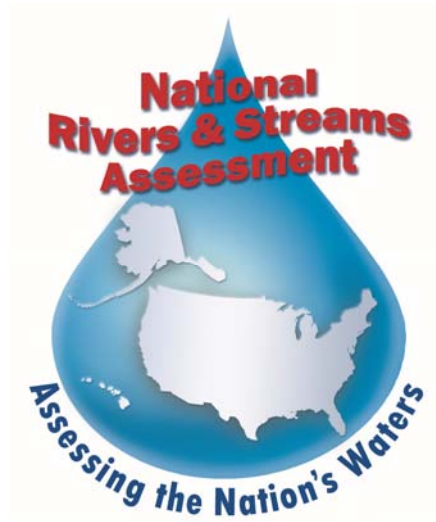




United States Environmental Protection Agency
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Washington, DC
EPA 841-B-12-010

National Rivers and Streams Assessment 2013-2014 Laboratory Operations Manual

Version 2.0 May 2014



NOTICE

The intention of the National Rivers and Streams Assessment 2013-2014 is to provide a comprehensive “State of Flowing Waters” assessment for rivers and streams across the United States. The complete documentation of overall project management, design, methods, quality assurance, and standards is contained in five companion documents:

National Rivers and Streams Assessment 2013-14: Quality Assurance Project Plan EPA-841-B-12-007

National Rivers and Streams Assessment 2013-14: Site Evaluation Guidelines EPA-841-B-12-008

National Rivers and Streams Assessment 2013-14: Non-Wadeable Field Operations Manual EPA-841-B-12-009a

National Rivers and Streams Assessment 2013-14: Wadeable Field Operations Manual EPA-841-B-12-009b

National Rivers and Streams Assessment 2013-14: Laboratory Operations Manual EPA 841-B-12-010

Addendum to the National Rivers and Streams Assessment 2013-14: Wadeable & Non-Wadeable Field Operations Manuals

This document (*Laboratory Operations Manual*) contains information on the methods for analyses of the samples to be collected during the project, quality assurance objectives, sample handling, and data reporting. These methods are based on the guidelines developed and followed in the Western Environmental Monitoring and Assessment Program (Peck et al. 2003). Methods described in this document are to be used specifically in work relating to the NRSA 2013-2014. All Project Cooperator laboratories should follow these guidelines. Mention of trade names or commercial products in this document does not constitute endorsement or recommendation for use. More details on specific methods for site evaluation, sampling, and sample processing can be found in the appropriate companion document.

The suggested citation for this document is:

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LIST OF ACRONYMS

A	absorbance	HCl	hydrogen chloride
ADT	analysis decision tree	HDPE	high density polyethylene
AFDM	ash-free dry mass	HNO ₃	nitric acid
ANC	acid neutralizing capacity	HQ	headquarters
ANS	Academy of Natural Sciences	HRP	antibody-Horseradish Peroxidase
AQM	absolute quantitation method	H ₂ S	hydrogen sulfide
ASTM	American Society for Testing and Materials	H ₂ SO ₄	sulphuric acid
Avg	Average	IBD	ionic balance difference
BHI	brain heart infusion	ID	Identification
BV	biovolume	IM	information management
Ca	Calcium	IPC	internal positive control
CCE	calibrator cell equivalents	ISBN	International Standard Book Number
CEQ	cell equivalent	ISO	International Organization for Standardization
Chl-a	chlorophyll-a	IT IS	Integrated Taxonomic Information System (IT IS)
CO ₂	carbon dioxide	K	potassium
Ct	threshold cycle	KC	kit control
CPR	cardiopulmonary resuscitation	LFB	lab fortified blanks
cv	curriculum vitae	LFM	lab fortified matrices
CV	coefficient of variation	LIMS	Laboratory Information Management System
DCF	dilution/concentration factor	LOM	Lab Operations Manual
DI	de-ionized	LRL	lower reporting limit
DIC	differential interference contrast	Mg	magnesium
DL	detection limit	MDL	method detection limit
DNA	Deoxyribo-nucleic Acid	MPCA	Minnesota Pollution Control Agency
DO	dissolved oxygen	MSDS	Materials Safety Data Sheet
DOC	dissolved organic carbon	N	nitrogen
DTH	depositional targeted habitat	Na	sodium
DW	distilled water	NABS	North American Benthological Society
ELISA	enzyme-linked Immunosorbent assay	NALMS	North American Lakes Management Society
EMAP	Environmental Monitoring and Assessment Program	NARS	National Aquatic Resource Surveys
ENT	enterococci	NAWQA	National Water Quality Assessment Program
EPA	Environmental Protection Agency	NC	negative control
ETOH	ethyl alcohol	ND	nondetect
FOM	Field Operations Manual		
GEQ	genomic equivalent		
GIS	geographic information system		
GPS	global positioning device		

NELAC	National Environmental Laboratory Accreditation Conference	qPCR	quantitative polymerase chain reaction
NELAP	National Environmental Laboratory Accreditation Program	QRG	Quick Reference Guide
NH ₄	ammonium	RL	reporting limit
NIST	National Institute of Standards	RMSE	root mean square error
NO ₂	nitrite	RO	reverse-osmosis
NO ₃	nitrate	RPD	relative percent difference
NRSA	National Rivers and Streams Assessment	RQM	relative quantitation method
NTL	no template control	RSD	relative standard deviation
NTU	Nephelometric Turbidity Units	RTH	richest targeted habitat
OD	optical density	S	standard deviation
OSHA	Occupational Safety and Health Administration	S0-S5	standards provided with microcystin kit
PBS	phosphate buffered saline	SFS	Society of Freshwater Science
PCB	polychlorinated biphenyl	SEG	Site Evaluation Guidelines
PctDIFF	percent difference	SiO ₂	silica
PDE	percent disagreement in enumeration	SO ₄	sulphate
PCR	polymerase chain reaction	SOPs	Standard Operating Procedures
PE	performance evaluation	SPC	sample processing control
PES	performance evaluation samples	S-R	Sedgewick-Rafter count
PHab	physical habitat	SRM	standard reference material
P-M	Palmer-Maloney (P-M) count	SS	salmon sperm
PDE	percent difference in enumeration	TMB	tetramethylbenzidine
PSE	percent sorting efficiency	TN	total nitrogen
PT	performance testing	TOC	total organic carbon
PTD	percent taxonomic disagreement	TP	total phosphorus
QA	quality assurance	TRANS	transect
QAPP	Quality Assurance Project Plan	TSN	taxonomic serial number
QA/QC	quality assurance/quality control	TSS	total suspended solids
QC	quality control	TVS	total volatile solids
QCCS	quality control check solution	UNK	unknown
QMP	Quality Management Plan	USGS	United States Geological Survey
		WSA	Wadeable Streams Assessment
		WQX	Water Quality Exchange

1.0 INTRODUCTION

This manual describes methods for analyses of the samples to be collected during the National Rivers and Streams Assessment (NRSA), including quality assurance objectives, sample handling, and data reporting. The NRSA is a probabilistic assessment of the condition of our Nation's rivers and streams and is designed to:

- Assess the condition of the Nation's rivers and streams
- Establish a baseline to compare future rivers and streams surveys for trends assessments
- Evaluate changes in condition from the 2004 Wadeable Streams Assessment
- Help build State and Tribal capacity for monitoring and assessment and promote collaboration across jurisdictional boundaries

This is one of a series of water assessments being conducted by states, tribes, the U.S. Environmental Protection Agency (EPA), and other partners. In addition to rivers and streams, the water assessments will also focus on coastal waters, lakes, and wetlands in a revolving sequence. The purpose of these assessments is to generate statistically-valid reports on the condition of our Nation's water resources and identify key stressors to these systems.

The goal of the NRSA is to address two key questions about the quality of the Nation's rivers and streams:

- What percent of the Nation's rivers and streams are in good, fair, and poor condition for key indicators of water quality, ecological health, and recreation?
- What is the relative importance of key stressors such as nutrients and pathogens?

EPA selected sampling locations using a probability based survey design. Sample surveys have been used in a variety of fields (e.g., election polls, monthly labor estimates, forest inventory analysis) to determine the status of populations or resources of interest using a representative sample of a relatively few members or sites. Using this survey design allows data from the subset of sampled sites to be applied to the larger target population, and assessments with known confidence bounds to be made.

With input from the states and other partners, EPA used an unequal probability design to select 900 wadeable streams and 900 non-wadeable rivers. To estimate change from the 2004 Wadeable Streams Assessment (WSA), 450 of the 900 wadeable sites were selected using an unequal probability design from the WSA original sites. Field crews will collect a variety of measurements and samples from randomly predetermined sampling reaches (located with an assigned set of coordinates), and from randomized stations along the sampling reach.

2.0 GENERAL LABORATORY GUIDELINES

2.1 Responsibility and Personnel Qualifications

All laboratory personnel shall be trained in advance in the use of equipment and procedures used for the standard operating procedure (SOP) in which they are responsible. All personnel shall be responsible for complying with all of the QA/QC requirements that pertain to the samples to be analyzed. Each lab should follow its institutional or organizational requirements for instrument maintenance. Specific lab qualification documentation required for analysis is contained in the Quality Assurance Project Plan (QAPP).

2.2 Roles and Contact Information

The **EPA Headquarters Project Management Team** consists of the Project Leader, Alternate Project Leaders, Project QA Lead, and Laboratory Review Manager. The Team is responsible for overseeing all aspects of the project and ensuring technical and quality assurance requirements are properly carried out. The Team is the final authority on all decisions regarding laboratory analysis.

The **NARS Information Management (IM) Coordinator** tracks the location of each NRSA 2013-2014 sample that involves post-processing. The coordinator will be the labs main point of contact in regards to sample tracking and data submission.

Table 2.1 Contact information

Title	Name	Contact Information
EPA HQ NRSA Project Lead	Ellen Tarquinio, OW	tarquinio.ellen@epa.gov 202-566-2267
EPA HQ NRSA Project QA Lead	Sarah Lehmann, OW	lehmann.sarah@epa.gov 202-566-1379
EPA HQ NRSA Laboratory Review Manager	Kendra Forde, OW	kendra.forde@epa.gov 202-564-0417
Information Management Center Coordinator	Marlys Cappaert, SRA International Inc.	cappaert.marlys@epa.gov 541-754-4467 541-754-4799 (fax)

2.3 Sample Tracking

Samples are collected by a large number of different field crews during the index period (May through September). The actual number of rivers and streams sampled on a given day will vary widely during this time. Field crews will submit electronic forms when they have shipped samples and the NARS IM Center will input each sample into the NARS IM database. Laboratories can track sample shipment from field crews by accessing the NARS IM database. Participating laboratories will be given access to the NARS IM system, where they can acquire tracking numbers and information on samples that have been shipped to them by field crews (either by overnight shipment for perishable samples or batch shipments for preserved samples). Upon sample receipt, the laboratory must immediately log in to the database and confirm that samples have arrived. Overnight samples may not be loaded into the database prior to sample arrival, but should be tracked by the laboratory and receipt information inputted into the database when sample information is loaded. Each lab will make arrangements with the NARS IM Coordinator, listed above, to ensure access is granted.

When the samples arrive from the field crews, laboratories should also receive tracking forms in the shipment (refer to the NRSA 2013-2014 FOM). These forms will list the samples that should be included in the shipment. Laboratory personnel should cross check the forms with the samples received to verify that there are not any inconsistencies. If any sample is missing or damaged, contact the NARS IM Coordinator immediately.

2.4 Reporting

All labs must provide data analysis information to the HQ Project Management Team and the NARS IM Center by **March 30, 2015** or as stipulated in contractual agreements. These reports must include the following information:

- Sample Type (indicator)
- Site ID (ex: CAS9-0918)
- Sample ID (ex: 999000)
- Pertinent information to the indicator
- Metadata for all fields

See Appendix C for reporting templates that labs will submit electronically.

The submitted file name must state the following:

- Indicator name (ex: microcystin)
- Date of files submission to NARS IM Center by year, month, and day (ex: 2013_11_01)
- Lab name (ex: MyLab)

Combined, the file name would look as follows: WaterChemistry_2013_11_01_MyLab.xlsx

As specified in the QAPP, remaining sample material and specimens must be maintained by the EPA's designated laboratory or facilities as directed by the NRSA 2013-2014 Project Lead. All samples and raw data files (including logbooks, bench sheets, and instrument tracings) are to be retained by the laboratory for 3 years or until authorized for disposal, in writing, by the EPA Project Lead. Deliverables from contractors and cooperators, including raw data, are permanent as per EPA Record Schedule 258. EPA's project records are scheduled 501 and are also permanent.

3.0 ALGAL TOXIN (MICROCYSTIN) IMMUNOASSAY PROCEDURE

This chapter describes an immunoassay procedure that measures concentrations of total microcystins in water samples. In applying the procedure, the laboratory uses Abraxis' Microcystins-ADDA Test Kits (Figure 3.1; "kits"). Each kit is an enzyme-linked immunosorbent assay (ELISA) for the determination of microcystins and nodularins in water samples. Microcystins refers to the entire group of toxins, all of the different congeners, rather than just one congener. Algae can produce one or many different congeners at any one time, including Microcystin-LR (used in the kit's calibration standards), Microcystin-LA, and Microcystin-RR. The different letters on the end signify the chemical structure (each one is slightly different) which makes each congener different.



Figure 3.1 Microcystin: Abraxis microcystin test kit
(from James, page 3, 2010)

3.1 Summary of the Procedure

The procedure is an adaptation of the instructions provided by Abraxis for determining total microcystins concentrations using its ELISA-ADDA kits.^a For freshwater samples, the procedure's reporting range is 0.15 µg/L to 5.0 µg/L, although, theoretically, the procedure can detect, not quantify, microcystins concentrations as low as 0.10 µg/L. For samples with higher concentrations of microcystins, the procedure includes the necessary dilution steps.

^a Abraxis, "Microcystins-ADDA ELISA (Microtiter Plate): User's Guide R021412." Retrieved on January 14, 2014 from http://www.abraxiskits.com/uploads/products/docfiles/278_Microcystin%20PL%20ADDA%20users%20R120214.pdf.

3.2 Health and Safety Warnings

The laboratory must require its staff to abide by appropriate health and safety precautions, because the kit substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. In addition to the laboratory's usual requirements such as a Chemical Hygiene Plan, the laboratory must adhere to the following health and safety procedures:

1. Laboratory facilities must properly store and dispose of solutions of weak acid.
2. Laboratory personnel must wear proper personal protection clothing and equipment (e.g. lab coat, protective eyewear, gloves).
3. When working with potential hazardous chemicals (e.g., weak acid), laboratory personnel must avoid inhalation, skin contact, eye contact, or ingestion. Laboratory personnel must avoid contacting skin and mucous membranes with the TMB and stopping solution. If skin contact occurs, remove clothing immediately. Wash and rinse the affected skin areas thoroughly with large amounts of water.

3.3 Definitions and Required Resources (Personnel, Laboratories, and Equipment)

This section provides definitions and required resources for using the procedure.

3.3.1 Definitions

The following terms are used throughout the procedure:

Absorbance (A) is a measure of the amount of light in a sample. A standard statistical curve is used to convert the absorbance value to the concentration value of microcystins.

Calibration Range is the assay range for which analysis results can be reported with confidence. For undiluted samples, it ranges from the reporting limit of 0.15 µg/L to a maximum value of 5.0 µg/L. Values outside the range are handled as follows. If the value is:

- < 0.10 µg/L, then the laboratory reports the result as being non-detected (“<0.10 µg/L”).
- Between 0.10 µg/L and the reporting limit of 0.15 µg/L (i.e., >0.10 µg/L and <0.15 µg/L), the laboratory should record the value, but assign a QC code to the value (i.e., DATA_FLAG=J).
- 5.0 µg/L, the laboratory must dilute and reanalyze the sample.

Coefficient of Variation (CV): The precision for a sample is reported in terms of the percent CV of its absorbance values. To calculate the %CV, first calculate *S* (standard deviation) as follows:

$$S = \left[\frac{1}{n-1} \sum_{i=1}^n (A_i - \bar{A})^2 \right]^{1/2}$$

where *n* is the number of replicate samples, *A_i* is the absorbance measured for the *i*th replicate. Per Section 3.5.3, samples are evaluated in duplicate (i=1 or 2); controls are either evaluated in duplicate or triplicate (i=1, 2, 3). \bar{A} is the average absorbance of the replicates. Then, calculate %CV as:

$$\%CV = \left| \frac{S}{\bar{A}} \right| \times 100$$

Dark or Dimly Lit: Away from sunlight, but under incandescent lighting is acceptable.

Detection Limit is the minimum concentration at which the analyte can be *detected* with confidence. In other words, the outcome can be reported with confidence that it is greater than zero (i.e., present in the sample). The detection limit is less than the reporting limit of 0.15 µg/L at which the *measured* value of the analyte can be reported with confidence. Also see “Sample-Specific Detection Limit.”

Duplicates are defined as two aliquots of the same sample which are analyzed separately using identical procedures. The results are used to evaluate the precision of the laboratory analyses. Per Section 3.5.3, controls are evaluated in duplicate or triplicate (i.e., three aliquots).

NARS: National Aquatic Resource Surveys. The National Rivers and Streams Assessment (NRSA) is part of the NARS program.

NARS Information Management System (NARS IM): The IM system established to support all surveys, including NRSA, in the NARS program. The IM system is used to track the samples from field collection to the laboratory.

NRSA: National Rivers and Streams Assessment. Freshwater samples will be collected during the field stage of NRSA.

Relative Standard Deviation (RSD) is the same as the coefficient of variation (%CV). Because many of the plate reader software programs provides the CV in their outputs, the procedure presents the quality control requirement in terms of %CV instead of RSD.

Reporting Limit: For undiluted freshwater sample, the reporting limit is 0.15 µg/L. A reporting limit is the point at which the measured value of the analyte can be reported with confidence.

Standard Deviation (S) shows variation from the average

Sample-Specific Detection Limit: Most samples will have a sample-specific detection equal to the method’s detection limit of 0.1 µg/L. For diluted samples, the sample-specific detection limit will be the product of the method’s detection limit of 0.1 µg/L and the dilution factor. Typical values for the dilution factor will be 10 or 100.

3.4 General Requirements for Laboratories

3.4.1 Expertise

To demonstrate its expertise, the laboratory shall provide EPA with one or more of the following:

- Memorandum that identifies the relevant services that the laboratory provided for the National Aquatic Resource Surveys in the past five years.
- Documentation detailing the expertise of the organization, including professional certifications for water-related analyses, membership in professional societies, and experience with analyses that are the same or similar to the requirements of this method.

3.4.2 Quality assurance and quality control requirements

To demonstrate its expertise in quality assurance and quality control procedures, the organization shall provide EPA with copies of the quality-related documents relevant to the procedure. Examples include Quality Management Plans (QMP), QAPPs, and applicable Standard Operating Procedures (SOPs).

To demonstrate its ongoing commitment, the person in charge of quality issues for the organization shall sign the NRSA QAPP Certification Page.

3.4.3 Personnel

Laboratory Technician: This procedure may be used by any laboratory technician who is familiar with the NRSA 2013-14 QAPP, and this procedure in the NRSA 2013-14 LOM (which differs from the Abraxis instructions). The laboratory technician also must be familiar with the use of a multichannel pipette and plate readers.

External QC Coordinator is an EPA staff person who is responsible for selecting and managing the “**QC contractor.**” To eliminate the appearance of any inherent bias, the QC contractor must be dedicated to QA/QC functions, and thus, must not be a primary laboratory or a field sampling contractor for NRSA. The QC contractor is responsible for complying with instructions from the External QC Coordinator; coordinating and paying for shipments of the performance samples to participating laboratories; comparing immunoassay results from the laboratories; and preparing brief summary reports.

3.4.4 Equipment/Materials

The procedures require the following equipment and information:

- Abraxis ADDA Test Kit, Product #520011 (see items in **Section 3.6.2**)
- Adhesive Sealing Film (Parafilm) for Micro Plates (such as Rainin, non-sterile, Cat. No. 96-SP-100): Used to cover plates during incubation.
- Data Template – See **Figure 3.2**
- Distilled or Deionized Water: For diluting samples when necessary.
- ELISA evaluation software
- Glass scintillation, LC, vials (two vials of 2 mL each)
- Glass vials with Teflon-lined caps of size:
 - 20 mL
 - 4 mL (for dilutions)
- Multichannel Pipette & Tips: A single-channel and an 8-channel pipette are used for this method.
- Norm-ject syringes (or equivalent)
- Paper Towels: For blotting the microtiter plates dry after washing.
- Permanent Marker (Sharpie Fine Point): For labeling samples, bottles, plates and covers.
- Plate Reader (e.g., Metertech Model M965 AccuReader; Chromate[®]; or equivalent readers with software to read the microtiter plates and measure absorbances).
- Reagent Reservoirs (e.g., Costar Cat Number 4870): Plain plastic reservoir for reagents that accommodate the use of a multi-channel pipette.
- Test tubes: For dilutions, if needed.
- Timer: For measuring incubation times.
- Vortex Genie: For mixing dilutions.
- Whatman Glass fiber syringe filter (25mm, GF 0.45 µm filter)

3.5 Sample Receipt

Field crews hold the microcystins samples on ice while in the field and then pack the samples in ice for delivery to a central facility (“batching laboratory”) or the State’s laboratory. The batching and State laboratories freeze the samples upon receipt. Periodically, the batching laboratory ships samples to the

microcystins laboratory. The batching and microcystins laboratory may retain the frozen samples for several months before analysis.

Because EPA initiates tracking procedures designed to recover any missing shipment, the laboratory personnel responsible for tracking samples must start the following login steps within 24 clock hours of receiving a delivery.

1. Report receipt of samples in the NARS IM sample tracking system (within 24 clock hours).
2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
 - a. Verify that the sample IDs in the shipment match those recorded on the:
 - i. Chain of custody forms when the batching laboratory sends the samples to the microcystins laboratory; or
 - ii. Sample tracking form if the field crew sends the shipment directly to the State laboratory.
 - b. Record the information in **Table 3.1** into NARS IM, including the Condition Code for each sample:
 - i. *OK*: Sample is in good condition
 - ii. *C*: Sample container was cracked
 - iii. *L*: Sample container is leaking
 - iv. *ML*: Sample label is missing
 - v. *NF*: Sample not frozen
 - c. If any sample is damaged or missing, contact the EPA HQ Laboratory Review Manager to discuss whether the sample can be analyzed. (See contact information in **Table 2.1**).
3. Store samples in the freezer until sample preparation begins.
4. Maintain the chain of custody or sample tracking forms with the samples.

Table 3.1 Microcystin: required data elements – login

FIELD	FORMAT	DESCRIPTION	
LAB ID	text	Name or abbreviation for QC laboratory	
DATE RECEIVED	MMDDYY	Date sample was received by lab	
SITE ID	text	NRSA site id as used on sample label	
VISIT NUMBER	numeric	Sequential visits to site (1 or 2)	
SAMPLE ID	numeric	Sample id as used on field sheet (on sample label)	
DATE COLLECTED	MMDDYY	Date sample was collected	
CONDITION CODE	text	Condition codes describing the condition of the sample upon arrival at the laboratory.	
		Flag	Definition
		OK	Sample is in good condition
		C	Sample container is cracked
		L	Sample or container is leaking
		ML	Sample label is missing
		NF	Sample is not frozen
		Q	Other quality concerns, not identified above

3.6 Procedure

The following sections describe the sample and kit preparation and analysis.

3.6.1 Sample Preparation

For each frozen sample (500 mL per sample), the laboratory technician runs it through a freeze-thaw cycle three times to lyse the cells as follows:

1. All cycles: Keep the samples in dark or dimly lit areas (i.e., away from sunlight, but under incandescent lighting is acceptable).
2. First freeze-thaw cycle:
 - a. Start with a frozen 500 ml sample.
 - b. Thaw the sample to room temperature (approximately 25° C). Swirl the sample to check for ice crystals. At this temperature, no ice crystals should be present in the sample.
 - c. Shake well to homogenize the sample, then transfer 10 mL to an appropriately labeled clean 20 mL glass vial.
3. Second freeze-thaw cycle:
 - a. Freeze the vial.
 - b. Keep the large sample bottle (from the 500 mL initial sample) frozen for future use.
 - c. Thaw the sample vial contents to room temperature.
4. Third freeze-thaw cycle:
 - a. Freeze the vial.
 - b. Thaw the vial contents to room temperature.
 - c. Filter the vial contents through a new, syringe filter (0.45 µm) into a new, labeled 20 mL glass scintillation vial. Norm-ject syringes and Whatman Glass fiber syringe filters (25mm, GF 0.45 µm filter) or other similar alternative are acceptable. One new syringe and filter should be used per sample.

3.6.2 Kit Preparation

The technician prepares the kits using the following instructions:

1. Check the expiration date on the kit box and verify that it has not expired. If the kit has expired, discard and select a kit that is still within its marked shelf life. (Instead of discarding the kit, consider keeping it for training activities.)
2. Verify that each kit contains all of the required contents:
 - Microtiter plate
 - Standards (6) referenced in this procedure as follows with the associated concentration:
 - S0: 0 µg/L
 - S1: 0.15 µg/L
 - S2: 0.40 µg/L,
 - S3: 1.0 µg/L
 - S4: 2.0 µg/L
 - S5: 5.0 µg/L
 - Kit Control (KC): 0.75 µg/L
 - Antibody solution
 - Anti-Sheep-HRP Conjugate

- Wash Solution 5X Concentrate
 - Color Solution
 - Stop Solution
 - Diluent
3. If any bottles are missing or damaged, discard the kit. This step is important because Abraxis has calibrated the standards and reagents separately for each kit.
 4. Adjust the microtiter plate, samples, standards, and the reagents to room temperature.
 5. Remove 12 microtiter plate strips (each for 8 wells) from the foil bag for each kit. The plates contain 12 strips of 8 wells. If running less than a whole plate, remove unneeded strips from the strip holder and store in the foil bag, ziplocked closed, and place in the refrigerator.
 6. Store the remaining strips in the refrigerator (4-8° C).
 7. Prepare a negative control (NC) using distilled water
 8. The standards, controls, antibody solution, enzyme conjugate, color solution, and stop solutions are ready to use and do not require any further dilutions.
 9. Dilute the wash solution with deionized water. (The wash solution is a 5X concentrated solution.) In a 1L container, dilute the 5X solution 1:5 (i.e., 100 mL of the 5X wash solution plus 400 mL of deionized water). Mix thoroughly. Set aside the diluted solution to wash the microtiter wells later.
 10. Handle the stop solution containing diluted H₂SO₄ with care.

3.6.3 Insertion of Contents into Wells

This section describes the steps for placing the different solutions into the 96 wells. Because of the potential for cross contamination using a shaker table, the following steps specify manual shaking of the kits instead mechanized shaking.

1. While preparing the samples and kit, turn the plate reader on so it can warm up. The plate reader needs a minimum of 30 minutes to warm up.
2. Turn on the computer so that it can control and access the plate reader.
3. Print the template (**Figure 3.2**) to use as reference when loading the standards, controls, and samples as described in the next step. Templates contain rows, labeled with a marking pen, of strips of 8 wells that snap into the blank frame. (If the laboratory wishes to use a different template, provide a copy to the EPA HQ Laboratory Review Manager for approval prior to first use. (See Section 2 of the manual for contact information.)
4. Using the 100-µL pipette, add 50 µL, each, of the standards, controls, and samples to the appropriate wells in the plate. Place all six standards (0.00, 0.15, 0.40, 1.00, 2.0 and 5.0 µg/L), the kit control (0.75 µL), and negative control, in pairs, starting in the well in the upper left-hand corner of the kit as shown in **Figure 3.2**. Verify that the software displays the same template or make any necessary corrections.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S4	NC	U4	U8	U12	U16	U20	U24	U28	U32	U36
B	S0	S4	NC	U4	U8	U12	U16	U20	U24	U28	U32	U36

C	S1	S5	U1	U5	U9	U13	U17	U21	U25	U29	U33	U37
D	S1	S5	U1	U5	U9	U13	U17	U21	U25	U29	U33	U37
E	S2	KC	U2	U6	U10	U14	U18	U22	U26	U30	U34	U38
F	S2	KC	U2	U6	U10	U14	U18	U22	U26	U30	U34	U38
G	S3	KC	U3	U7	U11	U15	U19	U23	U27	U31	U35	U39
H	S3	NC	U3	U7	U11	U15	U19	U23	U27	U31	U35	U39

Figure 3.2 Microcystin: sample template

Key:

S0-S5 = Standards;

KC = Control supplied with Kit (i.e., Kit Control);

NC = Negative Control;

U = Unknown (sample collected by the field crew);

5. Add 50 μL of the pink antibody solution to each well using the multi-channel pipettor and a reagent reservoir. Use dedicated reagent reservoirs for each reagent to avoid contamination from one reagent to another.
6. Place the sealing Parafilm over the wells.
7. Manually mix the contents by moving the strip holder in a rapid circular motion on the benchtop for 30 seconds. Be careful not to spill the contents.
8. Place the plate in an area away from light for 90 minutes.
9. After 90 minutes, carefully remove the Parafilm.
10. Empty the contents of the plate into the sink, pat inverted plate dry on a stack of paper towels, and then wash the wells of the plate three times with 250 μL of washing solution using the multi-channel pipette. After adding the washing solution each time, empty the solution into the sink and use the paper towels as before.
11. Add 100 μL of enzyme conjugate solution to all wells using the multi-channel pipettor.
12. Cover the wells with Parafilm.
13. Manually mix the contents by moving the strip holder in a rapid circular motion on the benchtop for 30 seconds. Be careful not to spill the contents.
14. Place the strip holder in an area away from light for 30 minutes.
15. After 30 minutes, remove the Parafilm, decant, and rinse the wells three times again with 250 μL of washing solution as described in step 10.
16. Add 100 μL of color solution to the wells using the multi-channel pipette and reagent reservoir. This color solution will make the contents have a blue hue.
17. Cover the wells with Parafilm.
18. Manually mix the contents by moving the strip holder in a rapid circular motion on the benchtop for 30 seconds. Be careful not to spill the contents.
19. Place the plate in an area away from light for 20 minutes.
20. After 20 minutes, remove the Parafilm and add 50 μL of stopping solution to the wells in the same sequence as for the color solution. This will turn the contents a bright yellow color. After adding the stopping solution, read the plate within 15 minutes.

21. Within 15 minutes of adding the stopping solution, use the microplate ELISA photometer (plate reader) to determine the absorbance at 450 nm. The software (i.e., commercial ELISA evaluation program) calculates the absorbance and concentration values of the samples from the calibration curve and the average values for each pair. Use a 4-parameter standard curve fit to determine the concentrations.
22. Dispose of solution in plates in a lab sink. Rinse plates and sink with water to dilute the weak acid present.
23. Perform QC evaluations of the data as follows:
 - a. If the following failures occur, then the laboratory must reanalyze all samples in the analytical run:
 - i. Standard curve with a correlation coefficient of less than 0.99 (i.e., $R < 0.99$)
 - ii. Standards S0-S5 must have decreasing absorbance values. First, calculate the average values for each standard. That is, if \bar{A}_i is the absorbance average for S_i , then the absorbance averages must be:
 - iii. $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5$
 - iv. The average absorbance of the standard S0 less than 0.8 (i.e., $\bar{A}_0 < 0.8$).
 - v. Two or more negative control samples with detectable concentrations of microcystins (i.e., values $> 0.1 \mu\text{g/L}$). If this occurs, then evaluate possible causes (e.g., cross-contamination between samples), and if appropriate, modify laboratory processes before the next analytical run.
 - vi. Results for control samples of outside the acceptable range of 0.75 ± 0.185 ppb. That is, results must be between 0.565 and 0.935.
 - b. If either, or both, of the following failures occur, then the sample must be reanalyzed (maximum of two analyses, consisting of the original analysis and, if necessary, one reanalysis):
 - i. The concentration value registers as HIGH (exceeds the calibration range). Dilute the sample for the reanalysis per **Section 3.6.4**.
 - ii. The %CV $> 15\%$ between the duplicate absorbance values for a sample.
24. Record the results, even if the data failed the quality control requirements in #23b, for each well in EPA's data template (see **Table 3.2** for required elements). The required entries are for the following columns:
 - a. **TYPE** should be one of the following codes: S0-S5 for standards; KC, NC, or SC for controls; U for unknown sample.
 - b. **CONC** contains the numeric concentration value. Two special cases:
 - i. Non-detected concentrations: If the sample is non-detected, then provide the sample-specific detection limit which is $0.1 \mu\text{g/L}$ if the sample is undiluted. See **Section 3.6.4** for calculating the sample-specific detection limit for a diluted sample.
 - ii. If the result shows that it is "HI," this indicates that the sample value is outside of the calibration range and must be diluted and re-run using another analytical run. Leave the CONC column blank and record 'HI' in the DATA FLAG column.
 - c. **DATA FLAGS** have codes for the following special cases:
 - i. **ND** if the sample was non-detected;

- ii. **J** if the value is detected but at a level below the reporting limit of 0.15 µg/L (for undiluted samples);
- iii. **HI** if the concentration value registers as HIGH (exceeds the calibration range).
- d. **QUALITY FLAGS** have codes for the following special cases:
 - i. **QCF** if there is a QC failure per step 23 above. The QCF code must be used for all failures to facilitate data analysis.
 - ii. **Q** for any other quality issue (describe in **COMMENTS**)
- e. **DILUTION FACTOR** is only required if the sample was diluted.
- f. **DUP AVG** and **DUP CV** are required for duplicate samples and control samples (use all three values if the controls are used in triplicate).

Table 3.2 Microcystin: required data elements – data submission

STAGE	FIELD	FORMAT	DESCRIPTION	
LOGIN	LAB ID	text	Name or abbreviation for QC laboratory	
	DATE RECEIVED	text	Date sample was received by lab	
	SITE ID	text	NRSA site ID code as recorded on sample label or tracking form (blank if standard or control)	
	VISIT NUMBER	numeric	Sequential visits to site (1 or 2) (blank if standard or control)	
	SAMPLE ID	numeric	6-digit Sample ID number as recorded on sample jar or tracking form (blank if standard or control)	
	DATE COLLECTED	MMDDYY	Date sample was collected (blank if standard or control)	
	CONDITION CODE	text	Sample condition upon arrival at the laboratory (blank if standard or control)	
			Flag	Definition
			Blank or N	Not a sample (blank, standard, or control)
			OK	Sample is in good condition
			C	Sample container is cracked
L			Sample or container is leaking	
ML			Sample label is missing	
NF			Sample is not frozen	
ANALYSIS	TECHNICIAN	text	Name or initials of technician performing the procedure	
	KIT EXPIRE DATE	MMDDYY	Expiration date on kit box	
	KIT ID	text	Kit identification code. If one does not exist, assign a unique code to each kit.	
	R2	numeric	R ² from curve fit to the average absorbance values for the standards. Value is between 0 and 1.	
	TYPE	text	Type of solution being tested in the well	
			Code	Definition
			KC	Kit Control
			NC	Negative Control
			S0,S1, S2,S3, S4, S5	Standard
	U	Sample of unknown concentration		
	LOCATION	text	Location of well in the kit (e.g., B5 would be the fifth well from the left in the second row B)	
SALINITY	numeric	If the sample vial has the salinity marked on the vial, record the value in units of parts per thousand. Otherwise, leave blank.		

CONC	numeric	Concentration or sample-specific detection limit of contents of well in µg/L. Sample-specific detection limit should be 0.1 µg/L if the sample hasn't been diluted.
ABSORBANCE	numeric	Absorbance value
DILUTION FACTOR	numeric	10, 100, etc for number of times the sample was diluted. If not diluted, leave blank or record 1
CV_ABSORB	numeric	Calculated %CV of duplicate values of absorbance for a sample. Only calculated for TYPE=U, KC, or NC. Enter %CV. Value is between 0 and 100%.
AVG_ABSORB	numeric	Calculated average of absorbance values for a sample. Only provided for TYPE=U, KC, NC, or SC. Average value of the original sample and its duplicate (or replicates for KC and NC).
AVG_CONC	numeric	Calculated average of concentration values for a sample. Substitute 0.15 µg/L for any result recorded as <0.15 µg/L
DATA FLAG (if appropriate)	text	Data qualifier codes associated with specific identifications of voucher samples. These codes provide more information than those used when reporting receipt of samples. A technician may use alternative or additional qualifiers if definitions are provided as part of the submitted data package (e.g., as a separate worksheet page of the data submission file).
		Flag Definition
		ND Concentration below detection. Unless the sample was diluted, the concentration will be 0.1 µg/L
		HI Result indicated that a high concentration (i.e., outside calibration range)
		J Concentration above detection but below reporting limit. Without dilution, these values are between 0.1 and 0.15 µg/L
QUAL_FLAG	QCF/Q	QCF QC failure
		Q Other quality concerns, not identified above
COMMENTS	text	Explanation for data flag(s) (if needed) or other comments.

3.6.4 Dilutions (if needed)

Dilutions if needed are prepared as follows (using clean glass tubes):

- 1:10 dilution
 - a. Add 900 µL of distilled water to a clean vial. (Note: Dilutions may also be made using the kit's diluent rather than distilled water.)
 - b. Pipette 100 µL from the sample into the vial. (To provide more accurate dilutions and less chance of contaminating the diluent, the diluent should be added to the vial before the sample.)
 - c. Mix by vortexing.
 - d. Multiply final concentration and Abraxis' detection limit of 0.1 µg/L by 10 to obtain the sample-specific detection limit of 1.0 µg/L.

- 1:100 dilution

- a. Add 3.96 mL of distilled water to a clean, appropriately labeled glass vial. (Note: Dilutions may also be made using the kit's diluent rather than distilled water.)
 - b. Vortex the sample to mix thoroughly, then pipette 40 µL from the sample and add to the water (or diluent) in the appropriate labeled vial. Vortex.
 - c. Multiply the final concentration and Abraxis' detection limit of 0.1 µg/L by 100 to obtain the sample-specific detection limit of 10 µg/L.
- Other dilutions can be calculated in the same manner as #1 and #2 if needed.

3.7 Quality Measures

This section describes the quality assurance and quality control measures used to ensure that the data will meet NRSA's requirements.

3.7.1 Assistance Visits

Assistance visits are intended to familiarize EPA with actual procedures being implemented by different laboratories; and to ensure a clear and consistent understanding of procedures and activities by both EPA and the laboratories. If EPA decides to conduct an assistance visit, a qualified EPA scientist or contractor will administer a checklist based upon the steps described in this chapter.

3.7.2 QC Samples

During the course of the survey, the External QC Coordinator will instruct the QC contractor to provide one or two identical sets of QC samples to all participating laboratories. Each set will contain up to five QC samples. As determined by the External QC Coordinator, the QC samples may be synthetic; aliquots of additional samples collected at NRSA reference sites; or reference samples obtained from an organization such as the National Institute of Standards. Each laboratory will run the QC samples following the same procedures used for the other samples. The QC contractor will compare the results and assess patterns in the data (e.g., one laboratory being consistently higher or lower than all others). Based upon the evaluation, the External QC Coordinator may request additional information from one or more laboratories about any deviations from the Method or unique laboratory practices that might account for differences between the laboratory and others. With this additional information, the External QC Coordinator will determine an appropriate course of action, including no action, flagging the data, or excluding some or all of the laboratory's data.

3.7.3 Summary of QA/QC Requirements

Table 3.3 provides a summary of the quality control requirements described in **Sections 3.5** and **3.6**.

Table 3.3 Microcystin: quality control – sample analysis

Quality Control Activity	Description and Requirements	Corrective Action
Kit – Shelf Life	Is within its expiration date listed on kit box.	If kit has expired, then discard or set aside for training activities.
Kit – Contents	All required contents must be present and in acceptable condition. This is important because Abraxis has calibrated the standards and reagents separately for each kit.	If any bottles are missing or damaged, discard the kit.
Calibration	All of the following must be met:	If any requirement fails:

	<ul style="list-style-type: none"> ○ Standard curve must have a correlation coefficient of ≥ 0.99; ○ Average absorbance value, \bar{A}_0, for S0 must be > 0.80; and ○ Standards S0-S5 must have decreasing average absorbance values. That is, if \bar{A}_i is the average of the absorbance values for S_i, then the absorbance average values must be: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5$ 	<ul style="list-style-type: none"> ● Results from the analytical run are not reported. ● All samples in the analytical run are reanalyzed until calibration provides acceptable results.
Kit Control	The average concentration value of the duplicates (or triplicate) must be within the range of 0.75 +/- 0.185 $\mu\text{g/L}$. That is, results must be between 0.565 and 0.935.	If either requirement fails: <ul style="list-style-type: none"> ● Results from the analytical run are not reported
Negative Control	The values for the negative control replicates must meet the following requirements: <ul style="list-style-type: none"> ○ All concentration values must be $< 0.15 \mu\text{g/L}$ (i.e., the reporting limit); and ○ One or more concentration results must be nondetectable (i.e., $< 0.10 \mu\text{g/L}$) 	<ul style="list-style-type: none"> ● The lab evaluates its processes, and if appropriate, modifies its processes to correct possible contamination or other problems. ● The lab reanalyzes all samples in the analytical run until the controls meet the requirements.
Sample Evaluations	All samples are run in duplicate. Each duplicate pair must have $\%CV \leq 15\%$ between its absorbance values.	<p>If $\%CV$ of the absorbances for the sample $> 15\%$, then:</p> <ul style="list-style-type: none"> ● Record the results for both duplicates. ● Report the data for both duplicate results as Quality Control Failure "QCF"; and ● Re-analyze the sample in a new analytical run. No samples are to be run more than twice. <p>If the second run passes, then the data analyst will exclude the data from the first run. If both runs fail, the data analyst will determine if either value should be used in the analysis (e.g., it might be acceptable to use data if the CV is just slightly over 15%).</p>
Results Within Calibration Range	All samples are run in duplicate. If both of the values are less than the upper calibration range (i.e., 5.0 $\mu\text{g/L}$ for undiluted samples), then the requirement is met.	If one or both duplicates register as 'HIGH,' then the sample must be diluted and re-run until both results are within the calibration range. No samples are to be run more than twice.
External Quality Control Sample	External QC Coordinator, supported by QC contractor, provides 1-2 sets of identical samples to all laboratories and compares results.	Based upon the evaluation, the External QC Coordinator may request additional information from one or more laboratories about any deviations from the Method or unique

		laboratory practices that might account for differences between the laboratory and others. With this additional information, the External QC Coordinator will determine an appropriate course of action, including no action, flagging the data, or excluding some or all of the laboratory's data.
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3.8 Sample and Record Retention

The laboratory shall retain:

1. The sample materials, including vials, for a minimum of 3 years from the date the EPA publishes the final report. During this time, the laboratory shall freeze the materials. The laboratory shall periodically check the sample materials for degradation.
2. Original records, including laboratory notebooks and the reference library, for a minimum of 10 years from the date that EPA publishes the final report.

After the stated time periods, the laboratory shall follow its internal protocols for disposal.

3.9 References

Abraxis, "Microcystins-ADDA ELISA (Microtiter Plate)," Product 520011, R021412, Undated. Retrieved January 2014 from http://www.abraxiskits.com/uploads/products/docfiles/278_Microcystin%20PL%20ADDA%20users%20R120214.pdf.

Abraxis, "Microcystin-ADDA ELISA Kit, Detailed Procedure," Undated. Retrieved January 2014 from http://www.abraxiskits.com/uploads/products/docfiles/253_PN520011FLOW.pdf.

James, R., et al., "Environmental Technology Verification Report: Abraxis Microcystin Test Kits: ADDA ELISA Test Kit; DM ELISA Test Kit; Strip Test Kit," in Environmental Technology Verification System Center 2010. Retrieved March 2013 from <http://nepis.epa.gov/Adobe/PDF/P100EL6B.pdf>

4.0 BENTHIC MACROINVERTEBRATES

4.1 Introduction

This chapter describes the steps for identifying benthic macroinvertebrate organisms in samples collected in the National Rivers and Streams Assessment (NRSA). The procedure is adapted from *Wadeable Streams Assessment: Benthic Laboratory Methods* (USEPA, 2004), and is modified to facilitate processing and identification of benthic organisms collected in the littoral zone of rivers and streams. Field crews preserve samples in the field with ethanol and ship them to a central holding facility or directly to the laboratory. Samples are sent to the laboratory on a regular basis to avoid delays in processing and sample identification.

In the following discussion, **Sections 4.1, 4.2, 4.3, and 4.4** summarize the procedure; health and safety concerns; and required resources. **Section 4.5** provides the steps for acknowledging sample receipt. **Section 4.6** and **Section 4.7** provide the steps for preparing and sorting the sample. **Sections 4.8 – 4.10** provide the steps for the taxonomy identification, data entry, and sample and record retention. **Section 4.11** describes EPA's external review of laboratory operations. **Section 4.12** identifies references used in developing the procedure.

Table 4.8 summarizes the quality control measures.

4.2 Summary of Method

The procedure is designed to pick and identify organisms from sediment samples. This section provides a summary of the procedure and quality control measures.

The sorter evenly distributes each sample across a sorting tray(s), and then places an evenly divided grid over the sample, usually 30 grids per tray (“Caton-style tray”). The sorter randomly selects a minimum of three grids for sorting and picking organisms. The sorter continues until all grids are sorted or the sorter has picked the 500th organism from a subsample. If a sorter reaches 500 organisms in the middle of a subsample, then the sorter continues until that the subsample is completed. **Figure 4.1** summarizes the sorting process.

During the identification step, a taxonomist identifies the picked organisms to the target taxonomic levels for the survey and discards materials that do not meet the identification criteria. If necessary, the sorter repeats the sorting and identification process to ensure that the target of 500 organisms is reached. The taxonomist also creates a reference collection with at least one organism from each genus or lowest taxonomic level identified.

As part of the quality control measures, a second taxonomist will re-identify a subset of the samples to quantify enumeration and taxonomic precision, or consistency, as percent difference in enumeration (PDE) and percent taxonomic disagreement (PTD), to help target corrective actions, and ultimately to help minimize problems during data analysis.

4.3 Health and Safety Warnings

In addition to the laboratory’s usual requirements, the following health and safety procedures must be followed for this procedure:

1. All proper personal protection clothing and equipment (e.g. lab coat, protective eyewear / goggles) must be worn or applied.
2. When working with potential hazardous chemicals (e.g. 95% ethanol) or biological agents (benthic organisms and sediments) avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs remove clothing immediately and wash / rinse thoroughly. Wash the affected skin areas thoroughly with large amounts of soap and water.

4.4 Definitions and Required Resources (Laboratory, Personnel, and Equipment)

This section provides definitions and required resources for using this procedure. **Section 4.4.1** defines the terms used throughout the procedure. **Section 4.4.2** describes the expertise required for each laboratory using the procedure. **Section 4.4.3** describes the roles and responsibilities of the personnel involved in the procedure. **Section 4.4.4** identifies the equipment necessary to apply the procedure in preparing, sorting, and identifying benthic macroinvertebrate organisms in samples.

4.4.1 Definitions

The following terms are used throughout the procedure:

Caton-style grid: Subsampling grid that consists of a solid outer tray, a mesh-bottomed inner tray, evenly spaced squares (e.g., 30 or 36), a square “cookie cutter” and a scoop.

Dissecting microscope: Microscope that is configured to allow low magnification of three-dimensional objects that are larger or thicker than the compound microscope can accommodate.

Distinct taxa: Data analysts use the number of distinct (i.e., unique) taxa within a given sample to evaluate the richness associated with the sample location. The distinctness attribute is assessed sample by sample, and not across all samples. To facilitate the data analyses, the database includes an additional variable (“flag”) that is used for the first identification of a particular taxon in a sample.

Section 4.8 provides the steps used to identify which taxa are flagged.

Good quality digital photograph: Good quality means that other taxonomists can readily identify the taxon from one or multiple photographs and the library can readily locate the photographs. To ensure that the photographs meet these objectives, the image must be:

- Taken through the microscope at a high enough resolution so that the key diagnostic features are distinguishable and clear. Include all features that would be necessary for an experienced taxonomist to identify the specimen, this may require multiple photographs and at different magnifications.
- Positioned so that it includes:
 - Only one taxon in the photo. If necessary, the laboratory may edit (e.g., crop) the digital photograph and save the file with a new filename. Both the original and edited files must be included in the digital library.
 - A scale bar or measurements in an appropriate location to indicate the size of the specimen.
 - One specimen that lies flat on the surface instead of tilted (to the extent practicable).
- Saved using a format that preserves the image in the highest resolution possible.
- Saved with a filename that is consistent within the digital library and shall include the following elements in the order listed below:
 - NRSA2 (for the second NRSA conducted in 2013-2014)
 - Laboratory name (or abbreviation)
 - Sample number
 - Taxa name
 - Magnification (if applicable, otherwise indicate no magnification as “1x”)
 - Date (format YYYYMMDD) that the photograph was taken.
 - Appendage of “e” if the photograph was edited (e.g., cropped).
- For example, on September 8, 2013, laboratory ABC identified the specimen in sample 1234 to be a *Homoeothrix janthina* and took a digital photograph at a resolution of 40x and then cropped the photograph to eliminate extraneous material. The filenames of the original and edited photographs would be: NRSA2_ABC_1234_homoeothrix janthina_40x_20130908.gif and NRSA2_ABC_1234_homoeothrix janthina_40x_20130908e.gif.

Elutriate: Circulate water over the sample in order to wash away the lighter or finer particles of the detritus.

Grid: Each individual square within the Caton tray

Inorganic material: Material that is not part of the animal or vegetable kingdom (e.g., gravel, sand, silt)

Integrated Taxonomic Information System (ITIS): Database with standardized, reliable information on species nomenclature and their hierarchical taxonomic classification. EPA has incorporated the ITIS information into classifications used by WQX.

NARS: National Aquatic Resource Surveys. The National Rivers and Streams Assessment (NRSA) is part of the NARS program.

NARS Information Management (IM) System: The IM system established to support all surveys, including NRSA, in the NARS program. The IM system is used to track the samples from field collection to the laboratory.

NRSA: National Rivers and Streams Assessment. The samples were collected during the field stage of NRSA.

Organic material: Material that is capable of decay or the product of decay (e.g., leaves, sticks, algae).

Percent sorting efficiency (PSE): Number of organisms recovered by sorter (A) compared to the combined (total) number of recoveries by the sorter (A) and QC Officer (B) for a sample.

$$PSE = \frac{A}{A + B} \times 100 \quad (1)$$

Percent disagreement in enumeration (PDE): measure of taxonomic precision comparing the number of organisms, n_1 , counted in a sample by the primary taxonomist with the number of organisms, n_2 , counted by the internal or external QC taxonomist.

$$PDE = \frac{|n_1 - n_2|}{n_1 + n_2} \times 100 \quad (2)$$

Percent taxonomic disagreement (PTD): measure of taxonomic precision comparing the number of agreements (positive comparisons, $comp_{pos}$) of the primary taxonomist and internal or external QC taxonomists. In the following equation, N is the total number of organisms in the larger of the two counts.

$$PTD = \left[1 - \frac{comp_{pos}}{N} \right] \times 100 \quad (3)$$

Pickate: Sort residue from all grids originally sorted. This is the remaining material left from the grid, after the sorter has removed all benthic macroinvertebrates. This could include small stones, sticks or leaves, etc.

Quarter: A subsection of a grid. If a sorter finds many organisms within a grid, they will evenly divide the grid into four “quarters”.

Sorting Bench Sheet: Form used by the laboratory to record information about the sample during the sample preparation and sorting procedure.

Primary laboratory: The laboratory that 1) sorts the sample; and 2) provides the first identification of benthic macroinvertebrates in the sample.

Secondary laboratory: The laboratory selected by the External QC Coordinator. It provides an independent identification of the benthic macroinvertebrates in the sample. The secondary laboratory must provide QC taxonomists who did not participate in the original identifications for the sample.

Subsample: Portion of the sample obtained by random selection and division.

Target number of organisms: 500 per sample. If the 500th organism is counted in a grid (or quarter), then the macroinvertebrates in the rest of the grid (quarter) are picked and counted until that grid (or quarter) is completed.

Target taxonomic levels:

Table 4.4 and **Table 4.5** provide the target taxonomic levels for typical benthic macroinvertebrates found in freshwater. EPA developed these tables considering its data analysis objectives, and thus, does not require more specific identifications. In other words, taxonomists need only identify benthic macroinvertebrates to the level specified in

Table 4.4 and Table 4.5.

Taxonomic Bench Sheet: Form used by the laboratory to record information about the sample during the identification procedure.

Taxonomic Serial Number (TSN): stable and unique identifier that the Integrated Taxonomic Information System (ITIS), Encyclopedia of Life, and/or Catalogue of Life couples with each scientific name to serve as the "common denominator" for accessing information. ITIS numbers are preferred, but when they are not available secondary sources are acceptable. WQX incorporates this identifier into its "ExternalID" variable in the database. For entries that are not present in the other databases, WQX assigns an identification number that should be used in the same manner as the TSNs. (This is *not* the identifier that should be used to identify organisms with this procedure. See WQX below for appropriate identifier.)

Water Quality Exchange (WQX): a database framework that allows for data submissions and sharing of water quality monitoring data, including data about benthic macroinvertebrates. It has assigned identification numbers using information from ITIS, Encyclopedia of Life, and Catalogue of Life. The following steps will retrieve the taxon information:

1. Access the WQX database at
http://www.epa.gov/storet/wqx/wqx_getdomainvalueswebservice.html
2. Click on the link for "Taxon" listed near the bottom of the webpage.
3. Select "Save" to the file download popup box that asks "Do you want to open or save this file?"
4. Extract the saved file using software such as WinZip.
5. To open the extracted file in Excel:
 - a. When prompted, select "Open the file with the following stylesheet applied (select one):"
The selection should show the default of
"http://www.epa.gov/storet/download/domain_value_use.xsl"
 - b. Click "yes" to the warning about format (i.e., "The file you are trying to open...is in a different format...Do you want to open the file now?")
6. Search for the taxon in the "Name" column. The "Description" column identifies a citation to a webpage with more information about the taxon. The "ExternalID" column provides an identifier commonly used by other references, and is left blank if none exists. Because the "UniqueIdentifier" column has a value for every taxon listed, its identifier is the one that should be used in recording the findings from this procedure.

4.4.2 Laboratory

The procedure may be used by any laboratory that has expertise in each of the following areas:

1. Analytical work: To demonstrate its expertise, the laboratory shall provide EPA with one or more of the following:
 - a. Memorandum that identifies the relevant services that the laboratory provided for the National Aquatic Resource Surveys in the past five years.
 - b. Dated copy of relevant Accreditation or Certification (NELAC, ISO, state, etc.) for the laboratory and/or its experts who will perform and/or oversee the analyses. The accreditation must be for the entirety of analysis that the laboratory will be performing.

- c. Memorandum that describes the laboratory's participation in round robin studies and/or performance studies.
- d. Report of findings from an on-site technical assessment or audit.
2. Quality procedures:
 - a. To demonstrate its expertise in quality assurance and quality control procedures, the laboratory shall provide EPA with copies of the quality-related documents relevant to the procedure. Examples include Quality Management Plans (QMP), QAPPs, and applicable Standard Operating Procedures (SOPs).
 - b. To demonstrate its ongoing commitment, the person in charge of quality issues for the laboratory shall sign the NRSA 13/14 QAPP Certification Page.
3. Reporting standardized data. To demonstrate its expertise, the laboratory shall provide EPA with a memorandum that confirms that the laboratory has a computerized Laboratory Information Management System (LIMS) that is routinely used to track samples and record laboratory results. The memorandum also shall confirm that the laboratory will use LIMS to record and report results from the procedure.

4.4.3 Personnel

The procedure may be used by any person who has received training in processing and identification of benthic macroinvertebrates; however, within a given laboratory, the work is generally parsed out to several personnel:

Quality Control (QC) Officers provide oversight of daily operations, sample processing, monitors QC activities to determine conformance, and conducts performance and systems audits of the procedures. Documentation for the QC Officer meeting these requirements must be kept at the lab, and made available to the EPA QC officer if requested. The following types of QC Officers are used by this procedure:

Sorting QC Officer is an experienced sorter who:

- Demonstrated an initial sorting proficiency (measured by PSE) of $\geq 90\%$ in 5 consecutive samples evaluated by an experienced sorter; and
- Maintains a sorting proficiency of $\geq 90\%$ in periodic QC checks (i.e., 1 in 10 samples).
- Verifies the completeness of every Preparation/Sorting Bench Sheet to ensure header information is correctly entered.
- Checks sorted grids of all inexperienced laboratory personnel (those who have not achieved a $\geq 90\%$ sorting efficiency) for missed organisms and records the number of missed organisms in the appropriate field of the Bench Sheet.
- Checks 1 in 10 of an experienced individual's samples, with a minimum of 1 NRSA sample. If the Sorting QC Officer performs the QC check more frequently, then the additional QC results must be submitted with the data for the required QC checks.
- Determines the sorting efficiency for each sample and sorter. The sorter's sorting efficiency is recorded on the Sorter Bench Sheet.

Internal Taxonomy QC Officer is an experienced taxonomist who:

- Demonstrated an initial enumeration and identification proficiency (as measured by $PDE \leq 5\%$ and $PTD \leq 15\%$).
- Maintains enumeration and identification proficiency in periodic QC checks (i.e., 1 in 10 samples with a minimum of one sample checked).

External QC Coordinator is an EPA staff person. Alternatively, the External QC Coordinator may be a contractor that has not previously been involved with field sampling and/or benthic macroinvertebrate analyses. Because the assigned duties are primarily logistical in nature, the QC Coordinator is not required to have laboratory experience, although such experience would be preferable.

External QC Taxonomists, are selected by the External QC Coordinator, and have demonstrated expertise and experience to be used as a quasi “gold standard” for taxonomic evaluations.

Taxonomists are trained, and have considerable experience, in identifying benthic macroinvertebrates, i.e., taxonomy. It is also important that the taxonomist maintains contact with other taxonomists through professional societies and other interactions, and keeps up with the pertinent literature, since systematics and species identifications change over time. EPA prefers, but does not require, that the taxonomists are certified by the Society of Freshwater Science (SFS). Each laboratory must submit the resume or *curriculum vitae* for the taxonomists who identify benthic macroinvertebrates for the NRSA samples to the EPA Project QC Officer.

Sorters are laboratory technicians who have basic training in laboratory procedures; and training in the use of the Caton-style tray. An “experienced” sorter is one that has achieved $\geq 90\%$ sorting efficiency in 5 consecutive samples. An experienced sorter can serve as the Sorting QC Officer.

4.4.4 Equipment/Materials

The procedure requires the following equipment and materials for sample preparation (subsampling), sorting, and taxonomic identifications.

