National Rivers and Streams Assessment

2023/24

Laboratory Operations Manual

Version 1.0

NOTICE

The intention of the National Rivers and Streams Assessment 2023/24 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 2023 - 2024: Quality Assurance Project Plan EPA-841-B-22-004

National Rivers and Streams Assessment 2023 - 2024: Site Evaluation Guidelines EPA-841-B-22-005

National Rivers and Streams Assessment 2023 - 2024: Wadeable Field Operations Manual EPA-841-B-22-006

National Rivers and Streams Assessment 2023 - 2024: Non-Wadeable Field Operations Manual EPA-841-B-22-007

National Rivers and Streams Assessment 2023 - 2024: Laboratory Operations Manual EPA-841-B-22-008

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 2023/24. 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. This document and associated QAPP ensure compliance with the EPA policies *Assuring the Competency of EPA Laboratories (2004)* and *Policy to Assure Competency of Laboratories, Field Sampling, and Other Organizations Generating Environmental Measurement Data under Agency-Funded Acquisitions (2011)*.

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A	absorbance	EMAP	Environmental Monitoring and Assessment Program
ADT	analysis decision tree		
AFDM	ash-free dry mass	ENT	enterococci
ANC	acid neutralizing capacity	EPA	Environmental Protection Agency
ANS	Academy of Natural Sciences	ETOH	ethyl alcohol
AQM	absolute quantitation method	FOM	Field Operations Manual
ASTM	American Society for Testing and Materials	GEQ	genomic equivalent
Avg	Average	GIS	geographic information system
BHI	brain heart infusion	GPS	global positioning device
Ca	Calcium	HCl	hydrogen chloride
CCE	calibrator cell equivalents	HDPE	high density polyethylene
CEQ	cell equivalent	HNO_3	nitric acid
Chl-a	chlorophyll-a	HQ	headquarters
CO_2	carbon dioxide	HRP	antibody-Horseradish Peroxidase
Ct	threshold cycle	H_2S	hydrogen sulfide
CPR	cardiopulmonary resuscitation	H_2SO_4	sulphuric acid
cv	curriculum vitae	IBD	ionic balance difference
CV	coeffecient of variation	ID	Identification
DCF	dilution/concentration factor	IM	information management
DI	de-ionized	IPC	internal positive control
DIC	differential interference contrast	ISBN	International Standard Book Number
DL	detection limit	ISO	International Organization for Standardization
DNA	Deoxyribo-nucleic Acid		
DO	dissolved oxygen	IT IS	Integrated Taxonomic Information System (IT IS)
DOC	dissolved organic carbon		
DTH	depositional targeted habitat	K	potassium
DW	distilled water	KC	kit control
ELISA	enzyme-linked Immunosorbent assay	LFB	lab fortified blanks
		LFM	lab fortified matrices

LIMS	Laboratory Information Management System
LOM	Lab Operations Manual
LRL	lower reporting limit
Mg	magnesium
MDL	method detection limit
MPCA	Minnesota Pollution Control Agency
MSDS	Materials Safety Data Sheet
N	nitrogen
Na	sodium
NABS	North American Benthological Society
NALMS	North American Lakes Management Society
NARS	National Aquatic Resource Surveys
NAWQA	National Water Quality Assessment Program
NC	negative control
ND	nondetect
NELAC	National Environmental Laboratory Accreditation Conference
NELAP	National Environmental Laboratory Accreditation Program
NH_4	ammonium
NIST	National Institute of Standards
NO_2	nitrite
NO_3	nitrate
NRSA	National Rivers and Streams Assessment
NTL	no template control
NTU	Nephelometric Turbidity Units
OD	optical density
OSHA	Occupational Safety and Health Administration
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PctDIFF	percent difference
PDE	percent disagreement in enumeration
PCR	polymerase chain reaction
PE	performance evaluation
PES	performance evaluation samples
PHab	physical habitat
P-M	Palmer-Maloney (P-M) count
PDE	percent difference in enumeration

PSE	percent sorting efficiency
PT	performance testing
PTD	percent taxonomic disagreement
QA	quality assurance
QAPP	Quality Assurance Project Plan
QA/QC	quality assurance/quality control
QC	quality control
QCCS	quality control check solution
QMP	Quality Management Plan
qPCR	quantitative polymerase chain reaction
QRG	Quick Reference Guide
RL	reporting limit
RMSE	root mean square error
RO	reverse-osmosis
RPD	relative percent difference
RQM	relative quantitation method
RSD	relative standard deviation
RTH	richest targeted habitat
S	standard deviation
S0-S5	standards provided with microcystin kit
SFS	Society of Freshwater Science
SEG	Site Evaluation Guidelines
SiO_2	silica
SO_4	sulphate
SOPs	Standard Operating Procedures
SPC	sample processing control
S-R	Sedgewick-Rafter count
SRM	standard reference material
SS	salmon sperm
TMB	tetramethylbenzidine
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
TRANS	transect
TSN	taxonomic serial number
TSS	total suspended solids
TVS	total volatile solids
UNK	unknown
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:

