Kernaghan et al (2005) (**5**) addressed several questions that have been raised regarding the experimental design or interpretation of data from toxicity tests conducted with glochidia or juvenile mussels. Glochidia and juvenile mussels of several genera are highly sensitive to some metals and ammonia in water exposures compared to many of the more sensitive genera of other invertebrates, fish, or amphibians that are commonly tested (for example, Augspurger et al 2003 (**6**), Keller et al 2005 (**7**); section 1.5). However, concerns have been expressed regarding the use of toxicity data generated with glochidia or juvenile mussels in the derivation of U.S. Environmental Protection Agency Water Quality Criteria; (Kernaghan et al 2005) (**5**). These concerns mainly include: *(1)* the duration of the toxicity tests conducted with glochidia, *(2)* the quality of organisms at the start of a test, and *(3)* test acceptability criteria. The following section summarizes information presented in Kernaghan et al (2005) (**5**) that addresses these concerns. Future research needs identified throughout the standard are highlighted in section A1.6.

A1.5.2 How long should a toxicity test be conducted with glochidia? There are nearly 300 species of freshwater mussels in North America and the length of time that glochidia remain viable after release from the marsupium of a female into the environment depends on the life history of the species and the temperature of the water (Table A1.2; section 10.1). Longevity of glochidia after release and before attachment to a host may exceed one week and may be dependent on temperature (Zimmerman and Neves 2002) (**42**); however, some reports are anecdotal (Murphy 1942 (**123**), Matteson 1948 (**124**), Tedla and Fernando 1969 (**125**)). Glochidia of some species released in conglutinates remain viable for days or weeks after release into the environment (Kernaghan et al 2005) (**5**). Glochidia of several species, including *Anodonta* spp., remain viable while free in the environment for 7 to 14 d (Howard and Anson 1922 (**126**), Mackie 1984 (**127**), Huebner and Pynnonen 1992 (**128**), Pynnonen 1995 (**129**)).

A1.5.2.1 Table A1.2 provides a summary of laboratory studies that have evaluated survival times of glochidia after removal from the marsupium of the female or survival time based on results reported in toxicity tests conducted with glochidia. For example, Zimmerman and Neves (2002) (**42**) report that the viability of glochidia of *V. iris* was >75 % for 8 d at 10°C and 2 d at 25°C and viability of glochidia of *A. pectorosa* was >75 % for 13 d at 10°C and 5 d at 25°C (Table A1.2). Similarly, glochidia of *Utterbackia imbecillis* may survive up to 19 d, but exhibit 50 % mortality within 13.5 d (Fisher and Dimock 2000) (**73**). Survival of isolated glochidia from many species listed in Table A1.2 is typically >90 % after 2 to 3 d; however, the viability of glochidia for a particular species should be determined before the start of an exposure. For example, glochidia of *Lampsilis teres* and *Epioblasma capsaeformis* were viable for only 4 to 6 h, glochidia of *Megalonaias nervosa* and *Quadrula quadrula* were viable for 1 d after removal from the marsupium of the female (Table A1.2). Therefore, 24 h is a reasonable time period to conduct toxicity tests with glochidia of many species at 20°C, although shorter or longer tests might be needed for a particular species depending on glochidia survival time and the life history characteristics of the species (that is, survival of glochidia in the control must be >90 % at the toxicity test Table A1.3).

A1.5.3 Short-term exposures with glochidia may be useful for screening of chemicals, but response of juvenile mussels would be more ecologically relevant (Kernaghan et al 2005) (**5**). Use of glochidia to screen the relative sensitivity of a particular mussel species to chemicals would be particularly useful when evaluating species where only a limited number of adult mussels are available for methods development or for generating juvenile mussels for toxicity testing. Moreover, the host fish for some species of mussels or techniques for transforming juvenile mussels in the laboratory may be unknown for some species.

A1.5.4 How long can glochidia survive and still be able to attach to a host? Glochidia of some species can still attach to a host for several days after release from a female depending on temperature (Kernaghan et al 2005) (**5**). The maximum time at which >50 % of *Utterbackia imbecillis* metamorphosed in a tissue culture medium was 9 d after isolation from a female (Fisher and Dimock 2002) (**73**). Zimmerman and Neves (2002) (**42**) reported that glochidia can successfully attach to a host 1 to 2 weeks after isolation from a female. A future research project could be to conduct a series of toxicity tests to determine if there is a change in sensitivity over time after glochidia have been released into the environment. Sensitivity of *Lampsilis siliquoidea* glochidia held for 24 h after isolation from a female was similar to newly-released glochidia in exposures to copper (Wang et al 2003) (**85**). The sensitivity of glochidia held in an extra piece of the marsupium in a refrigerator overnight was similar to the sensitivity of glochidia tested immediately after isolation from a female in toxicity tests conducted with zinc or copper (Kernaghan et al 2005) (**5**). Ultimately, it is more practical to base duration of exposure on survival of control organisms in the laboratory rather than on an estimate of the length of time glochidia can survive and still attach to a host (for example, Table A1.2).