4.4.4.1 Sample Preparation (Subsampling) and Sorting Equipment/Materials

- U.S. 35 sieve (500 μm)
- Round buckets
- Standardized gridded screen (40 Mesh (380- μm openings, T304 stainless steel wire, 34GA (0.010”))
- Mesh screen, square grids (e.g., 30 squares of 6 cm^2 each) with white plastic holding tray (e.g., Caton tray, 30cm x 36cm, 4cm deep)
- 6-cm scoop
- 6- cm^2 metal dividing frame (“cookie cutter”)
- White plastic or enamel pan (6" x 9") for sorting
- Scissors
- Teaspoon
- Permanent ink pen (e.g Pigma Micron® pen)
- Dropper
- Fine-tipped forceps (watchmaker type, straight and curved)
- Vials with caps or stoppers

- Sample labels for vials
- 70-80% ethanol (denatured or non-denatured)
- Sorting Bench Sheet or other Sheet or database approved by EPA's External QC Coordinator
- Stereo zoom microscope (6-10X magnification)

4.4.4.2 Taxonomy Identification Equipment/Materials

- Stereo dissecting microscope with fiber optics light source (50-60X magnification)
- Compound microscope (10, 40, and 100X objectives, with phase-contrast capability)
- Digital camera with high resolution capability mounted on a microscope
- Petri dishes
- Microscope slides (1" x 3" flat, precleaned)
- Cover slips (appropriately sized)
- CMCP-10 (or other appropriate mounting medium)
- Permanent ink pen (e.g Pigma Micron® pen)
- Dropper
- Fine-tipped forceps (watchmaker type, straight and curved)
- Vials with caps or stoppers
- Sample labels for vials
- 70 - 80% non-denatured ethanol in plastic wash bottle
- Taxonomic Bench Sheet (provided to each participating laboratory by EPA)
- Hand tally counter

4.5 Sample Receipt

Under U.S. regulations, samples preserved in ethanol are classified as "Dangerous Goods" and must be shipped according to hazardous material shipping requirements and regulations. Laboratory personnel receiving the shipment must be certified to handle hazardous material. Because EPA initiates tracking procedures designed to recover any missing shipment, the laboratory personnel should start the following login steps within 24 clock hours of receiving a delivery.

1. Record receipt of samples in the NARS IM system (within 24 clock hours) and the laboratory's Information Management System (LIMS). Assign the appropriate chronological bench number to each sample.
2. Inspect each jar THE SAME DAY THEY ARE RECEIVED:
 - a. Refill them with 70-80% ethanol if necessary.
 - b. Verify that the site identification and sample number on the label also appear on the chain of custody form in the shipment.
 - c. Notify the NARS IM team if any jars were broken and/or there are discrepancies between the custody form and jars.
3. After refilling the sample containers, store them at room temperature until sorting begins.
4. Maintain the chain-of-custody form with the samples; it will be needed if the samples are transported to any other location (e.g., for taxonomic identification, external QC evaluation).

5. Verify that the login information includes the required data elements in **Table 4.1**. After completing all required elements, provide the information to the data entry personnel.

Table 4.1 Benthic macroinvertebrate: required data elements – login

FIELD	FORMAT	DESCRIPTION	
LAB NAME	text	name of lab	
LAB ID (optional)	text	lab sample id	
DATE RECEIVED	MMDDYY	date sample was received by lab	
SITE ID	text	NRSA site identification code as used on sample label	
VISIT NUMBER	numeric	sequential visits to site (1 or 2, if specified on label)	
SAMPLE ID	numeric	sample number as used on field sheet (on sample label)	
DATE COLLECTED	MMDDYY	date sample was taken	
QA FLAG (if appropriate)	text	QA/QC flag (lab may use its own flags, if defined in QA_COMMENTS field or provided to NARS IM team)	
		Flag	Definition
		NP	Not enough preservative used
		S	Sample shipping problem (explain in QA_COMMENTS field)
		Q	Other quality concerns, not identified above
QA_COMMENTS	text	explanation for QA FLAG (if needed)	
LAB COMMENTS	text	general laboratory analysis comments	

4.6 Sample Preparation (Subsampling)

In preparation for sorting and picking organisms from the samples as described in **Section 4.5**, the sorters first prepare the sample as described in the following steps.

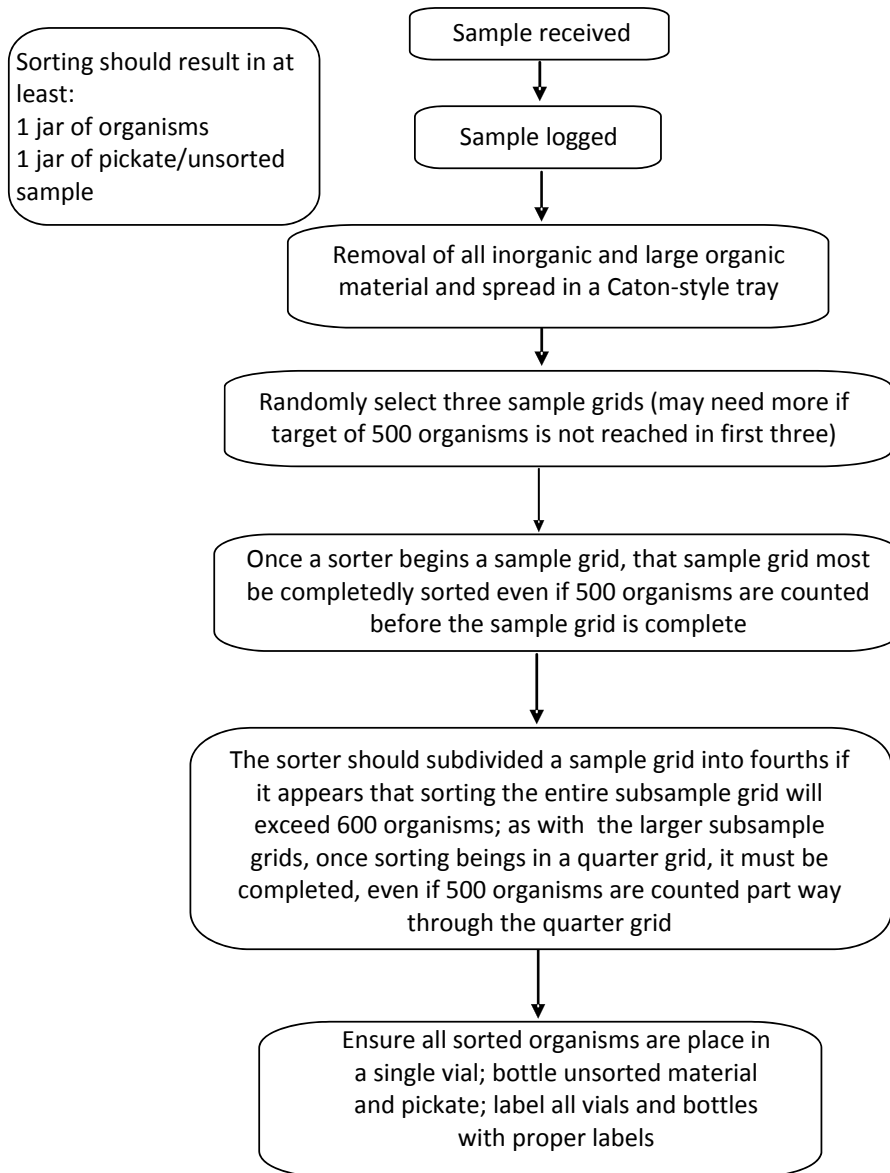


Figure 4.1 Benthic macroinvertebrate: summary of sorting

Figure 4.1 provides a simplified version that may be useful as a quick reference guide to the steps in **Sections 4.6 and 4.7. APPENDIX C: SAMPLE LABORATORY FORMS** provides an example of the **Benthic Macroinvertebrate: Sorting Bench Sheet**. If EPA's External QC Coordinator agrees, the laboratory may substitute its own Sorting Bench Sheet or computer format instead of using the example. The alternative must include the QC elements used for the evaluations described in **Table 4.6**.

1. Remove the lid from the sample container and remove the internal sample label (save the label—it will need to be returned to the sample container with the archived portion of the sample that does not get processed). Record the sample collection information on a Sorting Bench Sheet. Header information required includes station name, station location, station number, project name, bench number, sample type, date the sample was collected, and the

field team who collected the sample (e.g., Team 1). After the Sorting QC Officer verifies the information, set the bench sheet aside.

2. Consider the contents of the sample:
3. If the sample has heavy amounts of inorganic substrate (e.g., sample that has 4 or 5 jars total and 2 or 3 with gravel or sand), it is acceptable for the sorter to elutriate the sample, before subsampling. An example of an acceptable elutriation method is as follows:
4. Pour ethanol off of sample containers through sieve (500 μm or smaller). Also deposit leaf litter and any other organic material (leaves, sticks, algae) onto sieve.
5. Depending on amount of inorganic material (gravel, sand, silt), pour all or a portion of this material into a rectangular Tupperware®/Rubbermaid® container and cover with water.
6. Circulate (elutriate) sample with water and allow any organisms that might be in the gravel/sand to float to the top of the water and pour the water through a sieve (U.S. 35).
7. Repeat this until the water runs clear.
8. Fill the plastic container (that still has the inorganic material in it) with water one more time and take it to a well lit, flat surface. Inspect it here under a ring light with 3x magnification for any remaining organisms. Have another sorter double check for organisms.
9. Once no organisms remain in the plastic container, wash the water through the sieve and dump the inorganic material into a waste bucket.
10. Repeat this process until all of the inorganic material has been elutriated and checked for heavier organisms, such as clams, mussels, or worms.
 - a. For all other samples:
 - i. Carefully decant the ethanol from the sample container by pouring the fluid through a sieve (U.S. 35) into a separate container.
 - ii. Inspect the mesh of the sieve for any organisms and return any organisms found to the sample container so they can be included in the sample sort process.
11. Spread the sample now in the circular sieve over the Caton-style tray. Use multiple trays if necessary. If there is more than one jar for any particular sample, empty and wash each jar onto the tray one at a time, making sure to spread each jar's contents evenly across the gridded screen. If the amount of leaf litter or other detrital material fills the tray to the top of the wall panels, divide it among two or more trays.
12. Mix the sample around in the tray(s) so that large and small particles are evenly distributed throughout.
13. Add enough water to spread the sample evenly throughout the grid (the water level should be relatively close to the top of the white tray). Spread the sample material over the bottom of the pan as evenly as possible. Move the sample into the corners of the pan using forceps, spoon, or by hand. Vibrate or shake the pan gently to help spread the sample.
14. Lift the screen out of the white tray to drain. Pour off or siphon excess water from the white tray and set the screen back into the tray. Leave just enough water in the bottom of the tray so that it barely covers the screen once it is returned to the tray to allow the sample to remain moist.
15. Label the grids by marking the side of the gridded tray with letters on one side and numbers on the connecting side. For example, each grid could be labeled with one letter and one number, e.g., A-5, E-1, to represent its position in the tray as shown in the example of the sorting method

(Figure 4.2). If the sample is spread over more than one tray, label the grids identically in each tray. See Example.

16. Use a random number generator to select at least 10% of the grids to ensure that the subsample material is representative of the overall sample. For example, if the tray is subdivided into 30 squares (<6 cm² each), then 3 squares (“grids”) would be selected for sorting.
17. Make an initial assessment of the sample. If the number of organisms appears to exceed the target number (500 organisms) in the collective three grids (or however many grids were selected), quarter each grid, label the quarters (see example below) and randomly select a quarter for initial sorting.
18. Assign unique identifiers on the **Benthic Macroinvertebrate: Sorting Bench Sheet** for each grid and quarter processed. Provide a copy of the Sorting Bench Sheet, or its approved alternative, to EPA’s External QC Coordinator.
19. Remove all the material from the selected grid/quarter as follows:
20. Place the metal dividing frame or “cookie cutter” over the sample at the approximate location of the grid selected for processing (based on the letters and numbers marked on the sides of the gridded tray). Use a pair of rulers or other straight edges to facilitate lining up the cookie cutter at the intersection if necessary.
21. Remove the material within the “cookie cutter” using the 6-cm scoop, a teaspoon, forceps, or dropper. Depending on the consistency of what is in the sample, it might be necessary to cut the material along the outside of the “cookie cutter” with scissors or separate it with forceps so that only one grid’s (or quarter’s) worth of sample material is used. Inspect the screen for any remaining organisms. Use the following rules when dealing with organisms that lie on the line between two grids:
 22. An organism belongs to the grid containing its head.
 23. If it is not possible to determine the location of the head (i.e., for worms), the organism is considered to be in the grid containing most of its body.
 24. If the head of an organism lies on the line between two grids, all organisms on the top of a grid and those on the right side of a grid belong in that grid, and are picked with that grid.
25. Place the material from each selected grid(s) into a separate white plastic or enamel pan. Add the necessary amount of water to the pan to facilitate sorting.

Figure 4.2 Benthic Macroinvertebrate: sorting method example

EXAMPLE: The sorter spreads the sample into a single Caton tray and labels the sides with letters and numbers as shown below. The sorter randomly selects grids E-1, A-5, and B-4, in that order. The sorter processes grid E-1 and finds 50 organisms, and then continues to grid A-5 and finds 40 organisms. The sorter then assesses grid B-4 and decides that it will produce more than 410 organisms needed to reach the sample target of 500 organisms. The sorter then quarters grid B-4 and randomly selects the quarter labeled α -1. The quarter has 350 organisms. The sorter then randomly selects a second quarter β -2. The sorter processes the quarter and counts 75 organisms. The sorter now has picked a total of 515 organisms in the sample.

Place an X to indicate each grid sorted:
If more than one tray needed for the sample,
Tray #: 1 of 1

	1	2	3	4	5	6
A					X	
B				X		
C						
D						
E	X					

If a grid has been quartered, complete the following (see example in Step 10):

Grid #: B-4

Place an X to indicate each quarter sorted:

	1	2
α	X	
β		X

26. Set the subsampling device aside in case more grids need to be retrieved during the sorting process. Cover the sample with aluminum foil to prevent desiccation of the sample and damage to organisms (periodically moisten the sample with water from a spray bottle if the top layer begins to dry). Between each subsampling operation, be careful not to disturb the subsampling device to prevent redistribution of organisms, which could possibly change the probability of selection.

4.7 Sorting

After dividing the sample into grids as described in **Section 4.6**, the sorter follows the following steps to pick the organisms from the sample:

1. Remove the macroinvertebrates from the detritus with forceps.
 - a. In general, do not remove or count:
 - i. Empty snail or bivalve shells
 - ii. Organisms of surface-dwelling or strict water column² arthropod taxa (e.g., Collembola, Veliidae, Gerridae, Notonectidae, Corixidae, Culicidae, Cladocera, or Copepoda, see **Table 4.2**)
 - iii. Incidentally-collected terrestrial taxa.

²Strict water column taxa are those that do not have at least one life stage that is benthic (i.e., bottom-dwelling).

- iv. Fragments such as legs, antennae, gills, or wings.

Table 4.2 Benthic macroinvertebrate: list of taxa that are not to be sorted/counted

All terrestrial life history stages are to be excluded from the counts.

TAXON				
Phylum	Class (or Subclass)	Order	Family	
ANNELIDA	<i>Clitellata</i>	<i>Branchiobdellida</i>		
ARTHROPODA	<i>Cladocera and other Branchiopoda</i>			
	<i>Collembola</i>			
	<i>Insecta</i>	<i>Ephemeroptera (adults only)</i>		
		<i>Diptera (larvae of listed families, plus all adults)</i>		<i>Chaoboridae</i>
				<i>Culicidae</i>
		<i>Coleoptera</i>		<i>Dytiscidae (adult)</i>
				<i>Gyrinidae (adult)</i>
		<i>Heteroptera</i>		<i>Corixidae</i>
				<i>Gerridae</i>
				<i>Mesoveliidae</i>
				<i>Notonectidae</i>
			<i>Pleidae</i>	
		<i>Neuroptera (adults)</i>		
	<i>Odonata (adults)</i>			
	<i>Plecoptera (adults)</i>			
	<i>Trichoptera (adults)</i>			
	<i>Ostracoda</i>			
BRYOZOA				
NEMATODA				

- b. For Oligochaeta, attempt to remove and count only whole organisms and fragments that include the head; also, do not count fragments that do not include the head.
 - c. In case of uncertainties, place the organism in the sort vial without counting it (the final count is made by the taxonomist).
2. Place picked organisms for the selected grids and quarters into a single set of jars and vials for the sample.
 3. Sort all samples under a minimum of 6x (maximum of 10x) dissecting microscope. Once a grid, or quarter, enters the sorting process, all organisms must be sorted to minimize bias. For the survey, the target number of organisms is 500 per sample. If the 500th organism is counted in a grid (or quarter), then the macroinvertebrates in the rest of the grid (quarter) are picked until that grid (or quarter) is completed.
 - a. If the sample covers more than one tray, then sort the first selected grid in each tray, before moving to the second grid, etc. For example, assume that a sample covers 3 trays T1, T2, and T3. Further assume that grids E1, A3, and B5 were randomly selected in that order. Then, in this example, the sorting order would be: T1E1, T2E1, T3E1, T1A3, T2A3, T3A3, and T1B5, T2B5, and T3B5.

- b. If the grids have been quartered, then sort the first selected quarter in each grid. If the target count has not been reached after sorting the selected quarter in the selected grids, then randomly select another quarter for sorting. For example, if a sample covers a single tray; grids E1, A3, and B5 were randomly selected in that order; and quarter Q3 was randomly selected; then the sorting order would be: E1Q3, A3Q3, B5Q3 before randomly selecting another quarter.
4. Keep a rough count of the number of organisms removed and enter the number of organisms found in each grid under that column on the Sorting Bench Sheet.
5. When all randomly selected grids have been sorted, verify that the cumulative count is greater than 500 organisms.
6. This step is performed if: 1) the sorter has not reached 90% proficiency in 5 consecutive samples (“proficiency QC check”, see **Section 4.4.1** for definition and explanation); or 2) the Sorting QC Officer has selected this sample to use as the 1 in 10 sample QC check for experienced sorters (“periodic QC check”). (The Sorting QC Officer must check a minimum of one NRSA sample if an experienced sorter processes less than ten NRSA samples. If the laboratory performs QC checks more frequently, then the all of the QC data must be submitted to EPA.) The Sorting QC Officer:
 - a. Performs QC checks using the same power microscope as the sorter;
 - b. Counts any missed organisms found and places them into the sample vial, or other suitable sample vial;
 - c. Notes the number of organisms missed on the Sorting Bench Sheet; and
 - d. Adds that number to the final count of the sample.
 - e. Calculates the PSE for the sample (see **Section 4.4.1** for definition; equation 1). If the PSE is:
 - i. <90% and the sample is the:
 - 1) Proficiency QC check, the Sorting QC Officer must check the next 5 samples until the sorter has $PSE \geq 90\%$ for 5 consecutive samples.
 - 2) Periodic QC check, then the sorter’s samples since the last QC check are examined for organisms missed in sorted grids and quarters. The sorter must again demonstrate proficiency by achieving a $PSE \geq 90\%$ in 5 consecutive samples, unless he or she shows marked improvement in their sorting efficiency prior to completion of the next five samples, the Sorting QC Officer may, at his/her discretion, consider this individual to be “experienced” and check only 1 in the next 10 samples.
 - ii. $\geq 90\%$ and the sample is the:
 - 1) Proficiency QC check, the sample counts towards the 1 in 5 consecutive samples used to establish proficiency.
 - 2) Periodic QC check, no corrective action is required.
7. Place organisms removed from grid to an internally-labeled vial (or larger container, if necessary) containing 70-80% ethanol. Record the following information on internal sample labels used for vials of sorted material with Pigma Micron® pen on cotton rag paper or an acceptable substitute.
8. Remove the remaining material from that particular grid that was just sorted left on the sorting pan (i.e. material such as sticks, organic debris)and place it in a separate container with preservative (70-80% ethanol). Label the container “Pickate,” on both internal and external

labels. This material is kept by the lab until notified by TOPO. Internal sample labels should be made of cotton rag paper or an acceptable substitute. Include the following information on the label:

- a. Station Name
 - b. Station Location
 - c. Station Number
 - d. Date Sorted
 - e. Sorter's Initials
 - f. "1 of x" or "2 of x", etc. if the sample is sorted into more than one vial (where x is the total number of vials for the sorted sample)
9. Search the entire caton tray for 5-10 minutes, looking for large/rare organisms (Vinson and Hawkins, 1996). Large/rare is defined as any organism larger than 0.5" long and found in less than one eighth of the tray holding the entire sample. Place any organisms found into a vial labeled "L/R" for "Large/Rare."
 10. Return all material not subsampled (remaining on the Caton tray) to the original container with the preservative that was set aside. This container will include the original sample labels. Prepare two additional labels "Unsorted Sample Remains" and place one inside the container and attach the other to the outside of the container. Replace the lid and tighten securely. Archive the container until all appropriate QC checks are completed (subsampling and taxonomy), and in accordance with survey and laboratory requirements.
 11. Verify that the bench sheet includes the required data elements in **Table 4.3**. After completing all required elements, provide the sorting bench sheets to the data entry personnel.
 12. Thoroughly clean all sample preparation and sorting equipment and make sure all equipment is free of organisms prior to sorting the next sample.

Table 4.3 Benthic macroinvertebrate: required data elements – sorting

FIELD	FORMAT	DESCRIPTION
LAB NAME	Text	name of lab
LAB ID (optional)	Text	lab sample id
DATE RECEIVED	MMDDYY	date sample was received by lab
SITE ID	Text	NRSA site identification code as used on sample label
VISIT NUMBER	numeric	sequential visits to site (1 or 2, if specified on label)
SAMPLE ID	numeric	sample number as used on field sheet (on sample label)
DATE COLLECTED	MMDDYY	date sample was taken
DATE SORTED	MMDDYY	date that the sorter started working on the sample
PROPORTION SORTED	numeric	proportion of sample sorted based upon number of grids and quarters selected and available
CORRECTION FACTOR	numeric	subsampling correction factor
QA FLAG (if appropriate)	text	QA/QC flag (lab may use its own flags, if defined in QA_COMMENTS field or provided to NARS IM team)
		Flag Definition
		C Organism in poor condition or fragments
		DD Damaged Organism

		IM	Immature
		IN	Indeterminate (explain in QA_COMMENTS field)
		NP	Not enough preservative used
		NT	Not able to meet target level for identification (may be used with other codes, or explain in QA_COMMENTS field)
		S	Sample shipping problem (explain in QA_COMMENTS field)
		UN	Unknown. Identification is tentative. Organism has been sent to expert taxonomist for definitive identification.
		Q	Other quality concerns, not identified above
QA_COMMENTS	text	explanation for QA FLAG (if needed)	
LAB COMMENTS	text	general laboratory analysis comments	

4.8 Taxonomy Identification

The taxonomist performs the following steps in identifying the benthic macroinvertebrate organisms:

1. Upon receipt of a set of sample vials from the sorter:
 - a. Compare all site identification codes and sample numbers on the form with those entered on the labels of samples, and resolve any discrepancies with the sorter.
 - b. Determine if any vials are broken. For any broken vial, request that the sorting laboratory subsample its jar of unsorted sample and use the new vials to replace the original vials (broken and unbroken).
 - c. Maintain the chain-of-custody form with the sample vials; it will be needed to return/store them.
2. Empty one sample vial at a time into a small Petri dish. Add 70-80% ethanol to keep the organisms covered. Remove the internal sample label and complete the top portion of a Taxonomic Bench Sheet (**APPENDIX C: SAMPLE LABORATORY FORMS** or comparable system), using the information from the label. Depending on the type of organisms, select the appropriate step:
 - a. For all *Chironomidae* and *Oligochaeta* organisms, extract the organisms from the Petri dish.
 - i. Prepare slide mounts using CMCP-10 (or CMC-9, CMC-10, or other media) and applying a coverslip. All organisms should be visible, which generally means a maximum of 10-20 organisms per slide. Label the slides with the same sample identification code or log-in number as the ethanol organisms.
 - ii. If the laboratory prefers to use another method than slide mounting, the EPA External QC Coordinator will grant a waiver if the following applies:
 - 1) The request is for a laboratory located at a single location. For example, EPA would not consider the combined qualifications of a prime contract laboratory and its subcontract laboratories. Instead, for whichever laboratories met the requirements, EPA would evaluate and grant a waiver for the prime contract laboratory separate from each of its subcontractor laboratories.
 - 2) The request for a waiver must identify and describe a minimum of three studies. For each study, the external QC evaluation must demonstrate that the laboratory met or exceeded the NRSA QC requirements (i.e., PDE≤5% and PTD≤15%) for its *Chironomidae* and/or *Oligochaeta* organisms.

- 3) The laboratory agrees to mount the organisms on slides if it fails one of the periodic (NRSA) external QC evaluations, as follows:
 - a) It must mount all *Chironomidae* and *Oligochaeta* organisms in samples processed since the previous external QC evaluation (i.e., for which it met the PDE and PTD requirements).
 - b) It must continue to mount all *Chironomidae* and *Oligochaeta* organisms for the unprocessed samples.
- b. For all other organisms, remove similar organisms to other dishes (keep these covered with 70-80% ethanol).
3. View the sample to ensure that all necessary diagnostic characters have been observed, according to the taxonomic key or other literature using:
 - a. A stereo dissecting microscope for organisms in dishes.
 - b. A compound microscope for slides of *Chironomidae* and *Oligochaeta* organisms
4. To the extent possible, identify organisms to the target taxonomic level for the survey (usually genus, see

5. **Table 4.4** for the typical benthic macroinvertebrates found in freshwater and their target taxonomic levels). Add any necessary data qualifiers (see list provided with Required Data Elements in **Table 4.6**).
 - a. Enter the Taxonomic Serial Number (TSN) as it appears in the column “Unique Identifier” of EPA’s WQX taxon database. **Section 4.4.1** provides instructions in extracting the TSN values from WQX.
 - b. Note whether the identification of a group of organisms is distinct (Distinct=Y/N) from other organisms in the same sample as follows:
 - i. If the organisms can be identified to the target level, then Distinct=“Y.”
 - ii. If an organism cannot be identified to the target level then assign values as follows:
 - 1) If at least some of the organisms in the sample can be identified to the target level, then:
 - a) Distinct=“Y” for organisms identified at the target level; and
 - b) Distinct=“N” for organisms that were identified at a higher taxonomic level (e.g., family) that may contain a target level taxa already identified in a given sample (e.g., genus).
 - c) An example would be, if some organisms from a sample are identified to Baetis, but other organisms in the sample could only be identified to Baetidae and/or Ephemeroptera, then Baetis would be distinct, but Baetidae and/or Ephemeroptera would not be Distinct.
 - 2) If none of the organisms in the sample could be identified at the target level, then:
 - a) Distinct=“Y” for organisms identified at the lowest taxonomic level (e.g., family); and
 - b) Distinct=“N” for organisms identified at a higher level (e.g., order).
 - c) For example, if a taxonomist can identify a number of *Ephemeroptera* (Order) families, but a number of the organisms could not be taken past *Ephemeroptera*, then the individual families would be distinct, but the order would not be distinct.

Table 4.4 Benthic macroinvertebrate: target level of taxonomic identification – benthics commonly found in freshwater

TAXON				Target Level of Identification		
Phylum	Class (or Subclass)	Order	Family	Family	Genera	
ANNELIDA	(any, except <i>Hirudinea</i> , <i>Polychaeta</i>)				X	
	<i>Hirudinea</i>				X	
	<i>Oligochaeta</i>	(any, including <i>Tubificinae</i> , except as noted below)				X
		<i>Enchytraeidae</i>			X	
		<i>Lumbriculidae</i>			X	
	<i>Polychaeta</i>			X		
ARTHROPODA	<i>Arachnoidea</i>	(any)			X	
		<i>Acari</i>			X	
	<i>Insecta</i>	(any, except as noted)				X
		<i>Coleoptera</i>				X
		<i>Diptera</i> (any, except as noted)				X
			<i>Ceratogopogoninae</i>	subfamily		
		<i>Chironomidae</i>	see Table 4			
		<i>Dolichopodidae</i>	X			
		<i>Empididae</i>	X			
		<i>Ephydriidae</i>	X			
		<i>Muscidae</i>	X			
		<i>Phoridae</i>	X			
		<i>Scathophagidae</i>	X			
		<i>Sciomyzidae</i>	X			
		<i>Stratiomyidae</i>	X			
		<i>Syrphidae</i>	X			
		<i>Tabanidae</i>	X			
		<i>Ephemeroptera</i>				X
		<i>Hemiptera (Heteroptera)</i>				X
		<i>Lepidoptera</i>				X
	<i>Megaloptera</i>				X	
	<i>Odonata</i>				X	
	<i>Plecoptera</i>				X	
	<i>Trichoptera</i>				X	
	<i>Malacostraca</i>	(any)				X
		<i>Amphipoda</i>				X
		<i>Decapoda</i>				X
<i>Isopoda</i>					X	
<i>Mysidacea</i>					X	
COELENTERATA†						

MOLLUSCA	(any, except <i>Hydrobiidae</i>)				X
	<i>Bivalvia</i>				X
	<i>Gastropoda</i>	(any, except <i>Hydrobiidae</i>)			X
		<i>Hydrobiidae</i>		X	
NEMERTEA					X

†*Colelenterata* need only be identified to the phylum level.

Table 4.5 Benthic macroinvertebrate: target Level of taxonomic identification – chironomidae

FAMILY	GENUS	REQUIRED LEVEL OF IDENTIFICATION
<i>Chironomidae</i> (except as noted)		Genus
	<i>Cricotopus</i>	<i>Cricotopus/Orthocladius</i> if a clear distinction can not be made
	<i>Orthocladius</i>	<i>Cricotopus/Orthocladius</i> if a clear distinction can not be made
	<i>Conchapelopia</i>	<i>Thienemannimyia</i> genus group
	<i>Hayesomyia</i>	
	<i>Helopelopia</i>	
	<i>Meropelopia</i>	
	<i>Rheopelopia</i>	
	<i>Telopelopia</i>	
	<i>Thienemannimyia</i>	

6. Record the identifications. For example, using the taxonomic bench sheet in Appendix C, the identification would be recorded in the Column labeled “taxon.” Enter the number of larvae, pupae, and adults, or total count (e.g. mollusks), if appropriate life history column does not apply, of each taxon under the appropriate columns.
 - a. If the target taxonomic level cannot be achieved due to immature or damaged organisms this should be noted in the data file in the QA_FLAG field (e.g., QA_FLAG=IM). Appendix 2 provides other codes for the QA_FLAG field.
 - b. If damaged organisms can be identified, they are counted ONLY if the:
 - i. Fragment includes the head, and, in the case of arthropods, the thorax;
 - ii. Oligochaetes have a sufficient number of segments in the head;
 - iii. Mollusk shell (bivalve or gastropod) is occupied by an organism;
 - iv. Organism is the sole representative of a taxon in the sample.
 - c. If a unique taxon is determined for which the appropriate taxonomic level is not available in the literature and there are other taxa in that taxonomic level:
 - i. Provide good quality digital photographs of the organism to outside experts for identification; and
 - ii. Include the tentative identification in the database with a data qualifier code of QA_FLAG='UN' so that these organisms can be distinguished from other organisms in the data analysis.
 - iii. When the outside expert identifies the organism, update the database with the correct identification.
7. Compare taxa names from WQX (see **Section 4.4.1**) to the names used for the identifications. Check the non-matches for the following common problems and correct them.

- a. Abbreviations
 - b. Extra information identifiers (e.g., sp., spp., , nr., cf., genus 1, w/ hair chaetae)
 - c. Extra character (e.g., "?", "Acentrella ?turbida", blank space)
 - d. The word "probably" or "prob" (e.g., "Microcylloepus prob. similis")
 - e. Identifying to a lower level than in WQX (e.g. to species rather than genus)
 - f. Double names (e.g., Callibaetis callibaetis)
 - g. Common misspellings
 - h. Tribes/subfamilies/subgenus sometimes do not appear in WQX
 - i. Species with incorrect genus (Hydatopsyche betteni)
 - j. Split level taxonomy (e.g., Cricotopus/Orthocladus)
 - k. Invalid name (e.g., taxonomic change, synonym; Sphaeriidae vs. Pisiidae)
8. Complete the identification by entering the totals for each developmental stage and the total number of each taxon in the cells at the bottom of the sheet. Cross-check to be sure the totals were summed correctly.
 9. If the number of counted organisms is <500 and part of the sample is still unprocessed, return to the sorting steps to obtain more organisms for identifying and counting.
 10. Provide the data to the Internal Taxonomic Officer for another review to confirm that the identifications use the same nomenclature as the WQX database and the laboratory's reference collection.
 11. Make two copies of the bench sheet or computer file used to record the identifications. They are distributed as follows: 1) the project file; and 2) EPA's External QC Coordinator.
 12. Prepare a list of primary and secondary technical literature used in completing the identifications. Provide complete citations in bibliographic format, including authors' names, date of publication, title of document, name of journal or publisher, volume and page numbers, or ISBN number, as appropriate. These will be kept on file with the Internal Taxonomic QC Officer, who will periodically review the reference collection to ensure that it is complete.
 13. Verify that the reference collection contains at least one organism that represents each genus (or lowest taxonomic level) identified from all sample. For any missing references, choose an appropriate organism(s) from the sample to represent a taxon name in the master taxa list:
 - a. Place the physical specimen in the reference library.
 - b. Place two labels in the sample container to identify: organisms placed in the reference collection, and those in the non-reference organisms.
 - c. Obtain a good quality representative digital photographs of the specimen (see instructions in **Section 4.4.1**).
 14. If the Internal Taxonomy QC Officer selects the sample for a QC check, the Internal Taxonomy QC Officer re-counts and re-identifies the organisms in the sample following the same steps above for the original taxonomist. One in 10 of the taxonomist's samples must be checked. The Internal Taxonomy QC Officer records the independent verifications on a bench sheet or computer file. The Internal Taxonomy QC Officer will also supply a list of taxa that were found to be problematic during their QC sorting check, which can be submitted in an Excel or Word document format. (If the Internal Taxonomy QC Officer performs the QC check more frequently, then all QC data must be submitted.)

15. Carefully return the rest of the organisms to the original sample vial, fill with 70-80% ethanol, and cap tightly.
16. Re-package the samples and slide-mounted organisms carefully, and sign and date the chain-of-custody form. Return or store the samples according to laboratory protocols and any contractual requirements.
17. Verify that all required data elements in **Table 4.6** have been recorded by the taxonomist and Internal Taxonomy QC Officer. If the results were recorded on paper, provide the Taxonomic Bench Sheet to the data entry personnel.

Table 4.6 Benthic macroinvertebrate: required data elements – taxonomic identification

FIELD	FORMAT	DESCRIPTION	
LAB NAME	text	name of lab	
LAB ID (optional)	text	lab sample id	
DATE RECEIVED	MMDDYY	date sample was received by lab	
SITE ID	text	NRSA site identification code as used on sample label	
VISIT NUMBER	numeric	sequential visits to site (1 or 2, if specified on label)	
SAMPLE ID	numeric	sample number as used on field sheet (on sample label)	
DATE COLLECTED	MMDDYY	date sample was taken	
DATE TAXON	MMDDYY	date that the taxonomist started identifying organisms in the sample	
ANALYST NAME	text	name of taxonomist or Internal Taxonomy QC Officer (if record provides results of QC check)	
QC VERIFICATION	text	Y if the record provides the results from the QC check	
FAMILY	text	taxonomic family	
SUBFAMILY	text	taxonomic subfamily	
TRIBE	text	taxonomic tribe	
GENUS GROUP	text	taxonomic genus group (e.g., <i>Thienemannimyia</i>)	
GENUS	text	taxonomic genus	
SPECIES	text	taxonomic species	
WQX_TSN	numeric	Taxonomic Serial Number as defined by “UniqueIdentifier” in WQX. If taxon is not in WQX, provide citation for reference used to identify organism in CITATION field	
LAB TIN (OPTIONAL)	text	lab taxa ID number	
TAXANAME	text	unique taxon name in WQX	
ABUNDANCE LARVAE	numeric	number of individual larvae or immature bugs	
ABUNDANCE PUPAE	numeric	number of individual pupae	
ABUNDANCE ADULT	numeric	number of individual adults	
ABUNDANCE TOTAL	numeric	total number of individuals	
DISTINCT	text	distinct taxa in sample (y/n) (See description in Section 4.8)	
CITATION	text	citation for reference used to identify organism, if taxon not present in WQX database	
QA FLAG (if appropriate)	text	QA/QC flag (lab may use its own flags, if defined in QA_COMMENTS field or provided to NARS IM team)	
		Flag	Definition
		DD	Damaged Organism, poor condition or fragments
		IM	Immature
		IN	Indeterminate (explain in QA_COMMENTS field)
		NP	Not enough preservative used

		NT	Not able to meet target level for identification (may be used with other codes, or explain in QA_COMMENTS field)
		S	Sample shipping problem (explain in QA_COMMENTS field)
		UN	Unknown. Identification is tentative. Organism has been sent to expert taxonomist for definitive identification.
		Q	Other quality concerns, not identified above
QA_COMMENTS	text	explanation for QA FLAG (if needed)	
LAB COMMENTS	text	general laboratory analysis comments	

4.9 Data Entry

Table 4.1, **Table 4.3** and **Table 4.6** identify the required data elements that the sorting and taxonomic laboratories must provide to EPA, preferably in EPA’s data template provided to each of the laboratories and found in Appendix C, available separately from EPA. In addition, the laboratory must provide the resume or *curriculum vitae* for each taxonomist who identifies benthic macroinvertebrates for the NRSA samples. The resume or *cv* for each taxonomist is submitted once to EPA’s External QC Coordinator.

4.10 Sample and Record Retention

The laboratory shall retain:

1. The sample materials, including vials, slides, and sorting residuals, for a minimum of 3 years from the date the EPA publishes the final report. During this time, the laboratory shall store the materials in a cool location away from sunlight. The laboratory shall periodically check the sample materials for degradation and refill jars and vials with 70-80% ethanol if necessary.
2. Original records, including laboratory notebooks and the reference library, for a minimum of 10 years from the date that EPA publishes the final report.

After the stated time periods, the laboratory shall follow its internal protocols for disposal.

4.11 External Taxonomic Quality Control

Two laboratories and multiple experts together conduct the necessary work for the external taxonomic quality control. They perform the following steps:

1. The External QC Coordinator randomly selects 10% of each laboratory’s samples, subject to the following constraints:
 - a. If the primary laboratory received fewer than 30 samples, then the External QC Coordinator will randomly select three samples for the evaluation.
 - b. If the laboratory is responsible for processing 100 samples or more for the survey, the External QC Coordinator will perform the evaluation in batches (e.g., each 50 to 100 samples) so that performance can be evaluated and corrected as necessary. The External QC Coordinator will determine the size of the batches based upon the total number of samples that will be shipped to the laboratory, the delivery schedule, processing schedule, and availability of samples from other laboratories (e.g., the External QC Coordinator might combine samples from three laboratories into one batch at a convenient time for the QC taxonomist).

- c. The External QC Coordinator will arrange for a minimum of 4 QC evaluations in each year (2013, 2014). To the extent practicable, the QC Coordinator will schedule batch evaluations evenly throughout the project period.
2. The External QC Coordinator coordinates and pays for delivery of the selected samples, bench sheets, and chain-of-custody form.
3. The primary laboratory packs the selected samples for shipping. The QC taxonomist's review will be independent, and thus, will not have access to the original laboratory results. Although the External QC Coordinator may tailor the directions for the independent taxonomist's preferences, the directions are likely to include instructions such as:
 - a. Containers (e.g., jars, slides, vials) must include the site identification code and sample number.
 - b. Containers must not contain the complete identification of the organisms. If reference specimens were kept from a sample that is being QC'd, then these taxa must be noted and this information must be given to the External QC Coordinator. In the event that the reference specimens are the only organisms representing a given taxa for a given sample, then these specimens must be made available to the QC taxonomist, through the External QC coordinator.
 - c. Shipment must contain chain-of-custody documentation.
4. Upon receipt, the secondary laboratory follows the tracking procedures in **Section 4.5**.
5. The QC taxonomist:
 - a. Performs whole-sample re-identifications following the procedures in **Section 4.8**, taking care to ensure inclusion of all slide-mounted organisms.
 - b. Completes a separate copy of the taxonomic bench sheet for each sample.
 - c. Enters the data using EPA's template (Template found in **APPENDIX C: SAMPLE LABORATORY FORMS**, see **Section 4.9** for data entry instructions). The QC taxonomist shall label the bench sheet and database entries with the term "QC Re-ID."
6. The External QC Coordinator will compare the taxonomic results (counts AND identifications) generated by the primary and secondary laboratories. If either laboratory identified the organisms to a lower level than required by

7. **Table 4.4** and **Table 4.5** or by nomenclature different than used by WQX, the External QC Coordinator will substitute the required level or name, respectively, in the comparisons. The External QC Coordinator will calculate the percent difference in:
 - a. Enumeration as measured by PDE defined in **Section 4.4.1**; and
 - b. Taxonomy as measured by PTD defined in **Section 4.4.1**. Table 3 of Stribling (2003) provides examples of what would be considered agreements.
8. If any sample has PDE>5% and/or PTD>15%, the External QC Coordinator will facilitate a reconciliation call with EPA and the laboratories. The External QC Coordinator may decide that a reconciliation call is unnecessary if there appears to be an obvious explanation for differences, few samples are affected or other reasons.
9. After completing the reconciliation calls and/or documenting decisions to skip them, the External QC Coordinator will provide EPA with a report or memorandum with recommendations for resolving the differences. Significant differences may result in the re-identification of samples by the primary taxonomist and a second QC check by the secondary taxonomist.
10. As a final QC check on the data, the External QC Coordinator will arrange for an independent data validation of the database. For each sample, the data analyst will evaluate whether the reported taxa are known to occur in geographic area in which the sample site resides. For any discrepancy, the External QC Coordinator will arrange for another taxonomist to evaluate the specimen or its digital photograph.
11. As an additional verification on the generation of the data, EPA may conduct assistance visits at the laboratories. If EPA decides to conduct an assistance visit, a qualified EPA scientist or contractor will administer a checklist based upon the steps described in this LOM. The objective of the visit would be to:
 - Confirm the sorting and identification steps are properly implemented.
 - Assist with questions from laboratory personnel.
 - Suggest corrections if any errors are made.

4.12 Quality Assurance/Quality Control (QA/QC)

Equation 4.1 Percent sorting efficiency (PSE).

Number of organisms recovered by the sorter compared to the combined (total) number of recoveries by the sorter (A) and QC Officer (B) for a sample. PSE should be >90%.

$$PSE = \frac{A}{A + B} \times 100$$

Equation 4.2 Percent disagreement in enumeration (PDE).

Measure of taxonomic precision comparing the number of organisms, n_1 , counted in a sample by the primary taxonomist with the number of organisms, n_2 , counted by the internal or external QC taxonomist. PDE should be <5%.

$$PDE = \frac{|n_1 - n_2|}{n_1 + n_2} \times 100$$

Equation 4.3 Percent taxonomic disagreement (PTD).

Measure of taxonomic precision comparing the number of agreements (positive comparisons, $comp_{pos}$) of the primary taxonomist and internal or external QC taxonomists. In the following equation, N is the total number of organisms in the larger of the two counts. PTD should be $\leq 15\%$.

$$PTD = \left[1 - \left(\frac{comp_{pos}}{N} \right) \right] \times 100$$

Table 4.7 Benthic macroinvertebrate: measurement data quality objectives

Variable or Measurement	Precision	Accuracy
Sort and Pick	90% ^a	90% ^a
Identification	85% ^b	95% ^c

NA = not applicable; ^a As measured by PSE; ^b As measured by (100%-PTD); ^c As measured by (100%-PDE)

Table 4.8 Benthic macroinvertebrate: quality control – laboratory

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
SAMPLE PROCESSING AND SORTING			
Sample pickate examined by different analyst within lab	10% of all samples completed per analyst	PSE ≥ 90%	If < 90%, examine all residuals of samples by that analyst and retrain analyst
Sorting QC Officer counts number of organisms not found in sorted grids	All samples	Sorter achieves PSE ≥90% in 5 consecutive samples. Sorter is now considered “experienced”	Sorting QC Officer checks all samples until acceptance criteria met
Sorting QC Officer counts number of organisms not found in sorted grids for experienced sorters	1 in 10 samples completed per sorter	Sorter achieves PSE ≥90%	If <90%, examine all sorted grids in samples assigned to sorter since last achieving proficiency (i.e., PSE≥90%). Sorter loses “experienced” status and must again show proficiency by achieving PSE ≥90% in 5 consecutive samples. If the sorter shows marked improvement in their sorting efficiency prior to completion of the next five samples, the Sorting QC Officer may, at his/her discretion, consider this individual to be “experienced” and check only 1 in the next 10 samples.
External QC Coordinator evaluates grid and quarter data to determine if the sample was well mixed as demonstrated by consistency in counts between grids (or quarters)	All grids and quarters within each sample	Sorter demonstrates relative consistency for 90% of assigned samples	If <90%, evaluate whether: 1) the sorter’s consistency is similar to other sorters; or 2) few samples were assigned the sorter. If neither explanation applies, EPA’s External QC Coordinator contacts the laboratory to discuss possible corrective action (e.g., resorting of sorter’s samples)
IDENTIFICATION			
Duplicate identification by Internal Taxonomy QC Officer	1 in 10 samples per taxonomist,	PTD ≤15%	If PTD >15%, reidentify all samples completed by that taxonomist since last meeting the acceptance criteria, focusing on taxa of concern
Independent identification by outside, expert, taxonomist	All uncertain taxa	Uncertain identifications to be confirmed by expert in particular taxa	Record both tentative and independent IDs

External QC	10% of all samples completed per laboratory	PDE ≤ 5% PTD ≤ 15%	If PDE > 5%, implement recommended corrective actions. If PTD > 15%, implement recommended corrective actions.
Use of widely/commonly accepted taxonomic references by all NRSA labs	For all identifications	All keys and references used by each lab must be on bibliography prepared by one or more additional NRSA labs or in WQX (see Section 4.4.1 for retrieval instructions). This requirement demonstrates the general acceptance of the references by the scientific community.	If a lab proposes to use other references, the lab must obtain prior permission from Project QA Officer before submitting the data with the identifications based upon the references.
Prepare reference collection	Each new taxon per laboratory	Complete reference collection to be maintained by each individual laboratory	Internal Taxonomy QC Officer periodically reviews data and reference collection to ensure reference collection is complete and identifications are accurate
DATA VALIDATION			
Taxonomic "reasonable-ness" checks	All data sheets	Taxa known to occur in given rivers or streams or geographic area	Second or third identification by expert in that taxon

4.13 References

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5.0 FECAL INDICATOR: ENTEROCOCCI

5.1 Scope & Application

This document describes the application of Draft EPA Method 1606 for the processing and qPCR analysis of water sample concentrates from rivers and streams for the purpose of determining water quality by Real-Time Quantitative Polymerase Chain Reaction (qPCR) assays that determine the concentration of bacteria such as the fecal indicator, *Enterococcus*, by measuring the concentration of their DNA in the water sample.

This method facilitates the microbiological determination of water quality of water bodies at remote locations from which collected water samples cannot feasibly be analyzed for the enumeration of viable (culturable) indicator bacteria because they cannot be transported to an analytical laboratory within 6 hours of collection time for analysis by membrane filtration and / or selective media inoculation and incubation (e.g. MPN broth analysis) methods (EPA method 1600). Prior to qPCR analysis of the water samples, the bacterial cells present in a water sample will have been concentrated by “field” filtration within 6 hours after collection of the samples. The filter retentate preserved by freezing of the sample filters on dry ice and in < -20°C freezers will be subjected to DNA extraction (e.g. bead-beating) and purification processes leading up to qPCR analysis. This processing can be completed up to 1 year after cell concentration if the sample filter retentates are maintained frozen at -20 to -80°C.

5.2 Summary of Method

Each sub-sample has previously been filtered aseptically and folded inward in half three times to form an umbrella or in half and rolled up and then inserted into sterile sample extraction tubes containing sterile glass beads or Roche MagNA Lyser Green Beads™ (actually siliconized white ceramic beads in a green capped tube). Extraction tubes containing filter concentrates (retentates) have been stored on dry ice until transport to the analytical laboratory by air courier. Filter concentrates will be shipped by air courier on dry ice from the field to the analytical team at EPA New England Regional Laboratory. Filter concentrates received by NERL staff will be subjected to DNA extraction procedures and subsequently analyzed by Draft EPA Method 1606 or 1607 for Total *Enterococcus* along with modifications to the QA/QC procedures described below. The laboratory methods are summarized in **Table 5.4** of **Section 5.18**.

5.3 Definitions of Method

Batch Size: The number of samples that will be processed by filter extraction with the same batch (volume) of SAE buffer and analyzed by the same qPCR assay(s) using the same batch of qPCR master mix. A batch is covered for quantitation purposes by the same “batch” calibrator samples, a minimum of three, analyzed during the same week.

Bottle Blank: Analyte-free water is collected into a sample container, of the same lot number as the containers used for collection of the environmental samples. Analysis of this sample is performed to evaluate the level of contamination, if any, introduced into the environmental and control samples from the sample container(s) from a common vendor’s lot.

DNA: Deoxyribo-Nucleic Acid, double-stranded genetic molecules containing sequences of the four nucleotide bases, adenine, thymine, guanine, and cytosine that encode rRNA, mRNA, and tRNA involved in protein synthesis.

Field Filter Blank: A volume of sterile PBS, free of target organisms (i.e. *Enterococcus*) filtered through a sterile filter and processed in parallel with all other samples to serve as a sentinel for detection of reagent contamination or contamination transferred between samples by processing and analysis.

Field Replicates: Samples collected from rivers and streams that are collected at the same sampling site one right after the other with only slight temporal variation. They are not “splits” of the same sample volume.

Filtrate: Sample liquid or buffer rinsate passing through the filter into the vacuum flask.

Laboratory Quality Samples: Mock samples created in the lab such as lab blanks, lab-fortified blanks (LFBs), and Lab-Fortified Matrices (LFMs) used to assure lack of sample contamination and to measure analytical recovery during performance of sample processing and analysis methods.

Performance Testing (PT) / Performance Evaluation Sample (PES): Calibrator samples (filters spiked with *E. faecalis* grown in Brain Heart Infusion Broth) and Laboratory Fortified Blanks (Phosphate Buffered Saline; PBS) spiked with *Enterococcus faecalis* cells from BHI Broth suspension) will be assayed by EPA Method 1600 and Draft EPA Method 1606 to ascertain method performance. Ball-T Bioballs® which contain a specified number of *E. faecalis* cells may also be acquired to determine the performance of the Relative Quantitation Method. Purified *E. faecalis* DNA acquired from the American Type Culture Collection and TIB Mol Biol Inc. is used to test the performance of the Absolute Quantitation Method.

Retentate: The sample residue retained by the filter after the sample is vacuum-filtered. The retentate contains particulates, microbiota, and macrobiota from which the DNA is extracted into buffer by bead-beating for subsequent qPCR analysis.

Rinsate: The volume of phosphate buffered saline (PBS) applied to a sample’s filter retentate in order to “wash” any residual fine particles, smaller than the filter’s nominal pore size, through the retentate and the filter.

Sample Processing Control (SPC): A surrogate homologue analyte (e.g. Salmon DNA) spiked into each sample to determine the recovery of target analyte and/or detect assay inhibition caused by matrix effects.

Standards: Known amounts or numbers of copies of *Enterococcus* genomic DNA analyzed by the *Enterococcus* qPCR assay to generate a Standard Curve (Log Copy Number vs. Crossing Point Value) in order to determine *Enterococcus* genomic copy numbers in “Unknown” test sample extracts by Absolute Quantitation Method.

5.4 Interferences

- Low pH (acidic) water
- Humic and fulvic acid content
- Suspended solids (e.g. fecal matter) and particulates (sand, dirt)
- Excessive algal growth

5.5 Health & Safety Warnings

All proper personal protection clothing and equipment (e.g. lab coat, protective eyewear/goggles) must be worn or applied.

When working with potential hazardous chemicals (e.g. 95% ethanol) or biological agents (fecally-contaminated water) avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs

remove clothing immediately and wash/ rinse thoroughly. Wash the affected skin areas thoroughly with large amounts of soap and water. If available consult the MSDS for prompt action, and in all cases seek medical attention immediately. If inhalation, eye contact or ingestion occurs, consult the MSDS for prompt action, and in all cases seek medical attention immediately.

5.6 Personnel Qualifications

All laboratory personnel shall be trained in advance in the use of equipment and procedures used during the sample extraction and qPCR analysis steps of this SOP. All personnel shall be responsible for complying with all of the quality assurance/quality control requirements that pertain to their organizational/technical function. All personnel shall be responsible for being aware of proper health and safety precautions and emergency procedures.

5.7 Equipment and Supplies

- Clean powderless latex or vinyl gloves
- Goggles or Face Shield
- Roche MagNA Lyser
- Roche MagNA Pure LC (automated nucleic acid isolation and purification platform)
- High Speed Microfuge
- Micropipettors
- Semi-conical, screw cap microcentrifuge tubes (PGC, #506-636 or equivalent) pre-filled with 0.3 ± 0.02 g Acid-washed glass beads (Sigma, # G-1277 or equivalent). Filled tubes are autoclaved 15-min. Liquid Cycle (Slow Exhaust) OR
- Roche MagNA Lyser Green Bead tubes (Roche Applied Science, #03-358-941-001) sterile, siliconized 3-mm diameter ceramic beads in a siliconized 2-mL microfuge tube.
- Roche MagNA Lyser Rotor Cooling Block
- 2-mL tube racks
- Permanent marking pens (fine point and regular point) for labeling tubes
- Bench Sheets & Printouts of Computer Software Sampling Loading Screen

5.8 Reagents & Standards

- Qiagen AE buffer (Qiagen 19077)
- Salmon DNA (Sigma D1626)
- Frozen tubes of *Enterococcus faecalis* (ATCC #29212) calibrator cell stock
- Purified *Enterococcus faecalis* (ATCC #29212d) genomic DNA
- ABI TaqMan® Universal PCR Master Mix (ABI #4304437)
- *Enterococcus* PCR primers and TaqMan® probe
- Sketa PCR primers and TaqMan® probe
- Bovine Serum Albumen (BSA) Sigma Cat. #B-4287)
- Roche MagNA Pure LC DNA Isolation Kit III for Fungi & Bacteria

5.9 Preparations Prior to DNA Extraction & Analysis

Determine/estimate the sample batch size (number of samples) for one-week of sample processing and qPCR analysis. The batch size is the number of samples that will be processed by filter extraction with the same batch (volume) of SAE buffer and analyzed by the same qPCR assay(s) using the same batch of qPCR master mix. A batch is covered for quantitation purposes by the batch calibrator samples, (a minimum of three) whose 5-fold and 25-fold diluted extracts are analyzed at the outset of the week along with a reagent blank. The lab will fill out a batch sample analysis bench sheet.

1. Micropipettors are calibrated annually and tested for accuracy on a weekly basis. Follow manufacturer instructions for calibration check. Measure three replicate volumes per pipettor and keep log book of their weights on a calibrated balance scale.
2. **Preparation of stock Salmon Sperm (SS) DNA:** Dissolve Salmon DNA in PCR grade water at a concentration of $\sim 10 \mu\text{g}/\text{mL}$. Determine concentration of Salmon testes DNA stock by OD_{260} reading in a spectrophotometer. A DNA solution with an OD_{260} of 1.0 has a concentration equal to approximately $50 \mu\text{g}/\text{mL}$ depending on the GC content of the DNA's sequence(s).
3. Dilute Salmon testes DNA stock with AE buffer to make $0.2 \mu\text{g}/\text{mL}$ Salmon DNA Extraction Buffer (SAE). Extraction buffer may be prepared in advance and stored at 4°C for a maximum of 1 week.

Note: Determine the total volume of Salmon DNA Extraction Buffer required for each day or week by multiplying the volume ($600 \mu\text{L}$) times the total number of samples to be analyzed including controls, water samples, and calibrator samples. For example, for 18 samples, prepare enough Salmon/DNA extraction buffer for 24 extraction tubes ($18 / 6 = 3$, therefore, 3 extra tubes for water sample filtration blanks (method blanks) and 3 extra tubes for calibrator samples). Note that the number of samples is divided by 6 because you should conduct one method blank for every 6 samples analyzed. Additionally, prepare excess volume to allow for accurate dispensing of $600 \mu\text{L}$ per tube, generally 1 extra tube. Thus, in this example, prepare sufficient Salmon DNA Extraction Buffer for 24 tubes plus one extra. The total volume SAE needed per sample is $600 \mu\text{L}$. Hence for the SAE volume for 25 sample tubes is equal to $15,000 \mu\text{L}$. Dilute the Salmon DNA working stock 1:50, for a total volume needed ($15,000 \mu\text{L}$) $50 = 300 \mu\text{L}$ of $10 \mu\text{g}/\text{mL}$ Salmon DNA working stock. The AE buffer needed is the difference between the total volume and the Salmon testes DNA working stock. For this example, $15,000 \mu\text{L} - 300 \mu\text{L} = 14,700 \mu\text{L}$ AE buffer needed.

4. Make Dilution Series of *Enterococcus faecalis* purified genomic DNA for use as internal standards in individual qPCR runs and to generate the weekly *Enterococcus* qPCR Standard Curve for quantitation purposes.
5. *Enterococcus faecalis* DNA for Standards.
6. Frozen Reference Stock ($20\text{-}\mu\text{L}$) at 2.89×10^6 GEQs per μL .
7. Dilute $10\text{-}\mu\text{L}$ of the Frozen Reference stock 363-fold to a final volume of $3,630 \mu\text{L}$ AE buffer. Aliquot $20\text{-}\mu\text{L}$ volumes into many $200\text{-}\mu\text{L}$ microfuge tubes and store frozen at -20°C . The net concentration of *Enterococcus* GEQs is $8,000 / \mu\text{L}$. Each week perform a series of 10-fold and 4-fold dilutions from one thawed tube of the $8,000 \text{ GEQ}/\mu\text{L}$ standard solution to create $800 \text{ GEQ}/\mu\text{L}$, $80 \text{ GEQ}/\mu\text{L}$ and $20 \text{ GEQ}/\mu\text{L}$ standard solutions. The analyst performs *Enterococcus* qPCR upon duplicate $5\text{-}\mu\text{L}$ volumes of each of the four standards yielding a Standard Curve of Log

GEQs ENT versus Ct value from which the assays “efficiency” is subsequently calculated in the Relative Quantitation EXCEL Spreadsheet.

- a. Make *Enterococcus faecalis* calibrator filter samples:
 - i. Assemble calibrator positive control samples by thawing tubes of *E. faecalis* cell stocks, diluting their contents (10- μ L) up to 1-mL AE buffer and spotting 10- μ L on sterile PC filter previously folded and inserted into a pre-chilled Green Bead tube.
 - ii. Spot a sufficient number of calibrator filter samples for the entire study to insure uniform, consistent relative quantitation of study samples. Store the calibrator filter samples in -20°C freezer and thaw individual calibrators (three per week) for extraction with each week’s batch of samples.
8. The calibrator sample filters are spotted with 10^4 or 10^5 *Enterococcus faecalis* cells and this number is incorporated into the Relative Quantitation EXCEL spreadsheet.
9. Prior to and after conducting work with cells and / or genomic DNA standards, disinfect and inactivate (render non-amplifiable) DNA in the Sample Extraction Hood, the qPCR Cabinet, and the qPCR Sample Loading Hood with 10% bleach and \geq 15-min. exposure to high intensity germicidal (254 nm) ultraviolet light.

5.10 Procedures for Processing & qPCR Analysis of Sample Concentrates.

5.10.1 Sample Processing (DNA Extraction)

Typically, 100-mL volumes of surface water are filtered according to EPA Method 1606 for processing and analysis by PCR assays. Due to the limitations of field crew sampling time and the performance limitations of the manually-operated vacuum pumps used in the field sampling operations, only 50-mL surface water samples were filtered. Lower volumes (\leq 50-mL) are acceptable if suspended particulates hinder the filtering of the standard 50-mL volume but equivalent volumes for each filter replicate were requested. Filtration of lower sample volumes necessitated modifications to Method 1606 which are directed by the [Analysis Decision Tree](#) (ADT; **Section 5.18.1**).