1   Assess the condition of the Nation's rivers and streams

2   Establish a baseline to compare future rivers and streams surveys for trends assessments

3   Evaluate changes in condition from the 2004 Wadeable Streams Assessment, the NRSA 2008/09, NRSA 2013/14 and NRSA 2018/19

4   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:

1.   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?

2.   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.

EPA used an unequal probability to create two separate designs to address the dual objectives of (1) estimating current status and (2) estimating change in status for all flowing waters. There are 1028 new unique sites selected to estimate current satus and 790 unique sites that are resamples from a previous NRSA survey. 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.

INTRODUCTION

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 2023/24 sample that involves post-processing. The coordinator will be the labs main point of contact in regard to sample tracking and data submission.

Table 2.1 Contact information

Title	Name	Contact Information
EPA HQ NRSA Project Lead	Richard Mitchell, OW	mitchell.richard@epa.gov 202-566-0644
EPA HQ NRSA Project QA Lead	Sarah Lehmann, OW	lehmann.sarah@epa.gov 202-566-1379
EPA HQ Logistics Lead	Brian Hasty, OW	Hasty.brian@epa.gov 202-564-2236
EPA HQ NRSA Laboratory Review Manager	Kendra Forde, OW	kendra.forde@epa.gov 202-564-0417
Information Management Center Coordinator (NARS IM)	Michelle Gover, GDIT	Gover.michelle@epa.gov 541-754-4793
Contractor Logistics Coordinator	Chris Turner, GLEC	cturner@glec.com 715-829-3737

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 2023/24 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 1, 2024 for data collected in 2023; March 1st, 2025 for data collected in 2024; or as stipulated in contractual agreements. These reports must include the following information:

2. Sample Type (indicator)
3. Site ID (ex: CAS9-0918)
4. Sample ID (ex: 999000)
5. Pertinent information to the indicator
6. Metadata for all fields

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

The submitted file name must state the following:

7. Indicator name (ex: microcystin)
8. Date of files submission to NARS IM Center by year, month, and day (ex: 2024_11_01)
9. Lab name (ex: MyLab)

Combined, the file name would look as follows: WaterChemistry_2024_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 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.

2.0   GENERAL LABORATORY GUIDELINES

3.0 ALGAL TOXIN IMMUNOASSAY PROCEDURE: CYLINDROSPERMOPSIN

This chapter describes an enzyme-linked immunosorbent assay procedure that measures concentrations of total cylindrospermopsin in water samples. The laboratory uses Eurofins Technologies (formerly Abraxis) Cylindrospermopsin Test Kits ("kits") to conduct the analyses.

3.1 Summary of Method

Frozen cylindrospermopsin samples will be shipped on dry ice from the field crews to the contract batching laboratory. The contract batching laboratory will maintain frozen samples and send the batched samples to the analysis laboratory in coolers on ice. Samples will arrive in the analysis laboratory frozen, and they can be held in a freezer for several weeks. Cylindrospermopsin analyses laboratories will need to process the samples within the 90-day holding time and in accordance with timeframes outlined in contractual agreements.

The procedure is an adaption of the instructions provided by Eurofins Technologies (formerly Abraxis) for determining total cylindrospermopsin concentrations using its ELISA kits. For freshwater samples, the procedure's reporting range is 0.1 µg/L to 2.0 µg/L, although, theoretically, the procedure can detect, not quantify, cylindrospermopsin concentrations as low as 0.05 µg/L. For samples with concentrations higher than 2.0 µg/L of cylindrospermopsin, the procedure includes the necessary dilution steps.

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.
1. Laboratory personnel must wear proper personal protection clothing and equipment (e.g., lab coat, protective eyewear, gloves).
2. 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 Qualifications, 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 that is absorbed in a sample. A standard statistical curve is used to convert the absorbance value to the concentration value of cylindrospermopsin.