A1.5.5 What life stage should be used to start acute or chronic toxicity tests with juvenile mussels? Toxicity tests have been started with newly-transformed juvenile mussels that have either been transformed on a host or have been transformed with the use of an artificial medium (Table A1.4). Glochidia, newly-transformed juvenile mussels, and 2- to 4-month-old juvenile mussels have been successfully shipped via overnight carriers to other laboratories for use in toxicity

testing (for example, section 16.5, USGS 2004 (**112**), Bringolf et al 2005 (**108**)). Toxicity tests have been successfully conducted for 10 to 14 d starting with newly-transformed juvenile mussels (Table A1.4), but exposures conducted for longer periods of time have resulted in high mortality in controls at about 4 to 6 weeks, probably due to nutritional limitations of the diet (for example, Newton et al 2003) (**61**). Valenti et al (2005) (**107**) conducted 21-d exposures with 2-month old juvenile *Villosa iris* held in a small amount of sediment and fed algae (Neochloris). USGS (2005a,b) (**8, 9**) and Bringolf et al (2005) (**108**) conducted toxicity tests starting with 2- to 4-month-old juvenile *Actinonaias ligamentina*, *Lampsilis siliquoidea*, or *Villosa iris* and observed control survival >88 % in 21- to 28-d exposures when algae was used as a food source.

A1.5.6 Are there data that indicate that effect concentrations do not ehange very much during the last half of a toxicity test conducted with glochidia (that is, does the EC50 at 6, 24, 48, or 96 h differ)? There are limited studies with glochidia that have compared changes in toxicity over this timeframe. The toxicity of copper (Jacobson et al 1997 (**31**), Wang et al 2003 (**85**)), ammonia (Wang et al 2003) (**85**), and chlorine (Wang et al 2003) (**85**) decreased over 48 to 96-h exposures. In contrast, no cbange in the toxicity of several pestieides was observed in 24 to 48-h exposures (Keller and Ruessler 1997 (**58**), Bringolf et al 2005 (**108**)). If glochidia for a particular species are able to survive for more than 24 h, then a 24-h toxicity test should be considered. Importantly, researehers are encouraged to design studies that generate toxicity data throughout the exposure period (for example, reporting 6, 24, and 48-h responses; Guide E 729). However, generating data for a 6–h exposure period is logistically difficult in an 8-h day.

A1.5.7 How should death of juvenile mussels be determined at the end of a toxicity test? Laek of foot or shell movement, lack of ciliary activity on the foot, lack of a heart beat, or a wide gaped valve have been used to establish death in toxicity tests with juvenile mussels (Table A1.4). Lack of movement of the foot of a juvenile mussel is the primary endpoint recommended in this standard (section A1.4.8).

A1.5.8 How should the quality of glochidia be determined at the start of a toxicity test? Is the use of a solution of NaCl (or KCl) to determine the percentage of glochidia exhibiting valve closure an appropriate method to judge the acceptability of glochidia used to start a toxicity test? Does the response of glochidia to a solution of NaCl (or KCl) relate to the ability of glochidia to attach to a host? Is there an independent way of determining if glochidia are alive or healthy at the start (or end) of a toxicity test? Valve closure is an ecologically-relevant endpoint that is a critical for glochidia to successfully transform on the host. If glochidia do not snap shut, the glochidia should be considered ecologically dead (Huebner and Pynnonen 1992 (**128**), Goudreau et al 1993 (**130**), McMann 1993 (**131**), Jacobson et al 1997 (**31**)). The response of glochidia in toxicity tests was similar when either KCl or fish plasma was used to make glochidia close at the end of an exposure (Huebner and Pynnonen 1992) (**128**). Decreased response to KCl was considered an indication of reduced glochidia viability and thus reduced capability to attach to the fish host (Pynnonen 1995) (**129**). A significant correlation was observed between the response of glochidia to KCl and ability of glochidia of *Utterbackia imbecillis* to metamorphose to the juvenile life stage (Fisher and Dimock 2002) (**73**). Zimmerman and Neves (2002) (**42**) reported a correspondence between the response of glochidia of *Villosa iris* and *A. pectorosa* to NaCl and the ability to infest a host fish. Jacobson et al (1997) (**31**) reported glochidia of *Villosa iris* that responded to the addition of NaCl following an exposure to copper were able to attach to a host fish with no impairment of subsequent metamorphosis to juvenile mussels. Results of these studies indicate that addition of a solution of NaCl or KCl can be used to estimate the condition of glochidia. While either a solution of salt or fish plasma could be used to determine the percentage of organisms closing, it is easier to work with NaCl compared to KCl or fish plasma.

A1.5.9 Should there be a holding time for glochidia after harvesting but before application of a salt solution to determine if glochidia that are initially closed might open? Mature glochidia are not typically closed after being isolated from a female mussel. Glochidia that are elosed after isolation from a female may reopen after being held in clean water a few hours (Goudreau et al 1993 (**130**)).