In accordance with the ADT, if < 40-mL of a water sample is filtered per filter replicate, then the laboratory analyst extracts two replicate filters in parallel and combines equivalent volumes of the filter extracts to form one composite filter extract. Each individual filter is extracted with only 300- μ L of SAE Extraction Buffer instead of the usual prescribed 600- μ L volume of SAE buffer. Halving the SAE buffer volume enables the analyst to maintain an equivalent Method Detection Limit and maintain a similar Sample Equivalence Volume (SEQ; i.e. water sample volume per extract volume) in the extract volumes (e.g. 5- μ L) of each sample filter concentrate added to the PCR reactions.

1. Pre-chill MagNA Lyser Rotor Cooling Block in -20°C freezer. Label 1.7-mL sterile microfuge tubes with sample ID number to match them with Green Bead Tubes. Two supernatant recovery tubes and one “5-fold” dilution tube is needed per sample and should be labeled accordingly. The dilution tube shall be filled with 80- μ L AE buffer using a micropipettor.
2. To extract sample filters, uncap green bead tube (cold) and add 0.6-mL (600- μ L) SAE Buffer (Qiagen AE Buffer spiked with Salmon DNA). Re-cap tubes tightly.
3. Insert Green Bead tubes of samples into MagNA Lyser and bead-beat for 60-sec (1-min) at 5,000 rpm at Room Temperature. Transfer sample tubes to microfuge. Spin tubes at 12,000 rpm for 2-

min. Being careful to move filter aside, recover and transfer up to 400- μ L of supernatant (sans debris) to new tube with a P-200 or P-1000 micropipettor.

4. Spin the supernatant tubes for 5-min at 14,000 rpm at Room Temperature. Recover >350- μ L supernatant and transfer to new 1.7-mL tube. When all samples in a batch have been extracted transfer dilute 20- μ L of DNA extract (2nd supernatant) five-fold (5X) in 80- μ L AE buffer (sans SS-DNA) and store at 4°C for qPCR assays. (If supernatant, 5X and even 25X sample dilutions possess dark pigment and exhibit severe qPCR inhibition in Sketa assays, consider extracting replicate filters of samples using the Modified MagNA Pure LC DNA Isolation Protocol (see **Section 5.18.2**).

5.10.2 Sample Analysis by *Enterococcus* qPCR

5.10.2.1 Preparation of qPCR assay mix

1. To minimize environmental DNA contamination, routinely treat all work surfaces with a 10% bleach solution, allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. If available, turn on UV light for 15 minutes.
2. Using a micropipettor with aerosol barrier tips, add PCR grade water to the lyophilized primers and probe from the vendor to create stock solutions of 500 μ M primer and 100 μ M probe and dissolve by extensive vortexing. Pulse centrifuge to coalesce droplets. Store stock solutions at -20°C.
3. Prepare working stocks of *Enterococcus*, and Salmon DNA primer/probe mixes by adding 10 μ L of each *Enterococcus* or Salmon DNA primer stock and 4 μ L of respective probe stock to 676 μ L of PCR grade water, and vortex. Pulse centrifuge to create pellet. Use a micropipettor with aerosol barrier tips for all liquid transfers. Transfer aliquots of working stocks for single day use to separate tubes and store at 4°C.
4. Using a micropipettor, prepare assay mix of the *Enterococcus*, and Salmon DNA reactions in separate, sterile, labeled 1.7 mL microcentrifuge tubes as described in **Table 5.1**.
5. Finger vortex the assay mix working stocks; then pulse microcentrifuge to coalesce droplets. Return the primer/probe working stocks and other reagents to the refrigerator.
6. Thaw and finger vortex sample extract (dilution) tubes that will be assayed in PCR run. Microfuge a few seconds to coalesce droplets. Finger mix and spin the standards and calibrator samples (dilutions). Temporarily store all samples in 4°C refrigerators until use in assay or return to long term storage at -20°C. Discard disposable gloves and put on a new pair.
7. Set 32 Smart tubes in Cepheid Racks in PCR cabinet along with micro-pipettors and expose to germicidal UV lamp for 15-min.
8. Pipette 20- μ L of respective Master Mix into each labeled Smart tube. Transfer Smart tubes (racks) from PCR cabinet to disinfected Sample Loading Fume Hood.
9. Using P-10 or P-20 micro-pipettor load each Smart tube with 5- μ L volume of respectively designated sample extract (dilution), standard, or buffer blank (SAE). Cap each sample's Smart tube after loading.
10. Check to make sure each Smart tube is properly labeled and identifiable by sample number or I-core position (e.g. A4). Insert loaded Smart tubes into Smart Tube microfuge. Close lid and spin

5-sec. Pop lid to stop. Remove Smart Tubes from microfuge and insert into proper position in SmartCycler.

Enterococcus (Ludwig) and Salmon (Sketa) qPCR assays (EPA Method 1606) will be performed upon 5- μ L aliquots of un-diluted & 5X diluted extracts of sample unknowns, calibrator, field blank, and lab blank. A “No Template Controls” (NTC) shall be analyzed on an ongoing basis to ensure that the Master Mix PCR reagents are not contaminated. To minimize the number of *Enterococcus* qPCR reactions needed to be performed upon samples, Sketa qPCR assays will be performed upon the 5-fold diluted DNA extracts of samples before any *Enterococcus* qPCR assays are run in order to screen samples for the presence and dilution of PCR inhibitors by comparison with the undiluted and 5-fold dilution DNA extract of the calibrator samples and unused portions of SAE buffer. Each sample’s lowest dilution DNA extract not exhibiting PCR inhibition in the Sketa qPCR assay will be re-assayed by the *Enterococcus* qPCR assay and its results will be used for quantitation of *Enterococcus* DNA sequences and CCEs.

Detection of reduced levels of Salmon DNA (higher instrument Ct values) is indicative of technical error during extract dilution or excessive levels of PCR inhibitors or nuclease activity which could impact detection of the *Enterococcus* DNA target sequences in the *Enterococcus* PCR assay. Alternatively, the high Sketa Ct value may be indicative of the occurrence of a technical error during extract dilution. If a test sample’s Ct value is less than 3 cycles different than the blank negative control and calibrator samples, indicating only negligible or marginal inhibition (the Sketa Assay is more sensitive to inhibitors than the ENT Assay), an aliquot of its five-fold diluted extract is analyzed in the *Enterococcus* Assay. If an abundance of PCR inhibitors or DNA nucleases are present in a sample extract which are causing a greater increase in an extract’s Ct value (≥ 3 cycles increase), then the extract is diluted an additional five-fold (net 25-fold dilution) and re-assayed by both the Sketa and ENT assays. If the inhibition is not ameliorated by the additional dilution, which should restore the Sketa Ct value to that of the 25-fold diluted calibrator samples’ extracts, the following actions are taken by the analyst. First, the analyst re-dilutes the sample’s undiluted DNA extract five-fold and re-analyzes the dilution with the Sketa PCR assay to confirm that Ct variance is not due to a dilution error. If the Ct difference is not attributed to a dilution error, replicate sample filters of the “inhibited” samples are subjected to DNA extraction and purification by the MagNA Pure LC automated platform loaded with the Roche DNA Kit III (Bacteria; Fungi) reagents (see **Section 5.18.2**).

The EPA Modified MagNA Pure LC extraction process which includes the spiking of the Lysis Binding Buffer with the Salmon (IPC) DNA is more effective, but more costly, than EPA Method 1606 in neutralizing severe levels of PCR inhibitors and DNA nucleases present in some environmental samples, especially those containing high levels of algae or phytoplankton. The purified DNA extract yielded by MagNA Pure extraction of the few ($\leq 5\%$) “severely inhibited” samples is subsequently analyzed by the Sketa and *Enterococcus* qPCR assays and the number of *Enterococcus* CCEs per 100-mL determined by the delta Ct and delta delta Ct Relative Quantitation Methods. While the MagNA Pure LC extraction method is not 100% conservative (no partitioning or recovery issues) like EPA Method 1606, it typically exhibits DNA recoveries in the range of 25-50%. DNA recoveries and *Enterococcus* CCE concentrations are calculated using only the Delta-Delta Ct Relative Quantitation Method. The relative DNA recoveries are determined by comparison of the Sketa results from purified DNA eluates of each test sample with those of the extracted lab blank and calibrator samples. The absolute DNA recovery is calculated by comparison of the former Sketa results with those of elution buffer spiked with an amount of Salmon DNA equivalent to the amount in the Salmon-spiked Lysis Binding Buffer added to each sample filter lysate during the MagNA Pure LC DNA extraction process.

The “Unknown” and “Control” sample extracts whether processed using the SAE buffer or MagNA Pure LC Kit III reagents are analyzed according to the Cepheid SmartCycler *Enterococcus* and Sketa qPCR protocols described in Appendix A of the Draft EPA Method 1606 with Ct determination made by the software using Manual Determination (equivalent of Fit Points Method of Roche LightCycler) with the fluorescence threshold set at 8.0 units which enables uniform analysis and comparability of all samples’ qPCR results.

5.10.3 Sample analysis sequence for SmartCycler

Example: For analyses on a single 16-position SmartCycler, calibrator samples and water samples are analyzed in separate runs and a maximum of 6 water samples (or 2 replicates of 3 samples) are analyzed per run, as described in **Table 5.2** and **Table 5.3** of **Section 5.18**.

Enterococcus and Sketa (Salmon DNA = SPC) qPCR results are exported to an EXCEL spreadsheet in which relative quantitation calculations are performed by analysts. The Method 1606 results are reported in terms (units of measure) of Number of *Enterococcus* Sequences and Number of *Enterococcus* Calibrator Cell Equivalents (CCEs) per 100-mL sample volume. The qPCR results are converted to this standardized unit of measure based on the volume of water sample actually filtered (*e.g.*, 10-mL, 25-mL, or 50-mL). Non-detects are reported as below the reporting limit (RL) which varies proportionally to the volume of sample filtered by each sample crew at a specific site. Reporting limits and Method Detection Limits (MDLs) will be higher among samples for which a volume of water <50-mL was filtered.

Enterococcus qPCR results are flagged if some part of the sample collection, hold-time, processing, shipment, storage, sample extraction, or qPCR analysis are compromised and did not meet the requirements of the Sampling and Analysis SOPs.

5.11 Storage & Timing of Processing/Analysis of Filter Concentrates

When a sufficient number of water sample filter concentrates (filters and retentates) have been received by NERL and qPCR analytical reagents have been obtained the samples will be logged into LIMS. Sample processing and qPCR will commence and results will be entered into the LIMS upon completion of analysis.

5.12 Chain of Custody

Follow the Sample Control Procedures, Field Sampling Form/Enterococci Filtration/Sample Processing Standard Operating Procedures.

Field Sampling forms and NRSA 2013-2014 Sample Tracking EXCEL Spreadsheet shall be consulted to determine if a sample has been properly preserved during collection and transport prior to analysis and that it has passed all criteria permitting its analysis. The qPCR results of samples exceeding established criteria or whose associated field/lab blanks had positive *Enterococcus* qPCR detections of DNA shall be flagged.

5.13 Quality Assurance/Quality Control (QA/QC) Procedures

The Data Quality Objectives and the Laboratory QC Procedures are listed and summarized in **Table 5.5** and **Table 5.6** of **Section 5.18**.

The number of field blanks (dilution buffer only) shipped by field crews performing the resampling of 91 re-visited rivers and streams represents a frequency of 5-10% of the total number of samples extracted and analyzed by qPCR. All field blanks (negative controls) will be extracted and analyzed by qPCR for the

detection of *Enterococcus*. The blanks will be analyzed in these cases to insure that positive detections in field samples are not due to contamination by sampling crews.

One Lab / Method Blank (LB; sterile filters) will be run per batch week in order to insure the sterility (lack of DNA contamination) in the SAE buffer and pipette tips used to process all of the samples. The LB sample will be processed and diluted like all other "Unknown" samples.

Up to four replicate filter concentrates (retentates) derived from the field filtration of 50-mL (in some cases 10-mL and 25-mL) sample volumes of every sample will be received by NERL and stored at -20 to -80°C. One filter retentate of each sample (and duplicates for 10% of samples) will be extracted to obtain DNA lysates for *Enterococcus* qPCR analysis. The remaining filter concentrates will be archived for possible extraction and analysis at a later time if needed.

Enterococcus and Sketa qPCR analysis will be performed upon 5-µL volumes of the non-diluted and 5-fold diluted (in AE buffer) extracts which will be added to 20-µL qPCR Master Mix volumes and analyzed in the Cepheid SmartCycler qPCR instrument in accordance with draft EPA Method 1606.

Duplicate *Enterococcus* and Sketa qPCR assays will be performed upon 10% of the sample extracts (diluted and un-diluted) each week (batch) to determine qPCR assay variance.

5.14 Method Performance

Method Performance will be determined by the use of Performance Testing (PT)/Performance Evaluation Samples (PES). Calibrator samples (filters spiked with frozen stocks of *E. faecalis* grown in Brain Heart Infusion Broth) and Lab-Fortified Matrices (LFMs; duplicate sample filters spiked with frozen stocks of *E. faecalis* grown in Brain Heart Infusion Broth) will be extracted and assayed by EPA Method 1606 *Enterococcus* and Sketa qPCR assays in order to ascertain method performance. The LFMs are performed upon several samples (approx. 5% frequency) per batch, typically samples exhibiting non-detection of *Enterococcus*, in order to determine method performance and also to insure that non-detects are not due to poor DNA recovery caused by matrix effects.

5.15 Record Keeping & Data Management

Laboratory analysts shall follow the EPA OEME Laboratory Data Management SOP. Each lab analyst shall record all details pertaining to sample processing and analysis in a designated, bound laboratory notebook. Pertinent sample collection and analysis data shall be entered into the Laboratory Information Management System (LIMS) and SeaGate Crystal Reports shall be generated as required by the EPA (TOPO).

An EXCEL spreadsheet of sample analysis data and associated calculations used to derive a field sample's or control sample's *Enterococcus* genomic DNA (GEQ) and Cell Equivalent (CEQ) concentration shall be uploaded to the NRSA 2013-2014 database stored on a computer server in Corvallis, Oregon.

5.16 Waste Management & Pollution Prevention

During the sample processing procedures there may be hazardous waste produced. The waste must be handled and disposed of in accordance with federal, state, and municipal regulations. All recyclable and non-recyclable materials for disposal will be properly sorted for their respective waste streams and placed into proper containers for janitorial staff to collect and process according to EPA guidelines.

All ethanol used shall be consumed by ignition or evaporation. Volumes of ethanol remaining at the end of the project can be stored for later use in a flammable cabinet or disposed of through appropriate hazardous waste disposal vendors. Reagent ethanol shall be contained in screw cap tubes along with the

filter forceps to sterilize the latter and to prevent ethanol spillage during transport between sampling sites.

After the DNA extract is recovered from the sample filter after bead-beating in buffer and centrifugation, the filter and bead-tube will be discarded in autoclave bags and sterilized for 30-min at 121°C/30 psi to inactivate any potential pathogens that may be associated with the samples.

5.17 Literature Cited

USEPA Region 1 (New England) OEME NERL Standard Operating Procedure for the Collection of Chemical & Biological Ambient Water Samples (ECASOP-Ambient Water Sampling 2; January 31, 2007)

USEPA Draft Method 1606: Enterococci in Water and Wastewater by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. December 2006 (12/15/06 a)

USEPA NERL OEME Draft Bench SOP for Real-Time PCR Method Quantifying Enterococci in Recreational Water Samples (August 2006)

5.18 Tables, Diagrams, Flowcharts, Checklists, and Validation Data

Table 5.1 Enterococci: PCR assay mix composition (according to draft EPA method 1606)

Reagent	Volume/Sample (multiply by # samples to be analyzed per day)
Sterile H ₂ O	1.5 µL
Bovine Serum Albumen (20 mg/mL)	2.5 µL
TaqMan® master mix	12.5 µL
Primer/probe working stock solution	3.5 µL*

Note: This will give a final concentration of 1 µM of each primer and 80 nM of probe in the reactions. Prepare sufficient quantity of assay mix for the number of samples to be analyzed per day including calibrators and negative controls plus at least two extra samples. It is strongly recommended that preparation of assay mixes be performed each day before handling of DNA samples.

Table 5.2 Enterococci: batch calibrator & enterococcus standards PCR run – 7 samples

Sample Description*	Quantity		Quantity PCR Reactions
	Samples	PCR Assay Master Mix	
3 Calibrators (5- and/or 25-fold dilution)	3	Salmon DNA (Sketa)	6
3 Calibrators (5- and/or 25-fold dilution)	3	<i>Enterococcus</i>	6
4 <i>Enterococcus faecalis</i> DNA Standards	4	<i>Enterococcus</i>	8
No template control (reagent blank)	1	<i>Enterococcus</i>	1

* Diluted equivalently to the water samples

Table 5.3 Enterococci: sub batch test sample PCR run – 26 samples & 1 method blank

Sample Description*	Quantity		Quantity PCR Reactions
	Samples	PCR Assay Master Mix	
Water samples, (5-fold dilution)	26	<i>Enterococcus</i>	26

Method blank or Sample PCR Reaction Duplicate, (1- or 5-fold dilution)	1	<i>Enterococcus</i>	1
Non-diluted SAE Buffer	1	<i>Enterococcus</i>	1
Water samples, (1- or 5-fold dilution)	26	Salmon DNA	26
Method blank or Sample PCR Reaction Duplicate, (1- & 5-fold dilution)	1	Salmon DNA	1

* Use of 5-fold diluted samples for analysis is currently recommended if only one dilution can be analyzed. Analyses of undiluted water sample extracts have been observed to cause a significantly higher incidence of PCR inhibition while 25-fold dilutions analyses may unnecessarily sacrifice sensitivity.

Table 5.4 Enterococci: laboratory methods

Variable or Measurement	QA Class	Expected Range and/or Units	Summary of Method	References
Sample Collection	C	NA	Sterile sample bottle submerged to collect 250-mL sample 6-12" below surface at 1-m from shore	NRSA Field Operations Manual 2008
Sub-sampling	N	NA	4 x 50-mL sub-samples poured in sterile 50-mL tube after mixing by inversion 25 times.	NRSA Laboratory Methods Manual 2008
Sub-sample (& Buffer Blank) Filtration	N	NA	Up to 50-mL sub-sample filtered through sterile polycarbonate filter. Funnel rinsed with minimal amount of buffer. Filter folded, inserted in tube then frozen.	NRSA Laboratory Methods Manual 2008
Preservation & Shipment	C	-40C to +40 C	Batches of sample tubes shipped on dry ice to lab for analysis.	NRSA Laboratory Methods Manual 2008
DNA Extraction (Recovery)	C	10-141%	Bead-beating of filter in buffer containing Extraction Control (SPC) DNA. DNA recovery measured	EPA Draft Method 1606 <i>Enterococcus</i> qPCR
Method 1606 (<i>Enterococcus</i> & SPC qPCR)	C	<60 (RL) to >100,000 ENT CCEs /100-mL	5-µL aliquots of sample extract are analyzed by ENT & Sketa qPCR assays along with blanks, calibrator samples & standards. Field and lab duplicates are analyzed at 5% frequency. Field blanks analyzed along with test samples.	EPA Draft Method 1606 <i>Enterococcus</i> qPCR NERL NRSA 2008 qPCR Analytical SOP

C = critical, N = non-critical quality assurance classification.

Table 5.5 Enterococci: parameter measurement data quality objectives

Variable or Measurement	QA Class	Expected Range and/or Units	Summary of Method	References
DNA Extraction (Recovery)	C	10-141%	Bead-beating of filter in buffer containing Extraction Control (SPC) DNA. DNA recovery measured	EPA Draft Method 1606 <i>Enterococcus</i> qPCR
<i>Enterococcus</i> & SPC qPCR	C	<60 to >10,000 ENT CEQs /100-mL	5-µL aliquots of sample extract are analyzed by ENT & Sketa qPCR assays along with blanks, calibrator samples & standards. Field and lab duplicates are analyzed at 5% frequency. Field blanks analyzed at end of testing only if significant detections observed.	EPA Draft Method 1606 <i>Enterococcus</i> qPCR; NERL NRSA 2008 2009 qPCR Analytical SOP (QAPP)
SPC & ENT DNA sequence numbers of Calibrators & Standards by AQM	RSD = 30%	<u>80%</u>	95%	
ENT CCEs by dCt RQM	RSD = 55%	<u>40%</u>	95%	
ENT CCEs by ddCt RQM	RSD = 55%	50%	95%	

C = critical, N = non-critical quality assurance classification.

**AQM = Absolute Quantitation Method; RQM = Relative Quantitation Method;*

SPC = Sample Processing Control (Salmon DNA / Sketa); CCEs = Calibrator Cell Equivalents

Table 5.6 Enterococci: laboratory QC procedures – enterococci DNA sequences

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
SAMPLE PROCESSING			
Re-process sub-samples (duplicates)	10% of all samples completed per laboratory	Percent Similarity ≥70%	If <70%, re-process additional sub-samples
qPCR ANALYSIS			
Duplicate analysis by different biologist within lab	10% of all samples completed per laboratory	Percent Congruence ≤30% RSD	If >30%, determine reason and if cause is systemic, re-analyze all samples in question.
Independent analysis by external laboratory	None	Independent analysis TBD	Determine if independent analysis can be funded and conducted.

Use single stock of <i>E. faecalis</i> calibrator	For all qPCR calibrator samples for quantitation	All calibrator sample Cp (Ct) must have an RSD \leq 30%	If calibrator Cp (Ct) values exceed an RSD value of 30% a batch's calibrator samples shall be re-analyzed and replaced with new calibrators to be processed and analyzed if RSD not back within range.
DATA PROCESSING & REVIEW			
100% verification and review of qPCR data	All qPCR amplification traces, raw and processed data sheets	All final data will be checked against raw data, exported data, and calculated data printouts before entry into LIMS and upload to Corvallis, OR database.	Second tier review by contractor and third tier review by EPA.

5.18.1 *Enterococcus* qPCR Analysis Decision Tree (ADT)

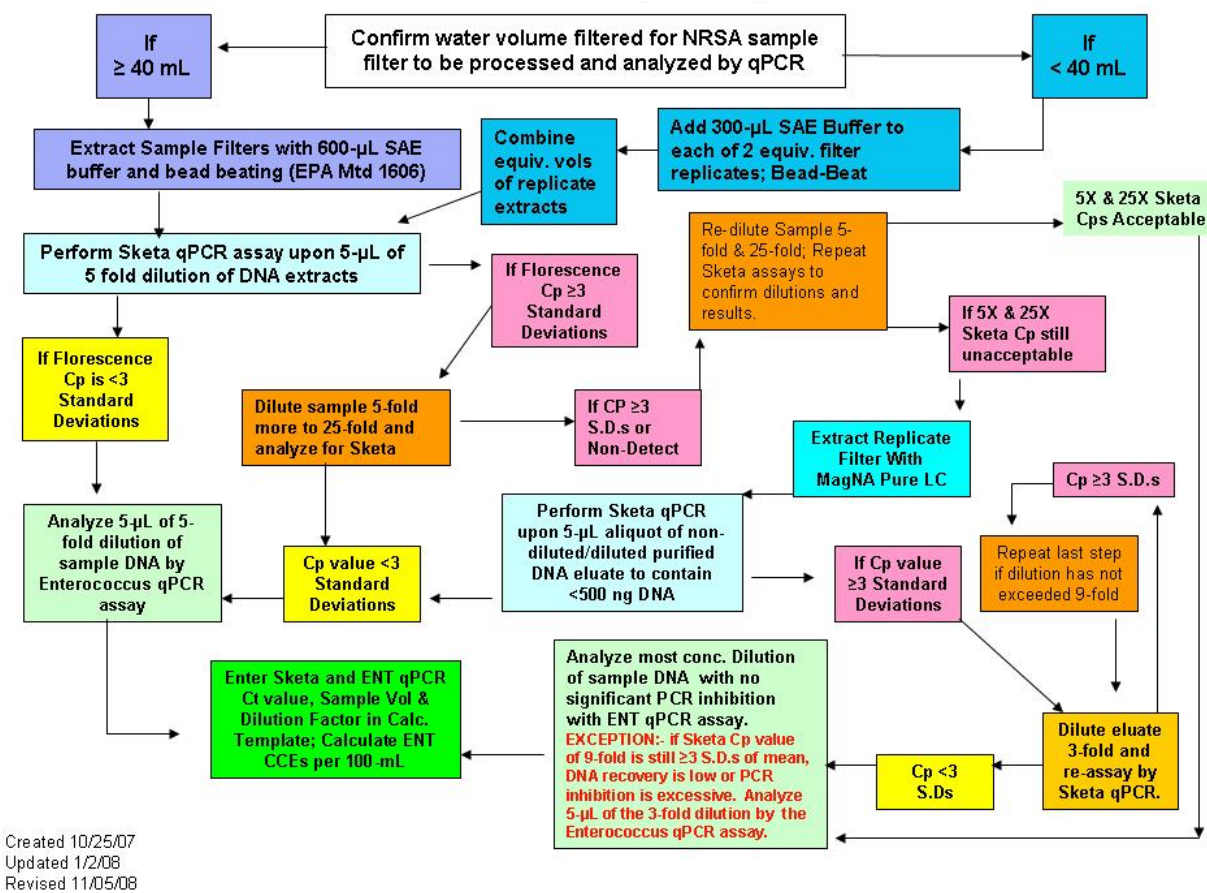


Figure 5.1 Enterococci: qPCR analysis decision tree (ADT)

5.18.2 “Modified” MagNA Pure LC DNA Purification Kit III Protocol

1. Pre-warm the MagNA Pure LC DNA Isolation Kit III Lysis Buffer to 65 °C in waterbath. Quickly pipette 260-µL of warm Lysis Buffer (un-amended) into each “Green Bead” tube with filter (preserved after filtration temporarily on ice or during long-term storage in freezer). Shake tube

- 5 to 10 seconds to mix buffer with beads and filter. Let stand at RT until batch of 16 samples (including positive control LFB or LFM and negative control LB samples) have all had Lysis Buffer and had their caps sealed tight. Leave water bath on to use during 30-minute Proteinase K treatment period.
2. Load the 16 samples into MagNA Lyser Rotor Plate and insert into MagNA Lyser. Tighten the three handscrews of the locking mechanism. Close the lid tightly. Set controls to shake for 60-sec at 5,000 rpm. Press the start button.
 3. When the shake cycle has ended press the Open Lid Button. Open the lid and unlock the locking mechanism screws. Remove tube plate and set on bench top MagNA Lyser tube ring hub. Remove tubes, insert into tube styrofoam water bath float and cool tubes in ice water for 2-min. or place directly into 24-place microfuge rotor, pre-chilled in freezer.
 4. Insert tubes into centrifuge rotor symmetrically in order to balance rotor. Close lid of centrifuge. Set spin parameters for 3,000 rpm for 1-min at 4°C. Press Start button. Centrifuge to collect drops and foam off of cap down into tube.
 5. When centrifuge stops, open lid and remove tubes from rotor. Uncap tubes in order and add 40- μ L of Proteinase K (dissolved in Lysis Buffer Elution Buffer). Re-cap tubes and mix lysate by inversion. Do not vortex. Knock beads and filter down from cap into bottom of tube by tapping tubes on bench countertop.
 6. Insert tubes into styrofoam floating rack. Incubate tubes 30-min at 65°C in water bath. Set timer for 15-min. At end of 15-min remove rack from water bath and inverts several times to mix samples and tap beads and filter back down into tube. Re-place rack in 65°C waterbath for 15-min. for total of 30-min.
 7. Repeat steps 3 to 8 to process 16 more samples in parallel for loading MagNA Pure LC sample cartridge with 32 DNA extracts for downstream processing in the robotic platform.
 8. After 30-min in 65 °C waterbath remove tubes from water bath and place in MagNA Lyser Bead Beater for 15 seconds at 5,000 rpm. After 15 seconds of bead-beating, place in ice bath for 5-min to cool.
 9. Insert tubes in centrifuge rotor and spin 3-min at 12,000 rpm and 4 °C to pellet sediment and cell debris. When spinning is complete, open lid of centrifuge and rotor and mark side of outer side of cap where pellet should have formed.
 10. Carefully remove rotor from centrifuge and set on bench. Remove tubes one at a time from rotor and use 200- μ L pipettor and sterile aerosol-proof tips to transfer approximately 150 μ L lysate supernatant from tube to wells in MagNA Pure LC Sample Cartridge in pre-designated order.
 11. When all 16 sample supernatants transferred to sample cartridge put adhesive film over cartridge to prevent contamination and evaporation. Put sample cartridge in ice water bath or fridge to maintain 4 °C.
 12. Repeat steps 9 to 13 for second batch of 16 samples (lysates). Re-cover sample cartridge with adhesive film for storage. Centrifuge sample cartridge opposite a balance cartridge for 75-sec (1-min, 15-sec) at 2800 rpm in IEC centrifuge (or equivalent) with rotor adaptors for microtiter plates in place. Insert the film-covered sample cartridge in MagNA Pure LC platform.

13. Load the MagNa Pure LC platform with volumes of extraction kit reagents prescribed by MagNA Pure LC computer software for the number of samples being extracted. Before closing the platform' lid and starting the extraction process add 1.34 μ L of 9.3 μ g/mL Salmon DNA Stock (10 μ g/mL nominal concentration) per 1mL Lysis Binding Buffer (blue soapy solution) as the Sample Processing Control (SPC). If the amount of Salmon DNA stock to be added is less than 10- μ L, dilute the Salmon DNA stock so that a volume \geq 10- μ L can be pipetted into the Lysis Binding Buffer. Rinse pipette tip up and down three times in Lysis Binding Buffer.
14. Remove film from top of sample cartridge and re-insert in Roche MagNA Pure LC platform set up with DNA Purification Kit III (Fungi; Bacteria) reagents in tubs, tips, tip holders, and processing / elution cartridges. Close platform lid and after checking off checklist of loaded items (e.g. reagents, tips) lock the lid and start the automated DNA III Extraction Protocol which purifies each sample's DNA and elutes it into 100- μ L Elution Buffer.
15. When extraction process is complete, unlock the MagNA Pure LC platform lid and remove the sample eluate cartridge. Cover the cartridge with adhesive film and store at 4 C until qPCR analysis. Store cartridge at \leq -20 $^{\circ}$ C for long term preservation.
16. Prepare Elution Buffer Control from 9.3 μ g/mL Salmon DNA Stock by diluting a small volume to 37.2pg/1000 μ L (1-mL). This control sample is only analyzed by the Sketa qPCR assay. The Ct value obtained represents that value expected in Sketa qPCR assays of each MagNA Pure LC purified sample if 100% of the Salmon DNA was recovered and detected. Vortex to mix on low speed briefly prior qPCR analysis. Centrifuge for 1.5-min to coalesce droplets. Remove film to aliquot sub-samples and re-place with new film cover to restore at cool temperatures.

6.0 FISH VOUCHER SPECIMENS

This chapter describes the procedures for taxonomic identification of fish voucher specimens collected from river and streams as part of the EPA's National Rivers and Streams Assessment. Two types of voucher samples are collected and sent to a laboratory.

The first type is the *QC voucher sample* prepared by the field crew at sites identified by EPA. The *QC voucher sample* includes specimens (preserved or digital images) of **all** types of fish, identified to the lowest practicable taxon level at the site by the field taxonomist. QC voucher samples are sent to the QC laboratory to assess the accuracy of the field identifications.

The second type is the *Unknown (UNK)* voucher sample that includes physical specimens or digital images for fish that the field fish taxonomist could not positively identify during the site visit. Field crews also label as "UNK" specimens for fish collected outside their usual or known range. In most cases, the field fish taxonomist will retain the UNK samples for further study and identification. However, in some instances, the field crew will opt to send the UNK voucher sample to the QC laboratory for identification.

This chapter describes the procedures and data reporting for the QC laboratory. The Field Operations Manual describes the procedures for the field fish taxonomist. In the following discussion, the first three sections summarize the procedure; health and safety concerns; and required resources. **Section 6.4** describes the sample receipt and login procedures. **Section 6.5** describes the identification steps. **Section 6.6** describes laboratory assistance visits. **Section 6.7** identifies the sample and record retention requirements. **Section 6.9** provides a summary of the quality control requirements. The final section provides references to aid in identifications.

6.1 Summary of Procedures

Each field crew includes a field fish taxonomist approved by EPA based upon a review of their qualifications and experience. At each site with sufficient fish, field crews collect a minimum of 500 fish of length greater than 25 millimeters (mm). The fish taxonomist on the field crew ("field fish taxonomist") quickly identifies and enumerates the fish, and then returns them to the water m.

In accordance with quality control requirements, EPA selected sites ("QC sites") for independent, taxonomic confirmation. At each QC site, the field fish taxonomist prepares a QC voucher sample that includes specimens, preserved or digital images, for each taxon of fish collected at the site. The field fish taxonomist identifies each fish taxon to the lowest practicable level. Instead of specimens, digital images are required for listed or trophy game specimens that must be released alive, specimens that are too large to preserve, or very common and easily identified fish types. EPA uses the QC taxonomists' identifications of the QC voucher samples as a "gold standard" in determining the accuracy of the field identifications.

In addition to providing vouchers for QC evaluations, the field fish taxonomist may opt to send the QC taxonomist any specimen, or its image, that cannot be identified in the field ("UNK voucher sample"). The QC taxonomist uses the same identification procedures, regardless of whether the specimens are from QC or UNK voucher samples.

6.2 Health and Safety Warnings

In addition to the laboratory's usual requirements, laboratories must adhere to the following health and safety procedures:

1. Laboratory facilities must properly store and dispose of solutions of 10% formalin and/or 75-95% ethanol.

2. Laboratory personnel must wear proper personal protection clothing and equipment (e.g. lab coat, protective eyewear, gloves).
3. When working with potential hazardous chemicals (e.g. formalin, ethanol), laboratory personnel must avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs, remove clothing immediately. Wash and rinse the affected skin areas thoroughly with large amounts of soap and water.

6.3 Definitions and Required Resources (Personnel, Taxonomy Laboratories, and Equipment)

This section provides definitions and required resources for using the procedure. **Section 6.3.1** defines the terms used throughout the procedure. **Section 6.3.2** describes the taxonomic expertise required for each QC taxonomist using the procedure. **Section 6.3.3** describes the roles and responsibilities of the personnel involved in the procedure. **Section 6.3.4** identifies the equipment necessary to apply the procedure in identifying fish voucher specimens in samples.

6.3.1 Definitions

The following terms are used throughout the procedure:

Bench Sheet: Optional form used by the QC taxonomist to record information about the voucher specimen. See **Figure 6.1** for an example.

Dissecting microscope: Microscope that is configured to allow low magnification of three-dimensional objects that are larger or thicker than the compound microscope can accommodate.

External QC Coordinator is an EPA staff person. For some activities, the External QC Coordinator may be supported by a contractor that is not involved with field sampling. The External QC Coordinator is responsible for arranging for laboratory assistance visits; comparing fish voucher identifications by the field and QC taxonomists; and assessing other quality control issues.

NARS: National Aquatic Resource Surveys. The National Rivers and Streams Assessment (NRSA) is part of the NARS program.

NARS Information Management System (NARS IM): The IM system established to support all surveys, including NRSA, in the NARS program. The NARS IM system is used to track the samples from field collection to the laboratory.

NRSA: National Rivers and Streams Assessment. The samples will be collected during the field stage of NRSA.

Photovoucher: Digital image of the fish specimen.

QC Site: site where a QC voucher specimen is prepared.

QC Voucher: Fish voucher sample collected at the QC site.

Reference Collection: A collection of voucher specimens and digital images that provide examples of each taxon identified from the NRSA samples sent to the laboratory. A reference collection is used to help with future identifications of unknown specimens, and to provide physical documentation of reported identifications.

Tagged lot: A group of voucher specimens believed to represent a single taxon based on field identifications. Tagged lots are maintained separately in a voucher sample, and are associated with a unique voucher tag number.

UNK voucher specimen: A voucher specimen of fish that could not be positively identified in the field.

Voucher collection: an assortment of preserved specimens maintained for validating taxonomic identifications. For each selected QC site, the field crew submits up to 20 examples of each taxon of fish identified at the site. If the fish is endangered, rare, or too large to preserve, the field crew will provide digital images instead of physical specimens.

Voucher sample: a collection of voucher specimens from a single site.

Voucher Specimens: Preserved specimens or digital images of one or more individual fish used to identify the taxa collected at a site.

Voucher Specimen Tag: A pre-numbered paper tag included in the mesh bag with each fish voucher specimen (or included with each digital image). Tags are used to distinguish between tagged lots in a sample.

6.3.2 General Requirements for Taxonomists and Taxonomy Laboratories

The procedures may be used by any fish taxonomist and associated taxonomy laboratory that has expertise in each of the following areas:

Taxonomic expertise. To demonstrate its expertise, the organization with the QC taxonomists shall provide EPA with one or more of the following:

1. Memorandum that identifies the relevant services that the organization or its taxonomists provided for a large national survey in the past five years.
2. A vitae, resume, or equivalent documentation detailing the taxonomic expertise of the organization and its taxonomists, including coursework, professional certifications, membership in professional societies, job experience specifically related to fish taxonomy (especially with taxa that are difficult to identify), and any experience with the curation of museum or other voucher collections.
3. Quality assurance and quality control requirements. To demonstrate its expertise in quality assurance and quality control procedures, the organization shall provide EPA with copies of the quality-related documents relevant to the procedure. Examples include Quality Management Plans (QMP), QAPPs, and applicable Standard Operating Procedures (SOPs).
4. To demonstrate its ongoing commitment, the person in charge of quality issues for the organization shall sign the NRSA QAPP Certification Page.

6.3.3 Personnel

QC Taxonomist: A **systematic ichthyologist** having considerable training and considerable experience in identifying fish taxa from large regions of the U.S., and experience with difficult groups of taxa. A QC taxonomist maintains contact with other taxonomists through professional societies and other interactions, and keeps up with the pertinent literature, since systematics and taxa identifications change over time. Taxa identifications by QC taxonomists represent the standard against which EPA evaluates identifications by field taxonomists. It is acceptable for the QC taxonomist to also serve as a field fish taxonomist, but not for the same sample. In other words, for any given sample, the field fish taxonomist and QC taxonomist must be two different persons.

6.3.4 Equipment/Materials

The procedures require the following equipment and information for taxonomic identifications.

- Fine-tipped forceps (watchmaker type, straight and curved)
- Dissecting microscope (3.5-12X magnification)
- Dividers and/or calipers for obtaining measurement and proportional measurement data from specimens
- Scalpel
- List of standard common and scientific names used in NRSA (Appendix D)
- Taxonomic keys or published descriptions in up-to-date, regional faunal works including online resources
- Keys or descriptions for the most recent, accepted taxonomic revisions of individual groups
- Descriptions in other taxonomic studies, especially original descriptions (preferably published).
- Voucher samples
 - Sample tracking form that accompanies each shipment of samples
 - Site location
 - Physical specimens or image file
 - Access to additional site information upon request to EPA
- Reporting data template file

6.4 Sample Receipt

Field crews ship the QC and UNK voucher specimens to a central facility (“batching laboratory”) or the State’s QC laboratory. Periodically, the batching laboratory ship voucher specimens to the QC laboratory.

Under U.S. regulations, samples preserved in formalin are classified as “Dangerous Goods” and must be shipped according to hazardous material shipping requirements and regulations. Personnel receiving the shipment must be certified to handle hazardous material.

Because EPA initiates tracking procedures designed to recover any missing shipment, start the following login steps within 24 clock hours of receiving a delivery.

1. Report receipt of QC voucher samples (including digital images) in the NARS IM sample tracking system (within 24 clock hours).
2. Inspect each QC voucher sample THE SAME DAY THEY ARE RECEIVED:
 - a. Verify that the sample IDs on the jar labels and the tag numbers in the shipment match those recorded on the:
 - i. Chain of custody forms when the batching laboratory sends the samples to the QC laboratory; or
 - ii. Sample tracking form if the field crew sends the shipment directly to the State QC laboratory.
 - b. Verify that all jars for each sample are included in the shipment (i.e., there may be cases where multiple jars are required to properly preserve specimens collected at a site).
 - c. Complete the Condition Code for each sample:
 - i. *OK*: Sample is in good condition
 - ii. *C*: Sample container was cracked
 - iii. *L*: Sample container is leaking

- iv. *ML*: Sample label is missing
 - v. *NP*: Sample is not or insufficiently preserved
 - vi. *S*: Sample shipping problem
 - vii. *Q*: Other quality concerns, not identified above
- d. A few samples may be preserved with ethanol or isopropyl alcohol rather than formalin. If the crew used either of these, replace the preservative with formalin.
3. Store preserved samples at room temperature until identification begins.
 4. Maintain the chain of custody or sample tracking forms with the samples.
 5. Transfer any photovoucher images included in the shipment to a suitable office computer for viewing.
 - a. Organize image files by site ID and then by file sequence.
 - b. The computer should have the graphics capability to view images in high-resolution, and regularly scheduled backup of files.
 6. If necessary to support the identifications, request site information (e.g., site coordinates, state, and river) for the samples from the NARS IM staff. You should receive a tabular file with this information.

Table 6.1 Fish voucher: required data elements – login

FIELD	FORMAT	DESCRIPTION
LAB ID	text	Name or abbreviation for QC laboratory
DATE RECEIVED	MMDDYY	Date sample was received by lab
SITE ID	text	NRSA site id as used on sample label
VISIT NUMBER	numeric	Sequential visits to site (1 or 2)
SAMPLE ID	numeric	Sample id as used on field sheet (and on sample label)
TAG NUMBER	numeric	Tag number as used on field sheet (and on sample label)
DATE COLLECTED	MMDDYY	Date sample was collected
CONDITION CODE	text	Condition codes describing the condition of the sample upon arrival at the laboratory.
		Flag Definition
		OK Sample is in good condition
		C Sample container is cracked
		L Sample or container is leaking
		ML Sample label is missing
		NP Sample is not or insufficiently preserved
		S Sample shipping problem (explain in QA_COMMENTS field)
Q Other quality concerns, not identified above		

6.5 QC Identification

The QC taxonomist identifies the common name at the lowest practical level, of the fish voucher specimens, or photovouchers, as described in the following steps. For each sample, the QC taxonomist will record the identifications on the bench sheet or in an electronic spreadsheet file. **Figure 6.1** provides an example of a bench sheet that may be used. Before submitting the results to EPA, the laboratory staff must provide the required information using EPA’s data template (required codes are provided in **Table 6.2**). The steps are described below:

1. Process the QC voucher sample by voucher tag.
2. For preserved specimens:
 - a. Remove a bag of specimens from the jar. Record the voucher specimen tag number on the bench sheet. Transfer the specimens from the bag to a dissecting tray. Arrange specimens by size and/or potential taxon (if you think the tagged lot includes more than one taxon).
 - b. View each specimen to identify necessary diagnostic characters using:
 - i. Well-lighted conditions; and
 - ii. A dissecting microscope, magnification 3.5X to 12X (larger fish may not require magnification).
 - c. Vary lighting (reflected or transmitted) and specimen conditions (wet or dry), as needed, to maximize the ability to observe different characters.
 - d. Dissect the specimen, if necessary, to observe internal characters. For example:
 - i. Remove pharyngeal teeth; or
 - ii. Incise abdomen to observe peritoneum or viscera.
3. For photovouchers:
 - a. For images of whole fish, examine size, color, and external characters. Use zoom feature to enlarge specific areas of the image if necessary.
 - b. Some specimens may have multiple images that include close-ups of specific morphological features (e.g., mouthparts, fins, etc.).
 - c. Each image of a whole fish should have some kind of measurement scale reference (e.g., ruler). Use dividers to estimate proportional measurements based on the scale represented in the image.
4. Unless thoroughly familiar with all regional species in the family (or genus), select appropriate taxonomic literature to use as references in identifying the specimens based upon the characters observed in the preceding steps. Appropriate references (see Walsh and Meador [1998] for examples) include:
 - a. Keys or descriptions in up-to-date, regional faunal works (including online resources), such as the references provided in Section 6.9 as examples;
 - b. Keys or descriptions in the most recent, accepted taxonomic revisions of individual groups;
 - c. Descriptions in other taxonomic studies, especially original descriptions.
 - d. Comparison with museum collections, if expert taxonomists consider the museum's identifications to be reliable.
 - e. If controversy exists for a particular taxon in the scientific literature, use the identification consistent with recent publications.
5. Use the written descriptions and illustrations in determining the taxon. For example, consider if the specimen has:
 - a. Diagnostic and other characters consistent with known characters for the taxon.
 - b. Size within known size ranges.
 - c. Site information data (e.g., geographic coordinates, drainage basin, stream or river name) indicate the collecting locality that falls within the known range of the taxon.

6. If identifications are problematic or difficult (e.g., specimens or images do not contain fully diagnostic characters), even after consulting the references in Step 4, consider the following aspects:
 - a. Mature males in breeding condition present colors and/or tuberculation that can be used to identify the taxon. Assume that their taxon identification applies to non-breeding male and female specimens with similar characters.
 - b. Mature specimens that have a more developed morphology can be used to identify the taxon. Assume that their taxon identification applies to less developed specimens with similar characters.
 - c. Location can be used as the basis of identification if the taxon is well-studied or allopatric (e.g., [speciation](#) that occurs when [populations](#) of the same species become isolated from each other such that they do not occur in any one place together.)
 - d. Hybrids are typically detected by presence of diagnostic characters of two closely related taxa.
 - e. Commonly-stocked hybrids, such as muskellunge x northern pike (tiger muskie), or striped bass x white bass (wiper), are diagnosed in many keys.
 - f. Backcrosses (i.e., progeny of crosses of hybrids and a parent species) may not be distinguishable from the parent species on the basis of visual and partially dissected characters. Because the evidence is consistent with non-hybrid characters, assume that the specimen is not a hybrid.
7. If you cannot confidently identify a specimen, consult with another taxonomist who specializes in the group. If necessary, provide them with the specimen(s) and associated information.
8. Record the final identification(s) on the bench sheet or data submission worksheet.
 - a. Use common or scientific names from the NRSA master taxa list (**APPENDIX D: OTHER PERTINENT ATTACHMENTS**).
 - b. For taxa that are not included on the master taxa list, provide the accepted name. Use the common name if available and well-recognized, scientific name if a common name is not available or if it is less likely to be confused with other taxa. Provide the taxonomic reference for the reported name in the *Citation* column of the submission worksheet e.g., Nelson et al. (2004), FishBase.org).
 - c. For hybrids that do not have accepted standard names (see 6.d.i above), record the common name of both taxa (e.g., *green sunfish x bluegill*, *cutthroat trout x rainbow trout*). Avoid using non-specific terms.
9. If a tagged lot from the field turns out to include multiple taxa (assuming multiple individuals were provided):
 - a. Record the name and count for one taxon on the current line of the bench sheet or submission worksheet
 - b. Record the revised name for the second taxon as a new line on the bench sheet or the submission worksheet.
 - i. Record the original existing specimen tag number on the next available blank line of the submission worksheet.
 - ii. Mark as *New Taxon*. Record the new name.

- c. Provide a comment for any taxa that could not definitively be identified using the list provided by EPA. Here are examples of situations that require comments:
 - i. Specimen cannot be identified to the lowest level provided in the master taxa list because:
 - 1) State of preservation prevented observation of diagnostic characters (e.g., loss of diagnostic characters prior to preservation).
 - 2) Inconsistencies among or between diagnostic characters and other aspects (e.g., other characters, size, range, habitat).
 - 3) Incomplete taxonomic or faunal knowledge for group in generally accepted references (keys).
 - 4) Other factors, explained in *Comments* column.
 - ii. Identification is tentative and based upon:
 - 1) Review of digital images (this qualifier is only necessary if the digital image did not provide enough information to make a definitive identification).
 - 2) Identification was based upon other specimens (e.g., breeding males) in the sample with similar characters.
 - 3) Location or habitat information provided from NARS IM staff.
10. Check and correct the final common (or scientific) names recorded on the bench sheet or submission worksheet for the following common problems.
 - a. Abbreviations
 - b. Extra information identifiers (e.g., sp., spp., , nr., cf., species A)
 - c. Extra characters (e.g., "?")
 - d. Extraneous words (e.g., "probably", "prob", "may be")
 - e. Common misspellings
 - f. Names not in the master list. Provide a taxonomic reference for these.
 - g. Incorrect citation or old name
11. Return the specimens to the bag along with the voucher tag. Place the bag back into the sample jar. See **Section 6.7** for retention requirements.
12. Select another bag and repeat Steps 1-10.
13. If using bench sheets, transfer the information to the data submission worksheet file that EPA has provided. The worksheet can be found at <https://nars.sharefile.com/> in the NRSA 2013-2014/Data Templates folder. **Table 6.2** identifies the contents and formats used by EPA's file.
14. Save the submission worksheet file using the following convention: Taxonomist name+affiliation-abbreviation+submission date (yyyymmdd).xlsx (e.g., JSmith_ABCfirm_20130615.xlsx).
15. Prepare a list of primary and secondary technical literature used in completing the identifications (Step 4). Provide complete citations in bibliographic format, including authors' names, date of publication, title of document, name of journal or publisher, volume and page numbers, or ISBN number, as appropriate. If using on-line resource, include a screen capture of the URL/data source. Keep the references on file, and provide to EPA if requested.

Table 6.2 Fish voucher: required data elements – data submission worksheet

FIELD	FORMAT	DESCRIPTION
LAB ID	text	Name or abbreviation for QC laboratory
DATE RECEIVED	text	Date sample was received by lab
SITE ID	text	NRSA site ID code as recorded on sample label or tracking form
VISIT NUMBER	numeric	Sequential visits to site (1 or 2)
SAMPLE ID	numeric	6-digit Sample ID number as recorded on sample jar or tracking form
TAG NUMBER	text	Voucher tag number (01, 02, etc.) included with tagged lot (bag in sample jar) or digital image
DATE COLLECTED	MMDDYY	Date sample was collected
TAXONOMIST NAME	text	Name of QC taxonomist
DATE IDENTIFIED	MMDDYY	Date first specimen identified in sample
NEW TAXON	Text (Y/blank)	Indicates a new taxon identified from multiple taxon presented in a single tagged lot in the sample. This may or may not represent a new taxon to the NRSA master taxa list.
COMMON OR SCIENTIFIC NAME	text	Unique common name from NRSA master taxa list (Appendix D). For taxa not included on the NRSA master taxa list, record the common or scientific name (whichever is more widely recognized and/or less ambiguous). Provide a citation for the reported name in the Citation column.
NUMBER OF INDIVIDUALS	numeric	The number of individuals of taxa noted
CONDITION CODE	text	Condition codes associated with the condition of the sample upon arrival at the laboratory.
		Flag Definition
		OK Sample is in good condition
		C Sample container is cracked
		L Sample or container is leaking
		ML Sample label is missing
		NP Sample is not or insufficiently preserved
		S Sample shipping problem (explain in QA_COMMENTS field)
		UN Unknown. Specimen has been sent to expert taxonomist for definitive identification.
		Q Other quality concerns, not identified above
DATA FLAG (if appropriate)	text	Data qualifier codes associated with specific identifications of voucher samples. These codes provide more information than those used when reporting receipt of samples. A QC taxonomist may use alternative or additional qualifiers if definitions are provided to the external QC Coordinator as part of the submitted data package (e.g., as a separate worksheet page of the data submission file).
		Flag Definition
		CF Tentative identification due to inconsistent characters; no comprehensive reference for genus; etc. (explain in COMMENTS field)
		DD Damaged Specimen
		IM Young-of year or too young/small to identify
		L Identification based upon location
		NP Not enough preservative used

		UN	Specimen/ or tagged lot could not be positively identified, even after consultations with other taxonomic specialists
		Q	Other quality concerns, not identified above
COMMENTS	text	Explanation for data flag(s) (if needed) or other comments regarding the taxon lot.	
CITATION	text	Citation for reference used to identify organism, if taxon not present in NRSA list of fish names (APPENDIX D: OTHER PERTINENT ATTACHMENTS)	

6.6 Assistance Visits

The External QC Officer may arrange for an assistance visit to one or more QC taxonomists. Assistance visits are intended to familiarize EPA with actual procedures being implemented by different QC taxonomists; and to ensure a clear and consistent understanding of procedures and activities by both EPA and QC taxonomists. If EPA decides to conduct an assistance visit, a qualified EPA scientist or contractor will administer a checklist based upon the steps described in this chapter. A checklist will be developed and attached to the LOM before conducting an assistance visit.

6.7 Sample and Record Retention

The laboratory shall retain:

1. The sample materials for a minimum of 3 years from the date the EPA publishes the final report. During this time, the laboratory shall store the materials in a cool location away from sunlight. The laboratory shall periodically check the sample materials for degradation and refill jars with preservative if necessary.
2. Original records, including laboratory notebooks and the reference library, for a minimum of 10 years from the date that EPA publishes the final report.

After the stated time periods, the laboratory shall follow its internal protocols for disposal.

6.8 Summary of QC Requirements for Fish Voucher Specimens

Percent taxonomic disagreement (PTD): measure of taxonomic precision comparing the number of agreements (positive comparisons, $comp_{pos}$) of the field taxonomist and QC taxonomist. In the following equation, N is the total number of specimens in the larger of the two counts. PTD should be $\leq 15\%$.

$$PTD = \left[1 - \frac{comp_{pos}}{N} \right] \times 100$$

Table 6.3 Fish voucher: measurement data quality objectives

Variable or Measurement	Precision	Accuracy	Completeness
Identification	85% ^a	85% ^a	99%

^a As measured by (100%-PTD)

Table 6.4 Fish Voucher: quality control – taxonomic identification

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
Use widely/commonly accepted taxonomic references	All identifications	All keys and references used must be on bibliography prepared by the field and QC taxonomists	For all field crew identifications, EPA will convert field crew's use of common names to taxonomic references
Independent identification by outside, expert, laboratory fish taxonomist ("QC taxonomist")	When field taxonomist cannot identify specimen	Identification by QC taxonomist (who must be a different individual than the field taxonomist)	Replace field crew's "unknown" identification with determination by QC taxonomist
External QC	Approximately 10% of all sites sampled by each field taxonomist	PTD ≤ 15%	If PTD > 15%, review data for possible explanations; otherwise, insert data qualifier for field crew identifications
Calculate average PTD for field taxonomist	Each sample submitted to the QC taxonomist	PTD ≤ 15%	If PTD > 15%, consult with NARS QA Officer for appropriate action.
Conduct assistance visit	EPA may choose to visit any laboratory	Visit conducted using checklist	Performance and any recommended improvements described in debrief with laboratory staff

Table 6.5 Fish voucher: data validation

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
Taxonomic "reasonable-ness" checks	All data sheets	Genera known to occur in given rivers/streams or geographic area	Data qualifiers on data that fail reasonableness check. No further corrective action steps.

6.9 References

Froese, R. and D. Pauly. Editors. 2011. FishBase. World Wide Web electronic publication. www.fishbase.org, version (10/2013).

Nelson, J. S., E. J. Crossman, H. Espinosa-Pérez, L. T. Findley, C. R. Gilbert, R. K. Lea, and J. D. Williams. 2004. Common and Scientific Names of Fishes from the United States Canada and Mexico. Sixth edition. Special Publication 29, American Fisheries Society, Bethesda, Maryland.

Stribling, J. B., K. L. Pavlik, S. M. Holdsworth, and E. W. Leppo. 2008. Data quality, performance, and uncertainty in taxonomic identification for biological assessments. *Journal of the North American Benthological Society* 27:906-919.

Walsh, S. J. and M. R. Meador. 1998. Guidelines for quality assurance and quality control of fish taxonomic data collected as part of the National Water-Quality Assessment Program. Water-Resource Investigations Report 98-4239, US Geological Survey, Raleigh, North Carolina.

Laboratory Information		Sample Information	
Labname		Sample ID	
QC Taxonomist		Site ID	
Project ID		Visit Number	
Internal Sample ID		Date Collected	

Taxonomist Name _____ Date 1st Specimen Identified in Sample: _____

Tag No.	New Taxon?	Common or Scientific Name	Condition Code	Data Flag	Comment Including Citation if Appropriate

Figure 6.1 Fish voucher: example QC fish taxonomy bench sheet (optional)

Additional Comments

7.0 FISH TISSUE FILLET (WHOLE FISH COLLECTION)

Laboratory Methods incorporated in OST Manuals.

8.0 FISH TISSUE PLUG

This procedure is applicable to the analysis of mercury in fish tissue plugs. The method is performance based. Laboratories may use any method that meets the requirements below to analyze the fish tissue samples (for example, EPA Method 1631). Example SOPs are provided in **APPENDIX F: EXAMPLE SOPs FOR MERCURY IN FISH TISSUE PLUG ANALYSES**.

Table 8.1 Fish tissue plug: measurement data quality objectives

Variable or Measurement	MDL	Quantitation Limit
Mercury	0.47 ng/g	5.0 ng/g

Table 8.2 Fish tissue plug: quality control

Activity	Evaluation/Acceptance Criteria	Corrective Action
Demonstrate competency for analyzing fish samples to meet the performance measures	Demonstration of past experience with fish tissue samples in applying the laboratory SOP in achieving the method detection limit	EPA will not approve any laboratory for NRSA sample processing if the laboratory cannot demonstrate competency. In other words, EPA will select another laboratory that can demonstrate competency for its NRSA samples.
Check condition of sample when it arrives.	Sample issues, such as punctures or rips in wrapping; missing label; temperature; adherence to holding time requirements; sufficient volume for test. All samples should arrive at the laboratory frozen.	Assign appropriate condition code identified in Appendix 3.
Store sample appropriately. While stored at the laboratory, the sample must be kept at a maximum temperature of -20° C.	Check the temperature of the freezer per laboratory's standard operating procedures.	Record temperature of sample upon arrival at the laboratory. If at any other time, samples are warmer than required, note temperature and duration in comment field.
Analyze sample within holding time	The test must be completed within the holding time (i.e., 1 year). If the original test fails, then the retest also must be conducted within the holding time.	Perform test, but note reason for performing test outside holding time. EPA expects that the laboratory will exercise every effort to perform tests before the holding time expires.
Maintain quality control specifications from selected method/SOP (that meets the measurement data quality objectives)	Data meet all QC specifications in the selected method/SOP.	If data do not meet all QC requirements, rerun sample or qualify data. If the lab believes the data are to be qualified without rerunning sample, the lab must consult with the EPA Survey QA Lead before proceeding.
Maintain the required MDL	Evaluate for each sample	If MDL could not be achieved, then provide dilution factor or QC code and explanation in the comment field.
Use consistent units for QC samples and field samples	Verify that all units are provided in wet weight units and consistently	If it is not possible to provide the results in the same units as most other analyses, then assign a QC code and describe the reason for different units in the comments field of the database.

Maintain completeness	Completeness objective is 95% for all parameters.	Contact the EPA Survey QA Lead immediately if issues affect laboratory's ability to meet completeness objective.
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9.0 PERIPHYTON

This procedure is applicable to the analysis of the diatom and soft algal components of algal samples collected for the National Rivers and Streams Assessment (NRSA) 2013-2014. NRSA field crews collect composite samples of the algae present on benthic and vegetative surfaces; preserve samples with formalin to obtain a pH of 7; and ship the samples to a central holding facility or directly to a state laboratory. Each 50mL composite sample contains both diatoms and soft algae from benthic and vegetative surfaces. This procedure describes the steps for identifying and enumerating periphyton organisms in the samples and has been adapted from *Protocols for the analysis of algal samples collected as part of the U.S. Geological Survey National Water-Quality Assessment program* (Charles et al., 2002).