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 0.1 µg/L to a maximum value of 2.0 µg/L. Please note, NARS IM cannot accept characters within numeric fields. Values outside the range are handled as follows. If the value is:

- < 0.05 µg/L, then the laboratory reports the result as is (without characters) and flags the sample as a non-detect (i.e. DATA_FLAG=ND).
- Between 0.05 µg/L and the reporting limit of 0.1 µg/L (i.e., >0.05 µg/L and <0.1 µg/L), the laboratory should record the value, but assign a Quality Control (QC) code to the value indicating that the result is between the detection limit and the reporting limit (i.e., DATA_FLAG=J).
- >2.0 µg/L, the result 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.

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:

Equation 3.1 Standard deviation

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

where n is the number of replicate samples, A_i, is the absorbance measured for the ith replicate. 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:

Equation 3.2 Percent (%) coefficient of variation

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

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

Duplicate samples (D): are defined as the second aliquot of an individual sample within a well plate. Each sample including the standards are run in pairs and both results for the primary and duplicate sample are reported in the result column of the lab deliverable.

Method Detection Limit (MDL) is the minimum concentration at which the analyte can be *detected* with confidence (0.05 µg/L). In other words, the outcome can be reported with confidence that it is greater than zero (i.e., present in the sample). The method detection limit is less than the reporting limit of 0.1 µg/L, at which the *measured* value of the analyte can be reported with confidence. Also see "Sample-Specific Detection Limit" below.

Primary samples (P): are defined as the first aliquot of a sample within a well plate. Each sample is analyzed in pairs. The results of both this aliquot and the secondary, duplicate aliquot are reported in the result column of the lab deliverable.

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

Reporting Limit (RL): For undiluted samples, the reporting limit is 0.1 µg/L. A reporting limit is the point at which the measured value of the analyte can be reported with confidence.

Sample-Specific Detection Limit: Most samples will have a sample-specific detection limit equal to the method detection limit of 0.05 µg/L. For diluted samples, the sample-specific detection limit will be the product of the method detection limit of 0.05 µg/L and the dilution factor. Typical values for the dilution factor will be 10 or 100.

Standard Deviation (S) shows variation from the average.

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 2023 - 2024 QAPP Certification Page.

3.0 ALGAL TOXIN IMMUNOASSAY PROCEDURE: CYLINDROSPERMOPSIN

3.4.3   Personnel

Laboratory Technician: This procedure may be used by any laboratory technician who is familiar with the NRSA 2023 - 2024 QAPP, and this procedure in the NRSA 2023 - 2024 LOM. 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 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:

- Eurofins Technologies Cylindrospermospin ELISA (Microtiter) Test Kit, Product # 522011.
- Adhesive Sealing Film (Parafilm) for Micro Plates: Used to cover plates during incubation.
- Data Template – See **Figure 3.1**
- Distilled or Deionized Water: For diluting samples when necessary.
- ELISA evaluation software.
- 2 glass scintillation vials (20 mL).
- Multichannel Pipette & Tips: An 8-channel pipette is used for this method. Proficient use of the multichannel pipette is necessary to achieve reliable results. Practice with water if you have never used this before.
- 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 (such as Metertech, Model M965 AccuReader): Complete with Metertech PC Mate software for operation of machine. This machine reads the microtiter plates.
- Project Quality Control Samples.
- Reagent Reservoirs (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

Cylindrospermopsin samples are kept on ice in the field, frozen as soon as possible and kept frozen until they are shipped on dry ice to a central facility ("batching laboratory"). Periodically, the batching laboratory will ship the frozen samples to the cylindrospermopsin laboratory. The batching and cylindrospermopsin laboratory may retain the frozen samples for several weeks but samples must be analyzed within the 90-day holding time.

Because USEPA 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 to the NARS IM Team by completing and emailing the sample tracking spreadsheet with the sample login and sample condition information. (See **Section 2.2 Roles and Contact Information** the manual for contact information).
2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
   o Verify that the sample IDs in the shipment match those recorded on the:
      ▪ Chain of custody forms when the batching laboratory sends the samples to the cylindrospermopsin laboratory; or
      ▪ Sample tracking form if the field crew sends the shipment directly to the state.
   o For each sample, record the date received and lab comment (including Condition Code as described below) in the sample tracking spreadsheet with the appropriate Site ID/ Sample ID for the NARS IM Team.
      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 is not frozen

   o If any sample is damaged or missing, contact the USEPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed.
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 Cylindrospermopsin login: required data elements

FIELD	FORMAT	DESCRIPTION	
LAB_NAME	Text	Name or abbreviation for QC laboratory	
DATE_RECEIVED	MMDDYY	Date sample was received by laboratory	
SITE_ID	text	NRSA site ID as used on sample label	
VISIT_NO	numeric	Sequential visits to site (1 or 2)	
SAMPLE_ID	numeric	Sample ID as used on field sheet (on sample label)	
DATE_COL	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
COND_COMMENT	text	Comments about the condition of the sample.	