A1.5.10 Will immature, stressed, or unhealthy glochidia close when exposed to a salt solution? Could glochidia be alive and successfully attaeh to a host but not close when exposed to a salt solution? Are broken glochidia frequently observed at the start of a test? Would the presence of broken glochidia be indicative of stress during harvesting? Immature glochidia that are free of an egg membrane or mature and healthy glochidia will close when cxposed to a salinity challenge. However, immature glochidia are generally enclosed in an egg membrane and are fragile and tend to fracture, thus should not be used for toxicity testing. The best approach for avoiding the use of immature glochidia in toxicity testing is to sample female mussels at a time of the year when the organisms would be expected to be releasing mature gloehidia (Kernaghan et al 2005) (**5**). Stressed or unhealthy glochidia could either be opened or closed before the start of a test. If stressed or unhealthy glochidia were to close when exposed to a salinity challenge, then these individuals would be used in a toxicity test. Measurement of the viability of glochidia in the control at the end of a toxicity test would help to identify stressed or unhealthy glochidia. Results of reference-toxicant tests should also be used to evaluate the health of the glochidia used to conduct the test (seetion 16.4). Broken gloehidia have not been observed at the start of a test (Kernaghan et al 2005) (**5**). The presence of broken glochidia may indicate that the glochidia are immature and should not be used for testing.

A1.5.11 Should glochidia be rinsed before use in a toxicity test? Would rinsing glochidia before the start of a test be stressful to the organisms? Glochidia should be rinsed with culture or dilution water after removal from marsupia to: *(1)* eliminate tissues or excess mucus from the excised glochidia that have a high potential for fungal growth and subsequently could affect the survival (toxicity tests) or transformation of glochidia (propagation) and *(2)* reduce the number of protozoans that may be present in the excised gill that could also affect

glochidia survival or transformation (10.5). Rinsed glochidia have been observed to successfully transform on fish or in artificial media and high control survival in toxicity tests has been reported using glochidia that have been rinsed (Huebner and Pynnonen 1992 (**128**), Johnson et al 1993 (**79**), Myers-Kinzie 1998 (**132**), Bishop et al 2005 (**47**)).

A1.5.12 Should glochidia be acclimated to test conditions before the start of a toxicity test? Glochidia are not typically acclimated to the water-quality characteristics of the dilution water before the start of a toxicity test (Table A1.1). Most of these exposures are started the same day that glochidia are isolated from marsupia of the females. Therefore, minimal time is available to acclimate glochidia to the dilution water before the start of a test. In order to maintain organisms in good condition and avoid unnecessary stress, Guide E 729 recommends that organisms should not be subjected to rapid changes in temperature or water quality before the start of a test. Wang et al (2003) (**85**) acclimated glochidia in a mixture of 50 % culture water and 50 % test water and gradually adjusted the temperature to the test temperature within about 2 h before the start of an exposure (A1.4.2.2). Investigators have held adult mussels under test conditions before isolation of glochidia (for example, Huebner and Pynnonen 1992 (**128**)) which would result in acclimating glochidia to the selected exposure temperature in the toxicity test. However, brooding glochidia in the marsupium are in contact with the hemolymph of the female that is physically isolated from direct contact with water (Silverman et al 1987) (**133**). In addition, glochidia are typically released instantaneously into the surrounding water from the marsupium of the female mussel. Therefore, holding the female mussels in the dilution water before isolating glochidia for toxicity testing would probably have a minimal influence on the ability of glochidia to acclimate to the conditions of the dilution water.

A1.5.13 What criteria should be used to judge acceptability of a toxicity test conducted with glochidia? Survival (measured as viability) of glochidia at the end of the exposure should be the primary endpoint to establish the acceptability of a toxicity test. Most investigators report >90 % survival of glochidia after 24 h (Tables A1.1 and A1.2). Therefore, setting test acceptability at >90 % survival seems appropriate for 24-h toxicity tests conducted with glochidia. Survival of glochidia was improved at cooler temperatures (Zimmerman and Neves 2002) (**42**) and may be different for short- versus long-term brooders (Kernaghan et al 2005) (**5**). Other investigators have observed inherently lower survival of some species (for example, Lasee 1991 (**134**); Keller and Ruessler 1997 (**58**); McMahon and Bogan 2001 (**29**); Table A1.2). Importantly, the viability of the glochidia should be established before the start of a toxicity test and the duration of the exposure should be established based on these data. For example, there are some species that exhibit <90 % survival for about 24 h after isolation from the female; therefore, toxicity tests with glochidia from these species should not be conducted for longer than this time period.

A1.5.14 What criteria should be used to judge acceptability of a toxicity test conducted with juvenile mussels? Survival of juvenile mussels at the end of the exposure is the primary endpoint to establish the acceptability of toxicity tests conducted for up to 14 d. Investigators have reported >90 % survival of newly-transformed juvenile mussels after the end of exposures conducted for up to 14 d (Table A1.4); however, additional research is needed to improve survival in tests conducted for >14 d with newly-transformed juvenile mussels including research on dietary requirements of juvenile mussels (10.5). Additional research is also needed with additional species to determine if tests started with juvenile mussels >2- to 4-months old will improve survival in chronic exposures. USGS (2005 a,b (**8,9**)) and Bringolf et al (2005) conducted toxicity tests starting with 2- to 4- month old juvenile *Actinonaias ligamentina, Lampsilis siliquoidea* or *Villosa iris* and observed control survival > 88% in 21– to 28–d exposures when algae was used as a food source (10.6.3.14). Klaine et al (1997) (**135**) report that shell length of newly-transformed juvenile mussels of *Utterbackia imbecillis* increased by 22 to 35 % in tests conducted from 5 to 15 d. Therefore, growth in should also be evaluated in future studies as a criterion to judge the acceptability of a toxicity tests conducted with juvenile mussels.