In the following discussion, **Sections 9.1, 9.2, and 9.3** summarize the procedure; health and safety concerns; and required resources. **Section 9.4** provides the steps for acknowledging sample receipt. **Section 9.5** provides the steps for subsampling and preparing the vials for the diatom and soft algal analyses. **Section 9.6** provides the steps for the diatom cleaning and slide mounting. **Section 9.7** describes the steps for diatom and all algal analyses. **Sections 9.8 and 9.9** provide the steps for the data entry, and sample and record retention. **Section 9.10** describes EPA's external quality control evaluation. **Section 9.11** identifies references used in developing the procedure. The Attachment summarizes the quality control measures.

9.1 Summary of Procedure

The procedure describes the extraction and separate analysis of the two subsamples of: 1) diatoms and 2) composite algae consisting of soft algae and diatoms. **Figure 9.1** provides a graphical summary of the procedure.

As described in **Section 9.5**, laboratory personnel subsample the liquid portion by volume and separate the heavier material that is difficult to suspend by mass. This step removes (oxidizes) organic matter from the siliceous frustules of diatoms and other material such as the soft algae in the subsample. Removing the organic matter is necessary so that all details of diatom structures essential to taxonomic identification are clearly visible.

As described in **Section 9.6**, laboratory personnel prepare slides by cleaning and mounting materials for identification and enumeration of diatoms. The laboratory technician mounts the cleaned material between a microscope slide and coverslip using a mounting medium with a high refractive index. With slides prepared with the mounting medium, the features of diatom frustules or valves are clearly visible at high magnification. The distribution of specimens on the final mounted coverslips will represent the samples contained within the cleaned material vials.

Also as described in **Section 9.6**, taxonomists spend a maximum of 8 hours to perform the diatom subsample analysis to estimate the proportion of diatom taxa found in a count of 600 valves (i.e., one-half of an individual diatom cell). For each observed species in the diatom subsample analysis, the taxonomist must verify that the laboratory's NRSA library includes the average biovolume measurement and a photograph.

As described in **Section 9.7**, the taxonomists identify, count, document, and measure all algae. Often this protocol is referred to as the soft-algal count because of the emphasis on identification of non-diatom algae. For this reason, this document refers to "soft algae" instead of "all algae." The procedure is designed to provide data on algal densities and amount of algal biovolume. It is the same procedure used by NRSA in 2008-2009.

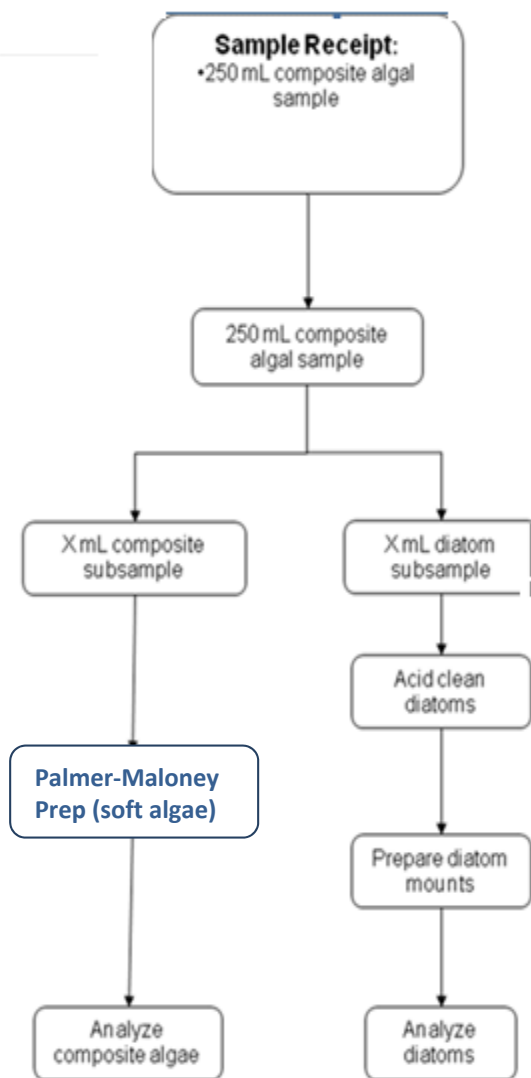


Figure 9.1 Periphyton: summary of procedure for identifying soft algae and diatoms

9.2 Health and Safety Warnings

In addition to the laboratory's usual requirements, personnel must implement the following health and safety procedures for this procedure:

1. Wear or apply proper personal protection clothing and equipment (e.g. lab coat, protective eyewear, gloves).
2. When working with potential hazardous chemicals (e.g. a mounting medium with a high refractive index such as Naphrax™) or biological agents (algae and sediments), avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs, remove clothing immediately, wash, and rinse the affected skin areas thoroughly with large amounts of soap and water.

9.3 Definitions and Required Resources (Laboratory, Personnel, and Equipment)

This section provides definitions and required resources for using this procedure. **Section 9.3.1** defines the terms used throughout the procedure. **Section 9.3.2** describes the expertise required for each laboratory using the procedure. **Section 9.3.3** describes the roles and responsibilities of the personnel involved in the procedure. **Section 9.3.4** identifies the equipment necessary to apply the procedure in preparing, sorting, and identifying algal samples.

9.3.1 Definitions

The procedure uses the following terms:

ANC: Academy of Natural Sciences

Biovolume: refers to the volume of algal cells.

Burn Mount: Rarely used. Refers to a procedure where a known portion of the untreated sample is dripped onto a coverslip and allowed to dry at room temperature and then placed on hot plate.

DCF: Dilution/Concentration Factor.

Digestion refers to the solubilization of organic material by strong acid oxidation.

Frustules are the siliceous, inorganic component (SiO₂) of diatom cells. Each diatom cell is composed of a frustule and organic cytoplasm. The frustule is composed of two valves. The taxonomist uses the features of the frustule to identify the taxon.

NARS: National Aquatic Resource Surveys. The National Rivers and Streams Assessment (NRSA) is part of the NARS program.

NARS Information Management (IM) System: The NARS IM system established to support all surveys, including NRSA, in the NARS program. The NARS IM system is used to track the samples from field collection to the laboratory.

Natural counting unit. Algae grow unicellularly or in multicellular filaments and colonies. Each natural grouping of algae (i.e., each individual filament, colony, or isolated cell) is defined as a natural counting unit. Diatoms are an exception; each diatom cell is always considered a natural counting unit, even if attached to other cells. The main purpose of using 'natural counting units' is to prevent a colonial or filamentous form from dominating a count. It also facilitates the counting of algal forms which have linked cells that may be hard to distinguish.

NRSA: National Rivers and Streams Assessment. The samples were collected during the field stage of NRSA.

Organic matter: Material that is capable of decay or the product of decay (e.g., leaves, sticks, algae).

Palmer-Maloney Fraction. A diluted or concentrated sample derived from the original sub-sample and used for the Palmer-Maloney count described in **Section 9.7**.

Reverse Osmosis (RO) is a membrane-technology filtration method that removes many types of large molecules and ions from solutions by applying pressure to the solution when it is on one side of a selective membrane.

Percent Difference (PctDiff) is a measure for soft algae that compares the enumerations for the taxa within a sample, as reported by the primary taxonomist (*a*) and secondary taxonomist (*b*) as follows:

$$PctDiff = \left[1 - \left(\frac{\sum_{i=1}^{total \# \text{ species}} \min(a_i, b_i)}{\sum_{i=1}^{total \# \text{ species}} \max(a_i, b_i)} \right) \right] \times 100$$

Percent disagreement in enumeration (PDE) is a measure of taxonomic precision comparing the number of organisms, n_1 , counted in a sample by the primary taxonomist with the number of organisms, n_2 , counted by the secondary taxonomist.

$$PDE = \frac{|n_1 - n_2|}{n_1 + n_2} \times 100$$

Percent taxonomic disagreement (PTD) is a measure of taxonomic precision comparing the number of agreements (positive comparisons, $comp_{pos}$) of the primary taxonomist and secondary taxonomists. In the following equation, N is the total number of organisms in the larger of the two counts.

$$PTD = \left[1 - \frac{comp_{pos}}{N} \right] \times 100$$

Primary laboratory: The laboratory that first receives the sample for analysis. The primary laboratory is responsible for: 1) preparing the subsamples and diatom slides; and 2) providing the original identification of diatoms and soft algae.

Secondary laboratory: The laboratory that performs a second, independent, quasi “gold standard” evaluation of a sample, as part of the external quality control evaluation.

Subsample: Portion of the sample obtained by randomly extracting volume from the composite sample collected by the field crew.

Target taxonomic level: species

Taxonomic nomenclature: All laboratories must identify taxon using the information provided in the BioData. The algae list is provided at the following website:

<https://aquatic.biodata.usgs.gov/domainDownloads.action>.

9.3.2 Laboratory

To participate in NRSA, each laboratory first must demonstrate to EPA that it has appropriate qualifications as follows:

1. Analytical work. To demonstrate its expertise, the laboratory shall provide EPA with one or more of the following:
2. Memorandum that identifies the relevant services that the laboratory provided for the National Aquatic Resource Surveys in the past five years.
3. Memorandum that describes the laboratory’s participation in relevant round robin studies and/or performance studies.
4. Report of relevant findings from an on-site technical assessment or audit.
5. Quality procedures.
6. To demonstrate its expertise in quality assurance and quality control procedures, the laboratory shall provide EPA with copies of the quality-related documents relevant to the procedure. Examples include Quality Management Plans (QMP), Quality Assurance Project Plans (QAPPs), and applicable Standard Operating Procedures (SOPs).

7. To demonstrate its ongoing commitment, the person in charge of quality issues for the laboratory shall sign the NRSA QAPP Certification Page.
8. Reporting standardized data.
9. To demonstrate its expertise, the laboratory shall provide EPA with a memorandum that confirms that the laboratory has a computerized Laboratory Information Management System (LIMS) that is routinely used to track samples and record laboratory results.
10. To demonstrate its ongoing commitment to use LIMS, the memorandum also shall confirm that the laboratory will use LIMS to record and report results from the procedure.

9.3.3 Lab Personnel

Any person who has received training in processing and identification of algae may use the procedure; however, the work is generally parsed out to several personnel:

Quality Control (QC) Officers provide oversight of daily operations, sample processing, monitors QC activities to determine conformance, and conducts performance and systems audits of the procedures. The procedure has separate responsibilities for the:

- **Internal QC Taxonomist** is a senior taxonomist at the laboratory who has at least 10 years of experience in analyzing algal samples.
- **External QC Coordinator** is an EPA staff person who manages the QC contractor. Because the assigned duties are primarily administrative in nature, the External QC Coordinator is not required to have laboratory experience, although such experience would be preferable.

Taxonomists are trained, and have considerable experience, in identifying algae, i.e., taxonomy. It is also important that the taxonomist maintains contact with other taxonomists through professional societies and other interactions, and keeps up with the pertinent literature, since systematics and species identifications change over time. Each laboratory must submit the resume or curriculum vitae for the taxonomists who analyze NRSA samples.

Algal Analysts are taxonomists who specialize in the identification of algal specimens.

Diatom Analysts, or diatomists, are taxonomists who specialize in the identification of diatoms.

Lab Technicians are technicians who have basic training in laboratory procedures.

9.3.4 Equipment/Materials

The procedure requires the following equipment and materials for sample preparation (subsampling) and taxonomic identifications:

9.3.4.1 *Subsampling Equipment/Materials (Section 9.5)*

- Distilled (DW) or reverse osmosis (RO) water
- Dispenser bottle for DW or RO water
- Beakers
- Beaker holding box (24 slots)
- Graduated cylinders
- Vials with 1 mL intervals marked on it for measuring volumes
- Positive-draw fume hood

- Screen cloth (210-um mesh)
- Screening apparatus
- Large plastic disposable weighing boats
- Analytical balance, capacity to 500 g, 0.2 g accuracy
- Spatulas
- Plastic disposable pipettes (tips of plastic pipettes can be cut)
- Small turkey baster

9.3.4.2 *Diatoms*

9.3.4.2.1 *Cleaning Equipment/Materials (Section 9.6 Diatom Cleaning and Mounting)*

See equipment and materials identified in ANS Protocol P-13-42 "Diatom Cleaning by Nitric Acid Digestion with a Microwave Apparatus" (<http://diatom.ansp.org/nawqa/pdfs/P-13-42.pdf>)

9.3.4.2.2 *Preparation of Diatom Slides (Section 9.6)*

- Corning ceramic-top hot plate with temperature control
- Positive-draw chemical hood
- Aluminum drying plate (25.5 x 20.0 x 0.5 cm, solid aluminum; lines forming 48 squares, each 3.2 cm on a side, are etched on the surface. Each square is etched with an identifying number)
- Glass microscope slides (1 x 3 inches; 2.5 x 7.5 cm)
- Glass coverslips (18 mm x 18 mm or 22 mm x 22 mm) - No. 1 thickness, stored in covered glass jar filled with 100 % ethanol.
- Mounting medium with a high refractive index (e.g., Zrax, Naphrax)
- Diamond scribe
- Disposable plastic pipettes
- Adjustable pipettor (0 - 250µl); adjustable pipettor (200 - 1000µl)
- Pipette tips for adjustable pipettors
- Round-style tooth picks
- Forceps
- Polished, rounded wooden splints
- Wash bottle filled with distilled (DW) or reverse osmosis (RO) water
- Single-edged razor blades
- Ethanol, 70%
- Acetone
- Tissues (e.g., Kimwipe®)
- Slide labels
- Wax (the kind commonly used for candle making and canning foods)
- 10% HCl

9.3.4.2.3 *Analysis of Diatoms (Section 9.7 Analysis of Diatoms and Soft Algae)*

- Compound microscope with transmitted light including:
 - Objective lenses of at minimum (10x, 40x, 100x)
 - Oil immersion objective (100x) with a minimum numerical aperture of 1.3

- Substage condenser lens with a minimum numerical aperture of 1.3
- Ocular lenses of 10-15x
- DIC (differential interference contrast) illumination
- Diamond objective marker mounted on the objective turret
- 100 watt halogen illumination, or equivalent
- Digital camera

9.3.4.3 *Soft algae (Section 9.7)*

- Compound microscope with 20x objective for a total system magnification of 200x and 40-45x objectives for a total system magnification of 400-450x.
- Glass microscope coverslips, rectangular, 26 x 60 mm, #1 thickness.
- Glass, wide-bore pipettes > 1 mm inside diameter, or eye-dropper.
- Two Palmer-Maloney Counting Cells with ceramic chamber (chamber depth of 0.4 mm; volume of 0.1 mL) or metal chambers
- Glass Pasteur pipettes, 5.25 inch, ≤ 1 mm diameter.
- Glycerin
- Digital camera

9.4 Sample Receipt

Under U.S. regulations, shippers must classify samples preserved in formalin as “Dangerous Goods” and handle according to hazardous material shipping requirements. At the laboratory, only personnel certified to handle hazardous material can receive the hazardous samples. Because EPA initiates tracking procedures designed to recover any missing shipment, the laboratory personnel start the following login steps within 24 clock hours of receiving a delivery.

1. Record receipt of samples in the NARS IM system (within 24 clock hours) and the Laboratory’s Information Management System (LIMS). Assign the appropriate chronological bench number to each sample.
2. Inspect each jar THE SAME DAY THEY ARE RECEIVED:
 - a. Verify that the site identification and sample number on the label also appear on the chain of custody form in the shipment.
 - b. Notify the NARS IM team if any jars were broken and/or if there are discrepancies between the custody form and the 50 mL bottles.
 - c. Verify that the bottles contain enough formalin. If not, then:
 - i. Add 2 mL of a 10% formalin solution
 - ii. Continue to add, if necessary, increments of 2 mL of the solution. Check after each addition to determine if additional solution is required.
 - iii. Record the amount of solution added to the bottle in the Comments_Arrival field of the Login spreadsheet (**Table 9.1**).
3. Maintain the chain-of-custody form with the samples; it will be needed if the samples are transported to any other location (e.g., for taxonomic identification, external QC evaluation).
4. Verify that the login information includes the required data elements in **Table 9.1**. After completing all required elements, provide the information to the data entry personnel.

Table 9.1 Periphyton: required data elements – login

FIELD	FORMAT	DESCRIPTION
LAB_ID	text	Name or abbreviation for QC laboratory
DATE_RECEIVED	MMDDYY	Date sample was received by lab
SITE_ID	text	NRSA site id as used on sample label
VISIT_NUMBER	numeric	Sequential visits to site (1 or 2)
SAMPLE_ID	numeric	Sample id as used on field sheet (on sample label)
DATE_COLLECTED	MMDDYY	Date sample was collected
CONDITION_CODE	text	Condition codes describing the condition of the sample upon arrival at the laboratory.
		CODE Definition
		OK Sample is in good condition
		C Sample container is cracked
		HM Sample contains heavy amounts of sand, silt, or other heavy material that may interfere with the algal analysis
		L Sample or container is leaking
		TL Too little sample left for the procedure
		ML Sample label is missing
		PA Preservative added, add amount in comments field
Q Other quality concerns, not identified above		
FORMALIN_ADDED	numeric	If the laboratory added formalin to the bottle, provide the amount in milliliters. If no formalin was added, then the value should be zero or blank.
COMMENTS_ARRIVAL	text	Any comments about the condition of the sample upon arrival.

9.5 Sample Preparation (Subsampling)

This section describes the steps to prepare subsamples from samples that have relatively small amounts of sand, silt, or other heavy material. EPA modified the following steps from ANS Protocol P-13-48 “Subsampling Procedures for USGS NAWQA Program Periphyton Samples” (<http://diatom.ansp.org/nawqa/pdfs/P-13-48.pdf>). If a sample contains large amounts of sand, silt, or other heavy material that the technicians determines could interfere with algal analysis, the technician should consult ANS P-13-48 for additional guidance (see Step 7.5).

In preparing the subsamples, the laboratory technician shall:

1. Abide by the following **Safety Precautions**:
 - a. Wear safety glasses, protective gloves and lab coats at all times when handling concentrated samples with formalin.
 - b. Use a positive-draw fume hood at all times to avoid exposure to formalin.
2. Keep the laboratory room and bench surfaces as clean as possible and free of debris to avoid cross-contamination of samples.
3. Verify that all necessary supplies and equipment are available. See **Section 9.3.4** for recommended supplies and equipment.
4. Examine the condition of the sample. If it leaked in transit, and thus, the bottle contains less than the required 50 mL:
 - a. If more than 20 mL remains:

- i. Reduce the volume sub-sampled for soft algal analysis as required. The minimum for any sub-sample, either for diatom slide preparation or soft algae analysis, is 10 mL.
 - ii. Advise the algae analyst of any reductions in sample volume on handover of the samples.
 - b. Otherwise, report the sample as 'lost' at login (**Section 9.4**).
5. Decide how much to subsample for soft algae and diatoms. This is somewhat of a judgment call, but, generally, for:
 - a. Diatoms: Subsample at least 20 mL.
 - b. Soft algae: Subsample at least 10 mL for soft algae. If the sample is very silty, it will be better to subsample a minimum of 10 mL for soft algae, whereas if the sample looks clear, at least 20 mL is recommended
 - c. Always save a small portion of the initial sample, even for soft algae, in case the taxonomist needs a "burn mount" as described in **Section 9.6**.
 - d. Record the volumes in the vials.
6. Select graduated cylinders of appropriate size for measuring subsamples. Verify that:
 - a. the desired subsample volume will be at least one-third the capacity of the graduated cylinder; and
 - b. the units of the graduated cylinder will allow estimation to the nearest milliliter.
7. Separate the sample into 2 subsamples as follows:
 - a. Suspend the algal material by shaking or swirling the sample
 - b. Obtain two subsamples by selecting a method that allows a representative subsample. Two possible methods include:
 - i. Carefully pour the determined amount of subsample into a graduated cylinder. Ensure the sample stays well mixed during subsampling.
 - ii. Use a wide-bore pipette or turkey baster, which allows pumping the pipette or baster to mix the sample, while withdrawing an aliquot of sample. Take multiple aliquots with the turkey baster to ensure a representative draw. Filaments and debris will block pipettes with small openings. Careful pouring or turkey basters may be more appropriate.
 1. Transfer each subsample to a vial. Label each of the two vials (i.e., one for diatoms and the other for soft algae) with a different Vial ID# using the sample number plus a "D" or "S." For each vial, record the Vial ID, subsample volume, and intended analysis (i.e., diatom or soft algae).
 2. If the laboratory intends to ship any vial to another location, pack it carefully for shipping and include the appropriate custody forms.

Verify that the subsampling information includes the required data elements in

Table 9.2. After completing all required elements, provide the information to the data entry personnel.

Table 9.2 Periphyton: required data elements – subsampling

FIELD	FORMAT	DESCRIPTION
LAB_ID	text	Name or abbreviation for QC laboratory
SITE_ID	text	NRSA site id as used on sample label
VISIT_NUMBER	numeric	sequential visits to site (1 or 2)
SAMPLE_ID	numeric	sample id as used on field sheet (on sample label)
VIAL_ID	numeric or text	Laboratory assigned ID to the vial holding the subsample
SAMPLE_VOLUME	numeric	Total volume of sample before any processing (from the sample label)
SUBSAMPLE_VOLUME	numeric	Volume of subsample in the vial
PURPOSE	D/S	Codes indicating whether the vial will be used for the diatom or soft algal analyses .
		CODE Definition
		D Diatom Analysis
		S Soft Algal Analysis
DESTINATION	text	Name of the receiving laboratory (i.e., if the vial will be shipped to another location)
COMMENTS_SUBSAMPLE	text	any comments about the subsampling

9.6 Diatom Cleaning and Mounting

The diatom cleaning steps remove both extracellular and intracellular organic material by digesting it with nitric acid. After removing the organic matter, all details of diatom structures essential to taxonomic identification should be clearly visible. To clean the diatoms, the laboratory technician may follow the cleaning steps in ANS Protocol P-13-42 “Diatom Cleaning by Nitric Acid Digestion with a Microwave Apparatus” (<http://diatom.ansp.org/nawqa/pdfs/P-13-42.pdf>) or the following nitric acid cleaning method:

1. Place the subsample of material for diatoms in a 250 mL beaker.
2. Place the beaker in a positive draw hood. Carefully add 50 mL of concentrated nitric acid.
3. Simmer on a hot plate or warmer for about 3 hours or until organic matter has dissolved. If small bits of coarser debris remain after acid-cleaning, they can be rinsed and removed at a later time with forceps.
4. Rinse the diatoms in the cleaned sample repeatedly to remove the nitric acid by repeatedly adding deionized water to the cleaned diatom sample to fill the beaker, letting the diatoms settle to the bottom, and then siphoning the water from the beaker before refilling again. This method is similar to instructions by ANSP, but draw from center of the water column, not the top. Also gently spin beakers and sprinkler water on the surface with a squirt bottle about midway through settling to dislodge diatoms adsorbed onto the water surface and sides of beakers (remember surface tension). We recommend using 1200 mL tall beakers.
5. Let samples in these beakers settle for a total of 8 hours or more.
6. After settling and no additional disturbance of the diatoms on the bottom of the beaker, slowly and carefully siphon water with a fine tip glass pipette, like a Pasteur pipette. Usually, 20 mL should be left above the settled diatoms in a 1200 mL tall beaker to minimize siphoning and discarding diatoms that were adsorbed to the beaker sides or bottom, or even the water surface. Be certain to avoid siphoning diatoms from the bottom. Watch for diatoms to move

from the water surface, sides or bottom of the beaker toward the pipette tip. Quickly remove the pipette from the water if clouds of suspended diatoms move toward the pipette tip. Rinse the pipette with a squirt bottle between samples.

7. Concentrate the cleaned diatom suspension to a volume appropriate for mounting on slides by settling into smaller and smaller containers.

After cleaning the materials, the laboratory technician produces high-quality diatom mounted slides from the cleaned diatom material. EPA modified the following cleaning steps in ANS Protocol P-13-49 “Preparation of Diatom Slides Using Naphrax™ Mounting Medium” (<http://diatom.ansp.org/nawqa/pdfs/P-13-49.pdf>).

To produce the slides, the laboratory technician shall:

1. Abide by the following **Safety Precautions**:
 - a. Wear safety glasses, protective gloves and lab coats at all times when handling liquid and/or solidified mounting medium. For example, Naphrax[®] is a hazardous substance because it contains toluene, which volatilizes readily when heated.
 - b. Use a positive-draw fume hood at all times for heating of the mounting medium.
 - c. Use extreme care when manipulating slides on the hot plate and when working close to the hot plate. Hot plate temperatures required for this procedure are high enough to cause severe burning of exposed skin.
2. Follow lab practices similar to those used for sterile experiments (bacteriological plating, etc.) to reduce the risk of cross contamination of samples. At a minimum:
 - a. Keep the laboratory room and bench surfaces as clean as possible and free of debris.
 - b. Use disposable pipettes, stirrers, etc. where feasible.
 - c. Rinse glass (non-disposable) pipettes, stirrers, etc. in DW or RO water at least three times after each sample. (Explanatory note: at times, tap water, because of algal blooms and use of diatomaceous earth filters, may contain diatoms.)
 - d. Dry all equipment before storing it. This step prevents growth of algae and fungi.
3. Verify that all necessary equipment is available. See **Section 9.3.4** for recommended supplies.
4. Determine if the vial’s contents will produce satisfactory slides. Consider the following:
 - a. The ideal density to be achieved on the final mount is somewhat subjective and is based on the amount of debris in the sample, the preferences of the slide analyst, and the way in which the slide is to be used (e.g., counting, documentation).
 - b. Generally, between 10 and 20 diatom valves or frustules should be present in a single high power microscope field (1000X).
 - c. The number of specimens per field will need to be reduced if samples contain considerable amounts of silt.
 - d. In many cases, analysts will request both a “heavy” slide (~40 cells/field at 400 – 450x magnification) and a “light” slide (~30 cells/field at 400 – 450x magnification).

b Naphrax™ is a commercially-available toluene-based mounting medium with high refractive index.

5. If the vial's contents will not produce satisfactory slides, because the number of diatoms is too sparse (this could be the result of too few diatoms in the sample and/or the sampled habitat), then:
 - a. Determine (i.e., judgement call) if concentrating the vial's contents 2-5 times will be sufficient to produce satisfactory slides. If yes, then increase the concentration of cleaned diatom material by two to five times as follows:
 - i. Allow the vial to settle for approximately 4 hours.
 - ii. Spin the vial carefully while still resting on the tabletop. This step dissociates diatoms that have adsorbed onto the side of the vial so they will settle.
 - iii. Allow the vial to settle for approximately 4 more hours (total of approximately 8 hours)
 - iv. Use a micropipettor to remove the required amount of water from the vial of material. Keep track of volume removed from the vial.
 - v. Record the SUBSAMPLE_DCF (i.e, subsample dilution/concentration factor).
 - b. If a concentration of cleaned material greater than two to five times is required, then re-subsample the original sample following the steps in **Section 9.5**. Use the entire remaining sample only if necessary. Digest the subsample as described previously (i.e., using ANS Protocol P-13-42).
 - c. If the concentration is still not sufficient, then combine two vials of cleaned subsample materials and concentrate as described in the previous two steps. Also revise the amount recorded for SUBSAMPLE_VOLUME.
 - d. If, after following the steps above to concentrate the cleaned material, the density of diatoms on a coverslip still does not meet the criteria of 30 to 40 cells per field at 400 - 450x magnification, proceed to make the densest slide possible and consult with a diatom analyst to determine whether it is practical to analyze the sample. This evaluation includes the following steps:
 - i. Scan the slide in its entirety under 100x magnification, and estimate the total number of individuals on the slide.
 - ii. Determine if the slide is countable (i.e., 100 specimens or more can be counted within 4 hours), considering factors such as:
 - 1) Density of diatoms.
 - 2) Evidence of dissolution
 - 3) Amount of debris (silt, clay, broken remains of diatoms and other siliceous organisms) that would make it difficult to identify specimens accurately.
 - iii. Determine if evidence suggests that lightly silicified diatoms in the sample may not have survived the digestion process in ANS Protocol P-13-42. If so, prepare a "burn mount" to view later to determine whether diatoms existed in the original sample and can be identified. (Burn mounts are seldom necessary.)
 - 1) If necessary, prepare a burn mount using the 1973 EPA procedure (USEPA 1973). It contains the following steps:
 - a) Drip a known portion of the untreated sample onto a coverslip.
 - b) Dry the coverslip at room temperature.

- c) When the sample is dry, place the coverslip onto a hot plate for about 30 min at approximately 570°C.
6. Deposit cleaned material on coverslip as follows:
 - a. Use forceps to remove single coverslips from the storage container.
 - b. Carefully clean each by wiping with a tissue (e.g., Kimwipe®c).
 - c. Place each coverslip on a marked space. (Be sure this space is clean and dry to avoid cross-contamination.)
 - d. Add enough diatom suspension to cover the entire surface of the coverslip, including the extreme edges. Usually, 1.0 mL is about right for a 22 x 22 mm coverglass. Add 10% HCl to the cleared slurry (diatom suspension) (2 drops per 20mL of material in vial) to achieve a more even distribution on the coverslip. Calculate and record the DRIP_DCF. Note:
 - i. If the intended drip count will be less than sufficient to cover the coverslip because the sample is dense (e.g., 1.0 mL of distilled water diatom suspension on a 22 x 22 mm coverslip or 600 µL of acidified suspension), then:
 - 1) Drip an amount of distilled water onto the coverslip with a disposable pipette that will be sufficient to form a thin layer of water over the entire coverslip when the diatom suspension is added.
 - 2) Agitate the sample vial to homogenize the diatom suspension.
 - 3) Using an adjustable pipettor, quickly withdraw the required amount from near the central portion of the sample.
 - 4) Eject this material smoothly and carefully into the layer of distilled water already on the coverslip.
 - 5) Repeat until achieving a homogeneous suspension on the coverslip.
 - 6) Record the subsample amount ejected.
 - ii. In the case where more than 1.0 mL of original sample is required, eject and mix the sample directly on the coverslip (i.e., the addition of distilled water is not necessary).
 - iii. If the coverslip overflows, discard the coverslip, wipe the drying area, and repeat the procedure with a freshly cleaned coverslip.
 - iv. Discard the pipette tip when finished with each vial.
 - e. Once the marked space is loaded with coverslip preparations, do not disturb until the coverslips are dry.
 - f. Dry the coverslips as follows:
 - i. Leave them undisturbed at room temperature for several hours;
OR
 - ii. Use gentle heat (warm to the touch only) to hasten evaporation using a:
 - 1) Crook-neck lamp with incandescent light bulb placed 15 - 30 cm over the coverslips;
 - 2) Slide maker that provides heat;

c Kimwipe® is a commercially-available product.

- 3) Or some other device that will gently dry the slides. Avoid any device that will rapidly evaporate the suspension. Rapid evaporation could produce strong patterns of diatoms settling on coverslips.
 - iii. Once completely dry, put the coverslips on the hot plate preheated to 250 to 300°F.
 - iv. Leave coverslips on hot plate for 3 - 5 minutes. Verify that:
 - 1) Heat has driven nearly all water from the material on the coverslips; and
 - 2) Diatom frustules adhere to the surface of the glass.
 - v. Remove the coverslips from the hotplate and inspect them. Consider:
 - 1) If the pattern of diatoms distributed on any coverslip is not even and smooth, discard and prepare a new coverslip.
 - 2) If coverslip distributions are still unsatisfactory after repeated attempts, consult an algal analyst.
7. Mount coverslip on slide in a **POSITIVE-DRAW FUME HOOD** using the following steps.
 - a. Use a rounded wooden splint or disposable pipette to transfer a small amount of the mounting medium (volume equivalent to two to four drops of water) to the central portion of the etched side of the microscope slide.
 - b. Remove the appropriate coverslip from the aluminum plate with forceps, being careful to handle the coverslip only at the extreme corners.
 - c. Invert the coverslip and place it gently on the portion of the slide covered with the mounting medium so the diatoms are in the medium between the coverslip and the microscope slide.
 - d. Place the slide (coverslip up) on the hotplate and apply gentle heat until the evolution of bubbles resulting from the evaporation of the toluene solvent first occurs, and then significantly diminishes. Heating the slide will allow the mounting medium to flow under the coverslip in a later step.
 - e. Remove the slide from the hot plate.
 - f. Gently position the coverslip using the rounded toothpicks.
 - g. Bring the edges of the coverslip parallel to the edges of the microscope slide.
 - h. Press the coverslip to form a uniform, thin layer of mounting medium beneath the entire coverslip that spreads to the edges of the coverslip. Take care at this stage to press gently to prevent breakage, warping of the coverslip, or dislodging diatoms. As this procedure is taking place, the mounting medium is “setting up” (becoming hard), and the ability to move the coverslip will diminish rapidly.
 - i. Set aside the mount to finish cooling.
1. Use a single-edge razor blade to carefully trim any excess mounting medium which has been squeezed out from beneath the coverslip. Take great care to avoid “lifting” the coverslip by inadvertently allowing the edge of the blade to move between the coverslip and the microscope slide.
2. After removing and discarding most of the excess mounting medium, and while still working under the hood, place the mount in successive baths of acetone, and then ethanol for no more than 10 or 15 seconds each.
3. Wipe the mount clean with a tissue (e.g., Kimwipe®).
4. Add a paper label to slides before analyzing the slides.

5. Mark the sample ID number on the side of the slide without a label with a diamond pencil.
6. Provide the slide to the Internal QC Taxonomist to verify that the slide has been properly made (i.e., does not have any obvious problems such as trapped bubbles). If the Internal QC Taxonomist identifies any problems with the slide, the laboratory discards the slide and the laboratory technician follows the above steps in preparing a replacement slide.
7. Preserve and store cleaned material.
8. Record the data elements described in **Table 9.3**. Comments should include information such as:
 - Observations about any interfering materials (sand, silt, etc.)
 - Rationale for using a sparsely populated slide.
 - Information on all burn mount attempts, successful or unsuccessful.

Table 9.3 Periphyton: required data elements – diatom cleaning & mounting

FIELD	FORMAT	DESCRIPTION
LAB_ID	text	Name or abbreviation for QC laboratory
SITE_ID	text	NRSA site id as used on sample label
VISIT_NUMBER	numeric	sequential visits to site (1 or 2)
SAMPLE_ID	numeric	sample id as used on field sheet (on sample label)
VIAL_ID	numeric or text	Laboratory assigned ID of the diatom vial used for the cleaning
SUBSAMPLE_DCF	numeric	Dilution or concentration factor. Record as 1 if sample was not diluted or concentrated.
SLIDE_ID	numeric or text	Laboratory assigned ID for the slide
SLIDE_DATE	date	Date that slide preparation was completed
TECHNICIAN	text	Name or initials of technician who prepared the slide
MOUNT_MEDIUM	text	Mounting medium used (e.g., Zrax, Naphrax)
INTERNAL_QC_TAXONOMIST	text	Name or initials of QC Taxonomist who reviewed the slide
SLIDE_CHECK	Y/N	Is slide acceptable for diatom analysis? If no, still record the slide number, but note in the comments that the slide has been destroyed.
EJECT_AMOUNT	numeric	Final amount of cleaned diatom material ejected on coverslip
EJECT_DCF	numeric	Dilution or concentration factor. Record as 1 if ejected amount was not diluted or concentrated separately from any dilution or concentration of the subsample.
DESTINATION	text	Name of the receiving laboratory (i.e., if the slide will be shipped to another location)
COMMENTS_DIATOM_PREP	text	any comments about the diatom cleaning and mounting

9.7 Analysis of Diatoms and Soft Algae

The taxonomist should complete the analyses of properly concentrated and prepared samples as described below within 8 hours or 6 transects (i.e., whichever limit is reached first) for each diatom slide, and within 4 hours for the soft algae subsample. However, EPA does not consider any research related to unfamiliar taxa to be part of the time limit. Within these time limits, the taxonomist should count 600 diatoms and 300 natural counting units of soft algae.

9.7.1 Taxonomic Nomenclature and Photographic Specifications

For both analyses of diatoms and soft algae for NRSA, EPA requires that all laboratories use the same sources for taxon names. EPA also requires photographs of each unique taxon. The following steps provide EPA's specifications:

1. Identify the species (i.e., the target level). Where it is not possible to identify the species, then identify the specimen to the lowest practical taxon level:
 - a. If the species/taxon is included in BioData (<https://aquatic.biodata.usgs.gov/domainDownloads.action>), then select the records corresponding to "Current" in *TaxonConceptState* and record:
 - i. *NADED_ID* for the taxon identification number
 - ii. *BiodataTaxonName* for the taxon name
 - iii. *BenchTaxonNameReferenceCode*, if provided in BioData. Otherwise, cite the reference used to identify the specimen. The reference only needs to be cited once for each taxon.
 - b. If the species/taxon is not included in BioData, then record:
 - i. *LAB_TAXON_ID*: an identifier assigned by the laboratory to provide a unique number for each taxon identified for NRSA
 - ii. *LAB_TAXON_NAME*: taxon name
 - iii. *LAB_REFERENCE*: reference used to identify the specimen. The laboratory only needs to cite a reference for the first identification of the taxon.
 - c. Photograph each species/taxon one or more times for the laboratory's NRSA library. The image must demonstrate the diagnostic feature(s) of the species/taxon so that they are distinguishable and clear to an algal taxonomist. The image should be:
 - i. Positioned vertically
 - ii. Tightly cropped
 - iii. Scaled with a 10 μm scale bar in the lower right corner of the image
 - iv. Saved with a tiff format
 - v. Have a filename of following elements in the order listed below:
 - NRSA2 (for the second NRSA conducted in 2013-2014)
 - Laboratory name (or abbreviation)
 - Sample number
 - Taxon name
 - NADED_ID (from BioData) or Laboratory's ID number for the taxon
 - Date (format YYYYMMDD) that the photograph was taken.
 - Last character is 'e' for an edited version of the photograph
 - For example, on September 8, 2013, laboratory ABC identified the specimen in sample 1234 to be a *Homoeothrix janthina*, for which the laboratory ID code is HJ0001, and took a digital photograph at a resolution of 40x, with a 10 μm scale bar present, and then cropped the photograph to eliminate extraneous material. The filenames of the original and edited photographs would be:

NRSA2_ABC_1234_homoeothrix_janthina_HJ0001_40x_20130908.tiff and
NRSA2_ABC_1234_homoeothrix_janthina_HJ0001_40x_20130908e.tiff.

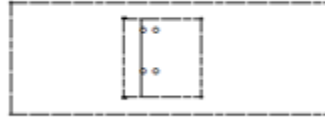
9.7.2 Analysis of Diatoms

This section describes the diatom subsample analysis used to estimate the proportion of diatom taxa found in a count of 600 valves (one-half of an individual diatom cell). The diatom analysis also includes biovolume measurements and imaging of all taxa encountered, including unknown taxon. In developing this section, EPA modified and expanded upon the steps on ANS Protocol P-13-39 "Analysis of Diatoms on Microscope Slides Prepared from USGS NAWQA Program Algae Samples" (<http://diatom.ansp.org/nawqa/pdfs/P-13-39.pdf>).

To analyze the diatoms, the taxonomist shall:

1. Verify that all necessary equipment is available. See **Section 9.3.4** for recommended supplies.
2. Verify that the slides can be used for diatom analysis:
 - a. Review comments from the subsampling, cleaning, and mounting steps for any deviations that may affect the outcome of diatom analysis.
 - b. Scan slides at low to medium magnification (100x to 450x) to confirm that diatoms are:
 - i. Evenly distributed on the coverslip.
 - ii. Present at a density appropriate for efficient counting. At high magnification (1000x), each field should have 5-10 diatoms.
 - 1) If diatoms on the slides are very sparse, refer to procedures in Section 9.6 for handling low-density samples.
 - c. If there are problems with dispersion or density that would compromise the quality and accuracy of the analysis, have new slides made. Always save any count data generated for a sample, even if the number of valves or frustules is low (e.g., <100).
3. Create the transect and count the diatoms as follows:
 - a. Secure the slide in the mechanical stage and use the microscope's diamond scribe to etch a horizontal or vertical line (depending on personal preference) on the coverslip to mark the edge of the first row to be counted. Clearly demarcate the areas of a slide scanned during a count. This is an important step because another taxonomist may need to recount the slide for QA/QC purposes.
 - i. If etching the slide before counting:
 - a) Etch at least three lines on the diatom slide.
 - b) Locate a starting point near one end of the etched line and make a circle with the scribe. This denotes the starting point of the count.
 - ii. If etching during the count:
 - a) Etch a line for each row counted. Rows are narrow rectangular areas (strips) of the slide adjacent to the scribed line, with width equal to the field of view. Start rows far enough from the coverslip edge to avoid optical distortion, and end them near the opposite coverslip edge where diatoms are no longer clearly visible (see diagram below).
 - b) Etch a circle around the last field counted in the first row and at the start and end of all other rows.

- iii. Verify that the etching is clearly visible so that another taxonomist can easily locate the circles and lines.



- b. Focus the first field under oil immersion.
- c. On a bench sheet and the upper right hand corner of the coverslip, record in mm the transect start coordinates from the microscope stage. Transects are narrow rectangular areas (strips) of the slide with width equal to the field of view. The transect width should be the maximum width common to all participating taxonomists (at least 90 microns).
- d. For each species/taxon, count diatom valves along the transect using the following rules:
 - i. One valve is:
 - a) A complete valve; or
 - b) A partial valve that is more than 50% of the valve. The valve must extend at least halfway into the transect (i.e., the center of the valve must be in the transect).
 - ii. Avoid counting valves in any disrupted areas of the mount, particularly edges that have optical aberrations.
 - iii. Identify and count 600 valves or as many as possible to complete the entire analysis within 8 hours or 6 transects (i.e., whichever limit is reached first).
- e. Record the level of magnification used to view the diatoms.
- f. Verify that photographs of the valves for the species/taxon exist in the NRSA library. If the valve is the first occurrence of the species/taxon or the valve is particularly notable, take a photograph following the specifications in **Section 9.7.1**.
- g. On a bench sheet, record the transect end coordinates from the microscope stage.
4. Calculate the total transect length and width (units are mm) evaluated for the sample. Record as TRANS_LENGTH and TRANS_WIDTH in the database.
5. For each species (or lowest practicable taxon), estimate the average biovolume for the species/taxon using information from one of the following sources:
 - a. Preferably, measurements reported in:
 - i. NRSA 2008-2009;
 - ii. Well-respected literature;
 - iii. Previous laboratory analyses for which the taxonomist is relatively confident provides a good estimate of the average biovolume for the taxon; or
 - iv. Databases with known sources.
 - b. When no other reasonable source is readily available, base the taxon's average biovolume on the measurements of the cells on the slide. Perform the following steps:
 - i. Determine the number of cells to measure:

- a) For each abundant taxon (i.e., occurring in more than 5% in any one sample), measure the biovolume of 10 or more cells.
 - b) For each common taxon (i.e., occurring 2-5% in any one sample), measure the biovolume for one or more cells.
 - c) For each rare taxon, use the biovolume measurements from literature descriptions of taxa or measure one or more cells.
- ii. Measure the biovolume (BV_i) of each cell i as follows:
- a) Select a simple geometric figure that matches the shape of the cell as best as possible, and measure its dimensions.
 - b) If a dimension of cannot be measured (e.g. depth), develop an estimate based on sizes of cells of the same taxon with similar known dimensions. To estimate depth, focus on tops and bottoms of cells.
 - c) Estimate the average biovolume by calculating the arithmetic average of the measured biovolumes of the cells on the slide:

$$Avg_{Biovolume} = \frac{(\sum_i BV_i)}{total\ number\ of\ cells\ measured}$$

6. Verify that the SUBSAMPLE_DCF value reflects all dilution and concentration of the subsample. Make any necessary corrections to the value.
7. Record the required data elements in described in **Section 9.7.4** (

8. **Table 9.4).**

9.7.3 Analysis of Soft Algae

This section describes the steps for identifying, counting, documenting, and measuring soft algae using the Palmer-Maloney counting cell method. The steps include re-processing a small portion of the subsample using final dilution/concentration factors. EPA adapted the steps from ANS Protocol P-13-50 “Preparation of USGS NAWQA Program Algal Samples for Analysis Using Palmer-Maloney Cells” (<http://diatom.acnatsci.org/nawqa/pdfs/P-13-50.pdf>) and ANS Protocol P-13-52 “Analysis of USGS NAWQA Program Phytoplankton Samples” (<http://diatom.anasp.org/nawqa/pdfs/P-13-52.pdf>).

In analyzing the soft algae, the taxonomist shall:

1. Follow lab practices similar to those used for sterile experiments (bacteriological plating, etc.) to reduce the risk of cross contamination of samples. At a minimum:
 - a. Keep the laboratory room and bench surfaces as clean as possible and free of debris.
 - b. Use disposable pipettes, stirrers, etc. where feasible.
 - c. Rinse glass (non-disposable) pipettes, stirrers, etc. in DW or RO water at least three times after each sample. (Explanatory note: at times, tap water, because of algal blooms and use of diatomaceous earth filters, may contain diatoms.)
 - d. Dry all equipment before storing it. This step prevents growth of algae and fungi.
2. Verify that all necessary equipment is available. See **Section 9.3.4** for recommended supplies.
3. Prior to taking a small portion of the subsample from the vial, verify that the sample is thoroughly mixed. If there are visible macro forms in the soft-algae subsample (i.e., visible filaments or colonies), break them up using scissors or small blender. This does not include maceration or grinding. The purpose of this step is to suspend algae so that fractions will have proportions of algal taxa equal to those in the original sample.
4. Concentrate the sample, if necessary. The original sample should usually be concentrated prior to adding to a Palmer-Maloney cell. The initial concentration should be approximately 5-10 times the original whole-water, leaving about 20 mL of concentrate for analysis. Concentrate the sample as follows:
 - a. Only concentrate/dilute a portion (10 mL or less) of the original subsample (i.e., from the vial).
 - b. Settle and decant (settle for at least 2 days) or use centrifugation (1000 g for 20 min)
 - c. Revise the subsample DCF using the original and final volumes, before and after concentration.
5. For samples with high amounts of sand and/or silt, dilute the sample if necessary, but do not dilute any sample to more than 20 % of its original volume. This step is valid ONLY for samples with large amounts of inorganic sand and silt. No settling should occur with samples that contain organic detritus or at any time during the dilution process.

- a. Determine if dilution is necessary by placing a portion of the subsample in the Palmer-Maloney Cell for assessment.
 - b. Dilute if necessary and recheck sample. If large inorganic particles are present (but NOT organic detritus) and blocking view of algal cells, again thoroughly mix the Palmer-Maloney fraction.
 - c. Allow the sample to stand while slowly counting to 5 or 6 (approximately 4-5 seconds).
 - d. Re-load the Palmer-Maloney cell with the subsample according to instructions below and count the sample or dilute again as necessary.
6. Use the concentrated/diluted fraction instead of the original in the following steps.
7. Calculate the subsample dilution/concentration factor (SUBSAMPLE_DCF) by dividing the final diluted or concentrated subsample volume by the initial volume. For example, a 10 mL subsample concentrated to 1 mL would have a subsample DCF=0.1; and a 10 mL subsample diluted to 100 mL would have a subsample DCF=10.0.
8. Prepare Palmer-Maloney counting cell as follows:
- a. Place a rectangular coverslip (#1 thickness, 22 x 50 mm) at 45° to the counting cell, covering about 1/3rd of the chamber, but not across the center of the cell.
 - b. Thoroughly mix the Palmer-Maloney fraction and draw into the micro-pipette 100 µl (plus suitable additional amount to allow for displacement from the chamber by the coverslip and prevent the occurrence of air bubbles).
 - c. Quickly add the 100 µL fraction, into the center of the chamber.
 - d. When the surface tension starts to draw the coverslip across the chamber, adjust the sides of the coverslip so that ends of the chamber are covered and the coverslip hangs over both sides of the ceramic portion of the counting cell.
 - e. Add glycerin to the area where the coverslip extends past the ceramic portion. This seals the coverslip to the counting cell temporarily (without excess heat or vibration, the counting cell can be used for a week or more).
9. Evaluate the Palmer-Maloney Fractions for suitability for the analysis:
- a. Assess 5 fields of view to determine if 15 – 30 natural counting units (both diatom and soft algae are visible per field of view.
 - b. Determine if it is appropriate to concentrate or dilute the subsample in the vial to create a new fraction. Such decisions are subjective and thus made on a case-by-case basis. Consider the following:
 - i. If the number exceeds 30 natural counting units, dilution may be appropriate.
 - ii. If the number is less than 15 natural counting units, concentration may be appropriate.
 - iii. In samples with high silt/detritus levels, even if there are < 15 natural units per field of view, consider dilution.

- iv. Avoid concentrating samples were the silt/detritus level would be raised to a level too high to complete a successful count. Include a comment in the database (see Table 9.4) if the silt/detritus levels prohibit concentration or lead to a dilution.
 - c. Once the Palmer-Maloney cell is prepared, allow chamber to stand for up to 10 minutes to allow contents to settle.
 - d. Revise the SUBSAMPLE_DCF if any step in this section required additional concentration or dilutions of the subsample. Because EPA will use the SUBSAMPLE_DCF in estimating whole sample values, it is extremely important that the recorded value for SUBSAMPLE_DCF reflects all of the dilution and concentration steps.
10. Use transects of varying length to enumerate 300 natural counting units.
 - a. Position the microscope stage so that the first field of view is in the top left hand area of the Palmer-Maloney cell at, but not overlapping, the cell edge.
 - b. Use transects, of varying length, with a width of 0.54 mm (or width of the field of view) scan up to, but not exceeding, a total transect length of 115.7 mm (or equivalent). Every other transect, moving down the Palmer-Maloney cell, is to be enumerated, i.e. each transect enumerated should be separated by a width of one field of view. This will prevent field of view overlap. For microscopes where the 40x field of view differs from 0.54 mm calculate the maximum transect length required. The full length of each transect should be scanned for algal enumeration. Record the start and end coordinates for each transect for the purpose of total transect length calculation on the Soft Algae Bench Sheet.
 - c. Identify and enumerate all soft algal forms within the field of view.
 - d. Record the level of magnification used to view the algal forms.
11. Enumerate 300 natural algal units and identify to species (or, if not possible, then the lowest practicable taxonomic level) as follows:
 - a. Using the pattern developed in the previous step, move the microscope stage to a new position in the pattern. Make all movements of the microscope stage without looking through the objectives.
 - b. Identify and enumerate all algal forms in the field of view: Enumerate algal forms using natural counting units. Natural counting units are defined as one for each colony, filament, diatom cell (regardless if colonial or filamentous) or unicell.
 - c. Differentiate diatoms as “living” or “dead” at type of collection. If there is any protoplast material in the frustules, consider the diatom to have been living when collected.
 - i. Identify all non-diatom taxa to species (or, if not possible, then the lowest practicable taxonomic level).
 - ii. Group all diatoms into one category – undifferentiated diatoms – instead of identifying their taxa.
 - d. Count the number of algal cells (living and dead) comprising each multicellular counting unit until reaching 300 natural algal units. Only “living” diatoms are part of the required 300 natural algal units.

- e. Record the number field or total transect length used for counting, the species identifications, and number of natural cells for each species.
 - f. For the undifferentiated diatoms, record their count and label the species “Dead diatoms” and leave all other taxonomic fields blank.
12. Enumerate larger, rarer taxa. There is an additional required procedure that can be used for samples with low concentrations (less than five natural counting units) of large cells or colonies (maximum dimension greater than 100 μm). Using a low-power objective (10-15x), scan 20 fields or 4 transects. Count the larger, rarer taxa. Enumerate as natural units and estimate the number of cells in each. Record the counts of each of the taxa, noting the scan area (i.e., total area for the 20 microscope fields or 4 transects). Multiply the number of larger, rarer taxa by the ratio of the total area scanned in the regular count to the area scanned in this count. Record that number as the total count for that taxon.
13. Calculate and record the estimated number of cells within a ‘typical’ natural unit. (This need only be a rough estimate.)
14. Verify that photographs exist in the NRSA library for the species/taxon. If this species/taxon is the first occurrence or particularly notable, take a photograph following the specifications in **Section 9.7.1**.
15. For each species (or lowest practicable taxon), estimate the average biovolume for the species/taxon using information from one of the following sources:
- a. Preferably, measurements reported in:
 - i. NRSA 2008-2009;
 - ii. Well-respected literature;
 - iii. Previous laboratory analyses for which the taxonomist is relatively confident provides a good estimate of the average biovolume for the taxon; or
 - iv. Databases with known sources.
16. When no other reasonable source is readily available, base the taxon’s average biovolume on the measurements of the cells. Perform the following steps:
- a. Determine the number of cells to measure:
 - i. For each abundant taxon (i.e., occurring in more than 5% in any one sample), measure the biovolume of 10 or more cells.
 - ii. For each common taxon (i.e., occurring 2-5% in any one sample), measure the biovolume for one or more cells.
 - iii. For each rare taxon, use the biovolume measurements from literature descriptions of taxa or measure one or more cells.
 - b. Measure the biovolume (BV_i) of each cell i as follows:
 - i. Select a simple geometric figure that matches the shape of the cell as best as possible, and measure its dimensions. When length within a filament cannot be determined, then use a standard length of 10 μm per cell. Note in the comments field (Table 9.4) when this standard length is applied.

- ii. If a dimension of cannot be measured (e.g. depth), develop an estimate based on sizes of cells of the same taxon with similar known dimensions. To estimate depth, focus on tops and bottoms of cells.
- iii. Estimate the average biovolume by calculating the arithmetic average of the measured biovolumes of the cells on the slide:

$$Avg_{Biovolume} = \frac{(\sum_i BV_i)}{total\ number\ of\ cells\ measured}$$

17. Verify that the SUBSAMPLE_DCF value reflects all dilution and concentration of the subsample. Make any necessary corrections to the value.

9.7.4 Internal Quality Control

For each sample, the laboratory must perform internal quality control evaluations. The Internal QC Taxonomist must randomly select 10% of the diatom slides and 10% of the soft algae subsamples for an independent count and identification. Calculate the PctDiff, PDE, and PTD using the equations in **Section 9.3**. If any samples do not meet the QA requirements listed in the Attachment, perform a third count and reidentification for the sample.

9.7.5 Required Data Elements for Diatom and Soft Algae Analyses

Table 9.3 identifies the required data elements for the analyses described in **Section 9.7.2** (diatom) and **Section 9.7.3** (soft algae).

Table 9.4 Periphyton: required data elements – analysis

FIELD	FORMAT	DESCRIPTION
LAB ID	text	Name or abbreviation for QC laboratory
SITE ID	text	NRSA site id as used on sample label
VISIT_NUMBER	numeric	sequential visits to site (1 or 2)
SAMPLE_ID	numeric	sample id as used on field sheet (on sample label)
SAMPLE_VOLUME	numeric	Original sample volume measured during subsampling step in Section 9.5. This value is used for both diatoms and soft algae analyses.
VIAL_ID	numeric or text	Laboratory assigned ID of the diatom vial
SUBSAMPLE_VOLUME	numeric	Volume of the subsample in the vial
SLIDE_ID	numeric or text	Laboratory assigned ID for the slide
TAXONOMIST	text	Name or initials of taxonomist or Internal QC Taxonomist analyzing the sample.
QC_CHECK	Y/N	Y if results were provided by the Internal QC Taxonomist for a sample selected for the 10% QC check.
ANALYSIS	DIA/SFT	Type of analysis: DIA=Diatoms; SFT=Soft Algae
DATE_ANALYSIS	date	Date when the analysis was completed
NADED_ID	numeric	BioData's identification number for the taxon
LAB_TAXON_ID	numeric or text	An identifier assigned by the laboratory to provide a unique number for each taxon identified for NRSA. If NADED_ID is available, this field is optional.
BiodataTaxonName	text	BioData's taxon name
LAB_TAXON_NAME	text	Taxon name. If BiodataTaxonName is available, this field is optional.
BenchTaxonNameReferenceCode	text	BioData's reference for the taxon. Identify the reference in <i>BenchTaxonNameReferenceCode</i> or <i>LAB_REFERENCE</i> the first time the lab identifies the taxon. Otherwise, this field is optional.
LAB_REFERENCE	text	Identify the reference in <i>BenchTaxonNameReferenceCode</i> or <i>LAB_REFERENCE</i> the first time the lab identifies the taxon. Otherwise, this field is optional.
MAGNIFICATION	numeric	Identify the magnification used to identify the taxon.
CLASS	text	taxonomic class
ORDER	text	taxonomic order
FAMILY	text	taxonomic family
GENUS	text	taxonomic genus
SPECIES	text	taxonomic species. For soft algae, label undifferentiated diatoms as "Dead diatoms" and leave other taxonomic fields blank.
TRANSECTS_COUNTED	numeric	Number of transects counted
TRANS_LENGTH	numeric	Length (mm) of transects counted
TRANS_WIDTH	numeric	Width (mm) of transects counted
CHAMBER_AREA	numeric	Chamber area in mm ²
CHAMBER_VOLUME	numeric	Volume of the chamber
COUNT	numeric	Number of valves (diatoms) or natural counting units (soft algal) identified for the taxon

COUNT_CENSORED	text	Code for a sample for which the taxonomist counted fewer than 600 valves (diatoms) or 300 natural counting units (soft algal)
		CODE Definition
		TL Time limit reached
		SS Sparse sample
		OT Other. Identify other reason in COMMENT_ANALYSIS
AVG_BIOVOLUME	numeric	Average biovolume for the species/taxon
BIOVOLUME_AVG_SOURCE	text	Source of the biovolume average value:
		CODE Definition
		NR8 From NRSA 2008-2009
		REF Cited in literature
		SS Sparse sample (i.e., fewer than 600 organisms in the sample)
		OT Other. Identify other reason in COMMENT_ANALYSIS
BIOVOLUME_REFERENCE	text	Identify the reference that provided the average biovolume value the first time the lab identifies the taxon. Otherwise, this field is optional. Leave blank if the laboratory estimated the biovolume from measuring the cells.
LAB_REFERENCE	text	The laboratory reference used to identify the taxon. Identify the reference in <i>BenchTaxonNameReferenceCode</i> or <i>LAB_REFERENCE</i> the first time the lab identifies the taxon. Otherwise, this field is optional.
PHOTOGRAPH	text	Filename of relevant photograph in the NRSA library. The filename is required the first time the lab identifies the taxon. Otherwise, this field is optional.
COMMENTS_ANALYSIS	text	any comments about the analysis
BV1	numeric	Biovolume of organism 1 (before measuring the biovolume, consider if the average biovolume is available from NRSA 2008-2009 or some other reliable source)
BV2	numeric	Biovolume of organism 2
BV3	numeric	Biovolume of organism 3
BV4	numeric	Biovolume of organism 4
BV5	numeric	Biovolume of organism 5
BV6	numeric	Biovolume of organism 6
BV7	numeric	Biovolume of organism 7
BV8	numeric	Biovolume of organism 8
BV9	numeric	Biovolume of organism 9
BV10	numeric	Biovolume of organism 10
BV11	numeric	Biovolume of organism 11
BV12	numeric	Biovolume of organism 12
BV13	numeric	Biovolume of organism 13
BV14	numeric	Biovolume of organism 14
BV15	numeric	Biovolume of organism 15

9.8 Data Entry

Table 9.1,

Table 9.2 and **Table 9.3** identify the required data elements that the laboratories must provide to EPA, in EPA's data template, available separately from EPA.