3.6   Procedure

The following sections describe the sample, 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:

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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:
   o Start with a frozen 500 ml sample.
   o 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.
   o Shake well to homogenize the sample, then transfer 10 mL to an appropriately labeled clean 20 mL glass vial.

3. Second freeze-thaw cycle:
   o Freeze the vial.
   o Keep the large sample bottle (from the 500 mL initial sample) frozen for future use.
   o Thaw the sample vial contents to room temperature.

4. Third freeze-thaw cycle:
   o Freeze the vial.
   o Thaw the vial contents to room temperature.
   o 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 similar alternatives 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:

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. (Optional: Instead of discarding the kit clearly mark all expired components as expired and consider keeping it for training activities.)

1. Verify that each kit contains all the required contents:
   - Microtiter plate
   - Standards (7) referenced in this procedure as follows with the associated concentration:
     o S0: 0 µg/L
     o S1: 0.05 µg/L
     o S2: 0.1 µg/L,
     o S3: 0.25 µg/L
     o S4: 0.5 µg/L
     o S5: 1.0 µg/L
     o S6: 2.0 µg/L
   - Kit Control (KC): 0.75 µg/L
   - Cylindrospermospin-HRP conjugate Solution (vortex before use)
   - Antibody solution (rabbit anti-Cylindrospermopsin)
   - Wash Solution 5X Concentrate
   - Substrate (Color) Solution

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- Stop Solution
- Dilutant
- Foil bag with 12 microtiter plate strips

2. 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.

3. Adjust the microtiter plate, samples, standards, and the reagents to room temperature.

4. 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 store in the refrigerator (4-8 $^{\circ}$C).

5. Prepare a negative control (NC) using distilled water.

6. The standards, controls, antibody solution, enzyme conjugate, color solution, and stop solutions are ready to use and do not require any further dilutions.

7. 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.

8. Handle the stop solution containing diluted H_2SO_4 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.

9. 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.

10. Turn on the computer so that it can control and access the plate reader.

11. Print the template (**Figure 3.1**) 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 USEPA HQ Laboratory Review Coordinator for approval prior to first use.

12. Using the 100-μL pipette, add 50 μL, each, of the standards, controls, and samples to the appropriate wells in the plate. Place all seven standards (0.00, 0.05, 0.10, 0.25, 0.50, 1.0 and 2.0 μg/L), the kit control (0.75 μL), and negative control, in pairs (duplicate), starting in the well in the upper left-hand corner of the kit as shown in **Figure 3.1**. Verify that the software displays the same template or make any necessary corrections. Laboratories with access to an autopipetter may use said machinery after proper documentation of set up, training and calibration has been provided and approved by EPA HQ Laboratory Review Coordinator prior to first use.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S4	NC	P4	P8	P12	P16	P20	P24	P28	P32	P36

B	S0	S4	NC	D4	D8	D12	D16	D20	D24	D28	D32	D36
C	S1	S5	P1	P5	P9	P13	P17	P21	P25	P29	P33	P37
D	S1	S5	D1	D5	D9	D13	D17	D21	D25	D29	D33	D37
E	S2	S6	P2	P6	P10	P14	P18	P22	P26	P30	P34	P38
F	S2	S6	D2	D6	D10	D14	D18	D22	D26	D30	D34	D38
G	S3	KC	P3	P7	P11	P15	P19	P23	P27	P31	P35	P39
H	S3	KC	D3	D7	D11	D15	D19	D23	D27	D31	D35	D39

Figure 3.1 Cylindrospermopsin: sample template

Key: S0-S6 = Standards;
KC = Control supplied with Kit (i.e., Kit Control);
NC = Negative Control (Laboratory Reagent Blank);
P = Primary run for each unknown sample collected by field crew;
D= "DUPLICATE" run for each matching unknown Primary sample

13. Add 50 µL of the conjugate solution to each well using the multi-channel pipettor and a reagent reservoir. Add 50 µL of the cylindrospermopsin 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.

14. Place the sealing Parafilm over the wells.

15. 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.

16. Place the plate in an area away from light for 45 minutes.

17. After 45 minutes, carefully remove the Parafilm.

18. 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 four 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.

19. Add 100 µL of substrate/ color solution to all wells using the multi-channel pipettor.

20. Cover the wells with Parafilm.

21. 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.