A1.6 *Future Research*—The methods outlined in Table A1.1 and Table A1.4 provide reliable estimates of toxicity of chemicals to glochidia and juvenile mussels in water-only exposures. The following list of research topics have been identified throughout the standard and in Kernaghan et al (2005) (**5**) for improving the reliability of results of toxicity tests conducted with glochidia or juvenile mussels. Results of this research may be included in future revisions of this standard.

A1.6.1 Further evaluate the influence of handling, holding, and acclimation on adult, glochidia, or juvenile mussels used to conduct toxicity tests (section 10.5).

A1.6.2 Determine the minimum number of female mussels that should be sampled to obtain glochidia or juvenile mussels used to start a toxicity test. These studies might include an evaluation of the variability in the sensitivity of glochidia or juvenile mussels obtained from individual females using a variety of chemicals with different toxie modes of action (section A1.4.9).

A1.6.3 Further evaluate the influence of contaminant exposure on immature glochidia developing within the marsupium of the female mussel (section 10.5.3.6).

A1.6.4 Establish methods for improving the performance of juvenile mussels in chronic toxicity tests (for example, test conducted for >14 d), focused on establishing feeding requirements for a variety of mussel species. Additional research is also needed with additional species to determine if tests started with juvenile mussels 2- to 4-months old will improve survival in chronic exposures. Ongoing research to improve culturing methods for propagation, holding, and feeding of newly-transformed juvenile mussels will hopefully provide additional information that can be adapted to establish methods for conducting chronic toxicity tests with juvenile mussels (section 10.6.3).

A1.6.5 Conduct additional intra- and inter-laboratory toxicity tests to evaluate variability in control and toxic responses of mussels to a variety of chemicals with different toxic modes of action (seetion 16.5).

A1.6.6 Further develop endpoints for establishing effects in toxicity tests with juvenile mussels (for example, behavior, biomarkers).

A1.6.7 Develop standard methods for conducting toxicity tests with *(1)* adult freshwater mussels and *(2)* contaminated sediments using various life stages of freshwater mussels.

A1.6.8 Evaluate the relative sensitivity of glochidia, newly-transformed juvenile mussels, older juvenile mussels, and adult mussels to a variety of different chemicals in acute or chronic toxicity tests.

A1.6.9 Compare the response of various species of mussels to the response of other surrogate species (for example, trout, cladocerans, *Corbicula*) in toxicity tests conducted using a variety of different chemicals.

A1.6.10 Compare the response of different populations of a species collected from different geographic regions to a variety of chemicals in laboratory toxicity tests.

A1.6.11 Compare the response of mussels tested in laboratory toxicity tests to the response of mussels exposed in the field (either using *in-situ* exposure containers or in a natural habitat).

TABLE A1.1 Summary of Test Conditions Used to Conduct Toxicity Tests with Glochidia of Freshwater Mussels (adapted from Kernaghan et al, 2005) (5)[A]

NOTE—The last column provides a summary of recommended conditions that can be used to conduct toxicity tests with glochidia.