Table 9.4 provides a consolidated list of required data elements. In addition to the database, the laboratory must provide the resume or curriculum vitae for each taxonomist who performs the diatoms and algal analyses for the NRSA samples. The resume or cv for each taxonomist is submitted once to EPA’s External QC Coordinator.

Table 9.5 Periphyton: required data elements – all

RECORDED DURING:	FIELD	FORMAT	DESCRIPTION	
LOGIN	LAB_ID	text	Name or abbreviation for QC laboratory	
	DATE_RECEIVED	MMDDYY	date sample was received by lab	
	SITE_ID	text	NRSA site id as used on sample label	
	VISIT_NUMBER	numeric	sequential visits to site (1 or 2)	
	SAMPLE_ID	numeric	sample id as used on field sheet (on sample label)	
	DATE_COLLECTED	MMDDYY	date sample was collected	
	CONDITION_CODE	text	Condition codes describing the condition of the sample upon arrival at the laboratory.	
			CODE	Definition
			OK	Sample is in good condition
			C	Sample container is cracked
			HM	Sample contains heavy amounts of sand, silt, or other heavy material that may interfere with the algal analysis
			L	Sample or container is leaking
			TL	Too little sample left for the procedure
			ML	Sample label is missing
PA			Preservative added, add amount in comments field	
Q			Other quality concerns, not identified above	
FORMALIN_ADDED	numeric	If the laboratory added formalin to the bottle, provide the amount in milliliters. If no formalin was added, then the value should be zero or blank.		
COMMENTS_ARRIVAL	text	any comments about the condition of the sample upon arrival.		
SUB-SAMPLING	VIAL_ID	numeric or text	Laboratory assigned ID to the vial holding the subsample	
	SAMPLE_VOLUME	numeric	Total volume of sample before any processing (from the sample label)	
	SUBSAMPLE_VOLUME	numeric	Volume of subsample in the vial	
	PURPOSE	D/S	Codes indicating whether the vial will be used for the diatom or soft algal analyses	
			CODE	Definition
			D	Diatom Analysis
S	Soft Algal Analysis			

	DESTINATION	text	Name of the receiving laboratory (i.e., if the vial will be shipped to another location)
	COMMENTS_SUBSAMPLE	text	any comments about the subsampling
DIATOM CLEANING AND MOUNTING	SUBSAMPLE_DCF	numeric	Dilution or concentration factor. Record as 1 if sample was not diluted or concentrated.
	SLIDE_ID	numeric or text	Laboratory assigned ID for the slide
	SLIDE_DATE	date	Date that slide preparation was completed
	TECHNICIAN	text	Name or initials of technician who prepared the slide
	MOUNT_MEDIUM	text	Mounting medium used (e.g., Zrax, Naphrax)
	INTERNAL_QC_TAXONOMIST	text	Name or initials of QC Taxonomist who reviewed the slide
	SLIDE_CHECK	Y/N	Is slide acceptable for diatom analysis? If no, still record the slide number, but note in the comments that the slide has been destroyed.
	EJECT_AMOUNT	numeric	Final amount of cleaned diatom material ejected on coverslip
	EJECT_DCF	numeric	Dilution or concentration factor. Record as 1 if ejected amount was not diluted or concentrated separately from any dilution or concentration of the subsample.
	DESTINATION	text	Name of the receiving laboratory (i.e., if the slide will be shipped to another location)
	COMMENTS_DIATOM_PREP	text	any comments about the diatom cleaning and mounting
ANALYSIS	TAXONOMIST	text	Name or initials of taxonomist or Internal QC Taxonomist analyzing the sample.
	QC_CHECK	Y/N	Y if results were provided by the Internal QC Taxonomist for a sample selected for the 10% QC check.
	ANALYSIS	DIA/SFT	Type of analysis: DIA=Diatoms; SFT=Soft Algae
	DATE_ANALYSIS	date	Date when the analysis was completed
	NADED_ID	numeric	BioData's identification number for the taxon
	LAB_TAXON_ID	numeric or text	An identifier assigned by the laboratory to provide a unique number for each taxon identified for NRSA. If NADED_ID is available, this field is optional.
	BiodataTaxonName	text	BioData's taxon name
	LAB_TAXON_NAME	text	Taxon name. If BiodataTaxonName is available, this field is optional.

	BenchTaxonNameReferenceCode	text	BioData's reference for the taxon. Identify the reference in <i>BenchTaxonNameReferenceCode</i> or <i>LAB_REFERENCE</i> the first time the lab identifies the taxon. Otherwise, this field is optional.			
	LAB_REFERENCE	text	Identify the reference in <i>BenchTaxonNameReferenceCode</i> or <i>LAB_REFERENCE</i> the first time the lab identifies the taxon. Otherwise, this field is optional.			
	MAGNIFICATION	numeric	Identify the magnification used to identify the taxon.			
	CLASS	text	taxonomic class			
	ORDER	text	taxonomic order			
	FAMILY	text	taxonomic family			
	GENUS	text	taxonomic genus			
	SPECIES	text	taxonomic species. For soft algae, label undifferentiated diatoms as "Dead diatoms" and leave other taxonomic fields blank.			
	TRANSECTS_COUNTED	numeric	Number of transects counted			
	TRANS_LENGTH	numeric	Length (mm) of transects counted			
	TRANS_WIDTH	numeric	Width (mm) of transects counted			
	CHAMBER_AREA	numeric	Chamber area in mm ²			
	CHAMBER_VOLUME	numeric	Volume of the chamber			
	COUNT	numeric	Number of valves (diatoms) or natural counting units (soft algal) identified for the taxon			
	COUNT_CENSORED	text		Code for a sample for which the taxonomist counted fewer than 600 valves (diatoms) or 300 natural counting units (soft algal)		
				CODE Definition		
				TL Time limit reached		
				SS Sparse sample		
	EST_CELLS_IN_NAT_UNIT	numeric		Estimated number of cells in a 'typical' natural counting unit for the species/taxon (soft algae only)		
CODE Definition						
NR8 From NRSA 2008-2009						
REF Cited in literature						
AVG_BIOVOLUME	numeric		Average biovolume for the species/taxon			
			BIOVOLUME_AVG_SOURCE	text		Source of the biovolume average value:
						CODE Definition
						SS Sparse sample (i.e., fewer than 600 organisms in the sample)
						OT Other. Identify other reason in COMMENT_ANALYSIS
REF Cited in literature						

	BIOVOLUME_REFERENCE	text	Identify the reference that provided the average biovolume value the first time the lab identifies the taxon. Otherwise, this field is optional. Leave blank if the laboratory estimated the biovolume from measuring the cells.
	LAB_REFERENCE	text	The laboratory reference used to identify the taxon. Identify the reference in <i>BenchTaxonNameReferenceCode</i> or <i>LAB_REFERENCE</i> the first time the lab identifies the taxon. Otherwise, this field is optional.
	PHOTOGRAPH	text	Filename of relevant photograph in the NRSA library. The filename is required the first time the lab identifies the taxon. Otherwise, this field is optional.
	COMMENTS_ANALYSIS	text	any comments about the analysis
	BV1	numeric	Biovolume of organism 1 (before measuring the biovolume, consider if the average biovolume is available from NRSA 2008-2009 or some other reliable source)
	BV2	numeric	Biovolume of organism 2
	BV3	numeric	Biovolume of organism 3
	BV4	numeric	Biovolume of organism 4
	BV5	numeric	Biovolume of organism 5
	BV6	numeric	Biovolume of organism 6
	BV7	numeric	Biovolume of organism 7
	BV8	numeric	Biovolume of organism 8
	BV9	numeric	Biovolume of organism 9
	BV10	numeric	Biovolume of organism 10
	BV11	numeric	Biovolume of organism 11
	BV12	numeric	Biovolume of organism 12
	BV13	numeric	Biovolume of organism 13
	BV14	numeric	Biovolume of organism 14
	BV15	numeric	Biovolume of organism 15

9.9 Sample and Record Retention

After the taxonomist analyzes the vials for a sample, retain the vials for possible selection into the external quality control evaluation. When the external evaluation is complete, process the vials containing the remaining acid-cleaned material for long-term storage. The lab technician shall:

1. Work under a fume hood to add two to four drops of 100% buffered formalin to each vial.
2. Add glass beads to the vials to help preserve the diatoms.
3. Tightly cap the vials and seal them by immersing the top 1/3 of the vial in melted wax.
4. Transfer the vials to long-term storage.

The laboratory shall retain:

1. The sample materials, including vials and slides, for a minimum of 3 years from the date the EPA publishes the final report. During this time, the laboratory shall store the materials in a cool location away from sunlight. The laboratory shall periodically check the sample materials for degradation and refill jars and vials with formalin if necessary.
2. Original records, including laboratory notebooks and the reference library, for a minimum of 10 years from the date that EPA publishes the final report.

After the stated time periods, the laboratory shall follow its internal protocols for disposal.

9.10 External Taxonomic Quality Control Evaluation

EPA requires that all NRSA laboratories (“primary laboratories”) participate in the External Taxonomic Quality Control Evaluation. Each taxonomist must participate in the QC evaluation, even if the taxonomist is under subcontract with, or consulting for, another firm.

In contrast to the internal QC evaluation in the earlier sections that verify adherence to the procedures and ensures in-laboratory consistency between taxonomists, the purpose of the external QC evaluation is to ensure consistency between laboratories and taxonomists. To achieve this objective, EPA compares the primary laboratory results to those from a second laboratory, considered a quasi “gold standard” for taxonomic evaluations.

The External QC Coordinator, who is an EPA staff member, is responsible for selecting and managing the “QC contractor.” To eliminate the appearance of any inherent bias, the QC contractor must be dedicated to QA/QC functions, and thus, must not be a primary laboratory or a field sampling contractor for NRSA. The QC contractor is responsible for complying with instructions from the External QC Coordinator; obtaining and managing the secondary laboratory; coordinating and paying for shipments of the QC samples between locations; comparing sample identifications by different laboratories; facilitating reconciliation teleconferences; and preparing brief summary reports.

The External QC Coordinator will arrange for the QC contractor to conduct a minimum of two QC evaluations in 2014. To the extent practicable, the External QC Coordinator and QC contractor will schedule batch evaluations evenly throughout the project period.

Each QC evaluation consists of the following steps:

1. In consultation with the QC contractor, the External QC Coordinator determines an appropriate time to conduct the evaluation based upon the total number of samples assigned to the laboratory, the delivery schedule, processing schedule, and the following constraints:
 - a. Availability of samples from other laboratories. For example, if three state laboratories are each processing less than 30, the External QC Coordinator might combine their samples into one batch for the QC evaluation.
 - b. If a primary laboratory is responsible for processing 100 samples or more for the NRSA, the External QC Coordinator will split their samples into several batches (e.g., each 50 to 100 samples) so that EPA can evaluate and correct performance on an ongoing basis.
2. The External QC Coordinator provides the QC contractor with a list of laboratories and processed samples. Sample identification includes the site identification code, sample number, and taxonomist who performed the identifications.

3. The QC contractor randomly selects 10% of the samples from each NRSA laboratory, subject to the following constraints:
 - a. If the primary laboratory received fewer than 30 samples, then the QC contractor randomly selects three samples for the evaluation.
 - b. For each taxonomist identified on the list, the QC contractor ensures that one or more of his/her samples are selected.
4. The QC contractor provides a list of the QC samples, and instructions, to the External QC Coordinator and each primary laboratory participating in the evaluation. Although the External QC Coordinator and QC contractor may tailor the instructions for the participating taxonomists' preferences, the instructions are likely to specify the following:
 - a. Pack and ship the QC samples to the central holding facility designated by the QC contractor. Instructions are likely to require that the:
 - i. Shipments contain chain-of-custody documentation for all slides and containers.
 - ii. Containers (e.g., slides, vials) include the site identification code and sample number.
 - iii. Containers cannot be marked in any way that might identify the taxonomic classification for any organism.
 - b. Use the NARS IM database to track the QC samples (see **Section 9.4**). Because of the potential impact to the QC contractor operations, the External QC Coordinator must approve any requests to use another tracking method than NARS IM.
 - c. Email a spreadsheet with the data for the QC samples to the External QC Coordinator. (EPA requires that all labs use its spreadsheet template for recording the taxonomic data.)
5. The QC contractor reviews the condition of the QC samples (e.g., verifies that the containers do not identify taxon for any organism) and ships the samples to the secondary laboratory along with instructions and the EPA template for reporting data.
6. Within 24 hours of receipt, the secondary laboratory:
 - a. Uses the NARS IM database to log in the sample (see **Section 9.4**);
 - b. Faxes or emails any additional receipt records, including discrepancies, within 24 hours; and
 - c. Completes any other instructions from the QC contractor.
7. The secondary laboratory:
 - a. Re-identifies and re-counts following the procedures in the Method.
 - b. Verifies that the secondary laboratory's NRSA library includes photographs for the species/taxon. If this species/taxon is the first occurrence or particularly notable, the secondary taxonomist takes a photograph following the specifications in **Section 9.7.1**.
 - c. Records the required data elements in **Section 9.8**.
 - d. Enters the data using EPA's spreadsheet template for the taxonomic data.
 - e. Emails the completed spreadsheet to the QC contractor.

8. The QC contractor compares the original taxonomic results (i.e., data) generated by the primary laboratory to the taxonomic results generated by the secondary laboratory for each sample. As part of this evaluation, the QC contractor calculates PctDiff, PDE, and PTD using the equations in **Section 9.3** and compares their values to the QC requirements in the Attachment.
9. If any samples exceed the PctDiff, PDE, or PTD limits in the QAPP, the QC contractor consults with the External QC Coordinator to determine if reconciliation calls are necessary to resolve differences. The External QC Coordinator may decide that a reconciliation call is unnecessary if there appears to be an obvious explanation for differences, few samples are affected, or other reasons.
10. The QC contractor schedules and facilitates reconciliation teleconferences with EPA and the laboratories.
 - a. In preparation for the teleconferences:
 - i. The QC contractor provides the participants with a spreadsheet that includes:
 - ii. List of samples and taxon identifications for discussion;
 - iii. Relevant data from the primary and secondary laboratories; and
 - iv. PctDiff, PDE, and PTD values.
 - v. The primary and secondary laboratories provide participants with the relevant reference (or citation) and photograph for each taxonomic identification for the discussion.
 - vi. The QC contractor emails a meeting announcement for a convenient time for all participants. The email identifies instructions for accessing the External QC Coordinator's toll-free teleconference line.
 - b. Within a week after the teleconference, the QC contractor sends an email to the External QC Coordinator and other teleconference participants that summarizes:
 - i. Agreements to use common nomenclature for discrepancies;
 - ii. Commitments to reevaluate identifications by reexamining samples;
 - iii. Application of changes that are appropriate for all samples, not just the QC samples (e.g., common nomenclature)
 - iv. Items that will not be resolved for some reason (e.g., sample degraded during shipment).
11. After completing the reconciliation calls, the participants complete the following steps:
 - a. Secondary laboratory:
 - i. Reexamines samples as deemed necessary during the reconciliation call
 - ii. Updates its database with changes to:
 - 1) QC samples per reexamination and other items in the QC contractor email; and
 - 2) Non-QC samples as appropriate (e.g., nomenclature changes apply to all samples, not just QC samples).
 - iii. Provides database to QC contractor.
 - b. QC contractor confirms that the secondary laboratory (i.e., its subcontractor) completed its assignments before allowing the secondary laboratory to move to the next step.

- c. Secondary laboratory stores its original records, including laboratory notebooks and the reference library, for a minimum of 10 years from the date that EPA publishes the final report.
- d. Secondary laboratory and QC contractor follow steps 4 and 5 above to return the samples to the primary laboratory.
- e. After receiving the samples (and tracking per step 4), the primary laboratory:
 - i. Reexamines samples as deemed necessary during the reconciliation call;
 - ii. Updates its database with changes to:
 - 1) QC samples per reexamination and other items in the QC contractor email; and
 - 2) Non-QC samples as appropriate (e.g., nomenclature changes apply to all samples, not just QC samples)
 - iii. Provides the revised database to the External QC Coordinator (not the QC contractor). It also confirms that it has completed all relevant items identified in the QC contractor's email summary of the teleconferences (from Step 10.b).
- f. QC contractor provides EPA with a report or memorandum that:
 - i. Identifies the participating laboratories, with the following information about each laboratory:
 - 1) Laboratory name
 - 2) Address
 - 3) Contact person (name, telephone, and email)
 - ii. Quantifies the taxonomic precision (PctDiff, PDE, and PTD) as they were prior to the reconciliation call;
 - iii. Assesses data acceptability;
 - iv. Highlights taxonomic problem areas;
 - v. Identifies any discrepancies for which the External QC Coordinator determined that a reconciliation teleconference was not necessary;
 - vi. Identifies primary and secondary laboratory commitments to change its identifications or provide additional review of any organisms; and
 - vii. Provides recommendations for improving precision for other samples not included in the QC evaluation.

12. After review, the External QC Coordinator:

- a. Submits the report, and draft technical direction with next steps for the laboratory, to the EPA staff managing or coordinating with the primary laboratory.
- b. Determines if significant differences within the batch of QC samples warrant re-identification of samples by the primary laboratory and a second QC evaluation by the secondary laboratory. If deemed necessary, EPA will instruct the primary laboratory to include the samples for review with the next batch of QC samples.

As an additional verification on the generation of the data, EPA may conduct assistance visits at the laboratories. If EPA decides to conduct an assistance visit, a qualified EPA scientist or contractor will

administer a checklist based upon the steps described in this chapter. EPA will develop the checklist and append it to the LOM prior to conducting assistance visits. The objective of the visit would be to:

- Confirm the laboratory is properly implementing the steps in the method.
- Assist with questions from laboratory personnel.
- Suggest corrections if any errors are made.

9.11 References

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Attachment: Summary of QC Requirements

Percent disagreement in enumeration (PDE): measure of taxonomic precision for diatoms comparing the number of organisms, n_1 , counted in a sample by the primary taxonomist with the number of organisms, n_2 , counted by the secondary taxonomist.

$$PDE = \frac{|n_1 - n_2|}{n_1 + n_2} \times 100$$

Percent taxonomic disagreement (PTD): measure of taxonomic precision for diatoms comparing the number of agreements (positive comparisons, $comp_{pos}$) of the primary taxonomist and internal or external QC taxonomists. In the following equation, N is the total number of organisms in the larger of the two counts.

$$PTD = \left[1 - \frac{comp_{pos}}{N} \right] \times 100$$

Percent Difference (PctDiff): measure of difference for soft algae that compares the enumerations for the taxa within a sample, as reported by the primary taxonomist (a) and secondary laboratory (b), as follows:

$$PctDiff = \left[1 - \left(\frac{\sum_{i=1}^{i=total \# \ species} \min(a_i, b_i)}{\sum_{i=1}^{i=total \# \ species} \max(a_i, b_i)} \right) \right] \times 100$$

Table 9.6 Periphyton: measurement data quality objectives – diatoms

Variable or Measurement	Precision	Accuracy	Completeness
Enumeration	75% ^a	85% ^b	99%
Identification	75% ^a	85% ^b	99%

^a As measured by (100%-PTD); ^b As measured by (100%-PDE)

Table 9.7 Periphyton: measurement data quality objectives – soft bodied algae

Variable or Measurement	Precision	Accuracy	Completeness
Identification	50% ^a	50% ^b	99%

^a As measured by PctDiff

Table 9.8 Periphyton: quality control – all activities

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
Internal QC Taxonomist verifies that diatom slide is appropriate for diatom analysis	All samples	No obvious problems such as bubbles under the coverslip	Slide is discarded and replaced with a new slide
Duplicate identification by Internal QC Taxonomists	1 in 10 samples per taxonomist, with a minimum of 1 NRSA sample	PctDiff ≤ 50% (soft algae) PDE ≤ 15% (diatoms) PTD ≤ 25% (diatoms)	If any criterion is exceeded, perform a third count and reidentification for the sample.
Independent identification by	All uncertain taxa	Uncertain identifications to be confirmed by expert in particular taxa	Record both tentative and independent IDs

outside, expert, taxonomist			
External QC	10% of all samples completed per laboratory	PctDiff ≤ 50% (soft algae) PDE ≤ 15% (diatoms) PTD ≤ 25% (diatoms)	Attempt to reconcile results during a conference call with EPA and the two laboratories. Document unresolved disagreements for the data analyst to determine if lowest practical level (e.g., species) is appropriate, or if identification should be to the level that both labs found (e.g., class)
Use of widely/commonly accepted taxonomic references by all NRSA labs	For all identifications	All keys and references used by each lab must be on bibliography prepared by one or more additional NRSA labs or in BioData (see Section 9.7 for retrieval instructions). This requirement demonstrates the general acceptance of the references by the scientific community.	If a lab proposes to use other references, the lab must identify them in the database.
Prepare reference collection	Each new taxon per laboratory	Complete reference collection to be maintained by each individual laboratory	Internal Taxonomy QC Officer periodically reviews data and reference collection to ensure reference collection is complete and identifications are accurate

Table 9.9 Periphyton: data validation

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
Taxonomic "reasonableness" checks	All data	Taxa known to occur in given rivers or streams or geographic area	Second or third identification by expert in that taxon

10.0 PERIPHYTON META-GENOMICS (PILOT RESEARCH EFFORT)

Laboratory methods incorporated in an ORD Quality Assurance Project Plan.

11.0 WATER CHEMISTRY AND CHLOROPHYLL A

11.1 Analytical Parameters

A total of 18 parameters are determined from each bulk water chemistry sample collected (**Table 11.1**). In addition, chlorophyll-*a* is determined from a separate, discrete sample following the same performance-based methods approach as proposed for water chemistry analytes.

Table 11.1 Water chemistry: NRSA 2013/14 parameters

Analyte	Units	Comments
Conductivity	µS/cm at 25°C	
pH (laboratory)	Standard (Std) Units	
Turbidity	Nephelometric Turbidity Units (NTU)	
Dissolved Organic Carbon (DOC)	mg C/L	
Ammonium (NH ₄)	mg N/L	
Nitrate-Nitrite (NO ₃ -NO ₂)	mg N/L	
Total Nitrogen (TN)	mg/L	
Total Phosphorus (TP)	µg P/L	
Sulfate (SO ₄)	mg SO ₄ /L	
Chloride (Cl)	mg Cl/L	
Nitrate (NO ₃)	mg N/L	
Calcium (Ca)	mg Ca/L	
Magnesium (Mg)	mg Mg/L	
Sodium (Na)	mg Na/L	
Potassium (K)	mg K/L	
Silica (SiO ₂)	mg SiO ₂ /L	
Total Suspended Solids (TSS)	mg/L	
True Color	PCU	
Chlorophyll a	µg/L (in extract)	

11.2 Sample Processing and Preservation

Due to the short holding time of these samples, samples will be shipped overnight by the field crews and must be preserved by close of business (COB) the day after sample collection. If expected samples do not arrive or arrive after the acceptable time frame (24 hours after the samples were collected), labs must notify the NARS IM Center (see **APPENDIX A: CONTACT INFORMATION**).

Upon receipt of samples, the assigned lab personnel inspects each sample and review the tracking form that was included with the samples. The assigned lab personnel will flag samples damaged during the shipping process in NARS IM upon receipt and inspection. The lab personnel then store samples at 4°C in darkness until aliquots are ready to be prepared. If possible, the lab will prepare aliquots the same day as samples are received, but no later than 48 hours after receipt. Labs should be familiar with and ensure that samples meet all defined target holding times. Any sample that does not meet holding time requirements is flagged and evaluated to determine if the exceedance impacts either sample integrity or any potential end uses of the data (USEPA 2002). The NRSA team and water chemistry analysts do not

reject results from samples that exceeded target holding times outright; the team will review the data and holding times and make a decision about whether EPA will use the data in the NRSA assessment.

11.2.1 Water Chemistry Samples

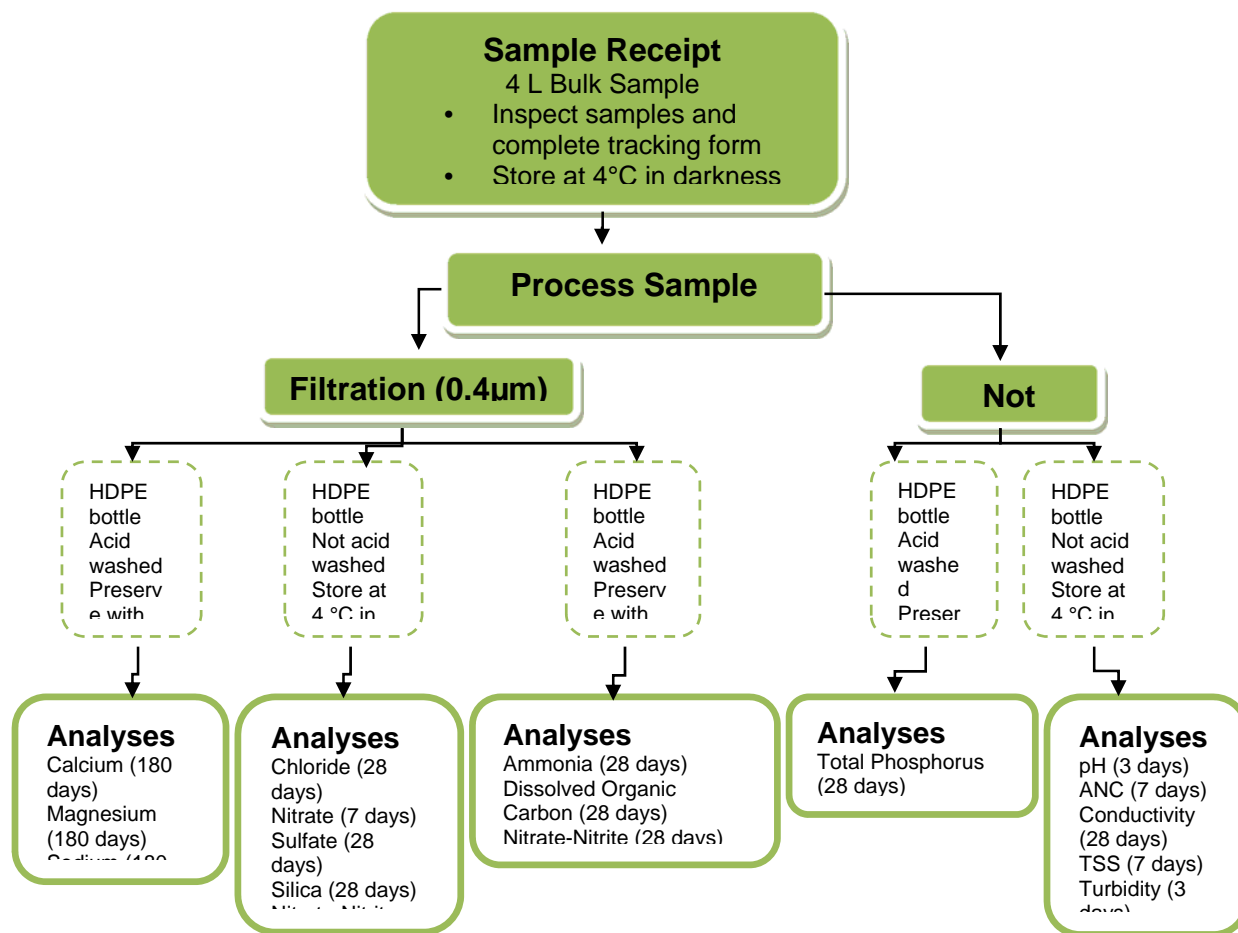


Figure 11.1 Water chemistry: sample processing procedures

Figure 11.1 illustrates sample preparation processing for the water chemistry indicators, including filtering and acidifying, for the various analytes.

1. Use 0.4µm pore size polycarbonate filters for all filtration.
2. Rinse vacuum filter funnel units thoroughly with reverse-osmosis (RO) or de-ionized (DI) water (ASTM Type II reagent water) five times before each use and in between samples. After placing a filter in the funnel unit, run approximately 100 mL of RO or DI water through the filter, with vacuum pressure, to rinse the filter. Discard the rinse water.

3. Place the appropriate sample bottle under the funnel unit and filter sample directly into the bottle. If a new filter is needed, remove the sample bottle, and rinse the new filter with 100 mL of RO or DI water before continuing.
4. After all filtered and unfiltered aliquots are collected, add ultra-pure acid (HNO_3 or H_2SO_4 , depending on the analyte, see **Table 11.2**) to the sample in the aliquot container. Cap tightly and invert the bottle several times to mix.
5. Store all aliquots except the cation aliquot (filtered, acidified with HNO_3) in a refrigerator at 4°C .

Table 11.2 Water chemistry: acid preservatives added for various analytes

Preservatives	
H_2SO_4	HNO_3
DOC	Ca
NH_4	Mg
Total N	Na
Total P	K
$\text{NO}_2\text{-NO}_3$	

11.2.2 Chlorophyll-a Samples

Chlorophyll-*a* samples are filtered in the field, placed in a labeled centrifuge tube in a dark cooler, and stored on ice until arrival at the laboratory. Store the filter in the centrifuge tube in the freezer at $-20 \pm 2^\circ\text{C}$ for no more than thirty days before analysis.

11.3 Performance-based Methods

As an alternative to specifying laboratory methods for sample analysis, a performance-based approach that defines a set of laboratory method performance requirements for data quality is utilized for this survey. Method performance requirements for this project identify lower reporting limit (LRL), precision, and bias objectives for each parameter (**Table 11.4**). The LRL is the lowest value that needs to be quantified (as opposed to just detected), and represents the value of the lowest non-zero calibration standard used. It is set to double the long-term method detection limit (LT-MDL), following guidance presented in Oblinger, Childress et al. (1999).

Precision and bias objectives are expressed in both absolute and relative terms following Hunt and Wilson (1986). The transition value is the value at which performance objectives for precision and bias switch from absolute (\leq transition value) to relative ($>$ transition value). For pH, the objectives are established for samples with higher and lower pH levels.

For duplicate samples, precision is estimated as the pooled standard deviation (calculated as the root-mean square) of all samples at the lower concentration range, and as the pooled percent relative standard deviation of all samples at the higher concentration range. For standard samples (of known concentration), precision is estimated as the standard deviation of repeated measurements across batches at the lower concentration range, and as percent relative standard deviation of repeated measurements across batches at the higher concentration range. Bias (i.e., systematic error) is estimated as the difference between the mean measured value and the target value of a performance evaluation and/or internal reference samples at the lower concentration range measured across sample batches, and as the percent difference at the higher concentration range.

Analytical methods used at the central laboratory (EPA ORD-Corvallis) are summarized in **Table 11.3**. Participating laboratories may use alternative analytical methods for each target analyte as long as they can satisfactorily demonstrate the alternative method is able to achieve the performance requirements

as listed in **Table 11.4**. Information is provided by the lab to the NRSA Quality Team. The team reviews the information to determine whether the labs meet the necessary requirements. The information from this process is maintained in the NRSA 2012 QA files by the EPA HQ NARS QA Lead.

Table 11.3 Water chemistry: NRSA 2013/14 analytical methods (Central Laboratory, EPA ORD-Corvallis)

Analyte	Summary of Method ^d	References ^e	WRS SOP ^f
pH (lab)	Automated, using ManSci PC-Titrate w/ Titra-Sip autotitrator and Ross combination pH electrode. Initial pH determination for ANC titration	EPA 150.6 (modified)	WRS 16A.0 (April 2011)
Specific conductance @ 25°C	Electrolytic, Man-Tech TitraSip automated analysis OR manual analysis, electrolytic	EPA 120.6	WRS 16A.0 (April 2011) WRS 11A.4 (April 2011)
Acid neutralizing capacity (ANC)	Automated acidimetric titration to pH _≤ 3.5, with modified Gran plot analysis	U.S. EPA (1987)	WRS 16A.0 (April 2011)
Turbidity	Nephelometric; Man-Tech TitraSip automated analysis, OR Manual analysis using Hach turbidimeter (high turbidity samples)	APHA 214 A, EPA 180.1 U.S. EPA (1987)	WRS 16A.0 (April 2011) WRS 13A.3 (April 2011)
Total suspended solids (TSS)	Gravimetric, dried at 104 °C	EPA 160.2; APHA 209-C	WRS 14B.4 (February 2011)
True color (Hach Kit)	Visual comparison to calibrated glass color disk.	APHA 204 A (modified), EPA 110.2 (modified), U.S. EPA (1987)	WRS 15A.3 (April 2011)
Dissolved Organic Carbon (DOC)g	UV promoted persulfate oxidation to CO ₂ with infrared detection	APHA 5310-C U.S. EPA (1987)	WRS 21A.4 (May 2011)
Nitrate+Nitrite, as N (fresh waters)	Ion Chromatography OR FIA automated colorimetric (cadmium reduction)	EPA 300.6; SW-846 9056A; APHA 4110B EPA 353.2 APHA 4500-NO ₃ -N-E Lachat 10-107-04-1-C	WRS 36A.0 (April 2011) WRS 40A.5 (May 2011)
Ammonia, as N (fresh waters)	FIA automated colorimetric (salicylate, dichloroisocyanurate)	Lachat 10-107-06-3-D	WRS 30A.4 (April 2011)
Silica, dissolved (SiO₂) Fresh waters	FIA automated colorimetric (molybdate, stannous chloride)	EPA366.0, APHA 425 C Lachat 10-114-27-1-B	WRS 32A.5 (February 2010)
Total nitrogen (TN)	Persulfate Digestion; FIA Automated Colorimetric Analysis (Cadmium Reduction, sulfanilamide)	EPA353.2 (modified) APHA 4500-N-C (modified) ASTM WK31786 U.S. EPA (1987) Lachat 10-107-04-1-C (modified)	WRS 34A.5 (April 2011)

^d FIA=Flow injection analysis. AAS=Atomic Absorption Spectrometry

^e U.S. EPA, 1987. *Handbook of Methods for Acid Deposition Studies: Laboratory Analyses for Surface Water Chemistry*. EPA/600/4-87/026. U.S. Environmental Protection Agency, Office of Research and Development, Washington D.C. APHA= American Public Health Association (*Standard Methods*). ASTM=American Society of Testing and Materials.

^f WRS= Willamette Research Station. References are to laboratory SOP being used at central laboratory. Available upon request. (contact the Project Lead)

^g For DOC, "dissolved" is defined as that portion passing through a 0.45 µm nominal pore size filter. For other analytes, "dissolved" is defined as that portion passing through a 0.4 µm pore size filter (Nucleopore or equivalent).

Analyte	Summary of Method ^d	References ^e	WRS SOP ^f
Total phosphorus (TP)	Persulfate Digestion; Automated Colorimetric Analysis (molybdate, ascorbic acid)	APHA 4500-P-E USGS I-4650-03 U.S. EPA (1987) Lachat 115-01-1-B (modified)	WRS 34A.5 (April 2011)
Major anions, dissolved chloride, nitrate, nitrite, sulfate	Ion Chromatography	EPA 300.6; SW-846 9056A; APHA 4110B	WRS 40A.5 (May 2011)
Major cations, dissolved calcium, sodium, potassium, magnesium	Inductively-coupled Plasma Atomic Emission Spectroscopy (ICP-AES) OR Flame AAS	EPA 200.7; EPA 6010B U.S. EPA (1987), EPA 215.1 EPA 273.1, EPA 258.1 EPA 242.1	WRS SOP 3.04 v3 (October 2011) WRS 50A.4 (March 2007)
Chlorophyll-a (Chl-a)	Extraction 90% acetone analysis by fluorometry	EPA 445.0 , EPA 446.0	WRS 71A.3 (April 2011)

11.4 Pertinent QA/QC Procedures

A single central laboratory and some State laboratories will analyze the water chemistry samples. The specific quality control procedures used by each laboratory are implemented to ensure that:

- Objectives established for various data quality indicators being met
- Results are consistent and comparable among all participating labs.

The central laboratory demonstrated in previous studies that it can meet the required LRL (USEPA 2004). QA/QC procedures outlined in this manual and the NRSA 2013/14 QAPP will be followed to ensure these LRLs are met for the NRSA 2013/14.

11.4.1 Laboratory Performance Requirements

Table 11.4 summarizes the pertinent laboratory performance requirements for the water chemistry and chlorophyll A indicators.

11.4.2 Laboratory Quality Control Samples

Table 11.5 summarizes the pertinent laboratory quality control samples for the water chemistry and chlorophyll A indicators.

Table 11.4 Water chemistry & chlorophyll-a: laboratory method performance requirements

Analyte	Units	Potential Range of Samples ^h	Lower Reporting Limit ⁱ	Transition Value ^j	Precision Objective ^k	Bias Objective ^l
Conductivity	µS/cm at 25°C	1 to 15,000	2.0	20	± 2 or ±10%	± 2 or 5%
pH (laboratory)	Std Units	3.5 to 10	N/A	5.75	>5.75= ±0.15 ≤5.75 =±0.07	>5.75 = ±0.05 ≤5.75 = ±0.15
Turbidity	Nephelometric Turbidity Units (NTU)	0 to 44,000	2.0	20	± 2 or ±10%	± 2 or ±10%
Dissolved Organic Carbon (DOC)	mg C/L	0.1 to 109	0.20	≤ 1 > 1	± 0.10 or ±10%	± 0.10 or ±10%
Ammonium (NH₄)	mg N/L	0 to 17	0.02 (1.4 µeq/L)	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Nitrate-Nitrite (NO₃-NO₂)	mg N/L	0 to 360 (as nitrate)	0.02	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Total Nitrogen (TN)	mg/L	0.1 to 90	0.02	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Total Phosphorus (TP)	µg P/L	0 to 22,000	4	20	± 2 or ±10%	± 2 or ±10%

^h Estimated from samples analyzed at the WED-Corvallis laboratory between 1999 and 2005 for TIME, EMAP-West, and WSA streams from across the U.S.

ⁱ The lower reporting limit is the lowest value that needs to be quantified (as opposed to just detected), and represents the value of the lowest nonzero calibration standard used. It is set to 2 times the long-term method detection limit, following USGS Open File Report 99-193 *New Reporting Procedures Based on Long-Term Method Detection Levels and Some Considerations for Interpretations of Water-Quality Data Provided by the U.S. Geological Survey National Water Quality Laboratory*.

^j Value at which performance objectives for precision and bias switch from absolute (≤ transition value) to relative (> transition value). Two-tiered approach based on Hunt, D.T.E. and A.L. Wilson. 1986. *The Chemical Analysis of Water: General Principles and Techniques*. 2nd ed. Royal Society of Chemistry, London, England.

^k For standard samples, precision is estimated as the standard deviation of repeated measurements across batches at the lower concentration range, and as percent relative standard deviation of repeated measurements across batches at the higher concentration range.

For pH precision, the looser criteria applies to more highly alkaline samples. For NRSA, that is less of a concern than the ability to measure acidic samples accurately and precisely.

^l Bias (systematic error) is estimated as the difference between the mean measured value and the target value of a performance evaluation and/or internal reference samples at the lower concentration range measured across sample batches, and as the percent difference at the higher concentration range.

Analyte	Units	Potential Range of Samples ^h	Lower Reporting Limit ⁱ	Transition Value ^j	Precision Objective ^k	Bias Objective ^l
Sulfate (SO₄)	mg SO ₄ /L	0 to 5,000	0.50 (10 µeq/L)	2.5	± 0.25 or ±10%	± 0.25 or ±10%
Chloride (Cl)	mg Cl/L	0 to 5,000	0.20 (6 µeq/L)	1	± 0.10 or ±10%	± 0.10 or ±10%
Nitrate (NO₃)	mg N/L	0 to 360	0.02 (4 µeq/L)	0.1	± 0.01 or ±10%	± 0.01 ±10%
Calcium (Ca)	mg Ca/L	0.04 to 5,000	0.10 (5 µeq/L)	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Magnesium (Mg)	mg Mg/L	0.1 to 350	0.10 (8 µeq/L)	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Sodium (Na)	mg Na/L	0.08 to 3,500	0.10 (4 µeq/L)	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Potassium (K)	mg K/L	0.01 to 120	0.10 (2 µeq/L)	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Silica (SiO₂)	mg SiO ₂ /L	0.01 to 100	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Total Suspended Solids (TSS)	mg/L	0 to 27,000	2	10	± 1 or ±10%	± 1 or ±10%
True Color	PCU	0 to 350	5	50	±5 or ±10%	±5 or ±10%
Chlorophyll a	µg/L (in extract)	0.7 to 11,000	0.5	15	± 1.5 or ±10%	± 1.5 or ±10%

Table 11.5 Water chemistry: quality control – laboratory samples

QC Sample Type and Description	Analytes	Description	Frequency	Acceptance Criteria	Corrective Action
Laboratory/ Reagent Blank	All except TSS (For TSS, the lab will filter a known volume of reagent water and process the filters per method)		Once per day prior to sample analysis	Control limits \leq LRL	Prepare and analyze new blank. Determine and correct problem (e.g., reagent contamination, instrument calibration, or contamination introduced during filtration) before proceeding with any sample analyses. Reestablish statistical control by analyzing three blank samples.
Filtration Blank	All dissolved analytes	ASTM Type II reagent water processed through filtration unit	Prepare once per week and archive. Prepare filter blank for each box of 100 filters, and examine the results before any other filters are used from that box.	Measured concentrations <LDL	Measure archived samples if review of other laboratory blank information suggest source of contamination is sample processing.
LT-MDL Limit Quality Control Check Sample (QCCS)	All analyses except true color and turbidity	Prepared so concentration is four to six times the LT-MDL objective	Once per day	Target LT-MDL value (which is calculated as a 99% confidence interval)	Confirm achieved LRL by repeated analysis of LT-MDL QCCS. Evaluate affected samples for possible re-analysis.
Calibration QCCS	For turbidity, a QCCS is prepared at one level for routine analyses (USEPA 1987). Additional QCCSs are prepared as needed for samples having estimated turbidities greater than 20 NTU.		Before and after sample analyses	$\pm 10\%$ or method criteria	Repeat QCCS analysis. Recalibrate and analyze QCCS. Reanalyze all routine samples (including PE and field replicate samples) analyzed since the last acceptable QCCS measurement.

QC Sample Type and Description	Analytes	Description	Frequency	Acceptance Criteria	Corrective Action
Laboratory Duplicate Sample	All analyses		One per batch	Control limits < precision objective	If results are below LRL: Prepare and analyze split from different sample (volume permitting). Review precision of QCCS measurements for batch. Check preparation of split sample. Qualify all samples in batch for possible reanalysis.
Standard Reference Material (SRM)	When available for a particular analyte		One analysis in a minimum of five separate batches	Manufacturers certified range	Analyze standard in next batch to confirm suspected imprecision or bias. Evaluate calibration and QCCS solutions and standards for contamination and preparation error. Correct before any further analyses of routine samples are conducted. Reestablish control by three successive reference standard measurements that are acceptable. Qualify all sample batches analyzed since the last acceptable reference standard measurement for possible reanalysis.
Matrix Spike Samples	Only prepared when samples with potential for matrix interferences are encountered		One per batch	Control limits for recovery cannot exceed 100±20%	Select two additional samples and prepare fortified subsamples. Reanalyze all suspected samples in batch by the method of standard additions. Prepare three subsamples (unfortified, fortified with solution approximately equal to the endogenous concentration, and fortified with solution approximately twice the endogenous concentration).

11.4.3 Data Reporting, Review, and Management

Checks made of the data in the process of review and verification are summarized in **Table 11.6**. Data reporting units and significant figures are given in **Table 11.7**. The NRSA 2013/14 Project QA Officer is ultimately responsible for ensuring the validity of the data, although performance of the specific checks may be delegated to other staff members.

Table 11.6 Water chemistry: quality control – data validation

Activity or Procedure	Requirements and Corrective Action
Range checks, summary statistics, and/or exploratory data analysis (e.g., box and whisker plots)	Correct reporting errors or qualify as suspect or invalid.
Review holding times	Qualify value for additional review
Ion balance: Calculate percent ion balance difference (%IBD) using data from cations, anions, pH, and ANC.	<ul style="list-style-type: none"> • If total ionic strength $\leq 100 \mu\text{eq/L}$ – %IBD $\leq \pm 25\%$. • If total ionic strength $> 100 \mu\text{eq/L}$ – %IBD $\leq \pm 10\%$. • Determine which analytes, if any, are the largest contributors to the ion imbalance. Review suspect analytes for analytical error and reanalyze. – Flag = unacceptable %IBD • If analytical error is not indicated, qualify sample to attribute imbalance to unmeasured ions. Reanalysis is not required. – Flag = %IBD outside acceptance criteria due to unmeasured ions
Conductivity check: Compare measured conductivity of each sample to a calculated conductivity based on the equivalent conductance of major ions in solution (Hillman et al., 1987).	<ul style="list-style-type: none"> • If measured conductivity $\leq 25 \mu\text{S/cm}$, – $([\text{measured} \ominus \text{calculated}] \div \text{measured}) \leq \pm 25\%$. • If measured conductivity $> 25 \mu\text{S/cm}$, – $([\text{measured} \ominus \text{calculated}] \div \text{measured}) \leq \pm 15\%$. • Determine which analytes, if any, are the largest contributors to the difference between calculated and measured conductivity. • Review suspect analytes for analytical error and reanalyze. • If analytical error is not indicated, qualify sample to attribute conductivity difference to unmeasured ions. Reanalysis is not required.
Review data from QA samples (laboratory PE samples, and inter-laboratory comparison samples)	Indicator QC Coordinator determines impact and possible limitations on overall usability of data based on the specific issue.

Table 11.7 Water chemistry: data reporting criteria

Measurement	Units	No. Significant Figures	Maximum No. Decimal Places
DO	mg/L	2	1
Temperature	°C	2	1
pH	pH units	3	2
Carbon, total & dissolved organic	mg/L	3	1
ANC	$\mu\text{eq/L}$	3	1
Conductivity	$\mu\text{S/cm}$ at 25 °C	3	1

Measurement	Units	No. Significant Figures	Maximum No. Decimal Places
Calcium, magnesium, sodium, potassium, ammonium, chloride, nitrate, and sulfate	µeq/L	3	1
Silica	mg/L	3	2
Total phosphorus	µg/L	3	0
Total nitrogen	mg/L	3	2
Nitrate-Nitrite	mg/L	3	2
Ammonium	mg/L	3	2
Turbidity	NTU	3	0
True color	PCU	2	0
TSS	mg/L	3	1
Chlorophyll a	ug/l	3	2

The ion balance for each sample is computed using the results for major cations, anions, and the measured acid neutralizing capacity. The percent ion difference (%IBD) for a sample is calculated as:

Equation 11.1 Percent ion difference (%IBD)

$$\% IBD = \frac{(\sum cations - \sum anions) - ANC}{ANC + \sum anions + \sum cations + 2[H^+]}$$

where ANC is the acid neutralization capacity; cations are the concentrations of calcium, magnesium, sodium, potassium, and ammonium (converted from mg/L to µeq/L); anions are the concentrations of chloride, nitrate, and sulfate (converted from mg/L to µeq/L), and H⁺ is the hydrogen ion concentration calculated from the antilog of the sample pH. Factors to convert major ions from mg/L to µeq/L are presented in **Table 11.8**. For the conductivity check, equivalent conductivities for major ions are presented in **Table 11.9**.

Table 11.8 Water chemistry: constants for converting major ion concentration from mg/L to µeq/L

Analyte	Conversion from mg/L to µeq/L ^m
Calcium	49.9
Magnesium	82.3
Potassium	25.6
Sodium	43.5
Ammonium	55.4
Chloride	28.2
Nitrate	16.1
Sulfate	20.8

^m Measured values are multiplied by the conversion factor.

Table 11.9 Water chemistry: factors to calculate equivalent conductivities of major ions.ⁿ

Ion	Equivalent Conductance per mg/L (μS/cm at 25 °C)	Ion	Equivalent Conductance per mg/L (μS/cm at 25 °C)
Calcium	2.60	Nitrate	1.15
Magnesium	3.82	Sulfate	1.54
Potassium	1.84	Hydrogen	3.5 x 10 ⁵ °
Sodium	2.13	Hydroxide	1.92 x 10 ⁵
Ammonium	4.13	Bicarbonate	0.715
Chloride	2.14	Carbonate	2.82

11.5 Literature Cited

Hunt, D.T.E. and A.L. Wilson. 1986. *The Chemical Analysis of Water: General Principles and Techniques*. 2nd ed.. Royal Society of Chemistry, London, England.

Youden, W.J. 1969. Ranking laboratories by round-robin tests. In *Precision Measurement and Calibration*. H.H. Ku, ed. NBS Special Publication 300, Vol. 1. U.S. GPO Washington, D.C.

ⁿ From Hillman et al. (1987).

^o Specific conductance per mole/L, rather than per mg/L.

APPENDIX A: CONTACT INFORMATION

Title	Name	Contact Information
EPA HQ NRSA Project Lead	Ellen Tarquinio, OW	tarquinio.ellen@epa.gov 202-566-2267
EPA HQ NRSA Project QA Lead	Sarah Lehmann, OW	lehmann.sarah@epa.gov 202-566-1379
EPA HQ NRSA Laboratory Review Manager	Kendra Forde, OW	forde.kendra@epa.gov 202-564-0417
Information Management Center Coordinator	Marlys Cappaert, SRA International Inc.	cappaert.marlys@epa.gov 541-754-4467 541-754-4799 (fax)

APPENDIX B: LABORATORY REMOTE EVALUATION FORMS

NRSA 2013-2014 Document Request Form – Chemistry Labs

EPA and its state and tribal partners will conduct a survey of the nation's rivers and streams. This National River and Streams Assessment (NRSA), is designed to provide statistically valid regional and national estimates of the condition of rivers and streams. Consistent sampling and analytical procedures ensure that the results can be compared across the country.

As part of the 2013-2014 NRSA, the Quality Assurance Team has been requested to conduct a technical assessment to verify quality control practices in your laboratory and its ability to perform chemistry analyses under this project. Our review will be assessing your laboratory's ability to receive, store, prepare, analyze, and report sample data generated under EPA's 2013-2014 NRSA.

The first step of this assessment process will involve the review of your laboratory's certification and/or documentation. Subsequent actions may include (if needed): reconciliation exercises and/or a site visit. All labs will need to complete the following forms:

All labs will be required to complete the following forms and check the specific parameter in which your lab will be conducting an analysis for the 2013-2014 NRSA:

- Water Chemistry, chlorophyll a, and Ash Free Dry Mass (AFDM) (all of the analytes identified in the LOM and QAPP)
- Microcystin
- Fish Tissue Plugs

If your lab has been previously approved within the last 5 years for the water chemistry indicator:

- A *signature* on the attached Lab Signature Form indicates that your lab will follow the quality assurance protocols required for chemistry labs conducting analyses for the 2013-2014 NRSA.
- A signature on the Quality Assurance Project Plan (QAPP) and the Laboratory Operations Manual (LOM) Signature Form indicates that you will follow both the QAPP and the LOM.

If you have not been approved within the last 5 years through the lab verification process for the water chemistry indicator, in order for us to determine your ability to participate as a lab in the NRSA, we are requesting that you submit the following documents (if available) for review:

- Documentation of a successful *quality assurance audit* from a prior National Aquatic Resource Survey (NARS) that occurred within the last 5 years (if you need assistance with this please contact the individual listed below).
- Documentation showing participation in a previous NARS for Water Chemistry for the same parameters/methods.

Additionally, we request that all labs provide the following information in support of your capabilities, (these materials are required if neither of the two items above are provided):

- A copy of your Laboratory's *accreditations and certifications* if applicable (i.e. NELAC, ISO, state certifications, NABS, etc.).
- An updated copy of your Laboratory's *QAPP*.
- Standard Operating Procedures (SOPs)* for your lab for each analysis to be performed (if not covered in 2013-2014 NRSA LOM).

- Documentation attesting to experience running all analytes for the 2013-2014 NRSA, including chlorophyll a and Ash Free Dry Mass (AFDM).

This documentation may be submitted electronically via e-mail to forde.kendra@epa.gov with a cc: to tarquinio.ellen@epa.gov and lehmann.sarah@epa.gov. Questions concerning this request can be submitted forde.kendra@epa.gov (202-566-0417) or tarquinio.ellen@epa.gov (202-566-2267).

Lab Signature Form – Chemistry Labs

I _____ certify that the _____ lab,
located in _____, will abide by the following standards in
performing the following data analysis and reporting for the 2013-2014 National Rivers
and Streams Assessment (NRSA).

This applies to the _____ chemistry indicator.

- 1.) Utilize procedures identified in the 2013-2014 NRSA Lab Operations Manual (or equivalent). If using equivalent procedures, please provide procedures manual and obtain approval from EPA.
- 2.) Read and abide by the 2013-2014 NRSA Quality Assurance Project Plan (QAPP) and related Standard Operating Procedures (SOPs).
- 3.) Have an organized IT tracking system in place for recording sample tracking and analysis data.
- 4.) Provide Quality Control (QC) data for internal QC check, on a quarterly basis.
- 5.) Provide data using the template provided on the NARS Sharefile.
- 6.) Provide data results in a timely manner. This will vary with the type of analysis and the number of samples to be processed. Sample data must be received no later than May 1, 2014 (for samples collected in 2013) and May 1, 2015 (for samples collected in 2014) or as otherwise negotiated with EPA.
- 7.) Participate in a lab technical assessment or audit if requested by EPA NRSA staff (this may be a conference call or on-site audit).
- 8.) Agree to analyze for all parameters specified in the LOM for the appropriate indicator(s) identified above, including Chlorophyll-a and AFDM for water chemistry.

Signature _____ Date _____

NRSA 2013-2014 Document Request Form - Biology Labs

EPA and its state and tribal partners will conduct a survey of the nation's rivers and streams. This National River and Streams Assessment (NRSA), is designed to provide statistically valid regional and national estimates of the condition of rivers and streams. Consistent sampling and analytical procedures ensure that the results can be compared across the country.

As part of the 2013-2014 NRSA, the Quality Assurance Team has been requested to conduct a technical assessment to verify quality control practices in your laboratory and its ability to perform biology analyses under this project. Our review will be assessing your laboratory's ability to receive, store, prepare, analyze, and report sample data generated under EPA's 2013-2014 NRSA.

The first step of this assessment process will involve the review of your laboratory's certification and/or documentation. Subsequent actions may include (if needed): reconciliation exercises and/or a site visit.

All labs will be required to complete the following forms and check the specific parameter in which your lab will be conducting an analysis for the 2013-2014 NRSA:

- Fish Voucher
- Benthic Macroinvertebrates
- Periphyton

If your lab has been previously approved within the last 5 years for the specific parameters:

- A *signature* on the attached Lab Signature Form indicates that your lab will follow the quality assurance protocols required for biology labs conducting analyses for the 2013-2014 NRSA.
- A signature on the Quality Assurance Project Plan (QAPP) and the Laboratory Operations Manual (LOM) Signature Form indicates you will follow both the QAPP and the LOM.

If you have not been approved within the last 5 years through the lab verification process for the specific parameters, in order for us to determine your ability to participate as a lab in the NRSA, we are requesting that you submit the following documents (if available) for review:

- Documentation of a successful *quality assurance audit* from a prior National Aquatic Resource Survey (NARS) that occurred within the last 5 years (if you need assistance with this please contact the individual listed below).
- Documentation showing participation in previous NARS for this particular indicator.

Additionally, we request that all labs provide the following information in support of your capabilities, (these materials are required if neither of the two items above are provided):

- A copy of your Laboratory's *accreditations and certifications* if applicable (i.e. NELAC, ISO, state certifications, NABS, etc.).
- Documentation of NABS (or other) *certification* for the *taxonomists* performing analyses (if applicable).
- An updated copy of your Laboratory's *QAPP*.
- Standard Operating Procedures (SOPs)* for your lab for each analysis to be performed (if not covered in 2013-2014 NRSA LOM).
- Resume of independent fish taxonomist who will verify fish vouchers (if applicable)

This documentation may be submitted electronically via e-mail to forde.kendra@epa.gov with a cc: to tarquinio.ellen@epa.gov and lehmann.sarah@epa.gov. Questions concerning this request can be submitted forde.kendra@epa.gov (202-566-0417) or tarquinio.ellen@epa.gov (202-566-2267).

Lab Signature Form – Biology Labs

I _____ certify that the _____ lab,
located in _____, will abide by the following standards in
performing biology data analysis and reporting for the 2013-2014 National Rivers and
Streams Assessment (NRSA).

This applies to the _____ biological indicator.

- 9.) Utilize procedures identified in the 2013-2014 NRSA Lab Operations Manual (or equivalent). If using equivalent procedures, please provide procedures manual and obtain approval from EPA.
- 10.) Read and abide by the 2013-2014 NRSA Quality Assurance Project Plan (QAPP) and related Standard Operating Procedures (SOPs).
- 11.) Have an organized IT tracking system in place for recording sample tracking and analysis data.
- 12.) Use taxonomic standards outlined in the 2013-2014 NRSA Lab Manual.
- 13.) Participate in taxonomic reconciliation exercises during the field and data analysis season, which include conference calls and other lab reviews.
- 14.) Provide Quality Control (QC) data for internal QC check, including for sorting, on a monthly basis.
- 15.) Provide data using the template provided on the NARS Sharefile.
- 16.) Provide data results in a timely manner. This will vary with the type of analysis and the number of samples to be processed. Sample data must be received no later than May 1, 2014 (for samples collected in 2013) and May 1, 2015 (for samples collected in 2014) or as otherwise negotiated with EPA. Samples results for independent taxonomic QC described in the LOM and QAPP must be provided to EPA prior to final datasets (fall and winter) to allow for reconciliation to take place.
- 17.) Participate in a lab technical assessment or audit if requested by EPA NRSA staff (this may be a conference call or on-site audit).
- 18.) Agree to utilize taxonomic nomenclature and hierarchical established for NRSA 2013-2014.

Signature _____ Date _____

APPENDIX C: SAMPLE LABORATORY FORMS

Benthic Macroinvertebrate: Sorting Bench Sheet

Laboratory Information		Sample Information	
Project ID		Sample ID	
Station Name		Site ID	
Station Location		Date Collected	
Station Number		Field Crew ID	

Sorter Name _____ Proficiency: ____%
Date Sorting Initiated _____ Proportion of Sample Sorted: _____

Sorting Order	Grid # (e.g., A5) Each grid has unique identifier	Quarter # (if any) Each quarter has unique identifier	Sorter's Results # Organisms		Sorting QC Officer (if QC check)	
			Per Grid	Cumulative	#Additional Organisms	Cumulative (include sorter#)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						

Is cumulative number between 500 and 600? Y / N (N permissible only if entire sample sorted)

If QC check, provide PSE: ____% Sorting QC Officer Initials _____

Benthic Macroinvertebrates: Taxonomy Bench Sheet (optional)

Laboratory Information		Sample Information	
Project ID		Sample ID	
Station Name		Site ID	
Station Location		Date Collected	
Station Number		Field Crew ID	

Taxonomist Name _____

Date 1st Organism Identified in Sample: _____ QC Check? Y / N

TSN (Use # in Unique Identifier from WQX)	Taxon (see target taxon in Table 3)	Distinct (Y/N)	Counts of Organisms in the Taxon:			Cumulative Number of Organisms in Sample	Data Qualifier (Codes in Table 4)
			Total (any stage)	Larvae	Pupae		

Is cumulative number of organisms ≥ 500 ? If not, unless the sample was completed sorted, obtain more sorted sample.