22. Place the strip holder in an area away from light for 30-45 minutes.

23. After 30-45 minutes, remove the Parafilm, add 100 µL of stop solution to the wells using the multi-channel pipette and reagent reservoir in the same sequence as the substrate solution.

24. Use a 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.

25. Dispose of solution in plates in a laboratory sink. Rinse plates and sink with water to dilute the weak acid present.

26. Perform QC evaluations of the data as follows:

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a. If the following failures occur in the standards and controls, 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-S6 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: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5 > \bar{A}_6$

  iii. The average absorbance of the standard S0 less than 0.8 (i.e., $\bar{A}_0 < 0.8$).

  iv. Two or more negative control samples with detectable concentrations of Cylindrospermopsin (i.e., values > 0.1 μg/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.

  v. Results for control samples of outside the acceptable range of 0.75 +/- 0.15 ppb. That is, results must be between 0.60 and 0.90.

b. If either, or both, of the following failures occur for the sample, 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 3.7.4 Dilutions** .

  ii. The %CV > 15% between the duplicate absorbance values for a sample.

27. Record the results, even if the data failed the quality control requirements in #26b, for each well in the USEPA's data template (**Table 3.2**). The required entries are for the following columns:

a. **SAM_CODE** should be one of the following codes: S0-S6 for standards; KC or NC, for controls; QC for quality control samples; P for primary run of unknown samples, D for duplicate/secondary run of unknown samples within a well plate.

b. **CONC** contains the numeric concentration value. Two special cases:

  i. Non-detected concentrations: If the sample is non-detected, provide the result within CONC column, record the data as 'ND' in the DATA FLAG column and provide the sample-specific detection limit in the method detection limit column (MDL). See **Section 3.3** for calculating the sample-specific detection limit for a diluted sample.

  ii. If the result shows that it is "HIGH," 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.[a]

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 0.1 μg/L (for undiluted samples);

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a EPA compares the cylindrospermopsin data values to 15 μg/L, which is the magnitude of the EPA criteria for recreational waterbodies in *Recommended Human Health Recreational Ambient Water Quality Criteria or Swimming Advisories for Microcystins and Cylindrospermopsin. 2019. EPA 822-R-19-001*. Retrieved June 5, 2019. https://www.epa.gov/sites/production/files/2019-05/documents/hh-rec-criteria-habs-document-2019.pdf

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iii. **H** if the sample did not meet the holding time and was not analyzed within 90 days.

iv. **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 26 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. **AVG_CONC and CV_ABSORB** are required for all duplicate runs (use all three values if the controls are used in triplicate).

Table 3.2: Cylindrospermopsin: required data elements- data submission

FIELD	COLUMN HEADING	FORMAT	DESCRIPTION	
LABORATORY ID	LAB_ID	Text	Name or abbreviation for QC laboratory	
DATE RECEIVED	DATE_RECEIVED	MMDDYY	Date sample was received by lab	
SITE ID	SITE_ID	Text	NRSA site ID code as recorded on sample label or tracking form (blank if standard or control)	
VISIT NUMBER	VISIT_NO	Numeric	Sequential visits to site (1 or 2) (blank if standard or control)	
SAMPLE ID	SAMPLE_ID	Numeric	6-digit Sample ID number as recorded on sample jar or tracking form (blank if standard or control)	
DATE COLLECTED	DATE_COL	MMDDYY	Date sample was collected (blank if standard or control)	
CONDITION CODE	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
CONDITION COMMENT	COND_COMMENT	Text	Any comment based on the condition code flags	
BATCH IDENTIFICATION	BATCH_ID	Numeric	Batch identification code; assigned by lab	
TECHNICIAN	TECHNICIAN	Text	Name or initials of technician performing the procedure	
DATE ANALYZED	DATE_ANALYZED	MMDDYY	Date when samples are inserted into the wells	
KIT EXPIRE DATE	KIT_EXPIRE_DATE	MMDDYY	Expiration date on kit box	
KIT ID	KIT_ID	Text	Kit identification code. If one does not exist, assign a unique code to each kit.	
R2	R2	Numeric	R^2 from curve fit to the average absorbance values for the standards. Value is between 0 and 1.	
SAMPLE CODE	SAM_CODE	Text	Type of solution being tested in the well	