	Conditions	Johnson et al (1990, 1993) (138, 79)	Lasee (1991) (134)	Huebner and Pynnonen (1992) (128)[B]	Goudreau et al (1993) (130)	Jacobson et al (1997) (31)	Keller and Russeler (1997) (58)	McCann (1993) (131)	Klaine et al (1997) (135)	USGS (2004) (112)	Recommended Test Conditions
1	Species tested	*Utterbackia imbecillis*[C]	*Lampsilis cardium*[D]	*Anodonta cygnea*, *A. anatina*	*Villosa iris*	Multiple species[E]	Multiple species[F]	*Villosa iris*	*Utterbackia imbecillis*	Mulitple species[G]	NA[H]
2	Test type	Static	Static	Static	Renewal	Static	Static	Static	Static	Static, renewal, flow-through	Static, renewal, or flow-through (depending on chemical tested)
3	Test duration (h)	24	48	24, 48, 72, 144	24	24, 48	4, 24, 48	24	24, 48	6, 24, 48	6, 24 (up to 48 depending on viability of glochidia)
4	Temperature, °C	20	21	13	22	10 to 25	25	20	25	20	20
5	Light quality	Ambient lab light	NR[H]	NR	NR	NR	NR	NR	Ambient lab light	Ambient lab light	Ambient lab light
6	Light intensity	NR	NR	NR	NR	NR	NR	NR	NR	200 lux	100 to 1000 lux
7	Photoperiod	16L:8D	24D	Natural regime	16L:8D	16L:8D	12L:12D	NR	16L:8D	16L:8D	16L:8D
8	Test chamber	100-mL beaker	250-mL crystallizing dish	400-mL beaker	Basket of mesh netting in 4-L chamber	12-well plate	6-well plate	12-well plate	12-well plate	200-mL crystallizing dish	100-mL glass chamber (minimum)
9	Test solution volume (mL)	50	200	200	NR	3.5	NR	5	3.5	100	75 (minimum)
10	Glochidia collection	shake piece of cut gill in water	flush gills with syringe	cut gills and press out glochidia using forceps	flush gills with syringe	cut gills and separate glochidia from marsupia	NR	flush gills with syringe	flush gills with syringe	flush gills with syringe	flush gills with syringe
11	Age of test organisms (h)	NR	NR	3 to 24	NR	NR	NR	<2	NR	<2 to <24	<24
12	No. organisms per test chamber	10	10	1000-3000	Several hundreds	50-75	50-100	40	50-100	about 1000	about 500 (1000 for repeated sampling during a toxicity test)
13	No. replicate chambers per treatment	2	3	2, counting 3 samples with about 100 glochidia	2, counting 3 samples with about 100 glochidia	3	3 or 4	3	3	3, counting a subsample with about 100 glochidia from each replicate	3, counting a subsample with about 100 glochidia from each replicate
14	Feeding	None	None	None	None	None	None	None	None	None	None
15	Aeration	None	None	Yes	None	None	NR	NR	NR	None	None, if dissolved oxygen is maintained above acceptable concentration
16	Dilution water	Reconstituted water, hardness 40-50 mg/L as $CaCO_3$	Hardness 150 mg/L as $CaCO_3$	Tap water	Dechlorinated effluent water	Dechlorinated tap water or Clinch River water, VA	Reconstituted water, hardness 47-76 mg/L as $CaCO_3$	Sinking Creek water, VA	Hardness 99-107 mg/L as $CaCO_3$	Reconstituted water, hardness 170 mg/L as $CaCO_3$	Depends on experimental design
17	Water quality	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	pH, Ca, Cu, Zn	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity

TABLE A1.1 *Continued*

	Conditions	Johnson et al (1990, 1993) (136, 79)	Lasee (1991) (134)	Huebner and Pynnonen (1992) (128)[B]	Goudreau et al (1993) (130)	Jacobson et al (1997) (31)	Keller and Russeler (1997) (58)	McCann (1993) (131)	Klaine et al (1997) (135)	USGS (2004) (112)	Recommended Test Conditions
18	Endpoint	Survival (valve closure with culture medium)	Survival (valve closure with NaCl)	Survival (valve closure with KCl)	Survival (valve closure with NaCl)	Survival (valve closure with NaCl)	Survival (valve closure with NaCl)	Survival (valve closure with salt solution)	Survival (valve closure with saline solution)	Survival (valve closure with NaCl)	Survival (valve closure with NaCl)
19	Control survival (%)	>95	>90	>80	80	>90	>80	>80	80	>90	>90 (must)

[A] Reprinted with permission of Kernaghan et al (2005) **(5)**. Copyright Society of Environmental Toxicology and Chemistry (SETAC).
[B] See also Pynnonen (1995) **(129)**, Hansten etal (1996) **(137)**.
[C] Formerly *Anodonta imbecillis*. See also Weinstein (2001) **(138)**.
[D] Formerly *Lampsilis ventricosa*.
[E] *Villosa iris, Actinonaias pectorosa, Pyganodon grandis, Lampsilis fasciola, Medionidus conradius*. See also Jacobson (1990) **(116)**, Cherry et al (2002).
[F] *Villosaosa lienosa, Villosa villosa, Utterbackia imbecillis, Megalonaias nervosa, Lampsilis teres, Lampsilis siliquoidea*. See also Jacobson (1990) **(116)**; McCann (1993) **(125)**, *Villosa iris, Actinonaias pectorosa, Medionidus conradius*.
[G] *Actinonaias ligamentina, Alasmidonta heterodon, Epioblasma capsaefotmis, Lampsilis siliquoidea, L. fasciola, L. abrupta, L. rafinesqueana, Potamilus ohiensis, Pleurobema plenum, Quadrula quadrula, Q. pustulosa, Leptodea fragilis, L. leptodon, Venustaconcha ellipsiformis, Villosa iris*.
[H] NA: not applicable. NR: not reported.