Comments:

Fish Voucher: Taxonomy Bench Sheet (optional)

Laboratory Information		Sample Information	
Labname		Sample ID	
QC Taxonomist		Site ID	
Project ID		Visit Number	
Internal Sample ID		Date Collected	

Taxonomist Name: _____

Date 1st Specimen Identified in Sample: _____

Tag No.	New Taxon?	Common or Scientific Name	Condition Code	Data Flag	Comment Including Citation if Appropriate

Additional Comments

APPENDIX D: OTHER PERTINENT ATTACHMENTS

Table D.0.1 Fish identification: standard common and scientific names

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
1	Perciformes	Cichlidae	<i>Hemichromis letourneuxi</i>	african jewelfish
2	Percopsiformes	Amblyopsidae	<i>Speoplatyrhinus poulsoni</i>	alabama cavefish
3	Perciformes	Percidae	<i>Etheostoma ramseyi</i>	alabama darter
4	Cypriniformes	Catostomidae	<i>Hypentelium etowanum</i>	alabama hog sucker
5	Clupeiformes	Clupeidae	<i>Alosa alabamae</i>	alabama shad
6	Cypriniformes	Cyprinidae	<i>Cyprinella callistia</i>	alabama shiner
7	Acipenseriformes	Acipenseridae	<i>Scaphirhynchus suttkusi</i>	alabama sturgeon
8	Esociformes	Umbridae	<i>Dallia pectoralis</i>	alaska blackfish
9	Clupeiformes	Clupeidae	<i>Alosa pseudoharengus</i>	alewife
10	Lepisosteiformes	Lepisosteidae	<i>Atractosteus spatula</i>	alligator gar
11	Cypriniformes	Cyprinidae	<i>Cyprinella xaenura</i>	altamaha shiner
12	Cypriniformes	Cyprinidae	<i>Gila alvordensis</i>	alvord chub
13	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon nevadensis</i>	amargosa pupfish
14	Cyprinodontiformes	Poeciliidae	<i>Poecilia formosa</i>	amazon molly
15	Siluriformes	Loricariidae	<i>Pterygoplichthys pardalis</i>	amazon sailfin catfish
16	Perciformes	Percidae	<i>Percina antesella</i>	amber darter
17	Petromyzontiformes	Petromyzontidae	<i>Lampetra appendix</i>	american brook lamprey
18	Anguilliformes	Anguillidae	<i>Anguilla rostrata</i>	american eel
19	Clupeiformes	Clupeidae	<i>Alosa sapidissima</i>	american shad
20	Cyprinodontiformes	Poeciliidae	<i>Gambusia amistadensis</i>	amistad gambusia
21	Cypriniformes	Cyprinidae	<i>Pteronotropis grandipinnis</i>	apalachee shiner
22	Perciformes	Percidae	<i>Percina gymnocephala</i>	appalachia darter
23	Salmoniformes	Salmonidae	<i>Salvelinus alpinus</i>	arctic char
24	Salmoniformes	Salmonidae	<i>Coregonus autumnalis</i>	arctic cisco
25	Salmoniformes	Salmonidae	<i>Thymallus arcticus</i>	arctic grayling
26	Petromyzontiformes	Petromyzontidae	<i>Lampetra camtschatica</i>	arctic lamprey
27	Perciformes	Percidae	<i>Etheostoma cragini</i>	arkansas darter
28	Cypriniformes	Cyprinidae	<i>Notropis girardi</i>	arkansas river shiner
29	Perciformes	Percidae	<i>Etheostoma euzonum</i>	arkansas saddled darter
30	Perciformes	Percidae	<i>Etheostoma sagitta</i>	arrow darter
31	Perciformes	Gobiidae	<i>Clevelandia ios</i>	arrow goby
32	Cypriniformes	Cyprinidae	<i>Gila orcuttii</i>	arroyo chub
33	Cyprinodontiformes	Goodeidae	<i>Empetrichthys merriami</i>	ash meadows poolfish
34	Perciformes	Percidae	<i>Etheostoma cinereum</i>	ashy darter
35	Synbranchiformes	Synbranchidae	<i>Monopterus albus</i>	asian swamp eel
36	Perciformes	Sciaenidae	<i>Micropogonias undulatus</i>	atlantic croaker
37	Beloniformes	Belonidae	<i>Strongylura marina</i>	atlantic needlefish
38	Salmoniformes	Salmonidae	<i>Salmo salar</i>	atlantic salmon

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
39	Myliobatiformes	Dasyatidae	<i>Dasyatis sabina</i>	atlantic stingray
40	Acipenseriformes	Acipenseridae	<i>Acipenser oxyrinchus</i>	atlantic sturgeon
41	Clupeiformes	Clupeidae	<i>Opisthonema oglinum</i>	atlantic thread herring
42	Gadiformes	Gadidae	<i>Microgadus tomcod</i>	atlantic tomcod
43	Perciformes	Percidae	<i>Etheostoma zonifer</i>	backwater darter
44	Perciformes	Sciaenidae	<i>Bairdiella icistia</i>	bairdiella
45	Perciformes	Cichlidae	<i>Heros severus</i>	banded cichlid
46	Perciformes	Percidae	<i>Etheostoma zonale</i>	banded darter
47	Cyprinodontiformes	Fundulidae	<i>Fundulus diaphanus</i>	banded killifish
48	Perciformes	Elassomatidae	<i>Elassoma zonatum</i>	banded pygmy sunfish
49	Scorpaeniformes	Cottidae	<i>Cottus carolinae</i>	banded sculpin
50	Perciformes	Centrarchidae	<i>Enneacanthus obesus</i>	banded sunfish
51	Cyprinodontiformes	Fundulidae	<i>Fundulus cingulatus</i>	banded topminnow
52	Perciformes	Percidae	<i>Etheostoma zonistium</i>	bandfin darter
53	Cypriniformes	Cyprinidae	<i>Luxilus zonistius</i>	bandfin shiner
54	Cypriniformes	Cyprinidae	<i>Cyprinella leedsii</i>	bannerfin shiner
55	Perciformes	Centrarchidae	<i>Lepomis symmetricus</i>	bantam sunfish
56	Perciformes	Percidae	<i>Etheostoma obeyense</i>	barcheek darter
57	Perciformes	Percidae	<i>Etheostoma forbesi</i>	barrens darter
58	Cyprinodontiformes	Fundulidae	<i>Fundulus julisia</i>	barrens topminnow
59	Clupeiformes	Engraulidae	<i>Anchoa mitchilli</i>	bay anchovy
60	Pleuronectiformes	Paralichthyidae	<i>Citharichthys spilopterus</i>	bay whiff
61	Perciformes	Percidae	<i>Etheostoma rubrum</i>	bayou darter
62	Cyprinodontiformes	Fundulidae	<i>Fundulus pulvereus</i>	bayou killifish
63	Cyprinodontiformes	Fundulidae	<i>Fundulus nottii</i>	bayou topminnow
64	Scorpaeniformes	Cottidae	<i>Cottus extensus</i>	bear lake sculpin
65	Salmoniformes	Salmonidae	<i>Prosopium abyssicola</i>	bear lake whitefish
66	Cypriniformes	Cyprinidae	<i>Cyprinella formosa</i>	beautiful shiner
67	Cypriniformes	Cyprinidae	<i>Notropis rupestris</i>	bedrock shiner
68	Salmoniformes	Salmonidae	<i>Coregonus laurettae</i>	bering cisco
69	Cyprinodontiformes	Poeciliidae	<i>Gambusia gaigei</i>	big bend gambusia
70	Cypriniformes	Cyprinidae	<i>Hybopsis amblops</i>	bigeye chub
71	Cypriniformes	Catostomidae	<i>Moxostoma ariommum</i>	bigeye jumprock
72	Cypriniformes	Cyprinidae	<i>Notropis boops</i>	bigeye shiner
73	Cypriniformes	Cyprinidae	<i>Hypophthalmichthys nobilis</i>	bighead carp
74	Cypriniformes	Catostomidae	<i>Ictiobus cyprinellus</i>	bigmouth buffalo
75	Cypriniformes	Cyprinidae	<i>Nocomis platyrhynchus</i>	bigmouth chub
76	Cypriniformes	Cyprinidae	<i>Notropis dorsalis</i>	bigmouth shiner
77	Perciformes	Eleotridae	<i>Gobiomorus dormitor</i>	bigmouth sleeper

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
78	Perciformes	Percidae	<i>Percina macrolepada</i>	bigscale logperch
79	Cypriniformes	Cyprinidae	<i>Rhodeus sericeus</i>	bitterling
80	Perciformes	Cichlidae	<i>Cichlasoma bimaculatum</i>	black acara
81	Cypriniformes	Catostomidae	<i>Ictiobus niger</i>	black buffalo
82	Siluriformes	Ictaluridae	<i>Ameiurus melas</i>	black bullhead
83	Perciformes	Centrarchidae	<i>Pomoxis nigromaculatus</i>	black crappie
84	Siluriformes	Ictaluridae	<i>Noturus funebris</i>	black madtom
85	Cypriniformes	Catostomidae	<i>Moxostoma duquesnei</i>	black redhorse
86	Scorpaeniformes	Cottidae	<i>Cottus baileyi</i>	black sculpin
87	Perciformes	Percidae	<i>Percina nigrofasciata</i>	blackbanded darter
88	Perciformes	Centrarchidae	<i>Enneacanthus chaetodon</i>	blackbanded sunfish
89	Cypriniformes	Cyprinidae	<i>Notropis heterodon</i>	blackchin shiner
90	Perciformes	Cichlidae	<i>Sarotherodon melanotheron</i>	blackchin tilapia
91	Salmoniformes	Salmonidae	<i>Coregonus nigripinnis</i>	blackfin cisco
92	Perciformes	Percidae	<i>Etheostoma nigripinne</i>	blackfin darter
93	Cypriniformes	Catostomidae	<i>Thoburnia atripinnis</i>	blackfin sucker
94	Cypriniformes	Cyprinidae	<i>Notropis melanostomus</i>	blackmouth shiner
95	Cypriniformes	Cyprinidae	<i>Notropis heterolepis</i>	blacknose shiner
96	Cypriniformes	Cyprinidae	<i>Phoxinus cumberlandensis</i>	blackside dace
97	Perciformes	Percidae	<i>Percina maculata</i>	blackside darter
98	Perciformes	Percidae	<i>Etheostoma duryi</i>	blackside snubnose darter
99	Cypriniformes	Cyprinidae	<i>Notropis atrocaudalis</i>	blackspot shiner
100	Cyprinodontiformes	Fundulidae	<i>Fundulus olivaceus</i>	blackspotted topminnow
101	Cyprinodontiformes	Fundulidae	<i>Fundulus notatus</i>	blackstripe topminnow
102	Cypriniformes	Catostomidae	<i>Moxostoma poecilurum</i>	blacktail redhorse
103	Cypriniformes	Cyprinidae	<i>Cyprinella venusta</i>	blacktail shiner
104	Cypriniformes	Catostomidae	<i>Moxostoma cervinum</i>	blacktip jumprock
105	Cypriniformes	Cyprinidae	<i>Lythrurus atrapiculus</i>	blacktip shiner
106	Cypriniformes	Cyprinidae	<i>Luxilus zonatus</i>	bleeding shiner
107	Perciformes	Percidae	<i>Etheostoma blennioides</i>	blenny darter
108	Salmoniformes	Salmonidae	<i>Coregonus hoyi</i>	bloater
109	Perciformes	Percidae	<i>Etheostoma sanguifluum</i>	bloodfin darter
110	Perciformes	Gobiidae	<i>Ctenogobius fasciatus</i>	blotchcheek goby
111	Cypriniformes	Cyprinidae	<i>Erimystax insignis</i>	blotched chub
112	Cyprinodontiformes	Poeciliidae	<i>Gambusia senilis</i>	blotched gambusia
113	Perciformes	Percidae	<i>Percina burtoni</i>	blotchside logperch
114	Siluriformes	Ictaluridae	<i>Ictalurus furcatus</i>	blue catfish
115	Cypriniformes	Cyprinidae	<i>Gila coerulea</i>	blue chub
116	Scorpaeniformes	Cottidae	<i>Cottus caeruleomentum</i>	blue ridge sculpin

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
117	Cypriniformes	Cyprinidae	<i>Cyprinella caerulea</i>	blue shiner
118	Cypriniformes	Catostomidae	<i>Cycleptus elongatus</i>	blue sucker
119	Perciformes	Cichlidae	<i>Oreochromis aureus</i>	blue tilapia
120	Clupeiformes	Clupeidae	<i>Alosa aestivalis</i>	blueback herring
121	Perciformes	Elassomatidae	<i>Elassoma okatie</i>	bluebarred pygmy sunfish
122	Perciformes	Percidae	<i>Etheostoma camurum</i>	bluebreast darter
123	Cyprinodontiformes	Fundulidae	<i>Lucania goodei</i>	bluefin killifish
124	Cypriniformes	Cyprinidae	<i>Campostoma pauciradii</i>	bluefin stoneroller
125	Perciformes	Centrarchidae	<i>Lepomis macrochirus</i>	bluegill
126	Cypriniformes	Cyprinidae	<i>Nocomis leptcephalus</i>	bluehead chub
127	Cypriniformes	Cyprinidae	<i>Pteronotropis hubbsi</i>	bluehead shiner
128	Cypriniformes	Catostomidae	<i>Catostomus discobolus</i>	bluehead sucker
129	Cypriniformes	Cyprinidae	<i>Pteronotropis welaka</i>	bluenose shiner
130	Perciformes	Percidae	<i>Etheostoma jessiae</i>	blueside darter
131	Perciformes	Centrarchidae	<i>Enneacanthus gloriosus</i>	bluespotted sunfish
132	Perciformes	Percidae	<i>Percina cymatotaenia</i>	bluestripe darter
133	Cypriniformes	Cyprinidae	<i>Cyprinella callitaenia</i>	bluestripe shiner
134	Cypriniformes	Cyprinidae	<i>Cyprinella camura</i>	bluntface shiner
135	Perciformes	Percidae	<i>Etheostoma chlorosoma</i>	bluntnose darter
136	Cypriniformes	Cyprinidae	<i>Pimephales notatus</i>	bluntnose minnow
137	Cypriniformes	Cyprinidae	<i>Notropis simus</i>	bluntnose shiner
138	Salmoniformes	Salmonidae	<i>Prosopium gemmifer</i>	bonneville cisco
139	Salmoniformes	Salmonidae	<i>Prosopium spilonotus</i>	bonneville whitefish
140	Cypriniformes	Cyprinidae	<i>Gila elegans</i>	bonytail
141	Cypriniformes	Cyprinidae	<i>Gila boraxobius</i>	borax lake chub
142	Perciformes	Percidae	<i>Etheostoma wapiti</i>	boulder darter
143	Amiiformes	Amiidae	<i>Amia calva</i>	bowfin
144	Cypriniformes	Cyprinidae	<i>Hybagnathus hankinsoni</i>	brassy minnow
145	Cypriniformes	Catostomidae	<i>Catostomus columbianus</i>	bridgelip sucker
146	Cypriniformes	Cyprinidae	<i>Notropis bifrenatus</i>	bridle shiner
147	Perciformes	Percidae	<i>Etheostoma lynceum</i>	brighteye darter
148	Siluriformes	Ictaluridae	<i>Noturus miurus</i>	brindled madtom
149	Salmoniformes	Salmonidae	<i>Coregonus nasus</i>	broad whitefish
150	Cypriniformes	Cyprinidae	<i>Pteronotropis euryzonus</i>	broadstripe shiner
151	Cyprinodontiformes	Fundulidae	<i>Fundulus euryzonus</i>	broadstripe topminnow
152	Perciformes	Percidae	<i>Percina palmaris</i>	bronze darter
153	Perciformes	Percidae	<i>Etheostoma burri</i>	brook darter
154	Atheriniformes	Atherinopsidae	<i>Labidesthes sicculus</i>	brook silverside
155	Gasterosteiformes	Gasterosteidae	<i>Culaea inconstans</i>	brook stickleback

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
156	Salmoniformes	Salmonidae	<i>Salvelinus fontinalis</i>	brook trout
157	Siluriformes	Ictaluridae	<i>Ameiurus nebulosus</i>	brown bullhead
158	Perciformes	Percidae	<i>Etheostoma edwini</i>	brown darter
159	Siluriformes	Callichthyidae	<i>Hoplosternum littorale</i>	brown hoplo
160	Siluriformes	Ictaluridae	<i>Noturus phaeus</i>	brown madtom
161	Salmoniformes	Salmonidae	<i>Salmo trutta</i>	brown trout
162	Perciformes	Percidae	<i>Etheostoma bison</i>	buffalo darter
163	Cypriniformes	Cyprinidae	<i>Nocomis raneyi</i>	bull chub
164	Carcharhiniformes	Carcharhinidae	<i>Carcharhinus leucas</i>	bull shark
165	Salmoniformes	Salmonidae	<i>Salvelinus confluentus</i>	bull trout
166	Cypriniformes	Cyprinidae	<i>Pimephales vigilax</i>	bullhead minnow
167	Perciformes	Channidae	<i>Channa marulius</i>	bullseye snakehead
168	Gadiformes	Gadidae	<i>Lota lota</i>	burbot
169	Cypriniformes	Cyprinidae	<i>Macrhybopsis marconis</i>	burrhead chub
170	Cypriniformes	Cyprinidae	<i>Notropis asperifrons</i>	burrhead shiner
171	Perciformes	Cichlidae	<i>Cichla ocellaris</i>	butterfly peacock bass
172	Siluriformes	Ictaluridae	<i>Noturus taylori</i>	caddo madtom
173	Cypriniformes	Cyprinidae	<i>Notropis cahabae</i>	cahaba shiner
174	Cypriniformes	Cyprinidae	<i>Hesperoleucus symmetricus</i>	california roach
175	Perciformes	Percidae	<i>Etheostoma osburni</i>	candy darter
176	Cypriniformes	Cyprinidae	<i>Notropis mekistocholas</i>	cape fear shiner
177	Cypriniformes	Cyprinidae	<i>Luxilus cardinalis</i>	cardinal shiner
178	Cypriniformes	Cyprinidae	<i>Notropis percobromus</i>	carmine shiner
179	Perciformes	Percidae	<i>Etheostoma collis</i>	carolina darter
180	Siluriformes	Ictaluridae	<i>Noturus furiosus</i>	carolina madtom
181	Perciformes	Elassomatidae	<i>Elassoma boehlkei</i>	carolina pygmy sunfish
182	Esociformes	Umbridae	<i>Umbra limi</i>	central mudminnow
183	Cypriniformes	Cyprinidae	<i>Campostoma anomalum</i>	central stoneroller
184	Esociformes	Esocidae	<i>Esox niger</i>	chain pickerel
185	Perciformes	Percidae	<i>Percina nevisense</i>	chainback darter
186	Siluriformes	Ictaluridae	<i>Ictalurus punctatus</i>	channel catfish
187	Perciformes	Percidae	<i>Percina copelandi</i>	channel darter
188	Cypriniformes	Cyprinidae	<i>Notropis wickliffi</i>	channel shiner
189	Siluriformes	Ictaluridae	<i>Noturus flavater</i>	checkered madtom
190	Perciformes	Percidae	<i>Etheostoma scotti</i>	cherokee darter
191	Perciformes	Percidae	<i>Etheostoma etnieri</i>	cherry darter
192	Cypriniformes	Cyprinidae	<i>Lythrurus roseipinnis</i>	cherryfin shiner
193	Petromyzontiformes	Petromyzontidae	<i>Ichthyomyzon castaneus</i>	chestnut lamprey
194	Perciformes	Percidae	<i>Etheostoma cervus</i>	chickasaw darter

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
195	Cypriniformes	Cyprinidae	<i>Gila nigrescens</i>	chihuahua chub
196	Cypriniformes	Cyprinidae	<i>Notropis chihuahua</i>	chihuahua shiner
197	Salmoniformes	Salmonidae	<i>Oncorhynchus tshawytscha</i>	chinook salmon
198	Cypriniformes	Cyprinidae	<i>Acrocheilus alutaceus</i>	chiselmouth
199	Perciformes	Percidae	<i>Etheostoma davisoni</i>	choctawhatchee darter
200	Perciformes	Percidae	<i>Etheostoma hopkinsi</i>	christmas darter
201	Cypriniformes	Cyprinidae	<i>Notropis potteri</i>	chub shiner
202	Salmoniformes	Salmonidae	<i>Oncorhynchus keta</i>	chum salmon
203	Salmoniformes	Salmonidae	<i>Coregonus artedii</i>	cisco
204	Cypriniformes	Cyprinidae	<i>Hybopsis winchelli</i>	clear chub
205	Cyprinodontiformes	Poeciliidae	<i>Gambusia heterochir</i>	clear creek gambusia
206	Cypriniformes	Cyprinidae	<i>Pogonichthys ciscaides</i>	clear lake splittail
207	Perciformes	Gobiidae	<i>Microgobius gulosus</i>	clown goby
208	Osteoglossiformes	Notopteridae	<i>Chitala ornata</i>	clown knifefish
209	Perciformes	Percidae	<i>Percina brevicauda</i>	coal darter
210	Perciformes	Percidae	<i>Etheostoma colorosum</i>	coastal darter
211	Cypriniformes	Cyprinidae	<i>Notropis petersoni</i>	coastal shiner
212	Scorpaeniformes	Cottidae	<i>Cottus aleuticus</i>	coastrange sculpin
213	Salmoniformes	Salmonidae	<i>Oncorhynchus kisutch</i>	coho salmon
214	Perciformes	Percidae	<i>Etheostoma ditrema</i>	coldwater darter
215	Cypriniformes	Cyprinidae	<i>Ptychocheilus lucius</i>	colorado pikeminnow
216	Scorpaeniformes	Cottidae	<i>Cottus hubbsi</i>	columbia sculpin
217	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon elegans</i>	comanche springs pupfish
218	Cypriniformes	Cyprinidae	<i>Notropis amoenus</i>	comely shiner
219	Cypriniformes	Cyprinidae	<i>Cyprinus carpio</i>	common carp
220	Cypriniformes	Cyprinidae	<i>Luxilus cornutus</i>	common shiner
221	Perciformes	Centropomidae	<i>Centropomus undecimalis</i>	common snook
222	Perciformes	Percidae	<i>Percina jenkinsi</i>	conasauga logperch
223	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon eximius</i>	conchos pupfish
224	Perciformes	Cichlidae	<i>Cichlasoma nigrofasciatum</i>	convict cichlid
225	Perciformes	Percidae	<i>Etheostoma coosae</i>	coosa darter
226	Cypriniformes	Cyprinidae	<i>Notropis xaenocephalus</i>	coosa shiner
227	Perciformes	Percidae	<i>Etheostoma aquali</i>	coppercheek darter
228	Perciformes	Percidae	<i>Etheostoma basilare</i>	corrugated darter
229	Cypriniformes	Cyprinidae	<i>Semotilus atromaculatus</i>	creek chub
230	Cypriniformes	Catostomidae	<i>Erimyzon oblongus</i>	creek chubsucker
231	Perciformes	Percidae	<i>Etheostoma collettei</i>	creole darter
232	Cypriniformes	Cyprinidae	<i>Luxilus cerasinus</i>	crescent shiner
233	Perciformes	Gobiidae	<i>Lophogobius cyprinoides</i>	crested goby

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234	Perciformes	Belontiidae	<i>Trichopsis vittata</i>	croaking gourami
235	Perciformes	Percidae	<i>Etheostoma corona</i>	crown darter
236	Perciformes	Percidae	<i>Crystallaria asprella</i>	crystal darter
237	Cypriniformes	Catostomidae	<i>Chasmistes cujus</i>	cui-ui
238	Perciformes	Percidae	<i>Etheostoma susanae</i>	cumberland darter
239	Perciformes	Percidae	<i>Etheostoma uniporum</i>	current darter
240	Cypriniformes	Cyprinidae	<i>Exoglossum maxillingua</i>	cutlip minnow
241	Salmoniformes	Salmonidae	<i>Oncorhynchus clarkii</i>	cutthroat trout
242	Perciformes	Percidae	<i>Etheostoma proeliare</i>	cypress darter
243	Cypriniformes	Cyprinidae	<i>Hybognathus hayi</i>	cypress minnow
244	Perciformes	Gobiidae	<i>Ctenogobius boleosoma</i>	darler goby
245	Salmoniformes	Salmonidae	<i>Coregonus johanna</i>	deepwater cisco
246	Scorpaeniformes	Cottidae	<i>Myoxocephalus thompsonii</i>	deepwater sculpin
247	Salmoniformes	Osmeridae	<i>Hypomesus transpacificus</i>	delta smelt
248	Cypriniformes	Cyprinidae	<i>Eremichthys acros</i>	desert dace
249	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon macularius</i>	desert pupfish
250	Cypriniformes	Catostomidae	<i>Catostomus clarkii</i>	desert sucker
251	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon diabolis</i>	devils hole pupfish
252	Cypriniformes	Cyprinidae	<i>Dionda diaboli</i>	devils river minnow
253	Cypriniformes	Cyprinidae	<i>Semotilus thoreauianus</i>	dixie chub
254	Perciformes	Centrarchidae	<i>Lepomis marginatus</i>	dollar sunfish
255	Salmoniformes	Salmonidae	<i>Salvelinus malma</i>	dolly varden
256	Perciformes	Percidae	<i>Percina sciera</i>	dusky darter
257	Cypriniformes	Cyprinidae	<i>Notropis cummingsae</i>	dusky shiner
258	Cypriniformes	Cyprinidae	<i>Luxilus pilsbryi</i>	duskystripe shiner
259	Perciformes	Percidae	<i>Etheostoma percnurum</i>	duskytail darter
260	Cypriniformes	Cyprinidae	<i>Rhinichthys atratulus</i>	eastern blacknose dace
261	Cyprinodontiformes	Poeciliidae	<i>Gambusia holbrooki</i>	eastern mosquitofish
262	Esociformes	Umbridae	<i>Umbra pygmaea</i>	eastern mudminnow
263	Perciformes	Percidae	<i>Ammocrypta pellucida</i>	eastern sand darter
264	Cypriniformes	Cyprinidae	<i>Hybognathus regius</i>	eastern silvery minnow
265	Perciformes	Percidae	<i>Etheostoma pseudovulatum</i>	egg-mimic darter
266	Siluriformes	Ictaluridae	<i>Noturus elegans</i>	elegant madtom
267	Perciformes	Percidae	<i>Etheostoma baileyi</i>	emerald darter
268	Cypriniformes	Cyprinidae	<i>Notropis atherinoides</i>	emerald shiner
269	Gasterosteiformes	Gasterosteidae	<i>Gasterosteus aculeatus</i>	espinucho
270	Perciformes	Percidae	<i>Etheostoma etowahae</i>	etowah darter
271	Salmoniformes	Osmeridae	<i>Thaleichthys pacificus</i>	eulachon
272	Perciformes	Elassomatidae	<i>Elassoma evergladei</i>	everglades pygmy sunfish

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273	Cypriniformes	Cyprinidae	<i>Semotilus corporalis</i>	fallfish
274	Perciformes	Percidae	<i>Etheostoma flabellare</i>	fantail darter
275	Perciformes	Eleotridae	<i>Dormitator maculatus</i>	fat sleeper
276	Cypriniformes	Cyprinidae	<i>Pimephales promelas</i>	fathead minnow
277	Cypriniformes	Cyprinidae	<i>Phenacobius crassilabrum</i>	fatlips minnow
278	Cypriniformes	Cyprinidae	<i>Cyprinella pyrrhomelas</i>	fieryblack shiner
279	Cypriniformes	Cyprinidae	<i>Phoxinus neogaeus</i>	finescale dace
280	Perciformes	Percidae	<i>Etheostoma pyrrhogaster</i>	firebelly darter
281	Perciformes	Cichlidae	<i>Cichlasoma meeki</i>	firemouth cichlid
282	Cypriniformes	Cyprinidae	<i>Pteronotropis signipinnis</i>	flagfin shiner
283	Cyprinodontiformes	Cyprinodontidae	<i>Jordanella floridae</i>	flagfish
284	Cypriniformes	Cyprinidae	<i>Hemitemia flamma</i>	flame chub
285	Cypriniformes	Catostomidae	<i>Catostomus latipinnis</i>	flannelmouth sucker
286	Siluriformes	Ictaluridae	<i>Ameiurus platycephalus</i>	flat bullhead
287	Siluriformes	Ictaluridae	<i>Pyloodictis olivaris</i>	flathead catfish
288	Cypriniformes	Cyprinidae	<i>Platygobio gracilis</i>	flathead chub
289	Perciformes	Centrarchidae	<i>Centrarchus macropterus</i>	flier
290	Lepisosteiformes	Lepisosteidae	<i>Lepisosteus platyrhincus</i>	florida gar
291	Perciformes	Percidae	<i>Ammocrypta bifascia</i>	florida sand darter
292	Cypriniformes	Cyprinidae	<i>Notropis edwardraneyi</i>	fluvial shiner
293	Perciformes	Percidae	<i>Etheostoma fonticola</i>	fountain darter
294	Scorpaeniformes	Cottidae	<i>Myoxocephalus quadricornis</i>	fourhorn sculpin
295	Gasterosteiformes	Gasterosteidae	<i>Apeltes quadracus</i>	fourspine stickleback
296	Perciformes	Percidae	<i>Percina stictogaster</i>	frecklebelly darter
297	Siluriformes	Ictaluridae	<i>Noturus munitus</i>	frecklebelly madtom
298	Perciformes	Percidae	<i>Percina lenticula</i>	freckled darter
299	Siluriformes	Ictaluridae	<i>Noturus nocturnus</i>	freckled madtom
300	Perciformes	Sciaenidae	<i>Aplodinotus grunniens</i>	freshwater drum
301	Perciformes	Gobiidae	<i>Ctenogobius shufeldti</i>	freshwater goby
302	Perciformes	Percidae	<i>Etheostoma crossopterum</i>	fringed darter
303	Cypriniformes	Cyprinidae	<i>Notropis buechanani</i>	ghost shiner
304	Cyprinodontiformes	Aplocheilidae	<i>Rivulus hartii</i>	giant rivulus
305	Cypriniformes	Cyprinidae	<i>Gila intermedia</i>	gila chub
306	Cyprinodontiformes	Poeciliidae	<i>Poeciliopsis occidentalis</i>	gila topminnow
307	Salmoniformes	Salmonidae	<i>Oncorhynchus gilae</i>	gila trout
308	Perciformes	Percidae	<i>Percina evides</i>	gilt darter
309	Clupeiformes	Clupeidae	<i>Dorosoma cepedianum</i>	gizzard shad
310	Perciformes	Percidae	<i>Etheostoma vitreum</i>	glassy darter
311	Perciformes	Percidae	<i>Etheostoma denoncourti</i>	golden darter

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312	Cypriniformes	Catostomidae	<i>Moxostoma erythrurum</i>	golden redhorse
313	Cypriniformes	Cyprinidae	<i>Notemigonus crysoleucas</i>	golden shiner
314	Cyprinodontiformes	Fundulidae	<i>Fundulus chrysotus</i>	golden topminnow
315	Hiodontiformes	Hiodontidae	<i>Hiodon alosoides</i>	goldeye
316	Cypriniformes	Cyprinidae	<i>Carassius auratus</i>	goldfish
317	Perciformes	Percidae	<i>Percina aurolineata</i>	goldline darter
318	Perciformes	Percidae	<i>Etheostoma parvipinne</i>	goldstripe darter
319	Cypriniformes	Cyprinidae	<i>Ctenopharyngodon idella</i>	grass carp
320	Cypriniformes	Cyprinidae	<i>Erimystax x-punctatus</i>	gravel chub
321	Cypriniformes	Catostomidae	<i>Moxostoma congestum</i>	gray redhorse
322	Perciformes	Lutjanidae	<i>Lutjanus griseus</i>	gray snapper
323	Cypriniformes	Catostomidae	<i>Moxostoma lachneri</i>	greater jumprock
324	Cypriniformes	Catostomidae	<i>Moxostoma valenciennesi</i>	greater redhorse
325	Acipenseriformes	Acipenseridae	<i>Acipenser medirostris</i>	green sturgeon
326	Perciformes	Centrarchidae	<i>Lepomis cyanellus</i>	green sunfish
327	Cyprinodontiformes	Poeciliidae	<i>Xiphophorus hellerii</i>	green swordtail
328	Perciformes	Percidae	<i>Etheostoma jordani</i>	greenbreast darter
329	Perciformes	Percidae	<i>Etheostoma chlorbranchium</i>	greenfin darter
330	Cypriniformes	Cyprinidae	<i>Cyprinella chloristia</i>	greenfin shiner
331	Cypriniformes	Cyprinidae	<i>Notropis chlorocephalus</i>	greenhead shiner
332	Perciformes	Percidae	<i>Etheostoma blennioides</i>	greenside darter
333	Perciformes	Percidae	<i>Etheostoma lepidum</i>	greenthroat darter
334	Perciformes	Centrarchidae	<i>Micropterus treculii</i>	guadalupe bass
335	Cyprinodontiformes	Fundulidae	<i>Fundulus parvipinnis</i>	guadalupe cardinalfish
336	Cypriniformes	Cyprinidae	<i>Dionda nigrotaeniata</i>	guadalupe roundnose minnow
337	Perciformes	Percidae	<i>Etheostoma oophylax</i>	guardian darter
338	Perciformes	Eleotridae	<i>Guavina guavina</i>	guavina
339	Perciformes	Percidae	<i>Etheostoma swaini</i>	gulf darter
340	Cyprinodontiformes	Fundulidae	<i>Fundulus grandis</i>	gulf killifish
341	Perciformes	Percidae	<i>Percina suttkusi</i>	gulf logperch
342	Gasterosteiformes	Syngnathidae	<i>Syngnathus scovelli</i>	gulf pipefish
343	Cyprinodontiformes	Poeciliidae	<i>Poecilia reticulata</i>	guppy
344	Cypriniformes	Cyprinidae	<i>Mylopharodon conocephalus</i>	hardhead
345	Siluriformes	Ariidae	<i>Ariopsis felis</i>	hardhead catfish
346	Cypriniformes	Catostomidae	<i>Moxostoma lacerum</i>	harelip sucker
347	Perciformes	Percidae	<i>Etheostoma histrio</i>	harlequin darter
348	Siluriformes	Ictaluridae	<i>Ictalurus lupus</i>	headwater catfish
349	Cypriniformes	Cyprinidae	<i>Gila nigra</i>	headwater chub
350	Perciformes	Percidae	<i>Etheostoma lawrencei</i>	headwater darter

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351	Clupeiformes	Clupeidae	<i>Alosa mediocris</i>	hickory shad
352	Cypriniformes	Cyprinidae	<i>Hybopsis hypsinotus</i>	highback chub
353	Cypriniformes	Catostomidae	<i>Carpiodes velifer</i>	highfin carpsucker
354	Cypriniformes	Cyprinidae	<i>Notropis altipinnis</i>	highfin shiner
355	Perciformes	Percidae	<i>Etheostoma kantuckeense</i>	highland rim darter
356	Cypriniformes	Cyprinidae	<i>Notropis micropteryx</i>	highland shiner
357	Cypriniformes	Cyprinidae	<i>Notropis hypsilepis</i>	highscale shiner
358	Cypriniformes	Cyprinidae	<i>Lavinia exilicauda</i>	hitch
359	Pleuronectiformes	Achiridae	<i>Trinectes maculatus</i>	hogchoker
360	Perciformes	Percidae	<i>Etheostoma brevirostrum</i>	holiday darter
361	Cypriniformes	Cyprinidae	<i>Nocomis biguttatus</i>	hornyhead chub
362	Cypriniformes	Cyprinidae	<i>Gila cypha</i>	humpback chub
363	Salmoniformes	Salmonidae	<i>Coregonus pidschian</i>	humpback whitefish
364	Cypriniformes	Cyprinidae	<i>Leuciscus idus</i>	ide
365	Salmoniformes	Salmonidae	<i>Stenodus leucichthys</i>	inconnu
366	Atheriniformes	Atherinopsidae	<i>Menidia beryllina</i>	inland silverside
367	Perciformes	Percidae	<i>Etheostoma exile</i>	iowa darter
368	Perciformes	Gerreidae	<i>Diapterus auratus</i>	irish pompano
369	Cypriniformes	Cyprinidae	<i>Notropis chalybaeus</i>	ironcolor shiner
370	Perciformes	Cichlidae	<i>Cichlasoma octofasciatum</i>	jack dempsey
371	Perciformes	Cichlidae	<i>Cichlasoma managuense</i>	jaguar guapote
372	Perciformes	Percidae	<i>Etheostoma nigrum</i>	johnny darter
373	Cypriniformes	Catostomidae	<i>Chasmistes liorus</i>	june sucker
374	Perciformes	Percidae	<i>Etheostoma kanawhae</i>	kanawha darter
375	Cypriniformes	Cyprinidae	<i>Phenacobius teretulus</i>	kanawha minnow
376	Perciformes	Percidae	<i>Etheostoma rafinesquei</i>	kentucky darter
377	Petromyzontiformes	Petromyzontidae	<i>Lampetra hubbsi</i>	kern brook lamprey
378	Cypriniformes	Cyprinidae	<i>Notropis ortenburgeri</i>	kiamichi shiner
379	Salmoniformes	Salmonidae	<i>Coregonus kiyi</i>	kiyi
380	Scorpaeniformes	Cottidae	<i>Cottus princeps</i>	klamath lake sculpin
381	Petromyzontiformes	Petromyzontidae	<i>Lampetra similis</i>	klamath lamprey
382	Cypriniformes	Catostomidae	<i>Catostomus snyderi</i>	klamath largescale sucker
383	Cypriniformes	Catostomidae	<i>Catostomus rimitulus</i>	klamath smallscale sucker
384	Elopiformes	Elopidae	<i>Elops saurus</i>	ladyfish
385	Cypriniformes	Cyprinidae	<i>Richardsonius egregius</i>	lahontan redside
386	Cypriniformes	Cyprinidae	<i>Couesius plumbeus</i>	lake chub
387	Cypriniformes	Catostomidae	<i>Erimyzon sucetta</i>	lake chubsucker
388	Acipenseriformes	Acipenseridae	<i>Acipenser fulvescens</i>	lake sturgeon
389	Salmoniformes	Salmonidae	<i>Salvelinus namaycush</i>	lake trout

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
390	Salmoniformes	Salmonidae	<i>Coregonus clupeaformis</i>	lake whitefish
391	Perciformes	Centrarchidae	<i>Micropterus salmoides</i>	largemouth bass
392	Cypriniformes	Cyprinidae	<i>Camptostoma oligolepis</i>	largescale stoneroller
393	Cypriniformes	Catostomidae	<i>Catostomus macrocheilus</i>	largescale sucker
394	Perciformes	Eleotridae	<i>Eleotris amblyopsis</i>	largescaled spinycheek
395	Cyprinodontiformes	Poeciliidae	<i>Gambusia geiseri</i>	largespring gambusia
396	Cypriniformes	Cyprinidae	<i>Rhinichthys deaconi</i>	las vegas dace
397	Cypriniformes	Cyprinidae	<i>Phoxinus saylora</i>	laurel dace
398	Petromyzontiformes	Petromyzontidae	<i>Lampetra aepyptera</i>	least brook lamprey
399	Cypriniformes	Cyprinidae	<i>Iotichthys phlegethontis</i>	least chub
400	Salmoniformes	Salmonidae	<i>Coregonus sardinella</i>	least cisco
401	Perciformes	Percidae	<i>Etheostoma microperca</i>	least darter
402	Cyprinodontiformes	Poeciliidae	<i>Heterandria formosa</i>	least killifish
403	Siluriformes	Ictaluridae	<i>Noturus hildebrandi</i>	least madtom
404	Cypriniformes	Cyprinidae	<i>Snyderichthys copei</i>	leatherside chub
405	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon bovinus</i>	leon springs pupfish
406	Cypriniformes	Cyprinidae	<i>Rhinichthys falcatus</i>	leopard dace
407	Perciformes	Percidae	<i>Percina pantherina</i>	leopard darter
408	Cypriniformes	Cyprinidae	<i>Hybopsis lineapunctata</i>	lined chub
409	Cyprinodontiformes	Fundulidae	<i>Fundulus lineolatus</i>	lined topminnow
410	Perciformes	Percidae	<i>Etheostoma chuckwachatte</i>	lipstick darter
411	Cypriniformes	Cyprinidae	<i>Lepidomeda vittata</i>	little colorado spinedace
412	Cypriniformes	Cyprinidae	<i>Rhinichthys cobitis</i>	loach minnow
413	Perciformes	Percidae	<i>Percina caprodes</i>	logperch
414	Perciformes	Percidae	<i>Etheostoma neopterum</i>	lollypop darter
415	Perciformes	Centrarchidae	<i>Lepomis megalotis</i>	longear sunfish
416	Cypriniformes	Cyprinidae	<i>Agosia chrysogaster</i>	longfin dace
417	Perciformes	Percidae	<i>Etheostoma longimanum</i>	longfin darter
418	Salmoniformes	Osmeridae	<i>Spirinchus thaleichthys</i>	longfin smelt
419	Perciformes	Percidae	<i>Percina macrocephala</i>	longhead darter
420	Perciformes	Gobiidae	<i>Gillichthys mirabilis</i>	longjaw mudsucker
421	Cypriniformes	Cyprinidae	<i>Rhinichthys cataractae</i>	longnose dace
422	Perciformes	Percidae	<i>Percina nasuta</i>	longnose darter
423	Lepisosteiformes	Lepisosteidae	<i>Lepisosteus osseus</i>	longnose gar
424	Cypriniformes	Cyprinidae	<i>Notropis longirostris</i>	longnose shiner
425	Cypriniformes	Catostomidae	<i>Catostomus catostomus</i>	longnose sucker
426	Cypriniformes	Catostomidae	<i>Deltistes luxatus</i>	lost river sucker
427	Elopiformes	Elopidae	<i>Elops affinis</i>	machete
428	Scorpaeniformes	Cottidae	<i>Cottus bendirei</i>	malheur sculpin

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429	Cypriniformes	Cyprinidae	<i>Dionda argentosa</i>	manantial roundnose minnow
430	Cyprinodontiformes	Poeciliidae	<i>Gambusia rhizophorae</i>	mangrove gambusia
431	Cyprinodontiformes	Aplocheilidae	<i>Rivulus marmoratus</i>	mangrove rivulus
432	Scorpaeniformes	Cottidae	<i>Cottus klamathensis</i>	marbled sculpin
433	Siluriformes	Ictaluridae	<i>Noturus insignis</i>	margined madtom
434	Scorpaeniformes	Cottidae	<i>Cottus marginatus</i>	margined sculpin
435	Cyprinodontiformes	Fundulidae	<i>Fundulus confluentus</i>	marsh killifish
436	Perciformes	Percidae	<i>Etheostoma sellare</i>	maryland darter
437	Perciformes	Cichlidae	<i>Cichlasoma urophthalmus</i>	mayan cichlid
438	Perciformes	Gobiidae	<i>Ctenogobius claytonii</i>	mexican goby
439	Cyprinodontiformes	Poeciliidae	<i>Poecilia sphenops</i>	mexican molly
440	Cypriniformes	Catostomidae	<i>Moxostoma austrinum</i>	mexican redbhorse
441	Cypriniformes	Cyprinidae	<i>Campostoma ornatum</i>	mexican stoneroller
442	Characiformes	Characidae	<i>Astyanax mexicanus</i>	mexican tetra
443	Perciformes	Cichlidae	<i>Cichlasoma citrinellum</i>	midas cichlid
444	Petromyzontiformes	Petromyzontidae	<i>Lampetra minima</i>	miller lake lamprey
445	Cypriniformes	Cyprinidae	<i>Notropis volucellus</i>	mimic shiner
446	Cypriniformes	Cyprinidae	<i>Notropis spectrunculus</i>	mirror shiner
447	Atheriniformes	Atherinopsidae	<i>Menidia audens</i>	mississippi silverside
448	Cypriniformes	Cyprinidae	<i>Hybognathus nuchalis</i>	mississippi silvery minnow
449	Perciformes	Percidae	<i>Etheostoma tetrazonum</i>	missouri saddled darter
450	Cypriniformes	Cyprinidae	<i>Moapa coriacea</i>	moapa dace
451	Perciformes	Percidae	<i>Percina kathae</i>	mobile logperch
452	Cypriniformes	Catostomidae	<i>Catostomus microps</i>	modoc sucker
453	Hiodontiformes	Hiodontidae	<i>Hiodon tergisus</i>	mooneye
454	Scorpaeniformes	Cottidae	<i>Cottus bairdii</i>	mottled sculpin
455	Petromyzontiformes	Petromyzontidae	<i>Ichthyomyzon greeleyi</i>	mountain brook lamprey
456	Siluriformes	Ictaluridae	<i>Noturus eleutherus</i>	mountain madtom
457	Mugiliformes	Mugilidae	<i>Agonostomus monticola</i>	mountain mullet
458	Cypriniformes	Cyprinidae	<i>Phoxinus oreas</i>	mountain redbelly dace
459	Cypriniformes	Cyprinidae	<i>Lythrurus lirus</i>	mountain shiner
460	Cypriniformes	Catostomidae	<i>Catostomus platyrhynchus</i>	mountain sucker
461	Salmoniformes	Salmonidae	<i>Prosopium williamsoni</i>	mountain whitefish
462	Perciformes	Cichlidae	<i>Oreochromis mossambicus</i>	mozambique tilapia
463	Perciformes	Percidae	<i>Etheostoma asprigene</i>	mud darter
464	Perciformes	Centrarchidae	<i>Acantharchus pomotis</i>	mud sunfish
465	Cyprinodontiformes	Fundulidae	<i>Fundulus heteroclitus</i>	mummichog
466	Esociformes	Esocidae	<i>Esox masquinongy</i>	muskellunge
467	Perciformes	Gobiidae	<i>Gobiosoma bosc</i>	naked goby

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468	Perciformes	Percidae	<i>Ammocrypta beanii</i>	naked sand darter
469	Siluriformes	Ictaluridae	<i>Noturus placidus</i>	neosho madtom
470	Cypriniformes	Cyprinidae	<i>Notropis scabriceps</i>	new river shiner
471	Perciformes	Percidae	<i>Etheostoma nianguae</i>	niangua darter
472	Perciformes	Cichlidae	<i>Oreochromis niloticus</i>	nile tilapia
473	Gasterosteiformes	Gasterosteidae	<i>Pungitius pungitius</i>	ninespine stickleback
474	Petromyzontiformes	Petromyzontidae	<i>Ichthyomyzon fossor</i>	northern brook lamprey
475	Percopsiformes	Amblyopsidae	<i>Amblyopsis spelaea</i>	northern cavefish
476	Cypriniformes	Catostomidae	<i>Hypentelium nigricans</i>	northern hog sucker
477	Siluriformes	Ictaluridae	<i>Noturus stigmosus</i>	northern madtom
478	Esociformes	Esocidae	<i>Esox lucius</i>	northern pike
479	Cypriniformes	Cyprinidae	<i>Ptychocheilus oregonensis</i>	northern pikeminnow
480	Cyprinodontiformes	Fundulidae	<i>Fundulus kansae</i>	northern plains killifish
481	Cypriniformes	Cyprinidae	<i>Phoxinus eos</i>	northern redbelly dace
482	Cyprinodontiformes	Fundulidae	<i>Fundulus catenatus</i>	northern studfish
483	Cypriniformes	Catostomidae	<i>Moxostoma collapsum</i>	notchlip redhorse
484	Cypriniformes	Cyprinidae	<i>Dionda serena</i>	nueces roundnose minnow
485	Cypriniformes	Cyprinidae	<i>Cyprinella callisema</i>	ocmulgee shiner
486	Petromyzontiformes	Petromyzontidae	<i>Ichthyomyzon bdellium</i>	ohio lamprey
487	Perciformes	Percidae	<i>Etheostoma okaloosae</i>	okaloosa darter
488	Perciformes	Elassomatidae	<i>Elassoma okefenokee</i>	okefenokee pygmy sunfish
489	Perciformes	Percidae	<i>Percina squamata</i>	olive darter
490	Esociformes	Umbridae	<i>Novumbra hubbsi</i>	olympic mudminnow
491	Gasterosteiformes	Syngnathidae	<i>Microphis brachyurus</i>	opossum pipefish
492	Perciformes	Percidae	<i>Etheostoma radiosum</i>	orangebelly darter
493	Perciformes	Percidae	<i>Etheostoma bellum</i>	orangefin darter
494	Siluriformes	Ictaluridae	<i>Noturus gilberti</i>	orangefin madtom
495	Cypriniformes	Cyprinidae	<i>Notropis ammophilus</i>	orangefin shiner
496	Perciformes	Sciaenidae	<i>Cynoscion xanthalmus</i>	orangemouth corvina
497	Perciformes	Centrarchidae	<i>Lepomis humilis</i>	orangespotted sunfish
498	Cypriniformes	Cyprinidae	<i>Pteronotropis merlini</i>	orangetail shiner
499	Perciformes	Percidae	<i>Etheostoma spectabile</i>	orangethroat darter
500	Cypriniformes	Cyprinidae	<i>Oregonichthys crameri</i>	oregon chub
501	Cypriniformes	Cobitidae	<i>Misgurnus anguillicaudatus</i>	oriental weatherfish
502	Siluriformes	Loricariidae	<i>Pterygoplichthys multiradiatus</i>	orinoco sailfin catfish
503	Perciformes	Cichlidae	<i>Astronotus ocellatus</i>	oscar
504	Siluriformes	Ictaluridae	<i>Noturus lachneri</i>	ouachita madtom
505	Cypriniformes	Cyprinidae	<i>Lythrurus snelsoni</i>	ouachita shiner
506	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon radiosus</i>	owens pupfish

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507	Cypriniformes	Catostomidae	<i>Catostomus fumeiventris</i>	owens sucker
508	Perciformes	Centrarchidae	<i>Ambloplites constellatus</i>	ozark bass
509	Percopsiformes	Amblyopsidae	<i>Amblyopsis rosae</i>	ozark cavefish
510	Cypriniformes	Cyprinidae	<i>Erimystax harrisi</i>	ozark chub
511	Perciformes	Percidae	<i>Percina fulvitaenia</i>	ozark logperch
512	Siluriformes	Ictaluridae	<i>Noturus albater</i>	ozark madtom
513	Cypriniformes	Cyprinidae	<i>Notropis nubilus</i>	ozark minnow
514	Scorpaeniformes	Cottidae	<i>Cottus hypselurus</i>	ozark sculpin
515	Cypriniformes	Cyprinidae	<i>Notropis ozarcanus</i>	ozark shiner
516	Petromyzontiformes	Petromyzontidae	<i>Lampetra tridentata</i>	pacific lamprey
517	Scorpaeniformes	Cottidae	<i>Leptocottus armatus</i>	pacific staghorn sculpin
518	Acipenseriformes	Polyodontidae	<i>Polyodon spathula</i>	paddlefish
519	Cypriniformes	Cyprinidae	<i>Lepidomeda altivelis</i>	pahranagat spinedace
520	Cyprinodontiformes	Goodeidae	<i>Empetrichthys latos</i>	pahrump poolfish
521	Scorpaeniformes	Cottidae	<i>Cottus beldingii</i>	paiute sculpin
522	Perciformes	Percidae	<i>Etheostoma pallididorsum</i>	paleback darter
523	Cypriniformes	Cyprinidae	<i>Notropis albizonatus</i>	palezone shiner
524	Cypriniformes	Cyprinidae	<i>Hybopsis amnis</i>	pallid shiner
525	Acipenseriformes	Acipenseridae	<i>Scaphirhynchus albus</i>	pallid sturgeon
526	Cypriniformes	Catostomidae	<i>Moxostoma pisolabrum</i>	pealip redhorse
527	Cypriniformes	Cyprinidae	<i>Mylocheilus caurinus</i>	peamouth
528	Cypriniformes	Cyprinidae	<i>Margariscus margarita</i>	pearl dace
529	Perciformes	Percidae	<i>Percina aurora</i>	pearl darter
530	Cyprinodontiformes	Poeciliidae	<i>Gambusia nobilis</i>	pecos gambusia
531	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon pecosensis</i>	pecos pupfish
532	Cypriniformes	Cyprinidae	<i>Macrhybopsis tetranema</i>	peppered chub
533	Cypriniformes	Cyprinidae	<i>Notropis perpallidus</i>	peppered shiner
534	Cypriniformes	Cyprinidae	<i>Notropis orca</i>	phantom shiner
535	Perciformes	Percidae	<i>Percina crassa</i>	piedmont darter
536	Perciformes	Haemulidae	<i>Orthopristis chrysoptera</i>	pigfish
537	Cyprinodontiformes	Poeciliidae	<i>Belonesox belizanus</i>	pike killifish
538	Perciformes	Percidae	<i>Etheostoma mariae</i>	pinewoods darter
539	Cypriniformes	Cyprinidae	<i>Lythrurus matutinus</i>	pinewoods shiner
540	Perciformes	Sparidae	<i>Lagodon rhomboides</i>	pinfish
541	Salmoniformes	Salmonidae	<i>Oncorhynchus gorbuscha</i>	pink salmon
542	Percopsiformes	Aphredoderidae	<i>Aphredoderus sayanus</i>	pirate perch
543	Scorpaeniformes	Cottidae	<i>Cottus pitensis</i>	pit sculpin
544	Petromyzontiformes	Petromyzontidae	<i>Lampetra lethophaga</i>	pit-klamath brook lamprey
545	Cyprinodontiformes	Fundulidae	<i>Fundulus zebrinus</i>	plains killifish

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546	Cypriniformes	Cyprinidae	<i>Hybognathus placitus</i>	plains minnow
547	Cyprinodontiformes	Fundulidae	<i>Fundulus sciadicus</i>	plains topminnow
548	Cypriniformes	Cyprinidae	<i>Cyprinella lepida</i>	plateau shiner
549	Salmoniformes	Osmeridae	<i>Hypomesus olidus</i>	pond smelt
550	Cypriniformes	Cyprinidae	<i>Notropis ariommus</i>	popeye shiner
551	Cyprinodontiformes	Poeciliidae	<i>Poeciliopsis gracilis</i>	porthole livebearer
552	Scorpaeniformes	Cottidae	<i>Cottus girardi</i>	potomac sculpin
553	Cypriniformes	Cyprinidae	<i>Macrhybopsis australis</i>	prairie chub
554	Cypriniformes	Cyprinidae	<i>Lythrurus bellus</i>	pretty shiner
555	Scorpaeniformes	Cottidae	<i>Cottus asper</i>	prickly sculpin
556	Cypriniformes	Cyprinidae	<i>Cyprinella proserpina</i>	proserpine shiner
557	Cypriniformes	Cyprinidae	<i>Opsopoeodus emiliae</i>	pugnose minnow
558	Cypriniformes	Cyprinidae	<i>Notropis anogenus</i>	pugnose shiner
559	Perciformes	Centrarchidae	<i>Lepomis gibbosus</i>	pumpkinseed
560	Cyprinodontiformes	Fundulidae	<i>Leptolucania ommata</i>	pygmy killifish
561	Siluriformes	Ictaluridae	<i>Noturus stanauli</i>	pygmy madtom
562	Scorpaeniformes	Cottidae	<i>Cottus paulus</i>	pygmy sculpin
563	Salmoniformes	Salmonidae	<i>Prosopium coulterii</i>	pygmy whitefish
564	Cypriniformes	Catostomidae	<i>Carpiodes cyprinus</i>	quillback
565	Cyprinodontiformes	Goodeidae	<i>Crenichthys nevadae</i>	railroad valley springfish
566	Perciformes	Percidae	<i>Etheostoma caeruleum</i>	rainbow darter
567	Cypriniformes	Cyprinidae	<i>Notropis chrosomus</i>	rainbow shiner
568	Salmoniformes	Osmeridae	<i>Osmerus mordax</i>	rainbow smelt
569	Salmoniformes	Salmonidae	<i>Oncorhynchus mykiss</i>	rainbow trout
570	Cyprinodontiformes	Fundulidae	<i>Lucania parva</i>	rainwater killifish
571	Cypriniformes	Catostomidae	<i>Xyrauchen texanus</i>	razorback sucker
572	Perciformes	Sciaenidae	<i>Sciaenops ocellatus</i>	red drum
573	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon rubrofluvialtilis</i>	red river pupfish
574	Cypriniformes	Cyprinidae	<i>Notropis bairdi</i>	red river shiner
575	Cypriniformes	Cyprinidae	<i>Cyprinella lutrensis</i>	red shiner
576	Perciformes	Percidae	<i>Etheostoma luteovinctum</i>	redband darter
577	Perciformes	Cichlidae	<i>Tilapia zillii</i>	redbelly tilapia
578	Perciformes	Centrarchidae	<i>Lepomis auritus</i>	redbreast sunfish
579	Perciformes	Centrarchidae	<i>Lepomis microlophus</i>	redeer sunfish
580	Perciformes	Centrarchidae	<i>Micropterus coosae</i>	redeye bass
581	Cypriniformes	Cyprinidae	<i>Notropis harperi</i>	redeye chub
582	Cyprinodontiformes	Fundulidae	<i>Fundulus rubrifrons</i>	redface topminnow
583	Perciformes	Percidae	<i>Etheostoma whipplei</i>	redfin darter
584	Esociformes	Esocidae	<i>Esox americanus</i>	redfin pickerel

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585	Cypriniformes	Cyprinidae	<i>Lythrurus umbratilis</i>	redfin shiner
586	Perciformes	Percidae	<i>Etheostoma rufilineatum</i>	redline darter
587	Cypriniformes	Cyprinidae	<i>Notropis chiliticus</i>	redlip shiner
588	Cypriniformes	Cyprinidae	<i>Clinostomus elongatus</i>	redside dace
589	Cypriniformes	Cyprinidae	<i>Richardsonius balteatus</i>	redside shiner
590	Cypriniformes	Cyprinidae	<i>Nocomis asper</i>	redspot chub
591	Perciformes	Percidae	<i>Etheostoma artesiae</i>	redspot darter
592	Perciformes	Centrarchidae	<i>Lepomis miniatus</i>	redspotted sunfish
593	Perciformes	Cichlidae	<i>Geophagus surinamensis</i>	redstriped eartheater
594	Cypriniformes	Cyprinidae	<i>Nocomis effusus</i>	redtail chub
595	Cypriniformes	Cyprinidae	<i>Relictus solitarius</i>	relict dace
596	Perciformes	Percidae	<i>Etheostoma chienense</i>	relict darter
597	Scorpaeniformes	Cottidae	<i>Cottus perplexus</i>	reticulate sculpin
598	Cypriniformes	Cyprinidae	<i>Lythrurus fumeus</i>	ribbon shiner
599	Cypriniformes	Cyprinidae	<i>Phenacobius catostomus</i>	riffle minnow
600	Scorpaeniformes	Cottidae	<i>Cottus gulosus</i>	riffle sculpin
601	Cypriniformes	Cyprinidae	<i>Gila pandora</i>	rio grande chub
602	Perciformes	Cichlidae	<i>Cichlasoma cyanoguttatum</i>	rio grande cichlid
603	Perciformes	Percidae	<i>Etheostoma grahami</i>	rio grande darter
604	Cypriniformes	Cyprinidae	<i>Notropis jemezanus</i>	rio grande shiner
605	Cypriniformes	Cyprinidae	<i>Hybognathus amarus</i>	rio grande silvery minnow
606	Cypriniformes	Catostomidae	<i>Catostomus plebeius</i>	rio grande sucker
607	Cypriniformes	Catostomidae	<i>Carpiodes carpio</i>	river carpsucker
608	Cypriniformes	Cyprinidae	<i>Nocomis micropogon</i>	river chub
609	Perciformes	Percidae	<i>Percina shumardi</i>	river darter
610	Perciformes	Gobiidae	<i>Awaous banana</i>	river goby
611	Petromyzontiformes	Petromyzontidae	<i>Lampetra ayresii</i>	river lamprey
612	Cypriniformes	Catostomidae	<i>Moxostoma carinatum</i>	river redhorse
613	Cypriniformes	Cyprinidae	<i>Notropis blennioides</i>	river shiner
614	Perciformes	Percidae	<i>Etheostoma podostemone</i>	riverweed darter
615	Perciformes	Centrarchidae	<i>Ambloplites cavifrons</i>	roanoke bass
616	Perciformes	Percidae	<i>Percina roanoka</i>	roanoke darter
617	Cypriniformes	Catostomidae	<i>Hypentelium roanokense</i>	roanoke hog sucker
618	Perciformes	Percidae	<i>Percina rex</i>	roanoke logperch
619	Cypriniformes	Catostomidae	<i>Moxostoma robustum</i>	robust redhorse
620	Perciformes	Centrarchidae	<i>Ambloplites rupestris</i>	rock bass
621	Perciformes	Percidae	<i>Etheostoma rupestre</i>	rock darter
622	Cypriniformes	Cyprinidae	<i>Notropis suttkusi</i>	rocky shiner
623	Cypriniformes	Cyprinidae	<i>Lythrurus ardens</i>	rosefin shiner