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FIELD	COLUMN HEADING	FORMAT	DESCRIPTION	
			Code	Definition
			KC	Kit Control
			NC	Negative Control
			S0, S1, S2, S3, S4, S5, S6	Standard
			QC	Quality Control
LOCATION	LOCATION	Text	Location of well in the kit (e.g., B5 would be the fifth well from the left in the second row B)	
PRIMARY OR DUPLICATE	PRIM_DUP	Text	Regular samples are listed as "P" for Primary/first run or "D" for second run (see Figure 3.1)	
CONCENTRATION	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.	
UNITS	UNITS	Text	The units of the concentration of the CONC column	
MDL*	MDL	Numeric	Method detection limit of the machine in same units as the CONC column	
RL	RL	Numeric	Reporting limit in same units as the CONC column	
ABSORBANCE	ABSORBANCE	Numeric	Absorbance value	
DILUTION FACTOR	DILUTION_FACTOR	Numeric	10, 100, etc for number of times the sample was diluted. If not diluted, leave blank or record 1	
CV ABSORBANCE	CV_ABSORB	Numeric	Calculated %CV of duplicate values of absorbance for all runs. Enter %CV. Value is between 0 and 100%.	
AVERAGE ABSORBANCE	AVG_ABSORB	Numeric	Calculated average of absorbance values for all samples and standards. Average value of the original sample and its duplicate (or replicates for KC and NC).	
AVERAGE CONCENTRATION	AVG_CONC	Numeric	Calculated average of concentration values for a sample. Substitute 0.1 µg/L for any result recorded as <0.1 µg/L	
QA FLAG (if appropriate)	QA_FLAG	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 0.05 µg/L
			H	Sample did not meet the holding time and was not analyzed within 90 days.

FIELD	COLUMN HEADING	FORMAT	DESCRIPTION	
			HI	Result indicated that a high concentration (i.e., outside calibration range)> 2.0 µg/L
			J	Concentration above detection but below reporting limit. Without dilution, these values are between 0.05 and 0.1 µg/L
			QCF	QC failure
			Q	Other quality concerns, not identified above
LABORATORY COMMENT	LAB_COMMENT	Text	Explanation for data flag(s) (if needed) or other comments.	

*In the event for sample dilution is necessary to overcome the matrix effect, please notify EPA Laboratory Coordinator

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 or deionized water to a clean 20 mL vial. (Note: Dilutions may also be made using the kit's diluent rather than distilled or deionized 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' method detection limit of 0.05 µg/L by 10 to obtain the sample-specific detection limit of .5 µg/L.

- 1:100 dilution

  a. Add 3.96 mL of distilled or deionized water to a clean, appropriately labeled glass vial. (Note: Dilutions may also be made using the kit's diluent rather than distilled or deionized 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 the sample again.
     Multiply the final concentration and Abraxis' method detection limit of 0.05 µg/L by 100 to obtain the sample-specific detection limit of 50 µg/L.

- Other dilutions can be calculated in the same manner as #1 and #2 if needed.

3.7   Pertinent QA/QC Procedures

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

3.7.1   QC Samples

The External QC Coordinator will instruct the QC contractor to provide one or two identical sets of freshwater QC samples (labeled as performance test (PT) samples) to all participating laboratories. Each set will contain five samples to test the expected range of concentrations in the NRSA samples.

For the contract laboratory, the QC contractor will provide the first set to be run with the first set of samples and a second set to be run at the midpoint of the assigned samples. If available, a third set will be run with the final batch of samples. Because most state laboratories will have relatively few samples that can be analyzed using a single kit, the QC contractor will send only one set to each state laboratory.

Each laboratory will run the QC samples following the same procedures used for the other samples. The External QC Coordinator 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, which may include no action, flagging the data, or excluding some or all the laboratory's data.

3.7.2   Summary of QA/QC Requirements

Table 3.3 provides a summary of the quality control requirements. For cylindrospermopsin, the precision for a sample is reported in terms of the percent coefficient of variation (%CV) of its absorbance values. Relative Standard Deviation (RSD) is the same as the %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. Accuracy is calculated by comparing the average concentration of the kit control with the required range (0.75 +/- 0.15).

Table 3.3: Cylindrospermopsin: 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: ○ 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-S6 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: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5 > \bar{A}_6$	If any requirement fails: Results from the analytical run are not reported. All samples in the analytical run are reanalyzed until calibration provides acceptable results. At its discretion, the laboratory may consult with USEPA for guidance on persistent difficulties with calibration.
Kit Control	The average concentration value of the duplicates (or triplicate) must be within the range of 0.75 +/-	If either requirement fails:

Quality Control Activity	Description and Requirements	Corrective Action
	0.15 µg/L. That is, results must be between 0.60 and 0.90.	• Results from the analytical run are not reported
Negative Control	The values for the negative control replicates must meet the following requirements: o All concentration values must be < 0.1 µg/L (i.e., the reporting limit); and o One or more concentration results must be nondetectable (i.e., <0.05 µg/L)	• The laboratory evaluates its processes, and if appropriate, modifies its processes to correct possible contamination or other problems. • The laboratory 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≤15% between its absorbance values.	If %CV of the absorbance for the sample>15%, then: • Record the results for both duplicates using different start dates and/or start times to distinguish between the runs. • Report the data for both duplicate results using Quality Control Failure flag "QCF"; and • Re-analyze the sample in a new analytical run. No samples are to be run more than twice without EPA approval. If the second run passes, then the data analyst will exclude the data from the first run (which will have been flagged with "QCF"). 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%).
Results Within Calibration Range	All samples are run in duplicate. If both of the values are less than the upper calibration range (i.e., 2.0 µg/L for undiluted samples), then the requirement is met.	If a result registers as "HIGH", then record the result with a data flag of "HI." If one or both duplicates register as 'HIGH,' then the sample must be diluted and re-run. **No samples are to be run more than twice without EPA approval.** If samples are re-run, do not enter concentration information of the first run.
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

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Quality Control Activity	Description and Requirements	Corrective Action
		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.

3.8   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.

3.0 ALGAL TOXIN IMMUNOASSAY PROCEDURE: CYLINDROSPERMOPSIN

4.0 ALGAL TOXIN IMMUNOASSAY PROCEDURE: MICROCYSTIN

This chapter describes an immunoassay procedure that measures concentrations of total microcystins in water samples using Eurofins Technologies (formerly Abraxis) Microcystins-ADDA Test Kits ("kits")b. 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.

4. 1   Summary of Method

Frozen microcystin samples will be shipped on dry ice from the field crews to the contract batching laboratory. The contract batching laboratory will send the batched frozen samples to the analysis laboratory in coolers on ice where they can be held in a freezer until ready for analysis. Microcystin analyses laboratories will need to process the samples within the 90-day holding time and in accordance with timeframes outlined in contractual agreements.

The procedure is an adaption of the instructions provided by Abraxis for determining total microcystins concentrations using its ELISA-ADDA kits.c 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.

4.2   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

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b Eurofins Technologies, "Microcystins-ADDA ELISA (Microtiter Plate): Product No. 520011." Retrieved on March 12, 2020 from https://www.eurofins-technologies.com/microcystins-nodularins-adda-epa-etv-epa-method-546-elisa-96-tests.html

occurs, remove clothing immediately. Wash and rinse the affected skin areas thoroughly with large amounts of water.

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

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

4.3.1   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 %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 ith replicate. 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.

Duplicate samples (D) are defined as the second aliquots of an individual sample within a well plate. Each sample, including the standards, are urn in pairs and both results for the primary (P) and duplicate aliquot are reported in the result column of the lab deliverable.

Method 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."

Primary samples (P) are defined as the first aliquot of a sample within a well plate. Each sample is analyzed in pairs. The result of both the primary aliquot and secondary, duplicate aliquot are reported in the result column of the lab deliverable.

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.

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.

Standard Deviation (S) shows variation from the average

4.4   General Requirements for Laboratories
4.4.1   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.

4.4.2   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 2023 - 2024 QAPP Certification Page.

4.4.3   Personnel

Laboratory Technician: This procedure may be used by any laboratory technician who is familiar with the NRSA 2023 - 2024 QAPP, and this procedure in the NRSA 2023 - 2024 LOM. 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.

4.4.4   Equipment/Materials

The procedures require the following equipment and information:

- Eurofins Technologies (formerly Abraxis ADDA Test Kit, Product #520011
- Adhesive Sealing Film (Parafilm) for Micro Plates (such as Rainin, non-sterile, Cat. No. 96-SP-100): Used to cover plates during incubation.

4.0   ALGAL TOXIN IMMUNOASSAY PROCEDURE: MICROCYSTIN

- Data Template – See Error! Reference source not found.**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)

4.5   Sample Receipt

Microcystins samples are kept on ice while in the field, frozen as soon as possible and kept frozen until they are shipped on dry ice to a central facility ("batching laboratory") or the State's laboratory. Periodically, the batching laboratory ships the frozen samples to the microcystins laboratory. The batching and microcystins laboratory may retain the frozen samples for several weeks but samples must be analyzed within the 90-day holding time.

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 (**Table 4.1** Microcystin: required data elements – login.

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 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 is not frozen

c. If any sample is damaged or missing, contact the EPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed.
3. Store samples in the freezer until sample preparation begins.
4. Maintain the chain of custody or sample tracking forms with the samples.