TABLE A1.2 Survival Time of Glochidia after Removal from Female Unionid Mussels (Kernaghan et al 2005) (5)[A]

Species	Temperature C	Duration of Viability Day (% Survival)	Reference
Actinonalas ligamentina	20	7 (>90); 8 (>75); 9 (>50)	USGS (2004) (112)
Actinonalas pectorosa	10	13 (>75)	Zimmerman and Neves (2002) (42)
	25	5 (>75)	Zimmerman and Neves (2002) (42)
	20	>2 (>90)*[B]	Jacobson et al (1997) (31)
Alasmidonta heterodon	20	2 (>90); 2 (>75); 2 (>50)	USGS (2004) (112)
Anodonta anatina	13	>3 (>90)	Huebner and Pynnonen (1992) (128)
Anodonta cataracta	10	>14 (>90)	Jacobson (1990) (118)
Anodonta cygnea	13	>3 (>90)	Huebner and Pynnonon (1992) (128)
Anodonta grandis	10	>14 (>90)	Jacobson (1990) (118)
Elliptio complanata	5	7 NR[C]	Matterson (1948) (124)
	20	<1 (>90); 3 (>75)	Bringolf et al (2005) (108)
Elliptio dilatata	20	<1 (>90); 1 (>75);<2 (>50)	Bringolf et al (2005) (108)
Epioblasma capsaeformis	20	0.3 (>90)	Wang et al (2003) (85)
Lampsilis abrupta	20	2 (>90); 5 (>75); 7 (>50)	USGS (2004) (112)
Lampsilis cardium	21	>2 (>90)*	Lasee (1991) (134)
Lampsilis fasciola	20	6 (>90); 7 (>75); 8 (>50)	Wang et al (2003) (85)
	20	>2 (>90)*	Jacobson et al (1997) (31)
	20	1 (>90); 2 (>75); 3 (>50)	Bringoll et al. (2005) (108)
	20	2 (>90; 4 (>75); 5 (>50)	Bringolf et al (2005) (108)
Lampsilis ratinesqueana	20	6 (>90); 6 (>75); 6 (>50)	USGS (2004) (112)
Lampsilis siliquoidea	10	9 NR	Tedla and Fernado (1969) (125)
	20	8 (>90); 9 (>75); 10 (>50)	Wang et al (2003) (85)
	25	>2 (>80)*	Keller and Ruessler (1997) (58)
	20	1 (>90); 3 (>75); 4 (>50)	Bringolf et al (2005) (108)
Lampsilis teres	25	0.2 (>80)	Keller and Ruessler (1997) (58)
Leptodea fragilis	20	1 (>90); 3 (>75); 4 (>50)	Wang et al (2003) (85)
Leptodea leptodon	20	1 (>90); 2 (>75)	Bringolf et al (2005) (108)
Laptodea leptodon	20	0.25 (>90); 1 (>75); 2 (>50)	USGS (2004) (112)
Margaritifera falcata	11	11 NR	Murphy (1942) (123)
Medionidus conradicus	20	>2 (>90)*	Jacobson et al (1997) (31)
Megalonalas nervosa	25	1 (>80)*	Keller and Ruessler (1997) (58)
Potamilus alatus	20	6 (>90) 6 (>75); 6 (>50)	Wang et al (2003) (85)
Potamilus ohiensis	20	5 (>90), 8 (>75); 7 (>50)	Wang et al (2003) (85)
Pyganodon grandis	20	>1 (>90)'	Jacobson etal. (1997) (31)
Quadrula quadrula	20	1 (>90); 1 (>75); 2 (>50)	Wang et al (2003) (85)
Quadrula pustulosa	20	<1 (>90): 1 (>75); 1 (>50)	Wang et al (2003) (85)
Utterbackia imbecillis	21	10 (>80); 14 (>50)	Fisher and Dimock (2000) (73)
	25	>2 (>80)'	Keller and Ruessler (1997) (58)
	25	>2 (>80)*	Klaineetal. (1997) (135)
	20	>1 (>90)*	Johnson et al (1990, 1993) (138, 79)
Venustaconcha ellipsiformis	20	2 (>90)l 3 (>75); 3 (>50)	Wang et al (2003) (85)
Villosa iris	10	8 (>75)	Zimmerman and Neves (2002) (42)
	20	5 (>90); 5 (>75): 6 (>50)	Wang et al (2003) (85)
	25	2 (>75)	Zimmerman and Neves (2002) (42)
	22	>1 (>80)*	Goudreau et al (1993) (130)
	20	>1 (>80)*	Scheller (1997) (139)
	20	>2 (>90)*	Jacobson et al (1997) (31)
Villosa lienosa	25	>2 (>80)*	Keller and Ruessler (1997) (58)
Villosa nebulosa	20	>2 (>90)*	Jacobson (1990) (116)
Villosa villosa	25	>2 (>80)*	Keller and Ruessler (1997) (58)

[A] Reprinted with permission of Kernaghan et al (2005) (5). Copyright Society of Environmental Toxicology and Chemistry (SETAC).
[B] An asterik indicates a value based on control survival in 24- or 48-h toxicity tests.
[C] NR: not reported.

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 ±10 % during the exposure and dissolved oxygen should be maintained above 4 mg/L.
4. The duration of an acute toxicity test should be no more than half of the length of time that 90% of the organisms survive in the dilution water under test conditions. Specifically, survival of control organisms in control water might be evaluated for an additional time period after the end of an acute test to further evaluate the quality of the test organisms (for example, control survival should be >90% for 24 h after the end of a 24-h glochidia toxicity test).

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 (for example, NaCl or $CuSO_4$, section 16.4). 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.