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
624	Cypriniformes	Cyprinidae	<i>Hybopsis rubrifrons</i>	rosyface chub
625	Cypriniformes	Cyprinidae	<i>Notropis rubellus</i>	rosyface shiner
626	Cypriniformes	Cyprinidae	<i>Clinostomus funduloides</i>	rosyside dace
627	Scorpaeniformes	Cottidae	<i>Cottus asperimus</i>	rough sculpin
628	Cypriniformes	Cyprinidae	<i>Notropis baileyi</i>	rough shiner
629	Atheriniformes	Atherinopsidae	<i>Membras martinica</i>	rough silverside
630	Cypriniformes	Cyprinidae	<i>Notropis semperasper</i>	roughhead shiner
631	Perciformes	Gobiidae	<i>Neogobius melanostomus</i>	round goby
632	Salmoniformes	Salmonidae	<i>Prosopium cylindraceum</i>	round whitefish
633	Cypriniformes	Cyprinidae	<i>Dionda episcopa</i>	roundnose minnow
634	Cypriniformes	Cyprinidae	<i>Gila robusta</i>	roundtail chub
635	Cypriniformes	Cyprinidae	<i>Scardinius erythrophthalmus</i>	rudd
636	Perciformes	Percidae	<i>Gymnocephalus cernuus</i>	ruffe
637	Perciformes	Percidae	<i>Etheostoma phytophilum</i>	rush darter
638	Cyprinodontiformes	Fundulidae	<i>Fundulus escambiae</i>	russetfin topminnow
639	Cypriniformes	Catostomidae	<i>Thoburnia hamiltoni</i>	rustyside sucker
640	Cypriniformes	Cyprinidae	<i>Notropis sabinae</i>	sabine shiner
641	Cypriniformes	Cyprinidae	<i>Orthodon microlepidotus</i>	sacramento blackfish
642	Perciformes	Centrarchidae	<i>Archoplites interruptus</i>	sacramento perch
643	Cypriniformes	Cyprinidae	<i>Ptychocheilus grandis</i>	sacramento pikeminnow
644	Cypriniformes	Catostomidae	<i>Catostomus occidentalis</i>	sacramento sucker
645	Perciformes	Percidae	<i>Percina vigil</i>	saddleback darter
646	Perciformes	Percidae	<i>Etheostoma flavum</i>	saffron darter
647	Cypriniformes	Cyprinidae	<i>Notropis rubricroceus</i>	saffron shiner
648	Cyprinodontiformes	Poeciliidae	<i>Poecilia latipinna</i>	sailfin molly
649	Cypriniformes	Cyprinidae	<i>Pteronotropis hypselopterus</i>	sailfin shiner
650	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon salinus</i>	salt creek pupfish
651	Cyprinodontiformes	Fundulidae	<i>Fundulus jenkinsi</i>	saltmarsh topminnow
652	Cyprinodontiformes	Poeciliidae	<i>Gambusia georgei</i>	san marcos gambusia
653	Percopsiformes	Percopsidae	<i>Percopsis transmontana</i>	sand roller
654	Cypriniformes	Cyprinidae	<i>Notropis stramineus</i>	sand shiner
655	Cypriniformes	Cyprinidae	<i>Notropis scepticus</i>	sandbar shiner
656	Cypriniformes	Cyprinidae	<i>Semotilus lumbee</i>	sandhills chub
657	Cypriniformes	Catostomidae	<i>Catostomus santaanae</i>	santa ana sucker
658	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon arcuatus</i>	santa cruz pupfish
659	Cypriniformes	Cyprinidae	<i>Cyprinella zanema</i>	santee chub
660	Cypriniformes	Cyprinidae	<i>Cyprinella analostana</i>	satinfish shiner
661	Perciformes	Percidae	<i>Sander canadensis</i>	sauger
662	Perciformes	Percidae	<i>Etheostoma fricksium</i>	savannah darter

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663	Perciformes	Percidae	<i>Etheostoma serrifer</i>	sawcheek darter
664	Clupeiformes	Clupeidae	<i>Harengula jaguana</i>	scaled sardine
665	Perciformes	Percidae	<i>Ammocrypta vivax</i>	scaly sand darter
666	Cypriniformes	Cyprinidae	<i>Lythrurus fasciolaris</i>	scarlet shiner
667	Siluriformes	Ictaluridae	<i>Noturus trautmani</i>	scioto madtom
668	Petromyzontiformes	Petromyzontidae	<i>Petromyzon marinus</i>	sea lamprey
669	Perciformes	Percidae	<i>Etheostoma thalassinum</i>	seagreen darter
670	Cyprinodontiformes	Fundulidae	<i>Fundulus seminolis</i>	seminole killifish
671	Perciformes	Centrarchidae	<i>Ambloplites ariommus</i>	shadow bass
672	Cypriniformes	Catostomidae	<i>Erimyzon tenuis</i>	sharpfin chubsucker
673	Perciformes	Percidae	<i>Etheostoma acuticeps</i>	sharphead darter
674	Perciformes	Percidae	<i>Percina oxyrhynchus</i>	sharpnose darter
675	Scorpaeniformes	Cottidae	<i>Clinocottus acuticeps</i>	sharpnose sculpin
676	Cypriniformes	Cyprinidae	<i>Notropis oxyrhynchus</i>	sharpnose shiner
677	Perciformes	Percidae	<i>Etheostoma tecumsehi</i>	shawnee darter
678	Perciformes	Sparidae	<i>Archosargus probatocephalus</i>	sheepshead
679	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon variegatus</i>	sheepshead minnow
680	Perciformes	Percidae	<i>Percina peltata</i>	shield darter
681	Perciformes	Gobiidae	<i>Tridentiger bifasciatus</i>	shimofuri goby
682	Perciformes	Embiotocidae	<i>Cymatogaster aggregata</i>	shiner perch
683	Perciformes	Centrarchidae	<i>Micropterus cataractae</i>	shoal bass
684	Cypriniformes	Cyprinidae	<i>Macrhybopsis hyostoma</i>	shoal chub
685	Perciformes	Gobiidae	<i>Tridentiger barbatus</i>	shokihaze goby
686	Cyprinodontiformes	Poeciliidae	<i>Poecilia mexicana</i>	shortfin molly
687	Cypriniformes	Catostomidae	<i>Moxostoma macrolepidotum</i>	shorthead redhorse
688	Scorpaeniformes	Cottidae	<i>Cottus confusus</i>	shorthead sculpin
689	Salmoniformes	Salmonidae	<i>Coregonus zenithicus</i>	shortjaw cisco
690	Salmoniformes	Salmonidae	<i>Coregonus reighardi</i>	shortnose cisco
691	Lepisosteiformes	Lepisosteidae	<i>Lepisosteus platostomus</i>	shortnose gar
692	Acipenseriformes	Acipenseridae	<i>Acipenser brevirostrum</i>	shortnose sturgeon
693	Cypriniformes	Catostomidae	<i>Chasmistes brevirostris</i>	shortnose sucker
694	Scorpaeniformes	Cottidae	<i>Cottus greeni</i>	shoshone sculpin
695	Acipenseriformes	Acipenseridae	<i>Scaphirhynchus platyrhynchus</i>	shovelnose sturgeon
696	Cypriniformes	Cyprinidae	<i>Macrhybopsis meeki</i>	sicklefin chub
697	Cypriniformes	Cyprinidae	<i>Hypophthalmichthys molitrix</i>	silver carp
698	Cypriniformes	Cyprinidae	<i>Macrhybopsis storeriana</i>	silver chub
699	Petromyzontiformes	Petromyzontidae	<i>Ichthyomyzon unicuspis</i>	silver lamprey
700	Perciformes	Sciaenidae	<i>Bairdiella chrysoura</i>	silver perch
701	Cypriniformes	Catostomidae	<i>Moxostoma anisurum</i>	silver redhorse

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702	Cypriniformes	Cyprinidae	<i>Notropis photogenis</i>	silver shiner
703	Cypriniformes	Cyprinidae	<i>Notropis shumardi</i>	silverband shiner
704	Cypriniformes	Cyprinidae	<i>Notropis buccatus</i>	silverjaw minnow
705	Cypriniformes	Cyprinidae	<i>Notropis candidus</i>	silverside shiner
706	Cypriniformes	Cyprinidae	<i>Notropis stilbius</i>	silverstripe shiner
707	Clupeiformes	Clupeidae	<i>Alosa chrysochloris</i>	skipjack herring
708	Cypriniformes	Cyprinidae	<i>Notropis uranoscopus</i>	skygazer shiner
709	Perciformes	Percidae	<i>Etheostoma smithi</i>	slabrock darter
710	Perciformes	Percidae	<i>Etheostoma boschungii</i>	slackwater darter
711	Perciformes	Gobiidae	<i>Ctenogobius pseudofasciatus</i>	slashcheek goby
712	Cypriniformes	Cyprinidae	<i>Erimystax cahni</i>	slender chub
713	Siluriformes	Ictaluridae	<i>Noturus exilis</i>	slender madtom
714	Scorpaeniformes	Cottidae	<i>Cottus tenuis</i>	slender sculpin
715	Perciformes	Percidae	<i>Percina phoxocephala</i>	slenderhead darter
716	Cypriniformes	Cyprinidae	<i>Pimephales tenellus</i>	slim minnow
717	Scorpaeniformes	Cottidae	<i>Cottus cognatus</i>	slimy sculpin
718	Perciformes	Percidae	<i>Etheostoma gracile</i>	slough darter
719	Cypriniformes	Cyprinidae	<i>Notropis buccula</i>	smalleye shiner
720	Perciformes	Centrarchidae	<i>Micropterus dolomieu</i>	smallmouth bass
721	Cypriniformes	Catostomidae	<i>Ictiobus bubalus</i>	smallmouth buffalo
722	Cypriniformes	Catostomidae	<i>Moxostoma breviceps</i>	smallmouth redhorse
723	Perciformes	Percidae	<i>Etheostoma microlepidum</i>	smallscale darter
724	Perciformes	Centropomidae	<i>Centropomus parallelus</i>	smallscale fat snook
725	Perciformes	Eleotridae	<i>Eleotris perniger</i>	smallscaled spinycheek
726	Pristiformes	Pristidae	<i>Pristis pectinata</i>	smalltooth sawfish
727	Siluriformes	Ictaluridae	<i>Noturus baileyi</i>	smoky madtom
728	Siluriformes	Ictaluridae	<i>Ameiurus brunneus</i>	snail bullhead
729	Perciformes	Percidae	<i>Percina tanasi</i>	snail darter
730	Cypriniformes	Catostomidae	<i>Chasmistes muriei</i>	snake river sucker
731	Perciformes	Percidae	<i>Etheostoma simoterum</i>	snubnose darter
732	Salmoniformes	Salmonidae	<i>Oncorhynchus nerka</i>	sockeye salmon
733	Cypriniformes	Cyprinidae	<i>Gila ditaenia</i>	sonora chub
734	Cypriniformes	Catostomidae	<i>Catostomus insignis</i>	sonora sucker
735	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon eremus</i>	sonoyta pupfish
736	Perciformes	Percidae	<i>Etheostoma olivaceum</i>	sooty darter
737	Cypriniformes	Catostomidae	<i>Cycleptus meridionalis</i>	southeastern blue sucker
738	Petromyzontiformes	Petromyzontidae	<i>Ichthyomyzon gagei</i>	southern brook lamprey
739	Percopsiformes	Amblyopsidae	<i>Typhlichthys subterraneus</i>	southern cavefish
740	Pleuronectiformes	Paralichthyidae	<i>Paralichthys lethostigma</i>	southern flounder

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741	Perciformes	Percidae	<i>Percina austroperca</i>	southern logperch
742	Cyprinodontiformes	Poeciliidae	<i>Xiphophorus maculatus</i>	southern platyfish
743	Cypriniformes	Cyprinidae	<i>Phoxinus erythrogaster</i>	southern redbelly dace
744	Siluriformes	Loricariidae	<i>Pterygoplichthys anisitsi</i>	southern sailfin catfish
745	Perciformes	Percidae	<i>Ammocrypta meridiana</i>	southern sand darter
746	Siluriformes	Doradidae	<i>Platydoras armatulus</i>	southern striped raphael
747	Cyprinodontiformes	Fundulidae	<i>Fundulus stellifer</i>	southern studfish
748	Cypriniformes	Cyprinidae	<i>Macrhybopsis aestivalis</i>	speckled chub
749	Cypriniformes	Cyprinidae	<i>Rhinichthys osculus</i>	speckled dace
750	Perciformes	Percidae	<i>Etheostoma stigmaeum</i>	speckled darter
751	Cyprinodontiformes	Fundulidae	<i>Fundulus rathbuni</i>	speckled killifish
752	Siluriformes	Ictaluridae	<i>Noturus leptacanthus</i>	speckled madtom
753	Cypriniformes	Cyprinidae	<i>Meda fulgida</i>	spikedace
754	Perciformes	Percidae	<i>Etheostoma barrenense</i>	splendid darter
755	Cypriniformes	Cyprinidae	<i>Pogonichthys macrolepidotus</i>	splittail
756	Scorpaeniformes	Cottidae	<i>Cottus ricei</i>	spoonhead sculpin
757	Perciformes	Sciaenidae	<i>Leiostomus xanthurus</i>	spot
758	Cypriniformes	Cyprinidae	<i>Erimonax monachus</i>	spotfin chub
759	Cyprinodontiformes	Fundulidae	<i>Fundulus luciae</i>	spotfin killifish
760	Cypriniformes	Cyprinidae	<i>Cyprinella spiloptera</i>	spotfin shiner
761	Perciformes	Percidae	<i>Etheostoma squamiceps</i>	spottail darter
762	Cypriniformes	Cyprinidae	<i>Notropis hudsonius</i>	spottail shiner
763	Perciformes	Centrarchidae	<i>Micropterus punctulatus</i>	spotted bass
764	Siluriformes	Ictaluridae	<i>Ameiurus serracanthus</i>	spotted bullhead
765	Perciformes	Percidae	<i>Etheostoma maculatum</i>	spotted darter
766	Lepisosteiformes	Lepisosteidae	<i>Lepisosteus oculatus</i>	spotted gar
767	Perciformes	Sciaenidae	<i>Cynoscion nebulosus</i>	spotted seatrout
768	Perciformes	Eleotridae	<i>Eleotris picta</i>	spotted sleeper
769	Cypriniformes	Catostomidae	<i>Minytrema melanops</i>	spotted sucker
770	Perciformes	Centrarchidae	<i>Lepomis punctatus</i>	spotted sunfish
771	Perciformes	Cichlidae	<i>Tilapia mariae</i>	spotted tilapia
772	Percopsiformes	Amblyopsidae	<i>Forbesichthys agassizii</i>	spring cavefish
773	Perciformes	Elassomatidae	<i>Elassoma alabamae</i>	spring pygmy sunfish
774	Perciformes	Percidae	<i>Percina uranidea</i>	stargazing darter
775	Cypriniformes	Cyprinidae	<i>Phenacobius uranops</i>	stargazing minnow
776	Cyprinodontiformes	Fundulidae	<i>Fundulus dispar</i>	starhead topminnow
777	Pleuronectiformes	Pleuronectidae	<i>Platichthys stellatus</i>	starry flounder
778	Cypriniformes	Cyprinidae	<i>Cyprinella whipplei</i>	steelcolor shiner
779	Perciformes	Percidae	<i>Etheostoma punctulatum</i>	stippled darter

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780	Cyprinodontiformes	Fundulidae	<i>Fundulus bifax</i>	stippled studfish
781	Perciformes	Percidae	<i>Etheostoma derivativum</i>	stone darter
782	Siluriformes	Ictaluridae	<i>Noturus flavus</i>	stonecat
783	Perciformes	Percidae	<i>Etheostoma fragi</i>	strawberry darter
784	Cypriniformes	Cyprinidae	<i>Erimystax dissimilis</i>	streamline chub
785	Perciformes	Percidae	<i>Etheostoma striatulum</i>	striated darter
786	Perciformes	Percidae	<i>Percina notogramma</i>	stripeback darter
787	Perciformes	Moronidae	<i>Morone saxatilis</i>	striped bass
788	Perciformes	Percidae	<i>Etheostoma virgatum</i>	striped darter
789	Cypriniformes	Catostomidae	<i>Moxostoma rupiscartes</i>	striped jumprock
790	Perciformes	Gerreidae	<i>Eugerres plumieri</i>	striped mojarra
791	Mugiliformes	Mugilidae	<i>Mugil cephalus</i>	striped mullet
792	Cypriniformes	Cyprinidae	<i>Luxilus chrysocephalus</i>	striped shiner
793	Perciformes	Percidae	<i>Etheostoma kennicotti</i>	stripetail darter
794	Cypriniformes	Cyprinidae	<i>Macrhybopsis gelida</i>	sturgeon chub
795	Siluriformes	Loricariidae	<i>Hypostomus plecostomus</i>	suckermouth catfish
796	Cypriniformes	Cyprinidae	<i>Phenacobius mirabilis</i>	suckermouth minnow
797	Salmoniformes	Osmeridae	<i>Hypomesus pretiosus</i>	surf smelt
798	Perciformes	Centrarchidae	<i>Micropterus notius</i>	suwannee bass
799	Cypriniformes	Cyprinidae	<i>Notropis procne</i>	swallowtail shiner
800	Perciformes	Percidae	<i>Etheostoma fusiforme</i>	swamp darter
801	Percopsiformes	Amblyopsidae	<i>Chologaster cornuta</i>	swampfish
802	Perciformes	Percidae	<i>Etheostoma swannanoa</i>	swannanoa darter
803	Perciformes	Centropomidae	<i>Centropomus ensiferus</i>	swordspine snook
804	Siluriformes	Ictaluridae	<i>Noturus gyrinus</i>	tadpole madtom
805	Cypriniformes	Catostomidae	<i>Catostomus tahoensis</i>	tahoe sucker
806	Cypriniformes	Cyprinidae	<i>Notropis maculatus</i>	taillight shiner
807	Perciformes	Percidae	<i>Etheostoma tallapoosae</i>	tallapoosa darter
808	Cypriniformes	Cyprinidae	<i>Cyprinella gibbsi</i>	tallapoosa shiner
809	Cypriniformes	Cyprinidae	<i>Notropis braytoni</i>	tamaulipas shiner
810	Perciformes	Percidae	<i>Percina aurantiaca</i>	tangerine darter
811	Elopiformes	Megalopidae	<i>Megalops atlanticus</i>	tarpon
812	Perciformes	Centropomidae	<i>Centropomus pectinatus</i>	tarpon snook
813	Perciformes	Percidae	<i>Etheostoma barbouri</i>	teardrop darter
814	Cypriniformes	Cyprinidae	<i>Notropis telescopus</i>	telescope shiner
815	Cypriniformes	Cyprinidae	<i>Tinca tinca</i>	tench
816	Cypriniformes	Cyprinidae	<i>Phoxinus tennesseensis</i>	tennessee dace
817	Cypriniformes	Cyprinidae	<i>Notropis leuciodus</i>	tennessee shiner
818	Perciformes	Percidae	<i>Etheostoma olmstedi</i>	tessellated darter

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819	Perciformes	Percidae	<i>Percina carbonaria</i>	texas logperch
820	Cypriniformes	Cyprinidae	<i>Notropis amabilis</i>	texas shiner
821	Cyprinodontiformes	Poeciliidae	<i>Gambusia speciosa</i>	tex-mex gambusia
822	Cypriniformes	Cyprinidae	<i>Cyprinella labrosa</i>	thicklip chub
823	Cypriniformes	Cyprinidae	<i>Gila crassicauda</i>	thicktail chub
824	Clupeiformes	Clupeidae	<i>Dorosoma petenense</i>	threadfin shad
825	Perciformes	Gobiidae	<i>Eucyclogobius newberryi</i>	tidewater goby
826	Perciformes	Gerreidae	<i>Eucinostomus harengulus</i>	tidewater mojarra
827	Perciformes	Percidae	<i>Etheostoma tippecanoe</i>	tippecanoe darter
828	Perciformes	Percidae	<i>Etheostoma lachneri</i>	tombigbee darter
829	Cypriniformes	Cyprinidae	<i>Exoglossum laurae</i>	tonguetied minnow
830	Siluriformes	Ictaluridae	<i>Trogloglanis pattersoni</i>	toothless blindcat
831	Cypriniformes	Cyprinidae	<i>Notropis topeka</i>	topeka shiner
832	Scorpaeniformes	Cottidae	<i>Cottus rhotheus</i>	torrent sculpin
833	Cypriniformes	Catostomidae	<i>Thoburnia rathoeca</i>	torrent sucker
834	Cypriniformes	Cyprinidae	<i>Cyprinella trichroistia</i>	tricolor shiner
835	Perciformes	Percidae	<i>Etheostoma trisella</i>	trispot darter
836	Percopsiformes	Percopsidae	<i>Percopsis omiscomaycus</i>	trout-perch
837	Perciformes	Gobiidae	<i>Proterorhinus marmoratus</i>	tubenose goby
838	Perciformes	Percidae	<i>Etheostoma gutselli</i>	tuckasegee darter
839	Cypriniformes	Cyprinidae	<i>Gila bicolor</i>	tui chub
840	Perciformes	Embiotocidae	<i>Hysteroecarpus traskii</i>	tule perch
841	Perciformes	Percidae	<i>Etheostoma inscriptum</i>	turquoise darter
842	Perciformes	Percidae	<i>Etheostoma tuscumbia</i>	tuscumbia darter
843	Perciformes	Percidae	<i>Etheostoma douglasi</i>	tuskaloosa darter
844	Cypriniformes	Cyprinidae	<i>Rhinichthys umatilla</i>	umatilla dace
845	Cypriniformes	Cyprinidae	<i>Oregonichthys kalawatseti</i>	umpqua chub
846	Cypriniformes	Cyprinidae	<i>Rhinichthys evermanni</i>	umpqua dace
847	Cypriniformes	Cyprinidae	<i>Ptychocheilus umpqua</i>	umpqua pikeminnow
848	Cypriniformes	Cyprinidae	<i>Gila atraria</i>	utah chub
849	Scorpaeniformes	Cottidae	<i>Cottus echinatus</i>	utah lake sculpin
850	Cypriniformes	Catostomidae	<i>Catostomus ardens</i>	utah sucker
851	Cyprinodontiformes	Poeciliidae	<i>Xiphophorus variatus</i>	variable platyfish
852	Perciformes	Percidae	<i>Etheostoma variatum</i>	variegated darter
853	Siluriformes	Loricariidae	<i>Pterygoplichthys disjunctivus</i>	vermiculated sailfin catfish
854	Perciformes	Percidae	<i>Etheostoma chermocki</i>	vermillion darter
855	Perciformes	Gobiidae	<i>Gobioides broussonetii</i>	violet goby
856	Cypriniformes	Cyprinidae	<i>Gila seminuda</i>	virgin chub
857	Cypriniformes	Cyprinidae	<i>Lepidomeda mollispinis</i>	virgin spinedace

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
858	Cypriniformes	Catostomidae	<i>Moxostoma pappillosum</i>	v-lip redbhorse
859	Perciformes	Percidae	<i>Etheostoma perlongum</i>	waccamaw darter
860	Cyprinodontiformes	Fundulidae	<i>Fundulus waccamensis</i>	waccamaw killifish
861	Atheriniformes	Atherinopsidae	<i>Menidia extensa</i>	waccamaw silverside
862	Salmoniformes	Osmeridae	<i>Hypomesus nipponensis</i>	wakasagi
863	Siluriformes	Clariidae	<i>Clarias batrachus</i>	walking catfish
864	Perciformes	Percidae	<i>Sander vitreus</i>	walleye
865	Perciformes	Cichlidae	<i>Oreochromis urolepis</i>	wami tilapia
866	Perciformes	Centrarchidae	<i>Lepomis gulosus</i>	warmouth
867	Cypriniformes	Catostomidae	<i>Catostomus warnerensis</i>	warner sucker
868	Cypriniformes	Cyprinidae	<i>Luxilus coccogenis</i>	warpaint shiner
869	Perciformes	Percidae	<i>Etheostoma bellator</i>	warrior darter
870	Cypriniformes	Cyprinidae	<i>Lythrurus alegnotus</i>	warrior shiner
871	Perciformes	Percidae	<i>Etheostoma nuchale</i>	watercress darter
872	Cypriniformes	Cyprinidae	<i>Notropis greenei</i>	wedgespot shiner
873	Cypriniformes	Cyprinidae	<i>Notropis texanus</i>	weed shiner
874	Cypriniformes	Cyprinidae	<i>Rhinichthys obtusus</i>	western blacknose dace
875	Petromyzontiformes	Petromyzontidae	<i>Lampetra richardsoni</i>	western brook lamprey
876	Cyprinodontiformes	Poeciliidae	<i>Gambusia affinis</i>	western mosquitofish
877	Perciformes	Percidae	<i>Ammocrypta clara</i>	western sand darter
878	Cypriniformes	Cyprinidae	<i>Hybognathus argyritis</i>	western silvery minnow
879	Cyprinodontiformes	Fundulidae	<i>Fundulus blairae</i>	western starhead topminnow
880	Perciformes	Moronidae	<i>Morone chrysops</i>	white bass
881	Siluriformes	Ictaluridae	<i>Ameiurus catus</i>	white catfish
882	Perciformes	Centrarchidae	<i>Pomoxis annularis</i>	white crappie
883	Mugiliformes	Mugilidae	<i>Mugil curema</i>	white mullet
884	Perciformes	Moronidae	<i>Morone americana</i>	white perch
885	Cypriniformes	Cyprinidae	<i>Lepidomeda albivallis</i>	white river spinedace
886	Cyprinodontiformes	Goodeidae	<i>Crenichthys baileyi</i>	white river springfish
887	Cyprinodontiformes	Cyprinodontidae	<i>Cyprinodon tularosa</i>	white sands pupfish
888	Cypriniformes	Cyprinidae	<i>Luxilus albeolus</i>	white shiner
889	Acipenseriformes	Acipenseridae	<i>Acipenser transmontanus</i>	white sturgeon
890	Cypriniformes	Catostomidae	<i>Catostomus commersonii</i>	white sucker
891	Cypriniformes	Cyprinidae	<i>Cyprinella nivea</i>	whitfin shiner
892	Cyprinodontiformes	Fundulidae	<i>Fundulus albolineatus</i>	whiteline topminnow
893	Cypriniformes	Cyprinidae	<i>Notropis alborus</i>	whitemouth shiner
894	Cypriniformes	Cyprinidae	<i>Cyprinella galactura</i>	whitetail shiner
895	Siluriformes	Ictaluridae	<i>Satan eurystomus</i>	widemouth blindcat
896	Scorpaeniformes	Cottidae	<i>Cottus leiopomus</i>	wood river sculpin

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
897	Perciformes	Percidae	<i>Etheostoma vulneratum</i>	wounded darter
898	Cypriniformes	Cyprinidae	<i>Plagopterus argentissimus</i>	woundfin
899	Siluriformes	Ictaluridae	<i>Ictalurus pricei</i>	yaqui catfish
900	Cypriniformes	Cyprinidae	<i>Gila purpurea</i>	yaqui chub
901	Cypriniformes	Catostomidae	<i>Catostomus bernardini</i>	yaqui sucker
902	Perciformes	Percidae	<i>Etheostoma raneyi</i>	yazoo darter
903	Cypriniformes	Cyprinidae	<i>Notropis rafinesquei</i>	yazoo shiner
904	Perciformes	Moronidae	<i>Morone mississippiensis</i>	yellow bass
905	Siluriformes	Ictaluridae	<i>Ameiurus natalis</i>	yellow bullhead
906	Perciformes	Percidae	<i>Perca flavescens</i>	yellow perch
907	Perciformes	Cichlidae	<i>Cichlasoma salvini</i>	yellowbelly cichlid
908	Perciformes	Percidae	<i>Etheostoma moorei</i>	yellowcheek darter
909	Perciformes	Gobiidae	<i>Acanthogobius flavimanus</i>	yellowfin goby
910	Siluriformes	Ictaluridae	<i>Noturus flavipinnis</i>	yellowfin madtom
911	Cypriniformes	Cyprinidae	<i>Notropis lutipinnis</i>	yellowfin shiner
912	Perciformes	Percidae	<i>Etheostoma juliae</i>	yoke darter
913	Perciformes	Percidae	<i>Sander lucioperca</i>	zander
914	Cypriniformes	Catostomidae	<i>Moxostoma cf. poecilurum</i>	apalachicola redhorse
915	Salmoniformes	Salmonidae	<i>Salmo salar</i>	atlantic salmon juvenile
916	Atheriniformes	Atherinopsidae	<i>Menidia menidia</i>	atlantic silverside
917	Cypriniformes	Cyprinidae	<i>Hypophthalmichthys nobilis</i>	bighead carp
918	Cypriniformes	Cyprinidae	<i>Mylopharyngodon piceus</i>	black carp
919	Perciformes	Pomatomidae	<i>Pomatomus saltatrix</i>	bluefish
920	Perciformes	Percidae	<i>Etheostoma meadiae</i>	bluespar darter
921	Salmoniformes	Salmonidae	<i>Oncorhynchus clarkii utah</i>	bonneville cutthroat trout
922	Cypriniformes	Catostomidae	<i>Moxostoma cf. lachneri</i>	brassy jumprock
923	Anura	Ranidae	<i>Rana catesbeiana</i>	bullfrog
924	Anura	Ranidae	<i>Rana catesbeiana</i>	bullfrog tadpole
925	Scorpaeniformes	Cottidae	<i>Cottus chattahoochee</i>	chattahoochee sculpin
926	Salmoniformes	Salmonidae	<i>Oncorhynchus tshawytscha</i>	chinook salmon (yoy)
927	Scorpaeniformes	Cottidae	<i>Cottus cf. broadband sculpin</i>	clinch sculpin
928	Salmoniformes	Salmonidae	<i>Oncorhynchus clarkii clarkii</i>	coastal cutthroat trout
929	Salmoniformes	Salmonidae	<i>Oncorhynchus clarkii pleuriticus</i>	colorado river cutthroat trout
930	Anguilliformes	Congridae	<i>Conger oceanicus</i>	conger eel
931	Cypriniformes	Cyprinidae	<i>Macrhybopsis cf. aestivalis</i>	coosa chub
932	Cypriniformes	Cyprinidae	<i>Semotilus X Luxilus atromaculatus x chrysocephalus</i>	creek chub x striped shiner
933	Salmoniformes	Salmonidae	<i>Oncorhynchus clarkii x mykiss</i>	cutbow
934	Perciformes	Percidae	<i>Etheostoma planasaxatile</i>	duck darter
935	Perciformes	Percidae	<i>Etheostoma orientale</i>	eastrim darter

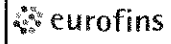
LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
936	Perciformes	Centropomidae	<i>Centropomus parallelus</i>	fat snook
937	Esociformes	Esocidae	<i>Esox americanus vermiculatus</i>	grass pickerel
938	Clupeiformes	Clupeidae	<i>Brevoortia patronus</i>	gulf menhaden
939	Scorpaeniformes	Cottidae	<i>Cottus kanawhae</i>	kanawha sculpin
940	Salmoniformes	Salmonidae	<i>Oncorhynchus clarkii henshawi</i>	lahontan cutthroat trout
941	Cypriniformes	Catostomidae	<i>Pantosteus lahontan</i>	lahontan sucker
942	Perciformes	Centrarchidae	<i>Micropterus salmoides</i>	largemouth bass (yoy)
943	Perciformes	Eleotridae	<i>Eleotris amblyopsis</i>	largescaled spinycheek sleeper
944	Cypriniformes	Catostomidae	<i>Catostomus cf. latipinnis</i>	little colorado river sucker
945	Cypriniformes	Cyprinidae	<i>Notropis amplamala</i>	longjaw minnow
946	Cypriniformes	Cyprinidae	<i>Pteronotropis stonei</i>	lowland shiner
947	Cypriniformes	Cyprinidae	<i>Cyprinus carpio</i>	mirror carp
948	Caudata	Ambystomatidae	<i>Dicamptodon tenebrosus</i>	pacific giant salamander
949	Salmoniformes	Salmonidae	<i>Oncorhynchus mykiss</i>	rainbow trout (steelhead)
950	Anura	Ranidae	<i>Rana aurora</i>	red-legged frog
951	Salmoniformes	Salmonidae	<i>Oncorhynchus mykiss gairdneri</i>	redband rainbow trout
952	Caudata	Salamandridae	<i>Taricha granulosa</i>	rough-skinned newt
953	Siluriformes	Ictaluridae	<i>Noturus fasciatus</i>	saddled madtom
954	Perciformes	Percidae	<i>Sander canadensis x vitreus</i>	saugeye
955	Cypriniformes	Cyprinidae	<i>Notropis cf. spectrunculus</i>	sawfin shiner
956	Cypriniformes	Cyprinidae	<i>Hypophthalmichthys molitrix</i>	silver carp
957	Cypriniformes	Catostomidae	<i>Moxostoma robustum</i>	smallfin redbhorse
958	Perciformes	Channidae	<i>Channa argus</i>	snakehead
959	Anguilliformes	Ophichthidae	<i>Myrophis punctatus</i>	speckled worm eel
960	Anura	Ascaphidae	<i>Ascaphus truei</i>	tailed frog
961	Anura	Ascaphidae	<i>Ascaphus truei</i>	tailed frog (tadpole)
962	Scorpaeniformes	Cottidae	<i>Cottus tallapoosae</i>	tallapoosa sculpin
963	Perciformes	Percidae	<i>Etheostoma tennesseense</i>	tennessee darter
964	Cypriniformes	Cyprinidae	<i>Cyprinella cf. zanema</i>	thinlip chub
965	Gasterosteiformes	Gasterosteidae	<i>Gasterosteus aculeatus</i>	threespine stickleback
966	Esociformes	Esocidae	<i>Esox lucius x masquinongy</i>	tiger muskellunge
967	Salmoniformes	Salmonidae	<i>Salmo X Salvelinus trutta x fontinalis</i>	tiger trout
968	Anura	Bufo	<i>Bufo boreas</i>	western toad
969	Perciformes	Percidae	<i>Etheostoma occidentale</i>	westrim darter
970	Salmoniformes	Salmonidae	<i>Oncorhynchus clarkii lewisi</i>	westslope cutthroat trout
971	Perciformes	Moronidae	<i>Morone na</i>	wiper
972	Perciformes	Gerreidae	<i>Gerres cinereus</i>	yellowfin mojarra
973	Perciformes	Percidae	<i>Percina burtoni</i>	blotchside darter
974	Anura	Pipidae	<i>Xenopus laevis</i>	african clawed frog

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
975	Salmoniformes	Salmonidae	<i>Oncorhynchus apache x mykiss</i>	apache x rainbow trout
976	Anura	Bufo	<i>Bufo microscaphus</i>	arizona toad
977	Anura	Hylidae	<i>Pseudacris maculata</i>	boreal chorus frog
978	Caudata	Ambystomatidae	<i>Dicamptodon ensatus</i>	california giant salamander
979	Caudata	Salamandridae	<i>Taricha torosa</i>	california newt
980	Anura	Hylidae	<i>Pseudacris cadaverina</i>	california treefrog
981	Anura	Bufo	<i>Bufo hemiophrys</i>	canadian toad
982	Anura	Hylidae	<i>Hyla arenicolor</i>	canyon treefrog
983	Anura	Ranidae	<i>Rana cascadae</i>	cascade frog
984	Anura	Ranidae	<i>Rana luteiventris</i>	columbia spotted frog
985	Caudata	Rhyacotritonidae	<i>Rhyacotriton kezeri</i>	columbia torrent salamander
986	Anura	Ranidae	<i>Rana boylei</i>	foothill yellow-legged frog
987	Salmoniformes	Salmonidae	<i>Oncorhynchus aguabonita</i>	golden trout
988	Anura	Ranidae	<i>Rana clamitans</i>	green frog
989	Caudata	Ambystomatidae	<i>Dicamptodon aterrimus</i>	idaho giant salamander
990	Petromyzontiformes	Petromyzontidae	<i>Lampetra similis</i>	klamath river lamprey
991	Anura	Ranidae	<i>Lithobates pipiens</i>	leopard frog
992	Caudata	Ambystomatidae	<i>Ambystoma macrodactylum</i>	longtoed salamander
993	Anura	Ranidae	<i>Rana yavapaiensis</i>	lowland leopard frog
994	Anura	Ranidae	<i>Rana muscosa</i>	mountain yellow-legged frog
995	Anura	Hylidae	<i>Pseudacris regilla</i>	pacific tree frog
996	Anura	Ranidae	<i>Lithobates blairi</i>	plains leopard frog
997	Salmoniformes	Salmonidae	<i>Oncorhynchus mykiss x aguabonita</i>	rainbow x golden trout
998	Anura	Bufo	<i>Bufo punctatus</i>	red-spotted toad
999	Cypriniformes	Cyprinidae	<i>Richardsonius X Rhinichthys balteatus x osculus</i>	redside shiner x speckled dace
1000	Anura	Ranidae	<i>Rana pretiosa</i>	spotted frog
1001	Anura	Ranidae	<i>Rana sylvatica</i>	wood frog
1002	Anura	Bufo	<i>Bufo woodhousii</i>	woodhouse's toad
1003	Anura	Ranidae	<i>Lithobates chiricahuensis</i>	chiricahua leopard frog

APPENDIX E: REPORTING TEMPLATES

Templates will be provided on the NARS Sharefile.

APPENDIX F: EXAMPLE SOPS FOR MERCURY IN FISH TISSUE PLUG ANALYSES

	Frontier Global Sciences	Document Title: Mercury in Water by Oxidation, Purge & Trap and CV-AFS (EPA Method 1631, Rev E)	Eurofins Document Reference: EFGS-SOP-137-R02
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Prepared by	Ryan Nelson
Reviewed and Approved by	Dave Wunderlich and Patrick Garcia-Strickland

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Approvals:

Prepared by: By [Signature]

Date: 6/17/13

Approved by: [Signature]

Date: 6/17/2013

Approved by: [Signature]

Date: 6/18/13

1 Revision Log:

Revision:	Effective Date: This version	
Section	Justification	Changes
Cover	Required change	Changed company name from Frontier Global Sciences to Eurofins Frontier Global Sciences.
All	Formatting requirement per LOM SOP-LAB-201	Reformatted document to new corporate specifications.
13.1, 13.2	Required	Added hardware and software components
14.8	Required	Updated mercury standard prep
14.9	Required	Updated standard and reagent documentation procedures
15.2 – 15.4	Required	Updated calibration information
16.7	Required	Added instrument maintenance and troubleshooting

2 Reference:

- 2.1 EPA Method 1631, Revision E: Mercury in Water by Oxidation, Purge and Trap, and Cold Vapor Atomic Fluorescence Spectrometry, 2002.
- 2.2 Method 1669, "Method for Sampling Ambient Water for Determination of Metals at EPA Ambient Criteria Levels," U.S. Environmental Protection Agency, Office of Water, Office of Science and Technology, Engineering and Analysis Division (4303), 401 M Street SW, Washington, DC 20460, April 1995 with January 1996 revisions.
- 2.3 Bloom, N.S.; and Tsalkitzis, E. Standard Operating Procedure FGS-012 Determination of Total Mercury in Aqueous Media (Modified EPA Method 1631). Frontier GeoSciences Inc., Quality Assurance Manual 1995.
- 2.4 Bloom, N.S.; Ultra-Clean Sample Handling, Environmental Lab 1995, March/April, 20.
- 2.5 Bloom, N.S.; Horvat M., and Watras C.J. Results of the International Mercury Speciation Intercomparison Exercise. Wat. Air Soil Pollut. 1995, 80, 1257.
- 2.6 Bloom, N.S.; Crecelius, E.A. Determination of Mercury in Seawater at Sub-nanogram per Liter Levels. Mar. Chem. 1983, 14, 49.
- 2.7 Bloom, N.S.; Crecelius, E.A. Distribution of Silver, Lead, Mercury, Copper, and Cadmium in Central Puget Sound Sediments Mar. Chem 1987, 21, 377-390.
- 2.8 Bloom, N.S.; Fitzgerald, W.F. Determination of Volatile Mercury Species at the Picogram Level by Low-Temperature Gas Chromatography with Cold-Vapor Atomic Fluorescence Detection. Anal. Chem. Acta. 1988, 208, 151.
- 2.9 Cossa, D.; Couran, P. An International Intercomparison Exercise for Total Mercury in Seawater. App. Organomet. Chem. 1990, 4, 49.
- 2.10 Fitzgerald, W.F.; Gill, G.A. Sub-Nanogram Determination of Mercury by Two-Stage Gold Amalgamation and Gas Phase Detection Applied to Atmospheric Analysis. Anal. Chem. 1979, 15, 1714.
- 2.11 Gill, G.A.; Fitzgerald, W.F. Mercury Sampling of Open Ocean Waters at the Picogram Level Deep Sea Res. 1985, 32, 287.
- 2.12 EPA Method 30.B, Determination of total vapor phase mercury emissions from coal-fired combustion sources using carbon sorbent traps.

- 2.13 Chemical Hygiene Plan, Eurofins Frontier Global Sciences, current version.
- 2.14 National Environmental Laboratory Accreditation Conference, NELAC Standard September 8, 2009.
- 2.15 Department of Defense Quality Systems Manual for Environmental Laboratories, prepared by DoD Environmental Quality Workgroup, Final Version 4.2, October 2010.

3 Cross Reference:

Document	Document Title
SOP FGS-003	Pipette Verification, Calibration and Maintenance
SOP FGS-007	Cleaning of Sampling Equipment and Bottles
SOP FGS-008	Ultra Clean Aqueous Sample Collection
SOP FGS-012	Oxidation of Aqueous Samples for Total Mercury Analysis
SOP FGS-061	Gold Trap Construction
SOP FGS-094, App F	Standard Operating Procedure Training Record
SOP FGS-099	Waste Disposal Procedure for Client Sample Waste
SOP FGS-121	Determination of Total Mercury by Flow Injection AFS (Mod 1631E)
SOP FGS-155	Calibration of Volumetric Dispensers

4 Purpose:

- 4.1 This SOP is designed to ensure that all reproducible traceable procedures in EPA 1631 are followed in the standardization of the total mercury analyzers and in the analysis of samples for total mercury, as well as to establish the limits wherein data will be considered acceptable.

5 Scope:

- 5.1 This Standard Operating Procedure (SOP) describes a method for the determination of total mercury (Hg) in filtered and unfiltered water by oxidation, purge and trap, desorption, and cold vapor atomic fluorescence spectrometry (CVAFS).
- 5.2 This method is designed for the determination of mercury in the range of 0.5-40 ng/L (ppt). Application may be extended to higher levels by selection of a smaller sample size, as long as the instrument value (intensity) remains within the calibration curve.
- 5.3 The Control Limits are established from EPA 1631E.

6 Basic Principles:

- 6.1 For analysis of aqueous samples, an aliquot of oxidized sample is neutralized with hydroxylamine-hydrochloride (NH₂OH-HCl) to destroy free halogens, and added to a bubbler.
- 6.2 Stannous chloride (SnCl₂) is added to the bubbler to reduce the Hg(II) to volatile Hg(0), and the bubblers are sealed with Keck clips. Blanked gold traps are placed at the end of soda-lime pre-traps. The bubbler is purged with nitrogen (N₂) for 20 minutes. All gas that flows into the bubbler should only leave the system through the soda-lime pre-trap and then the gold trap.
- 6.3 The gaseous mercury amalgamates to the gold traps, which are removed and individually placed in the analytical train. The gold trap is heated, thus releasing the mercury into the argon gas stream flowing into the instrument.

7 Reference Modifications:

7.1 There were no significant modifications to this method.

8 Definitions:

- 8.1 Analytical Duplicate (AD): A representative sample (that yielded a result within the calibration curve) is analyzed a second time during the analytical run. The second analysis should be at the same aliquot as the original.
- 8.2 Analytical Run – The continuous analysis of one or more batches during the same 12 hour-shift. Each analytical day requires a minimum five-point calibration curve, ICV, at least 3 IBLs, and CCV/CCB every ten runs. An analytical day must conclude with a CCV/CCB.
- 8.3 Analytical Spike and Analytical Spike Duplicate (AS/ASD): A representative sample is selected and spiked, with a dilution of the primary source, during the analytical run, at a target concentration of 1-5X the ambient concentration of the sample. These QC samples are used to indicate sample matrix effects on the analyte of interest. Non-detectable samples are spiked at 1 – 5 x of the MRL/PQL.
- 8.4 Batch: 20 client samples or less grouped for preparation. See Quality Assurance Section for batch requirements.
- 8.5 Calibration Standards (CAL) – a series of standards that will be used to calibrate the instrument, made from a primary source stock standard. A calibration blank plus at least five different concentrations are required, beginning with one at PQL concentration.
- 8.6 Certified Reference Material (CRM) – a standard of known composition that is certified by a recognized authority and representing a sample matrix. It is used to verify the accuracy of a method.
- 8.7 Continuing Calibration Blank (CCB): An instrument blank that is used to monitor the ambient blank concentration after the Continuing Calibration Verification (CCV).
- 8.8 Continuing Calibration Verification (CCV): An aliquot of standard from the same source as the calibration standard, at a value of 20ng/L (2.0ng in ~100mL bubbler water). This standard is analyzed after every 10 analytical runs, and determines whether the instrument is maintaining calibration.
- 8.9 Continuing Demonstration of Capability (CDOC)
- 8.10 Control Limit (CL) – the limit of the range of acceptability for the quality control samples
- 8.11 Equipment Blank (EB): Reagent water processed through the sampling devices and placed in a sample container prior to using the equipment to collect samples and used to demonstrate that the sampling equipment is free from contamination.
- 8.12 Field Blanks (FB): A sample of reagent water placed in a sample container in the field and used to demonstrate that samples have not been contaminated by sample collection or transport activities. EPA-1631E recommends the analysis of at least one field blank per 10 samples collected at the same site at the same time. Analyze the blank immediately before analyzing the samples in the batch.

- 8.13 Initial Calibration Verification (ICV): A standard that is prepared from a secondary source stock standard with a value of 15ng/L (1.5ng in ~100mL bubbler). This standard is run immediately following the calibration curve and verifies instrument calibration. It is always followed by the IBLs.
- 8.14 Initial Blank Level (IBL): An instrument blank that is used to demonstrate the ambient blank concentration of the instrument. One per bubbler is needed at the beginning of the analytical run.
- 8.15 Initial Demonstration of Capability (IDOC).
- 8.16 Laboratory Control Sample (LCS and LCSD) or Quality Control Sample (QCS): A sample (and duplicate) containing a known concentration of mercury that is used to monitor complete method performance. The preferred LCS is a matrix matched Certified Reference Material (CRM), but a blank spike meets the requirement also. In LIMS, the LCS is always referred to as a Blank Spike (BS), whether it is matrix matched or not.
- 8.17 Limit of Detection (LOD) – equal to MDL and verified on a quarterly/annual basis, depending on the preparation, by spiking within three times the established LOD and showing a positive result on the instrument.
- 8.18 Limit of Quantitation (LOQ) – equal to PQL and verified on a quarterly/annual basis, depending on the preparation, by spiking within 2 times the LOQ and showing a recovery between 70 – 130%.
- 8.19 LIMS: Laboratory Information Management System. Computer software used for managing samples, standards, and other laboratory functions.
- 8.20 May: This action, activity, or procedural step is optional.
- 8.21 May Not: This action, activity, or procedural step is prohibited .
- 8.22 Matrix Spike (MS) and Matrix Spike Duplicate (MSD): A representative sample is selected and spiked with a dilution of the primary source at a known concentration. The MS and MSD are run through the entire analytical process just as the samples are. These QC samples will indicate sample matrix effects on the analyte of interest.
- 8.23 Method Blank (MBLK) or Preparation Blank (PB): For waters, reagent water that is prepared and analyzed in a manner identical to that of samples. For digested solids, preparations blanks consist of the same reagents used to digest the samples, in the same volume or proportion and are carried through the complete sample preparation and analytical procedure. Boiling chips are used as a blank matrix for solids. Preparation blanks are referred to as BLK in LIMS.
- 8.24 Method Detection Limit (MDL): A limit derived from 40 CFR, Part 136, Appendix B. This method produces a defined value that is the minimum concentration that can be measured and reported with a 99% confidence that the analyte concentration is greater than zero from a given matrix.
- 8.25 Method Duplicates/Method Triplicates (MD/MT): A second or third separate sample dilution, taken from the same source sample, prepared and analyzed in the laboratory separately. An MSD may be used as a duplicate.

- 8.26 Reagent water: 18 M Ω minimum, reagent water starting from a pre-purified (distilled, Reverse Osmosis, etc.) source.
- 8.27 Must: This action, activity, or procedural step is required.
- 8.28 Ongoing Precision and Recovery (OPR): A dilution of a secondary source resulting in an instrumental concentration of 5.0 ng/L mercury.
- 8.29 PM: Project Manager.
- 8.30 Practical Quantitation Limit (PQL), Method Reporting Limit (MRL): The minimum concentration that can be reported quantitatively. The PQL is often described as 1-10 times higher than MDL. Eurofins Frontier defines the PQL as the lowest concentration that can achieve 70-130% recovery for 10 replicate sample preparations. In LIMS, the PQL is referred to as the MRL.
- 8.31 Primary Source: The stock standard used to make the calibration standard. Procedural Method: A method where standards and samples are run through the analytical procedure exactly the same. By NELAC definition, this SOP is a procedural method.
- 8.32 Secondary Source: The stock standard used to make the OPR standard.
- 8.33 Shall: This action, activity, or procedure is required.
- 8.34 Should: This action, activity, or procedure is suggested, but not required.
- 8.35 Stock Standard Solution (SSS) – a standard of analyte that is purchased from a certified source for the preparation of working standards.
- 8.36 Total mercury: As defined by this method, all bromine monochloride-oxidizable mercury forms and species found in aqueous solutions. This includes, but is not limited to, Hg(II), Hg(0), strongly organo-complexed Hg(II) compounds, adsorbed particulate Hg(P), and several tested covalently bound organomercurials (i.e. CH₃HgCl, (CH₃)₂Hg, and C₆H₅HgOOCCH₃). The recovery of mercury bound within microbial cells may require additional preparation steps (i.e. UV oxidation, or oven digestion).
- 8.37 Travel or Trip Blank (TB): A sample of reagent water placed in a sample container in the laboratory and used to demonstrate that samples have not been contaminated by transport activities.

9 Interferences:

- 9.1 Gold and iodide are known interferences. At a mercury concentration of 2.5 ng/L and at increasing iodide concentrations from 30 to 100 mg/L, test data have shown that mercury recovery will be reduced from 100 to 0 percent. At iodide concentrations greater than 3 mg/L, the sample should be pre-reduced with SnCl₂ (to remove brown color immediately prior to analysis) and additional or more concentrated SnCl₂ should be added to the bubbler containing sample. If samples containing iodide concentrations greater than 30 mg/L are analyzed, it may be necessary to clean the analytical system with 4N HCl after the analysis.
- 9.2 Water vapor has the potential to create recovery interferences. To prevent interference from water, ensure that soda-lime pre-traps and gold traps remain dry.

- 9.3 The presence of high concentrations of silver and/or gold can cause SnCl_2 to precipitate out of solution and adhere to the bubbler walls. High concentrations of these metals can sometimes be found in the matrix spike samples from the digestion sets that are shared with the trace metals group. When analyzing digestates where the matrix spike samples have been spiked with silver or gold, the matrix-spiked samples must not be used for mercury analysis. Instead, an alternate matrix spike and matrix spike duplicate (MS/MSD) should be prepared and analyzed. If this is not possible, an Analytical Spike/Analytical Spike Duplicate (AS/ASD) must be analyzed on the ambient sample.

10 Safety Precautions, Pollution Prevention and Waste Handling:

- 10.1 Personnel will don appropriate laboratory attire according to the Chemical Hygiene Plan. This includes, but is not limited to, laboratory coat, safety goggles and nitrile gloves under clean gloves.
- 10.2 The toxicity or carcinogenicity of reagents used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure to these compounds should be as low as reasonably achievable. Chemists should refer to the MSDS (Material Safety Data Sheets) for each chemical they are working with.
- 10.2.1 Note: Use particular caution when preparing and using BrCl , as it releases extremely irritating, corrosive fumes similar in effect to free chlorine. Always handle this reagent in an approved fume hood
- 10.3 All personnel handling environmental samples known to contain or to have been in contact with human waste should be immunized against known disease-causative agents. Eurofins Frontier will reimburse the expense of Hepatitis A and B immunizations for any laboratory staff member who desires this protection.
- 10.4 Hydrochloric acid: Very hazardous in case of skin contact (corrosive, irritant, permeator), of eye contact (irritant, corrosive), of ingestion. Slightly hazardous in case of inhalation (lung sensitizer). Non-corrosive for lungs. Liquid or spray mist may produce tissue damage particularly on mucous membranes of eyes, mouth and respiratory tract. Skin contact may produce burns. Inhalation of the spray mist may produce severe irritation of respiratory tract, characterized by coughing, choking, or shortness of breath. Severe over-exposure can result in death. Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering. For more information see MSDS.
- 10.5 See Eurofins Frontier Global Sciences Chemical Hygiene Plan (CHP) for general information regarding employee safety, waste management, and pollution prevention.
- 10.6 Pollution prevention information can be found in the current Eurofins Frontier Global Sciences Chemical Hygiene Plan (CHP), which details and tracks various waste streams and disposal procedures.
- 10.7 All laboratory waste is accumulated, managed, and disposed of in accordance with all federal, state, and local laws and regulations. Any waste generated by this procedure should be disposed of according to SOP FGS-099 "Waste Disposal Procedure for


Client Sample Waste,” which provides instruction on dealing with laboratory and client waste.

11 Personnel Training and Qualifications:

- 11.1 An analyst must perform an initial demonstration of capability (IDOC) that includes four replicates of a secondary source before being qualified to analyze samples without supervision. Continuing DOC will be maintained and monitored via performance on CRMs and other QC samples, as well as obtaining acceptable results on proficiency testing exercises.
- 11.2 The analyst/laboratory technician must have read this SOP and other relevant SOPs and have the training documented on the applicable form(s). The analysis may be questioned on SOP by supervisor(s) and/or trainers.
- 11.3 Training is documented by the employee and supervisor, and is kept on file in the QA Office. The employee must read, understand, and by signing the training document, agree to perform the procedures as stated in all Standard Operating Procedures (SOPs) related to this method.
- 11.4 Reading of the SOP must be documented on the correct form such as “Standard Operating Procedure Training Record,” Appendix F in FGS-094, the last page of this SOP, Appendix A “Standard Operating Procedure Training Record” or a similar document.”
- 11.5 All employees must also, on a yearly basis, read the Quality Manual (QM), and complete the yearly Ethics training.
- 11.6 All training documents including IDOCs, CDOCs, SOP reading, Initial QA orientation, and Ethics training are stored by the Quality Assurance Manager in the employees training file for ten years after the employee is no longer working for Eurofins Frontier Global Sciences.
- 11.7 Chemical Safety Training, Compressed Gas Training, Chemical Hygiene Plan documentation, and Shipping of Hazardous goods, are stored by the Health and Safety Officer for ten years after the employee is no longer working for Eurofins Frontier Global Sciences.

12 Sample Collection, Preservation, and Handling:

- 12.1 Aqueous samples are collected in rigorously cleaned fluoropolymer (e.g. Teflon) or PETG bottles and caps (as described in FGS-007 “Cleaning of Sampling Equipment and Bottles for Mercury Analysis”). Certified clean glass bottles with fluoropolymer lids may be used if mercury is the only analyte of interest.
 - 12.1.1 Aqueous samples are preserved upon receipt with 0.2N BrCl that has tested low in mercury. Samples are typically preserved to 1% BrCl v/v, but may require further oxidation due to high levels of organic matter or mercury. Refer to FGS-012 “Oxidation of Aqueous Samples for Total Mercury Analysis” for oxidation of aqueous samples. Samples requiring greater than 10% BrCl must have a method blank prepared at the time of preservation. Preservation levels should be limited to 1%, 2%, 3%, 5%, 10%, and 100%.

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12.1.2 Preservation levels other than 1% are written on the LIMS label of the sample bottle. Preservation levels are also documented in the LIMS bench sheet by adjusting the initial and final volumes. For example, a sample preserved at 2 % BrCl must say "2" on the LIMS label, and have an initial volume of 100mL and a final volume of 102mL in the bench sheet.

12.2 All samples should be collected utilizing clean techniques, so as not to cross-contaminate samples with mercury. See FGS-008 "Ultra Clean Aqueous Sample Collection" and EPA Method 1669 for aqueous sample techniques.

13 Apparatus and Equipment:

- 13.1 *LIMS – Element, version 5.85 or higher; Computer – Windows XP, 7 or 8*
- 13.2 *Tekran 2500 Atomic Fluorescence Spectrophotometer (AFS) or equivalent: A high sensitivity AFS Detector (IDL<1pg) with a required wavelength of 253.7 nm and associated software.*
- 13.3 Flow meter/needle valve: A unit capable of controlling and measuring gas flow to the cold vapor generator at 200-500 mL/min.
- 13.4 Teflon Fittings: Connections between components and columns are made using Teflon FEP tubing and Teflon friction fit tubing connectors.
- 13.5 Soda-Lime pre-trap: A 10cm x 0.9cm diameter Teflon tube containing 2-3 g of reagent grade, non-indicating 8-14 mesh soda-lime ($\text{Ca}(\text{OH})_2 + \text{NaOH}$) aggregates, packed between portions of silanized glass wool. This trap is purged of mercury by placing it on the output of a clean cold vapor generator and purging it with ~3-5% HCl and ~600 μL of SnCl_2 for approximately 20 minutes with N_2 at 40 mL/min.
- 13.6 Cold-vapor generator (bubbler): A 150 mL, tall, flat-bottom borosilicate flask with standard taper 24/40 neck, fitted with a sparger having a coarse glass frit which extends to within 0.2 cm of the flask bottom.
- 13.7 Gold Traps: Made from 12 cm lengths of 6 mm OD quartz tubing, with a 4-way crimp 3.0 cm from one end. The tube is filled with approximately 2.5 cm of 20/40 mesh gold-coated quartz sand, the end of which is then plugged with quartz wool. Gold-coated sand traps are heated to 450-500°C (the coil should have a barely visible red glow when the room is darkened) with a coil consisting of 75 cm of 24-gauge nichrome wire at a potential of 10 VAC. Potential is applied and finely adjusted with an auto-transformer. Refer to SOP FGS-061 regarding the construction of gold traps used for total mercury analysis.
- 13.8 Agilent Integrator Recorder or equivalent: Any multi-range chart recorder or integrator with 0.1-5.0 mV input and variable speeds is acceptable. Data capture software may also be used.
- 13.9 Pipettes: Calibrated variable pipettes with a range of 5 μL – 10 mL. Used to make solutions and sample dilutions. Pipettes are to be calibrated weekly according to SOP FGS-003 and FGS-155.

14 Reagents and Standards:

All reagents, except those made daily, must be entered into LIMS

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- 14.1 Reagent Water: 18-M Ω ultra pure deionized water starting from a pre-purified (distilled, R.O., etc.) source is used. To remove any remaining trace metals and organics, an activated carbon cartridge is placed between the final ion exchange bed and the 0.2- μ m filter. Reagent water used in the mercury lab is checked weekly for total mercury concentrations, and must test below 0.25ng/L.
- 14.2 Hydrochloric Acid (HCl): Concentrated (36-38% weight basis). Must be trace-metal purified and reagent grade. HCl is typically monitored through performance of the BrCl. Sometimes it will be necessary to test the HCl directly. To do so, add 1 mL, using a calibrated pipette, of HCl to approximately 100mL of purged bubbler water. Enter 1mL as aliquot in the Excel spreadsheet. Do not prep blank correct. Analyze one replicate per bottle. This reagent should test below 5.0 ng/L. This solution is considered stable until the expiration date on the bottle, set by the manufacturer.
- 14.3 0.2N Bromine Monochloride (BrCl):
- 14.3.1 37.5 g of KBr is added to a 2.5-L bottle of concentrated HCl (pre-analyzed and found to be below 0.25 ng/L Hg). The bottle is then inverted in a fume hood to mix the acid and KBr. The solution then sits overnight allowing for the KBr to be dissolved.
- 14.3.2 27.5 g of KBrO₃, certified to be low in Hg, is slowly added to the acid. When all of the KBrO₃ has been added, the solution should have gone from yellow to red to orange.
- 14.3.3 Loosely cap the bottle, and allow to sit for 30 minutes in a fume hood before tightening the lid. Once capped invert bottle to make sure all of the solids goes into solution. **CAUTION: This process generates copious quantities of free halogens (Cl₂, Br₂, BrCl) which are released from the bottle. Add the KBrO₃ SLOWLY and in a well operating fume hood.**
- 14.3.3.1 To test the BrCl, add 1 mL, using a calibrated pipette, of the BrCl to a prep blank vial containing approximately 4 mL reagent water. Add 200 μ L Hydroxylamine-HCl to the vial; pour the entire contents into a bubbler containing approximately 100 mL of purged water. Assume a 100 mL aliquot in the Excel spreadsheet. This reagent must test below 0.20ng/L. Do not prep blank correct. Analyze one replicate per bottle.
- 14.3.3.2 The expiration time for this reagent is set by default to six months in LIMS. There is no suggested holding time in EPA method 1631E, therefore the holding time can be extended, as long as the primary reagent has not expired. The mercury concentration of the BrCl is monitored through the preparation of water preparation blanks.
- 14.4 Hydroxylamine hydrochloride: dissolve 300g of NH₂OH-HCl in reagent water and bring the volume up to 1L. This solution may be purified by the addition of 1mL SnCl₂ solution and purging overnight at 500mL/min with mercury-free N₂. The working reagent is a 25% solution that is made by adding one part reagent water to one part 50% hydroxylamine hydrochloride. This reagent must test below 0.25ng/L.
- 14.4.1 To test the Hydroxylamine-HCl (NH₂OH-HCl), add 1 mL of the 50% reagent, using a calibrated pipette, to approximately 100 mL of purged bubbler water.

Assume a 100 mL aliquot in the Excel spreadsheet. This reagent must test below 0.20 ng/L. Do not prep blank correct. Analyze one replicate per bottle.

- 14.4.2 The expiration time for this reagent is set by default to six months in LIMS. There is no suggested holding time in EPA method 1631E; therefore the holding time can be extended, as long as the primary reagent has not expired.
- 14.5 Stannous Chloride (SnCl_2): Weigh out 500 g SnCl_2 using a calibrated balance that also has been verified for the day. Dissolve with three 100 mL aliquots of concentrated HCl and transfer to a 1L I-CHEM glass bottle, which contains approximately 300 mL of reagent water. Bring this solution up to approximately 1 L of volume and purge overnight with mercury-free N_2 at 500 mL/min to remove all traces of mercury. Store tightly capped. The working reagent is a 25 % solution that is made by adding one part reagent water to one part 50 % stannous chloride.
- 14.5.1 To test the Stannous Chloride (SnCl_2), add 1 mL of the 50% reagent, using a calibrated pipette, to approximately 100 mL of purged bubbler water. Assume a 100 mL aliquot in the spreadsheet. This reagent must test below 0.20 ng/L. Do not prep blank correct. Analyze one replicate per bottle.
- 14.5.2 The expiration time for this reagent by default is set to six months in LIMS. There is no suggested holding time in EPA method 1631E; therefore the holding time can be extended, as long as the primary reagent has not expired.
- 14.6 Argon Grade 4.7 or better (ultra high-purity grade): Argon that has been further purified by the removal of mercury using a gold trap that is located in line between the gas output and the analyzer gas input.
- 14.7 Nitrogen Grade 4.5 (standard laboratory grade): Nitrogen that can be further purified of mercury using a gold trap that is located in line between the gas output and bubbler
- 14.8 Preparation of Total Mercury Standard Solutions:
- 14.8.1 Mercury standard solutions are prepared in ultra clean volumetric glassware and gravimetrically calibrated pipettes. Resulting solutions must be stored in glass or Teflon bottles and preserved to at least 2 % BrCl. All working standards must be tested prior to use.
- 14.8.1.1 New working standards and standard dilutions are tested prior to use. Three reps of the new standard are analyzed in the same run as three reps of the current NIST 1641D standard. Analyze 200 μl of the NIST 1641D and assume 100 ml in the bubbler. The mean percent recovery of the three standards should be $\pm 5\%$ (95-105 %) of the true value and also within 5 % of the average NIST 1641D recovery (e.g. If the average of NIST 1641D recovery is 97 %, the range for the standard is 95-102 %). If the standard does not test within this control limit, it is retested. If it still does not meet the control limit, it is discarded and remade, unless otherwise approved by the Quality Assurance Officer. NOTE: When making serial dilutions to create various standard levels; the lowest concentration may be used to test any of the higher concentration steps (for example: if a 10ng/mL calibration standard is created from a 1000ng/mL spiking standard, only the 10ng/mL standard requires testing.

If the 10ng/mL standard passes, then both standards are considered to be passing within the control limits.)

- 14.8.2 Total Mercury Stock Standard Solution (Stock): Certified mercury standard purchased from High Purity Standards (1000 µg/mL (1 000 000 ng/mL) primary source) or Absolute Standards (100 µg/mL (100 000 ng/mL) secondary source), or any equivalent standard.
- 14.8.3 Total Mercury Spiking Standard Solutions (Spiking Standard): Spiking standards are made from either the primary or secondary sources.
- 14.8.3.1 To make standards, use an ultra clean volumetric flask and a calibrated pipette. Add reagent water until flask is about half full. Add 2 % 0.2N BrCl and the specific spike volume noted below (these volumes may be changed as long as ratio and resulting concentration remains the same). Bring up to the mark with reagent water and mix well prior to testing. When spiking samples, no more than 200 µL of any spiking standard is added to the sample to minimize effects on volume. It is also recommended that staff pipette no less than 25 µL. If possible, minimize headspace during standard storage. Expiration date is currently set at 6 months or when the stock standard expires, whichever is shorter.
- 14.8.3.2 100,000 ng/mL Spiking Standard: Made from the Primary Stock Standard (High Purity, or equivalent vendor). Dilute 10 mL of the stock standard to 100 mL of reagent water containing 2 % BrCl. (Can also be made by preserving Secondary Stock Standard to 2% BrCl).
- 14.8.3.3 10,000 ng/mL Spiking Standard: If made from the Primary Stock Standard (High Purity, or equivalent vendor). Dilute 1.0 mL of the stock standard to 100 mL of reagent water containing 2 % BrCl. If made from Secondary Stock Standard, dilute 10mL of stock standard to 100mL with reagent water containing 2% BrCl.
- 14.8.3.4 1,000 ng/mL Spiking Standard: If made from the Primary Stock Standard (High Purity, or equivalent vendor). Dilute 0.250 mL of the stock standard to 250 mL RO water containing 2 % BrCl. If made from Secondary Stock Standard dilute 2.5mL of stock standard to 250mL with RO water containing 2% BrCl.
- 14.8.3.5 100 ng/mL Spiking Standard: Made from a stock standard or dilution of a stock standard with a concentration of 100,000 ng/mL. Dilute 0.100 mL of the 100,000 ng/mL dilution to 100 mL of reagent water containing 2 % BrCl. Expiration date is currently set at 3 months or when the stock standard expires, whichever is shorter.
- 14.8.4 Calibration Standard (10 ng/mL): Must be made from a dilution of the Primary Stock Standard (High Purity, or equivalent vendor). Typically made by diluting 0.5mL of a 10,000 ng/mL Primary Spiking Standard to 500 mL of reagent water containing 2 % BrCl. Transfer to glass or Teflon bottle. The calibration standard is considered stable for three months or until the stock standard expires.

- 14.8.5 Calibration Standard (1 ng/mL): Must be made from a dilution of a Primary Stock Standard. Typically made by diluting 1.0mL of a 100 ng/mL Primary Spiking Standard to 100mL with Reagent water containing 2% BrCl.
- 14.8.6 Initial Calibration Verification (ICV): A 10 ng/mL ICV solution is prepared using the Secondary Stock Standard (Absolute Standards, or equivalent vendor). Use 0.100 mL (100 µL) of the Secondary Stock Standard to 1000 mL Milli-Q containing 2 % BrCl. Transfer to one 1000 mL glass or Teflon bottle. The ICV standard is considered stable for three months or until the stock standard expires. It is recommended to alternate expiration date with the CAL standard.
- 14.8.7 Continuing Calibration Verification (CCV): For CCV analysis, use 200 µL of the 10 ng/mL CAL standard (documented in LIMS as SEQ-CAL3). The True Value is 20 ng/L.
- 14.8.8 Certified Reference Material (CRM) for Total Mercury in Water: A 1.5679 mg/L solution (1.557 mg/kg at a density of 1.007 g/mL) is prepared by adding a 5.0 mL of CRM NIST 1641d (from ampoule) into a 1000 mL flask containing of reagent water. This solution is diluted to 1000 mL, and an additional 10 mL of 0.2N BrCl is added, resulting in a final volume of 1010 mL. Preparing the solution in this manner makes a 1:200 dilution of the stock CRM. This solution is considered stable for one year, or until the stock standard expires. Results are corrected for the additional 1 % BrCl in the analysis Excel spreadsheet and in LIMS.
- 14.8.9 Ongoing Precision and Recovery (OPR) for "Strict" 1631E: A 5.0 ng/L solution is prepared by adding 100 µL of the 100 ng/mL secondary spiking standard into 2000 mL reagent water. An additional 1 % BrCl (20 mL) of BrCl is added, so that the final volume is 2020 mL. This standard is analyzed at 100 mL at the instrument, and preparation blank corrected exactly in the same manner as samples
- 14.9 Documentation of Standards and Reagents:
- 14.9.1 Standards and Reagents are documented in LIMS upon receipt or creation. A LIMS generated label is affixed to each standard and reagent that has the name of the solution, the person who prepared or received it, the date it was prepared or received, and the expiration date.
- 14.9.2 Each bottle of standard must be labeled with the following: the date of receipt or creation, the initials (or name) of who entered the standard into LIMS, the concentration and analyte, the expiration date and the LIMS ID. This information must also appear on the certificate of analysis of stock standards.
- 14.9.3 Stock standards and CRMs are logged into LIMS upon receipt by Shipping and Receiving (S&R) or the Quality Assurance department (QA). These do not require testing, provided there is a Certificate of Analysis on file in QA. When receiving a solid CRM, QA shall generate a work order in LIMS for total solids analysis.
- 14.9.4 For all standards, LIMS documentation must include the following: a description of the standard, department, expiration date of the standard (not to exceed the expiration of the parent standard), the name of the person who made (or

received) the standard or reagent, the date it was prepared (or received), final volume, a reference date (date entered into LIMS), concentration units ($\mu\text{g/mL}$), the vendor and vendor lot. The solvent lot is used to document the Lot Number or LIMS ID of the BrCl that was used. In the comments section, the analyst must enter the sequence and applicable results for documentation of standard testing. Other notes may be entered in here as well. The correct parent standard must be noted, as well as the amount used. Analytes are entered individually from the list. LIMS will calculate the true value of the standard based on the amount of the parent used and the final volume. Click the appropriate radio button under Standard type. A Spike Mix is a standard that is used in a bench sheet, and a Calibration standard is a standard used only in sequences. A Reference Standard is a Certified Reference Material (CRM). The standard must not be used until it has passed control limits and is approved by the mercury supervisor, mercury laboratory manager, or QA for use.

- 14.9.4.1 If the new standard is a calibration standard, a separate standard ID must be created for each calibration point based on the final concentration in the sequence (example: THg CAL1 0.10 ng or THg CAL2 0.50 ng). These are given the same expiration as the standard they are made from, and will need to be generated every three months as each new working calibration standard is made and tested.
- 14.9.4.2 To generate new "CAL" standards in LIMS, go to the Laboratory drop down menu and select Standards. Open the current CAL1 standard and click "Copy". Update the appropriate information, including the Prepared Date, Expiration Date, Prepared By, and the Reference Date. For these standards, which are to be used in the sequence, the final volume is equal to the assumed aliquot in the bubbler (100 mL). Check that the vendor lot is correct. Remove the old (expired) parent standard. Choose the new parent standard, and enter the amount of standard added to the bubbler for that calibration point. All depleted or expired standards are moved into the Expired Standards Department once they are no longer being used.
- 14.9.4.3 Each bottle of standard must be labeled with the following: the date of receipt or creation, the initials (or name) of who entered the standard into LIMS, the concentration and analyte, the expiration date and the LIMS ID. This information must also appear on the certificate of analysis of stock standards.
- 14.9.5 Neat reagents are logged into LIMS with a unique identifier upon receipt by Shipping and Receiving Department and given a default expiration of 3 years, unless otherwise noted by the manufacturer.
- 14.9.6 Working reagents are prepared by the analyst, logged into LIMS and assigned a unique identifier. Reagents entered into LIMS must have the information listed in section 14.9.2. In addition the parent neat reagents are added by their unique identifier and the amount of each reagent is entered. It is not necessary to enter analytes from the list for reagents. The Solvent Lot is not applicable to working reagents. The radio button must be clicked to Reagent. If the reagent

requires testing, it must test clean prior to using. All reagents used during analysis and prep should be added to bench sheet.

- 14.9.7 Depleted or expired standards and reagents are segregated and removed from use.

15 Calibration:

- 15.1 The analyst should label the strip chart/integrator printout with the corresponding dataset ID as well as print and sign their name. For strip chart printouts, the analyst should label the baseline ratios accordingly (usually X=1 and X=20) and label with the analysis day start time and strip chart drum speed (usually 1 mm/min). The analyst should note the end time as well. If using an integrator, the date and time should be checked and corrected if necessary.
- 15.2 The calibration sequence determines the range of sample concentrations that are reportable. The calibration sequence starts with a 5-point curve using the total mercury calibration standard solution. The five points are: *0.05ng (0.50 ng/L)*, *0.10 ng (1.00 ng/L)*, *0.50 ng (5.00 ng/L)*, *2.00 ng (20.00 ng/L)*, and *4.00 ng (40.0 ng/L)*. An ICV/OPR and IBLs (one for every bubbler used are analyzed immediately following the standard curve.
- 15.2.1 Using the 10 ng/mL calibration standard, add 5 μ L, 10 μ L, 50 μ L, and 200 μ L to the bubblers sequentially from the left to right. Add 300 μ L SnCl₂ to the bubblers and seal bubbler tops using Keck Clips.
- 15.2.2 Place blanked gold traps securely at the end of soda-lime traps (pinched section of gold trap closest to the soda-lime trap). Purge bubblers with N₂ for a minimum of 20 minutes.
- 15.2.3 Attach individual gold traps to the analytical train and burn in sequential order. Peaks produced should be labelled, as well as recorded in the Excel spreadsheet in real time.
- 15.3 For the second round, add 400 μ L of the 10 ng/mL mercury calibration standard to the first bubbler. Add 50 μ L of the 10 ng/mL ICV(OPR) standard to the second bubbler (*5.0 ng/L*). *The third and fourth bubblers are used for the first and second IBLs and nothing should be added to these bubblers.* To ensure that nothing is added, keep it sealed with a Keck Clip. Add 300 μ L SnCl₂ to all bubblers except the fourth and seal bubbler tops with Keck Clips.
- 15.4 For the third round, use the first and second bubbler to finish the IBLs needed for 1631. The third and fourth bubbler can be used for the first portion of the batch. If the curve does not pass or needs to be investigated any batch portions analyzed in this round will need to be reanalyzed.
- 15.5 Once the instrument is calibrated and the ICV/IBLs are analyzed and judged to be in control, the instrument is operational. The sample concentrations must fall within the range of the calibration standards or be diluted and reanalyzed.
- 15.6 The purge efficiency of the bubbler system is 100 % and is independent of volume at the volumes used in this method. Calibration of this system is typically performed using units of mass. For purposes of working in concentration, the volume is assumed to be 100 mL.

15.7 This completes the instrument calibration for total mercury analysis.

16 Procedure:

16.1 *When analyzing on the Tekran 2600, follow the procedure in EFGS-121 while still adhering to the QA/QC criteria of this method.*

16.2 Pre-analysis and Organization:

16.2.1 Prior to analyzing samples it is imperative to reference LIMS for all project specific information, such as QC requirements, suggested dilutions, project manager information, and specifics regarding spike levels.

16.2.2 The analyst should then locate samples and check the work order in LIMS for notes about specific project requirements.

16.2.3 The analyst should compare the sample IDs to the work order and see that the samples are accounted for, and notify the project manager of any discrepancies in analysis required, sample identification, etc.

16.2.4 All mercury analyses receive a unique dataset identifier. This is comprised of the instrument type and number, the date and the calibration number for that day. The format is as follows: THg8-091218-1, where "THg" refers to a total mercury analysis; "8" refers to the analyzer number 8; 091218 refers to the date (December 18, 2009 in the YYMMDD format); and "1" refers to the first calibration of the day.

The sequence number is assigned by LIMS when the data gets imported into LIMS. The alpha-numeric code is based on the following format: 3B02001, where the 3 refers to the year (2013), the "B" is the month (A= January, B=February...L=December), "02" is the day of the month (February 2nd) and the final 3 digits is the nth sequence created on that particular year/month/day combination.

16.2.5 In general, the analyst should organize their samples in the order listed on the bench sheet. The first samples analyzed should be the preparation blanks, then the LCS if analyzing solid samples, followed by actual samples. If possible, run total and dissolved samples side by side to facilitate verification that total concentration is greater than dissolved concentration. See QA section.

16.2.6 All samples specified as being *High QA* should be analyzed prior to any Standard QA projects that are being analyzed on the same instrument on the same day. However, if concentrations are known, analyze samples with low concentrations prior to samples with high concentrations

16.3 Instrument Start Up:

16.3.1 Begin blanking gold traps. To do this, attach one trap at a time to the analytical train and burn to the instrument. Ensure the Argon is flowing at appropriate levels (~25-40 mL/min). The pinched portion of the gold trap should be on the left (closest to the analytical trap). Continue to burn traps in sequential order.

- 16.3.2 Rinse out the bubbler three times with reagent water and fill with about 100 mL of reagent water. Using a pre-purged pipette, add 3-5 mL HCl. Initially add 600 μ L of SnCl_2 .
- 16.3.3 Prepare one soda-lime trap for each bubbler. To prepare soda-lime traps, hold soda-lime between two glass wool plugs in a Teflon tube. Cap the tubes with Teflon plugs and attach to the bubbler. Once the soda-lime traps have been attached, the bubbler system (soda lime trap and bubbler water/acid/ SnCl_2) must purge for a minimum of 20 minutes before beginning the instrument calibration sequence.

16.4 Analyzing Aqueous Samples:

- 16.4.1 All aqueous samples should be preserved with BrCl according to FGS-012 at least 24 hours prior to analysis. In the event a sample requires further oxidation prior to analysis, additional BrCl is added and the sample should not be analyzed for at least 12 additional hours. In special cases where rush turn-around-time is required and an oxidation period of less than 24 hours may be used, a heated oven digestion procedure can be utilized.
- 16.4.2 While bubbling and burning the standard curve, the analyst should prepare a minimum of three BrCl method blanks (BLK) at 1% BrCl. Add 1 mL BrCl and 200 μ L hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$) to each bubbler. The aliquot is assumed to be 100 mL. Any sample requiring an increased amount of reagent must be accompanied by at least one method blank that includes an identical amount of reagent.
- 16.4.3 After the instrument calibration sequence, preparation blanks and the LCS/LCSD are analyzed.
- 16.4.4 All known field, equipment, and trip blanks should be analyzed before any other sample types, usually after the BLKs. Aliquots of 100 mL should be analyzed, provided there is adequate collected sample volume. Sample aliquot sizes of 125 mL can be analyzed upon request by the project manager.
- 16.4.5 For all waters, select the appropriate dilution (refer to LIMS, historical data, etc.).
- 16.4.5.1 For sample aliquots of 25 μ L to 10.0 mL, use calibrated pipettes to dispense the aliquots directly into bubbler. Due to minimal amounts of BrCl in aliquots of 10 mL or less, $\text{NH}_2\text{OH}\cdot\text{HCl}$ is not added. It is highly recommended that the analyst should not pipette less than 25 μ L. A dilution of the sample should be made to allow a larger aliquot to be analyzed.
- 16.4.5.2 For sample aliquots greater than 10 mL, gravimetrically weigh out the selected volume (± 0.2 g) into a clean 125 mL Teflon bottle. Once quantity is weighed out, neutralize BrCl with 200 μ L $\text{NH}_2\text{OH}\cdot\text{HCl}$ no more than five minutes prior to adding the sample to bubblers. The sample should turn from a yellowish color to a clear/cloudy solution, depending on the matrix.
- 16.4.6 If the material is a seawater or highly dense liquid, it may be necessary to account for the density if the aliquot is gravimetrically determined. Density

checks can be performed at the time of analysis to determine if further determinations are necessary.

16.4.7 The procedure for analysis is similar to that of the calibration. Samples to be analyzed are pipetted or poured into the bubbler (one sample per bubbler) along with 300 μ L SnCl₂. Bubbler tops are sealed with Keck Clips to ensure nominal sample leakage. Blanked gold traps are securely placed at the end of the soda-lime trap. Purge bubblers with N₂ for a minimum of 20 minutes, remove gold traps, and sequentially place in the analytical train. Burn individual traps to analyzer, labeling resulting peaks with corresponding sample in real time.

16.4.7.1 Sample IDs, aliquot volume, BrCl percentage (group ID), peak height/peak area, and dilution factor (if applicable) associated with each sample should be entered into the THg Waters Template Excel spreadsheet.

16.4.7.2 While purging one set of samples, the analyst should begin preparing the next round of water samples in the same fashion to maximize efficiency.

16.5 End of analysis close-down procedure:

16.5.1 Turn off gas flow.

16.5.2 Carryout all end of day cleaning and restocking tasks.

16.6 The analytical data is compiled into an Excel file. The data is then copied and pasted into an Excel template that is LIMS compatible.

16.7 *Maintenance and Troubleshooting*

16.7.1 *ISSUE: No peaks at all*

16.7.1.1 *Ensure that the system is powered.*

16.7.2 *ISSUE: Low sensitivity*

16.7.2.1 *Make sure that you have freshly changed soda lime in the soda lime trap, and that it is from a good source.*

16.7.2.2 *Do not use old calibration standards to calibrate the system.*

16.7.2.3 *Make sure you are running fresh SnCl₂ solution.*

16.7.2.4 *Make sure that your stock Hg standard has not expired and is from a reliable source and that it is not compromised.*

16.7.2.5 *Check the lamp voltage*

16.7.3 *ISSUE: High blanks*

16.7.3.1 *Check reagent (including water) quality*

16.7.3.2 *Check for system contamination*

16.7.4 ISSUE: Nonlinearity of the calibration curve

16.7.4.1 *Check and investigate high blanks.*

16.7.4.2 *Contaminated and expired soda lime. Change soda lime.*

16.7.4.3 *Make sure your calibration standards are fresh and properly prepared.*

17 Calculations:

17.1 Average all instrument blanks (PH_x) using the peak area values from the TekMDS software. Subtract the average (IB) from the peak area for each standard and sample.

17.2 Calculate the calibration factor (CF_x) for mercury in each of the five standards using the mean instrument-blank-subtracted peak area and the following equation:

$$CF_x = PA_x - IB / C_x$$

Where:

17.2.1 PA_x =peak area (or peak height) for mercury in standard

17.2.2 IB= mean peak height (or peak area) for mercury in bubbler blank

17.2.3 C_x =mass in standard analyzed (ng/L)

17.2.4 CF_x =Calibration Factor of each concentration

17.2.4.1 Average the five calibration factors to establish mean value: $CF_{(Avg)}$ (units/ng/L).

17.3 Sample results are then corrected for the average peak area values of at least three preparation blanks (PBs), unless otherwise requested. This result is shown as the Initial Result on the Excel spreadsheet and in LIMS.

17.4 Total Mercury in Water:

$$\text{Instrument Value (ng/L)} = (\text{Peak Height} - \text{BB}) / CF_{(Avg)}$$

$$\text{Final Result (ng/L)} = [(\text{Instrument Value} \times \text{DF}) - (\text{BLK})] \times (V_f / V_i)$$

Where:

17.4.1 $CF_{(avg)}$ = average calibration factor for curve (in units/ng/L).

17.4.2 BB = average bubbler blank peak area or peak height (in units)

17.4.3 V_f = Final volume of sample (in mL) from bench sheet.

17.4.4 V_i = initial volume of sample analyzed in mL prior to addition of BrCl.

17.4.5 DF = Dilution Factor - takes into account any instrumental dilution of the sample

17.4.6 BLK = average of the preparation blanks in ng/L.

- 17.5 A linear regression can be used as alternate calibration. A linear regression will not change values significantly. If linear regression is used, the correlation coefficient (R) must be ≥ 0.995 .

18 Statistical Information/Method Performance:

- 18.1 The Method Detection Limit (MDL) is determined according to 40 CFR Part 136 Section B. Ten replicates (9 degrees of freedom) spiked 3-10 times the expected MDL are run. The standard deviation (s) is taken from the resulting data and the MDL is calculated as follows: $MDL = 2.821 * s$. This value should not be interpreted as the method reporting limit.
- 18.2 The Practical Quantitation Limit (PQL) is the reporting limit for this method and is included as the lowest calibration point (2003 NELAC regulation 5.5.5.2.2.1.h.3). The PQL is determined by running ten samples with a concentration that will produce a recovery of 70-130 %. The PQL is referred to as the Method Reporting Limit (MRL) in LIMS.
- 18.3 Using clean handling techniques and reagents tested low for Hg content, the LOD value for Total Hg in water is typically less than 0.2 ng/L, while the PQL is 0.50 ng/L.
- 18.4 Current LODs, LOQs, MDLs, and PQLs are stored at: Cuprum\General and Admin\Quality Assurance\MDLs & PQLs.

19 Quality Assurance/Quality Control:

- 19.1 A minimum of three preparation blanks and one LCS/LCSD (preferably NIST 1641d), must be analyzed per preparation batch. The upper control limit for each preparation blank is equal to the PQL.
- 19.2 Matrix Spikes: One Matrix Spike/Matrix Spike Duplicate (MS/MSD) must be performed for every 10 samples. The recovery of the MS/MSD must be between 71%-125% recovery, and the Relative Percent Difference (RPD) below 24%. If an MS/MSD is out of control, the analyst should investigate to identify the source of the failure. The MS and MSD may be used as duplicates. Some failures may be qualified using QA Qualification Flow Charts (Appendix A).
- 19.2.1 For aqueous samples, the MS/MSD is spiked at 1 to 5 times the ambient concentration, with 0.25 ng, in the bubbler, being the minimum spiking level. Sample aliquots for the MS/MSD should be the same as the ambient sample aliquot, if sufficient sample volume exists. Spikes are added to the split aliquots for volumes of 10mL or greater. For less than 10mL aliquots, spikes are added directly to the bubbler. NEVER ADD SPIKE DIRECTLY TO THE ORIGINAL SAMPLE VESSEL UNLESS OTHERWISE STATED.
- 19.3 Matrix Duplicates – One Matrix Duplicate (MD) may be analyzed for every batch of 20 samples. Upon request, a Matrix Triplicate (MT) may be performed. The MSD may serve as the MD if necessary. The Relative Percent Difference (RPD) and the Relative Standard Deviation (RSD) of duplicate samples must be less than 24%. Some failures may be qualified using QA Qualification Flow Charts.

- 19.3.1 For aqueous samples, analyze the parent, duplicate and triplicate at the same dilution.
- 19.4 Laboratory Control Standard (LCS) or Quality Control Sample (QCS): For every batch of samples, at least one LCS is processed and analyzed. The recovery of the LCS must be within 80-120% for the aqueous NIST 1641d. An LCS Duplicate (LCSD) should accompany the LCS.
- 19.4.1 A Certified Reference Material (CRM) is the preferred LCS, but a Blank Spike may serve as an LCS if an appropriate CRM does not exist. The spiking level is based on client request, historical data, or a default of mid-curve. A duplicate blank spike must also be prepared as an LCSD.
- 19.5 Ongoing Precision and Recovery (OPR): An OPR must be analyzed at the beginning and end of each analytical batch, or at the end of each 12-hour shift. The recovery of the OPR must be within 77-123% to be considered in control.
- 19.6 All calibration standards must be traceable to the original standard source. The calibration curve must be established at the beginning of the analytical run. It must include at least five different concentrations, with the lowest concentration equal to the PQL. The average response factor of each calibration standard is used to calculate the sample values. The RSD of the response factors must be less than 15% of the mean or the calibration fails.
- 19.7 ICV and CCV control limit is 77-123%. The CCV is analyzed every 10 analyses, and at the end of an analytical run. CCBs are always analyzed after the CCVs.
- 19.8 Field Blanks: To be compliant with EPA 1631, clients must submit a field blank for each set of samples (samples collected from the same site at the same time, to a maximum of 10 samples).
- 19.8.1 If no field blanks are submitted by the client, their data will be flagged with "FB-1631." "Required equipment/field/filter blank not submitted by the client. The sample has been analyzed according to 1631E, but does not meet 1631E criteria."
- 19.9 Method or Preparation Blanks (BLK): Method blanks are used to demonstrate that the analytical system is free from contamination that could otherwise compromise sample results. Method blanks are prepared and analyzed using sample containers, labware, reagents, and analytical procedures identical to those used to prepare and analyze the samples.
- 19.9.1 A minimum of three 1 % BrCl method blanks per analytical batch are required. Any sample requiring an increased amount of reagent must be accompanied by at least one method blank that includes an identical amount of reagent.
- 19.9.2 If the result for any 1 % BrCl method blank is found to contain ≥ 0.50 ng/L Hg (0.25 ng/L for DOD), the system is out of control. Mercury in the analytical system must be reduced until a method blank is free of contamination at the 0.50 ng/L level.
- 19.9.3 For method blanks containing more than 1% BrCl, the control limit is equal to 0.50 ng/L multiplied by the final preservation percentage of BrCl. For example, for a method blank preserved to 2 % BrCl, the control limit for the blank is 0.50

ng/L * (102/101), or 0.50 ng/L. For 3% BrCl the control limit is (103/101)*0.50ng/L, or 0.51ng/L.

- 19.10 Instrument Blanks (IBL): A minimum of three instrument blanks must be analyzed with each analytical batch. To analyze an instrument blank, attach a clean gold trap to the bubbler. Purge and analyze as previously described and determine the amount of Hg remaining in the system.
- 19.10.1 An instrument blank must be performed on all bubblers used during the analytical run (normally four, but three at a minimum).
- 19.10.2 If the instrument blank is found to contain more than 0.50ng/L, the system is out of control. The problem must be investigated and remedied and the samples run on that bubbler must be reanalyzed. If the blanks from other bubblers contain less than 0.50 ng/L, the data associated with those bubblers remain valid, provided that all other QC criteria are met.
- 19.10.2.1.1 The mean result for all instrument blanks must be <0.25ng/L with a standard deviation of 0.10 ng/L.
- 19.11 The analytical day must close with a CCV/OPR/CCB.
- 19.12 Because the method is done in real-time, it is EFGS' position that a single non-compliant QC sample result does not automatically invalidate a data set. All data points that can be explained and rerun with a passing result can be qualified. If the source of error cannot be corrected for a QC standard that day, none of the data can be validated. In the event that the system becomes out of control during the analysis day, all results bracketed between valid QC data points shall still be considered valid (CCV, OPR, CCB, etc).
- 19.13 The Control Limits are established from EPA 1631E.

20 Corrective Action

- 20.1 The data is reviewed as in the QC section (or matrix specific QC section) for all parameters that pass specific requirements. If the data does not meet QC requirements it is qualified or submitted for reruns. Data may be qualified (based on scientific peer review) by the Group Supervisor, Project Manager, Lab Manager, or QA Officer.
- 20.2 Control Chart data is generated through LIMS to monitor the performance of the CCV, LCS, MS, and MSD. This is done by the QA department.
- 20.3 Due to the real-time nature of the CVAFS method, failures must be investigated as they happen. If the source of the problem can be identified, and corrected, the samples may be rerun. If source of problem cannot be isolated, see the Senior Analyst, Group Supervisor, or Laboratory Manager for instructions.
- 20.4 *The Senior Analyst, Group Supervisor, Laboratory Manager, or QA Officer must be informed if QC fails. It is also advisable to always alert the Project Managers.*

21 List of Attachments

Table 1: QC Requirements for Total Mercury

Appendix A: Example - Standard Operating Procedure Training Record



Table 1: QC Requirements for Total Mercury

QC Parameter	Acceptance Criteria
Initial Calibration Verification (ICV)	77-123% Recovery
Continuing Calibration Verification (CCV)	77-123% Recovery
Ongoing Precision and Recovery (OPR)	77-123% Recovery
Initial Calibration Blank (ICB)/ Continuing Calibration Blank (CCB)	Individually, IBL and CCB $\leq 0.50\text{ng/L}$, but the mean of all the IBLs shall be $< 0.25\text{ng/L}$ with a standard deviation of 0.10ng/L .
Laboratory Control Standard (LCS) or Quality Control Standard (QCS)	80-120% Recovery for NIST1641d and 75-125% for all other CRMs. RSD $< 24\%$
Calibration Curve RSD (Referred to as "Corr. RSD CF" in Excel spreadsheet).	RSD of Calibration Response Factor $\leq 15\%$
Lowest Calibration Point	75-125%
1% BrCl Method Blank (BLK)	Less than 0.50ng/L (0.25ng/L for DOD projects) (individually)
Matrix Duplicate (MD) and Analytical Duplicate (AD)	$< 24\%$ RPD
Matrix Spike and Matrix Spike Duplicate (MS/MSD) ; Analytical Spike (AS) and Analytical Spike Duplicate (ASD)	71-125% Recovery $< 24\%$ RPD

Appendix A: Example - Standard Operating Procedure Training Record

By signing this document, I the employee, certifies to have read, understood and agreed to follow the test method and quality procedure as described in this procedure.

Reading of SOP FGS-137.02:

Mercury in Water by Oxidation, Purge & Trap and CV-AFS (EPA Method 1631, Rev E).

SOP name and Revision number

Employee name (print)

Employee name (sign)


Date:

Supervisor name (sign)

Date:

Initial SOP Training (leave blank if not applicable)

Initial reading of method and training	Initials	Date	Supervisor
1. Read method			
2. Observe the method			
3. Detailed review of method and associated literature			
4. Supervised practice of method with trainer			
5. Unsupervised practice of the method with trainer			
6. Review of work with trainer and/or peer-review			
7. IDOC to determine precision and accuracy			
8. Determination of blanks			


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Eurofins Document Reference	EFGS-SOP-011-R05	Revision	5
Effective Date	5/20/2013	Status	Final
Historical/Local Document Number	FGS-SOP-011.05		
Local Document Level	Level 3		
Local Document Type	SOP		
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Prepared by	Ryan Nelson
Reviewed and Approved by	Dave Wunderlich and Patrick Garcia-Strickland

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Approvals:

Prepared by: Ray Nelson Date: 5/20/13

Approved by: David A. Wundt Date: 5/16/13

Approved by: Pat Date: 5/20/13

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1 Revision Log:

Revision:	Effective Date:	
05	This version	
Section	Justification	Changes
Cover	Required change	Changed company name from Frontier Global Sciences to Eurofins Frontier Global Sciences.
All	Formatting requirement per LOM SOP-LAB-201	Reformatted document to new corporate specifications.
8.9	Required	Updated spiking levels for the matrix spike
14.3, 14.4	Required	Updated max contamination levels of reagent acids
17.3	Required	Replaced MDL with LOD
18.2 – 18.5	Required	Updated QC limits
18.3	Required	Incorporated QA MOC 2011-007

2 Reference:

- 2.1 Chemical Hygiene Plan, Eurofins Frontier Global Sciences, current version.
- 2.2 EPA Method 1631, Revision E: Mercury in Water by Oxidation, Purge and Trap, and Cold Vapor Atomic Fluorescence Spectrometry, 2002.
- 2.3 National Environmental Laboratory Accreditation Conference, NELAC Standard September 8, 2009.
- 2.4 Department of Defense Quality Systems Manual for Environmental Laboratories, prepared by DoD Environmental Quality Workgroup, Final Version 4.2, October 2010

3 Cross Reference:

Document	Document Title
SOP FGS-003	Pipette Verification, Calibration and Maintenance
SOP FGS-008	Ultra Clean Aqueous Sample Collection
SOP FGS-038	Data Review and Validation
SOP FGS-094, App F	Standard Operating Procedure Training Record
SOP FGS-099	Waste Disposal Procedure for Client Sample Waste
SOP FGS-121	Determination of Total Mercury in Various Matrices by Flow Injection Atomic Fluorescence Spectrometry (EPA Method 1631E)
SOP FGS-155	Calibration of Volumetric Dispensers


4 Purpose:

- 4.1 The purpose of this Standard Operating Procedure (SOP) is to describe the method for digesting biological tissue samples prior to analysis by CV-AFS for total mercury.

5 Scope:

- 5.1 This method is for the preparation of biological tissue samples for the determination of total mercury at concentrations less than 1 ng/g. Through the analysis of smaller digestate aliquots, contaminated tissues of up to 10,000 ng/g can be directly measured. Using clean handling techniques and low-level reagents, the typical detection limit for samples prepared by this method is less than 1 ng/g.
- 5.2 Total mercury, as defined by this method, is all HNO₃/H₂SO₄/BrCl-oxidizable mercury forms and species found in tissue matrices. This includes, but is not limited to, Hg(II), Hg(O), HgS, strongly organo-complexed Hg(II) compounds, adsorbed particulate Hg,

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and several covalently bound organo-mercurials (i.e., CH_3HgCl , $(\text{CH}_3)_2\text{Hg}$, and $\text{C}_6\text{H}_5\text{HgOOCCH}_3$).

6 Basic Principles:

- 6.1 Samples are collected using clean sample handling protocols into commercially available clean glass containers with Teflon-lined caps (i.e., I-Chem glass jars) or 125 mL or 250 mL HDPE jars. Freezing ($< -15^\circ\text{C}$) preserves tissue samples until sample preparation is performed.
- 6.2 A subsample of homogenized sample is digested with 10 mL of 70:30 $\text{HNO}_3/\text{H}_2\text{SO}_4$.
- 6.3 The digested sample is diluted up to 40 mL with 10% (v/v) BrCl.


7 Reference Modifications:

- 7.1 No significant modifications were made to this method.

8 Definitions:

- 8.1 Batch – no more than 20 client samples grouped for preparation. 3 Preparation Blanks, 1 CRM or 1 LCS/LCSD (or BS/BSD) set and 1 MD are prepared per every 20 samples; 1 MS/MSD set is prepared for every 10 samples.
- 8.2 Celsius (C), conversion of Celsius to Fahrenheit: $(C * 1.8) + 32$.
- 8.3 Fahrenheit (F), conversion of Fahrenheit to Celsius: $(F - 32) * 5/9$.
- 8.4 Method Detection Limit (MDL) – the limit derived from an exercise as described in 40 CFR, Part 136, Appendix B. The exercise produces a defined value that is the minimum concentration that can be measured and reported with 99% confidence that the analyte concentration is greater than zero from a given matrix.
- 8.5 Certified Reference Material (CRM) – a standard of known composition that is certified by a recognized authority and representing a sample matrix. It is used to verify the accuracy of a method.
- 8.6 Laboratory Control Sample (LCS) and Laboratory Control Sample Duplicate (LCSD), is a sample containing known concentrations of the analytes of interest that is taken through the entire preparation and analysis process in the same manner as the samples to monitor complete method performance. A Certified Reference Material (CRM) is preferred as the LCS, but a blank spiked sample also meets the requirement.
- 8.7 Preparation Blank (BLK) – Method blanks consist of the same reagents used to digest the samples, in the same volume or proportion, and are carried through the complete sample preparation and analytical procedure. Teflon boiling chips are added to the preparation blanks.
- 8.8 Matrix Duplicate (MD) – a representative sample is selected and digested in the same manner. This QC sample will indicate sample homogeneity on the analytes of interest
- 8.9 Matrix Spike (MS) and Matrix Spike Duplicate (MSD) – a representative sample is selected and spiked with a secondary source at two to five times the ambient

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concentration or at *two* to five times the MRL, whichever is greater. These QC samples will indicate sample matrix effects on the analytes of interest.

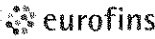
- 8.10 May: This action, activity or procedure is optional.
- 8.11 May Not: This action, activity or procedure is prohibited.
- 8.12 Shall: This action, activity or procedure is required.
- 8.13 Should: This action, activity or procedure is suggested, but is not required.

9 Interferences:

- 9.1 Due to the high levels of halogens (i.e., iodine) typically found in tissue digestates, it is recommended that aliquots of no more than 5.0 mL of the digestate be analyzed. Otherwise, soda-lime traps may be overloaded and the gold traps may lose the ability to amalgamate and retain mercury.
- 9.2 The high acidity and halogen levels that are found in tissue digestates necessitate the changing of the bubbler water after every 10 mL of digestate analyzed. Failure to do so can lead to low recoveries that would be reflected in the analysis of QC samples.

10 Safety Precautions, Pollution Prevention and Waste Handling:

- 10.1 Personnel will don appropriate laboratory attire according to the Chemical Hygiene Plan. This includes, but is not limited to, laboratory coat, safety goggles, and nitrile gloves under clean gloves.
- 10.2 The toxicity or carcinogenicity of reagents used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure to these compounds should be as low as reasonably achievable. Chemists should refer to the MSDS (Material Safety Data Sheets) for each chemical they are working with.
 - 10.2.1 Note: Use particular caution when preparing and using BrCl, as it releases extremely irritating, corrosive fumes similar in effect to free chlorine. Always handle this reagent in an approved fume hood.
 - 10.2.2 Note: Use particular caution when preparing and using the Nitric/Sulfuric Mixture. Always handle this reagent in an approved fume hood.
- 10.3 All personnel handling environmental samples known to contain or to have been in contact with human waste should be immunized against known disease-causative agents. Eurofins Frontier will reimburse the expense of Hepatitis A and B immunizations for any laboratory staff member who desires this protection.
- 10.4 Nitric acid (HNO₃): Corrosive. Strong oxidizer. Contact with other material may cause a fire. Causes eye and skin burns. May cause severe respiratory tract irritation with possible burns. May cause severe digestive tract irritation with possible burns. For more information see MSDS.
- 10.5 Sulfuric acid (H₂SO₄): Corrosive. Causes eye and skin burns. May cause severe eye irritation with possible burns. May cause severe respiratory tract irritation with possible burns. May cause severe digestive tract irritation with possible burns. Cancer hazard.


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Animal studies suggest this acid may cause fetal effects. May cause kidney damage. May cause lung damage. May be fatal if inhaled. Hygroscopic. Strong oxidizer. Contact with other material may cause a fire. For more information see MSDS.

- 10.5.1 Eyes: Get medical aid immediately. Do NOT allow victim to rub or keep eyes closed. Extensive irrigation with water is required (at least 30 minutes).
- 10.5.2 Skin: Get medical aid immediately. Flush skin with soap and water for at least 15 minutes while removing contaminated clothing and shoes. Wash clothing before reuse. Destroy contaminated shoes.
- 10.6 See Eurofins Frontier Global Sciences Chemical Hygiene Plan (CHP) for general information regarding employee safety, waste management, and pollution prevention.
- 10.7 Pollution prevention information can be found in the current Eurofins Frontier Global Sciences Chemical Hygiene Plan (CHP), which details and tracks various waste streams and disposal procedures.
- 10.8 All laboratory waste is accumulated, managed, and disposed of in accordance with all federal, state, and local laws and regulations. Any waste generated by this procedure should be disposed of according to SOP FGS-099 "Waste Disposal Procedure for Client Sample Waste," which provides instruction on dealing with laboratory and client waste.

11 Personnel Training and Qualifications:

- 11.1 An analyst must perform an initial demonstration of capability (IDOC) that includes four replicates of a secondary source before being qualified to analyze samples without supervision. Continuing DOC will be maintained and monitored via performance on CRMs and other QC samples, as well as obtaining acceptable results on proficiency testing exercises.
- 11.2 The analyst/laboratory technician must have read this SOP and other relevant SOPs and have the training documented on the applicable form(s). The analyst may be questioned on SOP by supervisor(s) and/or trainers.
- 11.3 Training is documented by the employee and supervisor, and is kept on file in the QA Office. The employee must read, understand, and by signing the training document, agree to perform the procedures as stated in all Standard Operating Procedures (SOPs) related to this method.
- 11.4 Reading of the SOP must be documented on the correct form such as "Standard Operating Procedure Training Record," Appendix F in FGS-094, the last page of this SOP, Appendix A "Standard Operating Procedure Training Record" or a similar document."
- 11.5 All employees must also, on a yearly basis, read the Quality Manual (QM), and complete the yearly Ethics training.
- 11.6 All training documents including IDOCs, CDOCs, SOP reading, Initial QA orientation, and Ethics training are stored by the Quality Assurance Manager in the employees training file for ten years after the employee is no longer working for Eurofins Frontier Global Sciences.

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11.7 Chemical Safety Training, Compressed Gas Training, Chemical Hygiene Plan documentation, and Shipping of Hazardous goods, are stored by the Health and Safety Officer for ten years after the employee is no longer working for Eurofins Frontier Global Sciences.

12 Sample Collection, Preservation, and Handling:

- 12.1 Samples must be collected in accordance with established ultraclean sampling techniques (see FGS-008 "Ultra Clean Aqueous Sample Collection"). Samples may be in commercially available clean glass containers with Teflon-lined caps (i.e., I-Chem glass jars), or 125 mL or 250 mL HDPE jars.
- 12.2 Tissue sample preservation - The tissue sample must be frozen in the sampling container at less than -15°C or freeze-dried and stored at room temperature. The holding time for tissue samples is 1 year.
- 12.3 Just prior to digestion, samples are thawed and if necessary homogenized. The sample is well mixed to ensure the most representative sample possible.

13 Apparatus and Equipment:


- 13.1 *LIMS – Element, version 5.85 or higher; Computer – Windows XP, 7 or 8*
- 13.2 40 mL or 20 mL I-Chem Vials: Borosilicate glass, series 300 vials with Teflon-lined septa in lids. The size used depends on the amount of sample available. The vials are volumetrically accurate to ± 0.5 mL when filled such that the meniscus is just to the bottom of the vial neck. The person performing the preparation should verify this.
- 13.3 Hot plate: A hot plate with the ability to achieve and maintain a temperature of 75 °C.
- 13.4 *Pipettors: All-plastic, pneumatic, fixed volume and variable pipettes in the range of 5 μ L to 10 mL. Pipettes are to be calibrated weekly according to SOP FGS-003 and FGS-155.*
- 13.5 Clean hood.
- 13.6 Analytical Balance: A laboratory analytical balance capable of weighing to ± 1 mg, with documented calibration.
- 13.7 Calibrated thermometer: Submerged in water in a 20 mL I-Chem vial. This vial is placed on the hotplate during the digestion process. The analysts must record the actual digestion temperature and the serial number of the thermometer used in the digestion logbook.
- 13.8 Sample Digestion Log.
- 13.9 Stainless steel tools for homogenization
- 13.10 Tissue Homogenization Log.
- 13.11 Disposable spatula.
- 13.12 Teflon boiling chips.
- 13.13 Teflon reflux cap to fit the 40 mL and 20 mL I-Chem vials.

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14 Reagents and Standards:

- 14.1 **Reagent Water:** 18 MΩ ultra-pure deionized water starting from a pre-purified (distilled, R.O., etc.) source. As a final mercury and organic removal step, the activated carbon cartridge on the 18-MΩ system is placed between the final ion exchange bed and the 0.2 μm filter.
- 14.2 **Nitric Acid (HNO₃):** Trace metal purified reagent-grade HNO₃ is pre-analyzed and lot sequestered. Several brands (Baker, Fisher, Omnitrace) have been found to have lots with acceptably low levels of trace metals. This reagent should be from a lot number that has been previously tested to be low for the analytes of interest. This reagent shall be entered into LIMS and the expiration date is set to the same as the manufacturer's expiration date.
- 14.3 **Sulfuric acid (H₂SO₄) -** Trace metal purified reagent-grade H₂SO₄ is pre-analyzed to < 50 ng/L Hg and lot sequestered before purchase. This reagent shall be entered into the LIMS and is considered stable until the expiration date on the bottle (set by the manufacturer).
- 14.4 **Nitric/Sulfuric Acid Mixture:** Carefully add 300 mL of pre-analyzed, low mercury (< 50 ng/L) concentrated sulfuric acid to 700 mL of pre-analyzed, low mercury concentrated nitric acid to a pre-marked Teflon bottle. Stir constantly. This reagent shall be entered into the LIMS with an expiration date of six months. **CAUTION: THIS MIXTURE BECOMES VERY HOT AND EMITS CAUSTIC FUMES.**
- 14.5 **Potassium Bromide (KBr), neat:** this reagent is pre-certified by the vendor to be low in mercury and is entered into the LIMS with a five year expiration date.
- 14.6 **Potassium Bromate (KBrO₃), neat:** this reagent is pre-certified by the vendor to be low in mercury and is entered into the LIMS with a five year expiration date.
- 14.7 **0.2N Bromine Monochloride (BrCl):**
- 14.7.1 37.5 g of KBr is added to a 2.5 L bottle of concentrated HCl (pre-analyzed and below 5 ng/L Hg). The bottle is inverted in a fume hood to mix the acid and KBr. The solution sits overnight, allowing the KBr to dissolve.
- 14.7.2 27.5 g of KBrO₃ (certified to be low in Hg) is slowly added to the acid. As the KBrO₃ is added, the solution should go from yellow to red to orange.
- CAUTION: This process generates copious quantities of free halogens (Cl₂, Br₂, BrCl) which are released from the bottle. Add the KBrO₃ SLOWLY in a well operating fume hood.**
- 14.7.3 Loosely cap the bottle and allow to sit for 30 minutes (in a fume hood) before tightening. Once tightly capped, invert bottle to make sure all of the solids go into solution.
- 14.7.4 This reagent shall be entered into the LIMS with a six month expiration date.
- 14.8 **10% (v/v) of 0.2N BrCl:** 200 mL of 0.2N BrCl is diluted up to 2.0 L with reagent water in a clean, empty HCl bottle. This bottle is fitted with a 10 mL repipettor. The expiration time for this reagent is set by default to six months in the LIMS.

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15 Procedure:

- 15.1 If needed, the sample is dissected and homogenized with acid-washed stainless steel tools.
- 15.1.1 The process used for homogenization, number of samples, work order number, client name, and initials of the technician are entered into the Tissue Homogenization Log.
- 15.2 Weigh at least a 0.5 g aliquot (but not more than 0.65 g) for common and unknown samples, and up to 1.0 g \pm 0.025 g for low-level or large-grain samples. This aliquot is placed into a 40 mL I-Chem glass vial.
- 15.2.1 If limited sample is available, use 20 mL glass vials and drop the initial mass of the samples to 0.25g \pm 0.025 g.
- 15.2.2 It is imperative that all biological tissue samples are thoroughly homogenized. The importance of representativeness cannot be understated.
- 15.2.3 Batch requirements for this digestion limit the number of samples to 20. In each batch, there must be three method blanks (BLKs), a Blank Spike and Blank Spike Duplicate (BS/BSD) that is *preferably a Certified Reference Material (CRM) or a Laboratory Control Spike (LCS, prepared at 8 ng/g)*, a Matrix Duplicate (MD), and a Matrix Spike and Matrix Spike Duplicate (MS/MSD).
- 15.3 10.0 mL of 70:30 (v/v) HNO₃/H₂SO₄ solution is pipetted in and the sample is swirled. *Note: 5.0 mL of 70:30 (v/v) HNO₃/H₂SO₄ solution is used for limited samples prepared in 20 mL vials (15.2.1).*
- 15.4 The vial is placed on a hot plate operating at 75 \pm 5°C with a Teflon reflux can in place instead of the vial's lid. An aluminum rack is often used to keep the vials from tipping over while on the hot plate.
- 15.4.1 A calibrated thermometer submerged in water is placed in a 20 mL I-Chem vial. This I-Chem vial with a calibrated thermometer is placed on the hot plate during the digestion process. The analysts must record the actual digestion temperature and the serial number of the thermometer used in the digestion logbook.
- 15.5 After the samples start to reflux, the samples are heated at 75 \pm 5°C for an additional 2 hours or until all organic matter is dissolved.
- 15.6 The samples are allowed to cool and are diluted to 40 mL (or to 20 mLs for limited sample digestions as described in 15.2.1) with a 10% (v/v) solution of 0.2N BrCl, capped with their respective lids, and are thoroughly shaken. Sample digestates should be allowed to settle prior to an aliquot being taken for analysis.
- 15.7 Analysis for total mercury is according to Eurofins Frontier SOP FGS-121.

16 Calculations:

- 16.1 This preparation procedure does not involve calculations.

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17 Statistical Information/Method Performance:

- 17.1 Method Detection Limit (MDL) and Practical Quantitation Limit (PQL) studies are based on 40 CFR 136, Appendix B. The MDL and PQL must be performed for each analyte/matrix/preparation combination.
- 17.2 The Practical Quantitation Limit (PQL) is the reporting limit for this method and is included as the lowest calibration point (2003 NELAC regulation 5.5.5.2.2.1.h.3). The PQL is determined by running ten replicate samples with a concentration that will produce a recovery of 70-130% for most analytes, but the recovery requirements are analyte dependent. The PQL is referred to as the Method Reporting Limit (MRL) in LIMS.
- 17.3 The current LOD value for Total Hg in tissue prepared by the Nitric and Sulfuric Acids (70:30) Digestion is 0.16 ng/g, while the PQL is 0.8 ng/g.
- 17.4 Current LODs and PQLs are stored at: \General and Admin\Quality Assurance\MDLs & PQLs.


18 Quality Assurance/Quality Control:

- 18.1 Maximum Sample Batch Size: 20 samples.
- 18.2 Preparation Blanks: Minimum of three per batch. Each preparation blank must be less than *one-half* the PQL for the method.
 - 18.2.1 The preparation blanks are prepared with a similar mass of Teflon boiling chips as the samples, with the same reagents, and put through the same preparation process as the samples.
- 18.3 *Certified Reference Material (CRM, representing the sample matrix when commercially available); a Laboratory Control Spike (LCS) and Laboratory Control Spike Duplicate (LCSD) prepared at 8 ng/g is used when a suitable CRM is not available: One per batch in duplicate. The control limits are 77-123% recovery.*
- 18.4 Matrix Duplicate (MD) Sample: One per batch. The control limit for the RPD is $\leq 24\%$.
- 18.5 Matrix Spike/Matrix Spike Duplicate (MS/MSD) Samples: One set per 10 samples. The control limits are 71-125% recoveries and an RPD of $\leq 24\%$.
- 18.6 Follow the flow charts in SOP FGS-038 "Data Review and Validation" to determine if any QC falling outside the established control limits can be qualified.
- 18.7 All of the quality control limits for the analysis method are included on the "Data Review Checklist".
 - 18.7.1 The data review checklists are located at: \cuprum\General and Admin\Quality Assurance\Data Review\Current Data Review Checklists.

19 Corrective Action:

- 19.1 Limiting the source of contamination/error in the preparatory stage can decrease QC problems during analysis. Limiting such contamination/error sources may include: cleaning all digestion tools in a 10% HCl solution, ensuring all samples are thoroughly homogenized, changing gloves whenever appropriate, flushing repipettors at least

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three times before dispensing into vials and, in general, following ultra-clean procedures.

- 19.2 A failing QC point does not necessary fail the entire dataset. If upon analysis a QC sample is out of control, some investigation must be performed to assess if the difficulties are related to matrix effects. The cause and method of determining the set's failure must be documented on the checklist and in the MMO notes, and the Group Supervisor shall be informed. See SOP FGS-038 "Data Review and Validation" for flow charts regarding analytical issues.
- 19.3 Additional corrective actions are listed in the SOP for total mercury analysis (Eurofins Frontier SOP FGS-121).

20 List of Attachments

Appendix A: Example - Standard Operating Procedure Training Record

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Appendix A: Example - Standard Operating Procedure Training Record

By signing this document, I the employee, certifies to have read, understood and agreed to follow the test method and quality procedure as described in this procedure.

Reading of SOP EFGS-011.05:
 Digestion of Tissues for Total Mercury Analysis Using Nitric and Sulfuric Acids (70:30).

SOP name and Revision number

Employee name (print)

Employee name (sign)

Date:

Supervisor name (sign)

Date:

Initial SOP Training (leave blank if not applicable)

Initial reading of method and training	Initials	Date	Supervisor
1. Read method			
2. Observe the method			
3. Detailed review of method and associated literature			
4. Supervised practice of method with trainer			
5. Unsupervised practice of the method with trainer			
6. Review of work with trainer and/or peer-review			
7. IDOC to determine precision and accuracy			
8. Determination of blanks			