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 combine 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 test solution should not exceed 0.5 mL/L. A surfactant should not be used in the preparation of a test solution.
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, *(1)* no treatment other than a control treatment killed or affected less than 37 % of the organisms or, *(2)* no treatment killed or affected more than 63 % of the organisms.

TABLE A1.4 Summary of Test Conditions Used to Conduct Toxicity Tests with Juvenile Freshwater Mussels (adapted from Kemaghan et al, 2005[A]) (5)

NOTE—The last column provides a summary of recommended conditions that can be used to conduct toxicity tests with juvenile mussels. In the last column, acute tests are tests conducted for up to 96 h and chronic tests are tests conducted for at least 21 d.

	Conditions	Johnson et al (1990, 1993) (136, 79)	Jacobson (1990) (116), Jacobson et al (1993) (75)	Lasee (1991) (134)	Keller and Zam (1991) (67)	Klaine et al (1997) (135)	Scheller (1997) (138)	Myers-Kinzie (1998) (132)	Dimock and Wright (1993) (48)	Newton et al (2003) (61)	Lasee (1991) (134)	Wade et al (1993) (119)[B]	Jacobson (1990) (116)	Valenti et al (2005) (107)	USGS (2004) (112)	USGS (2005b) (9)	Recommended Test Conditions
1	Species tested	*Utterbackia imbecillis*[C]	*Villosa nebulosa, V. iris, Anodonta grandise*[D]	*Lampsilis cardium*[E]	Mulitple species[F]	*Utterbackia imbecillis*	*Villosa iris*	*Lampsilis siliquoidea*	*Utterbackia imbecillis, Pyganodon cataracta*	*Lampsilis cardium*	*Lampsilis ventricosa*	*Utterbackia imbecillis*	*Villosa nebulosa*	*Villosa iris*	Mulitple species[G]	*Villosa iris*[H]	NA[I]
2	Test type	Renewal	Static	Static	Static	Static	Static	NR	Static	Flow through	Renewal	Renewal	Artificial stream	Renewal	Renewal, flow through	Flow through	Static, renewal or flow-through (depending on duration of exposure and chemical tested)
3	Test duration (d)	2	1	2	1-4	1-4	4	1, 2, 4	1-4	4. 10	7	9	14	21	2, 4, 10	28	Acute:≤4 Chronic:21 to 28
4	Temperature, °C	20	20	21	22, 25, or 32	25	25	24	20	21	21	24	20	20	20	20	20
5	Light quality	Ambient lab light	NR[I]	NR	NR	NR	NR	NR	NR	Fluorescent	NR	NR	NR	NR	Fluorescent	Fluorescent	Ambient lab light
6	Light intensity	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	200 lux	200 lux	100 to 1000 lux
7	Photo period	16L:8D	16L:8D	24 D	12L:12D or 16L:8D	16L:8D	NR	NR	NR	18L:8D	24D	24D	16L:8D	12L:12D	16L:8D	16L:8D	16L:8D
8	Test chamber	125-mL beaker	12-well plate	Covered 250-mL crystallizing dish	Petri dish	Petri dish	12-well plate	Petri dish	120-mm diam. tub with mesh bottom in 4-L chamber	132 by 90 by 130 mm chamber	Covered 250-mL crystallizing dish	50-mm diam. glass tub with mesh bottom in 250-mL chamber	Dish covered with mesh	30-mL beakers submerged in a 1-L glass beaker	50- or 300-mL beaker	300-mL beaker	Static or renewal: 50-mL beakers (minimum) Flow-through: 300 mL beakers (minimum)
9	Test solution volume (mL)	100	3.5	NR	15	15	5	10	NR	1200	NR	200	150	950	30 or 200	200	Static or renewal: 30 (minimum) Flow-through: 200 (minimum)
10	Procedure for obtaining juveniles	Artificial media	Fish host	Fish host	Fish host or artificial media	Fish host or artificial media	Fish host	Artificial media	Artificial media	Fish host	Fish host	Artificial media	Fish host	Fish host	Fish host	Fish host	Fish host
11	Age of test organisms (day)	1-10	1-3	0, 7, 14	1-2	1-3	<3, 5, 9	<10	7-10	3-5	0	6-10	1-3	60	3-5, 60	60	Acute:<5 Chronic:60 to 120

TABLE A1.4 *Continued*

	Conditions	Johnson et al (1990, 1993) (136, 79)	Jacobson (1990) (116), Jacobson et al (1993) (75)	Lasee (1991) (134)	Keller and Zam (1991) (67)	Klaine et al (1997) (135)	Scheller (1997) (138)	Myers-Kinzie (1998) (132)	Dimock and Wright (1993) (48)	Newton et al (2003) (81)	Lasee (1991) (134)	Wade at al (1993) (119)[B]	Jacobson (1990) (116)	Valenti et al (2005) (107)	USGS (2004) (112)	USGS (2005b) (9)	Recommended Test Conditions
12	No. organisms per test chamber	10	10	10	10-20	1	5	NR	10	20	50	15	15	5	5	10	Acute:<5 (minimum) Chronic:10 (minimum)
13	No. replicate chambers per treatment	2	2 or 3	3	2-4	10	4	NR	3	6	2	3	3	4	4	4	Acute:4 (minimum) Chronic:3 (minimum)
14	Feeding	None	None	None	None	None	None	None	Nona	None	Lab cultured phytoplankton	Algae and silt	Algae	Algae and sediment	None	Instant algae mixture[J]	Acute:none Chronic: Algae
15	Aeration	None	None	Yes	NR	None	NR	NR	Yes	Yes	None	None	None	Yes	None	None	None, if dissolved oxygen a maintained above acceptable concentration
16	Dilution water	Reconstituted water, hardness 40-50 mg/L as $CaCO_3$	Clinch River water, VA	Hardness 150 mg/L as $CaCO_3$	Reconstituted water, hardness 47-76 mg/L as $CaCO_3$	Reconstituted water, hardness 99-107 mg/L as $CaCO_3$	Sinking Creek water, VA	Hardness 100 or 200 mg/L as $CaCO_3$	NR	Hardness 133 mg/L as $CaCO_3$	Hardness 150 mg/L as $CaCO_3$	Tennessee River water	Clinch River water, VA	Reconstituted water, hardness 100 mg/L as $CaCO_3$	Reconstituted water, hardness 170 mg/L as $CaCO_3$	Reconstituted water- hardness 170 mg/L as $CaCO_3$	Depends on experimental design
17	Water quality	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	pH, hardness	NR	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	DO, pH, hardness, alkalinity, conductivity	NR	NR	DO, pH, ammonia, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity	DO, pH, ammonia, hardness, alkalinity, conductivity
18	Endpoints	Survival (movement)	Survival (gaped valves, foot activity or stained with neutral red)	Survival (foot or ciliary movement)	Survival (activity and heartbeat)	Survival (gaped valves with foot and ciliary activity)	Survival (heartbeat and ciliary action)	Survival (foot or valve movement)	Survival (foot, valve or ciliary activity, heartbeat)	Survival, growth, ratio of stressed to alive	Survival (foot or ciliary movement), growth (length and height)	Survival (Ciliary action)	Survival (extruded foot and gaping valves)	Survival, growth	Survival (foot or shell movement) and growth (shell length)	Survival (foot or shell movement) and growth (shell length)	Survival (foot movement), growth (shell length)
19	Control survival (%)	>95	100	96	NR	>90	>80	99	>90	>95	97	>90	100	90	>90	>88	Acute:>90 (must) Chronic:>80 (should)

[A] Reprinted with permission of Kernaghan et al (2005) (5). Copyright Society of Environmental Toxicology and Chemistry (SETAC).
[B] See also Masnado et al (1995) (140), McKinney and Wade (1996) (120), Keller et al (1999) (141).
[C] Formerly *Anodonta imbedilis*.
[D] See also McCann (1993) (131) for 2- to 4-d exposures with *Villosa iris*, *Actinonaias pectomsa*, *Medionidus conradius*.
[E] Formerly *Lampsilis ventricosa*.
[F] *Anodonta imbecillis*, *Villosa lienosa*, *V. villosa*, *Utterbackia imbecillis*, *Lampsilis straminea daibomensis*, *L. subangulata*, *Elliptic icterina*. See also Keller 1993 (142), Keller and Ruessler 1997 (58).
[G] *Villosa iris*, *Epioblasma capsaeformis*, *Lampsilis fasciola*, *L. siliquoidea*, *L. abrupta*, *L. rafinesqueana*, *Leptodea leptodon*.
[H] Bringotf et al (2005) (108) and USGS (2005a) (8) have adapted this method to conduct 21- to 28-d toxicity tests with 2- to 4-month old juvenile *Actinonaias ligamentina* or *Lampsilis siliquoidea*.
[I] NA: not applicable. NR: not reported.
[J] See section A1.4.5.3 for a description of the procedure used to prepare this instant algae mixture.

TABLE A1.5 Test Acceptability Requirements for Toxicity Tests Conducted with Freshwater 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 ±10 % during the exposure and dissolved oxygen should be maintained above 4 mg/L.
3. The duration of an acute toxicity test should be no more than half of the length of time that 90% of the organisms survive in the dilution water under test conditions. Specifically, survival of control organisms in control water might be evaluated for an additional time period after the end of an acute test to further evaluate the quality of the test organisms (for example, control survival should be >90% for 98 h after the end of a 96-h juvenile toxicity test).

B. Performance-based criteria for culturing and handling of juvenile 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 (for example, NaCl or $CuSO_4$, section 16.4). 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 juvenile and adult mussels in the cultures. Records should also be kept on procedures used to collect and hold juvenile and 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.

C. Additional requirements:
1. All organisms in a test must be from the same source and should be acclimated to the dilution water for at least 24 h before the start of 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 test solution should not exceed 0.5 mL/L in 96-h tests or 0.1 mL/L in longer-term tests. A surfactant should not be used in the preparation of a test solution.
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, *(1)* no treatment other than a control treatment killed or affected less than 37 % of the organisms or, *(2)* no treatment killed or affected more than 63 % of the organisms.

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