Table 4.1 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	LA 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**
		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
		Q	Other quality concerns, not identified above
CONDITION COMMENT	text	Comments about the condition of the sample. Required for "Q". Optional for others.	

4.6   Procedure

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

4.6.1   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 similar alternative are acceptable. One new syringe and filter should be used per sample.

4.6.2   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 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
- Foil bag with 12 microtiter plate strips

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 (4-8º 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.

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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 H_2SO_4 with care.

4.6.3   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.

11. 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.

12. Turn on the computer so that it can control and access the plate reader.

13. Print the template (**Figure 4.1 Microcystin: sample template**) 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 Coordinator for approval prior to first use.

14. 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 4.1**. Verify that the software displays the same template or make any necessary corrections. Laboratories with access to an autopipetter may use said machinery after proper documentation of set up, training and calibration has been provided and approved by EPA HQ Laboratory Review Coordinator prior to first use.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S4	P1	P5	P9	P13	P17	P21	P25	P29	P33	P37
B	S0	S4	D1	D5	D9	D13	D17	D21	D25	D29	D33	D37
C	S1	S5	P2	P6	P10	P14	P18	P22	P26	P30	P34	P38
D	S1	S5	D2	D6	D10	D14	D18	D22	D26	D30	D34	D38
E	S2	KC	P3	P7	P11	P15	P19	P23	P27	P31	P35	P39
F	S2	KC	D3	D7	D11	D15	D19	D23	D27	D31	D35	D39
G	S3	NC	P4	P8	P12	P16	P20	P24	P28	P32	P36	P40
H	S3	NC	D4	D8	D12	D16	D20	D24	D28	D32	D36	D40

Figure 4.1 Microcystin: sample template

Key: S0-S5 = Standards; KC = Control supplied with Kit (i.e., Kit Control);
NC = Negative Control (Laboratory Reagent Blank);
P = Primary aliquot for each unknown sample collected by field crew;
D= "DUPLICATE" aliquot for each matching unknown Primary sample.

15. 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.

16. Place the sealing Parafilm over the wells.

17. 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.

18. Place the plate in an area away from light for 90 minutes.

19. After 90 minutes, carefully remove the Parafilm.

20. 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.

21. Add 100 µL of enzyme conjugate solution to all wells using the multi-channel pipettor.

22. Cover the wells with Parafilm.

23. 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.

24. Place the strip holder in an area away from light for 30 minutes.

25. 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.

26. 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.

27. Cover the wells with Parafilm.

28. 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.

29. Place the plate in an area away from light for 20 minutes.

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30. 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.

31. 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.

32. Dispose of solution in plates in a lab sink. Rinse plates and sink with water to dilute the weak acid present.

33. 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:
        $$\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5$$

        iii. The average absorbance of the standard S0 less than 0.8 (i.e., $\bar{A}_0 < 0.8$).

        iv. Two or more negative control samples with detectable concentrations of microcystins (i.e., values > 0.1 μg/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.

        v. 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 4.6.4.**

        ii. The %CV > 15% between the duplicate absorbance values for a sample.

34. Record the results, even if the data failed the quality control requirements in #23b, for each well in EPA's data template (see **Table 4.2** 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; P (primary) or D (for duplicate) of 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 μg/L if the sample is undiluted with a salinity <3.5 ppt. See **Section 4.3.1** for calculating the sample-specific detection limit for a diluted sample.

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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. **H** if sample did not meet the holding time and was not analyzed within 90 days.
   iv. **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).

g.

Table 4.2 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
			Q	Other quality concerns, not identified above
	CONDITION COMMENT	text	Comments about the condition of the sample.	

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STAGE	FIELD	FORMAT	DESCRIPTION
ANALYSIS	BATCH ID	Numeric	Batch identification code, assigned by lab
	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
QC	Quality control sample
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)
	PRIM_DUP	text	Primary or duplicate run of a sample of unknown concentration (see Figure 4.1)
	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 for a sample with salinity <3.5 ppt which hasn't been diluted.
	UNITS	text	The units of the concentration of the CONC column
	MDL	numeric	Minimum detection limit in the same units as the CONC column
	RL	numeric	Reporting Limit in same units as the CONC column
	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.
	QA FLAG (if appropriate)	text	Data qualifier codes associated with specific identifications of voucher samples. These codes provide more information that 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).

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STAGE	FIELD	FORMAT	DESCRIPTION	
			Flag	**Definition**
			ND	Concentration below detection. Unless the sample was diluted, the concentration will be 0.1 µg/L
			H	Sample did not meet the holding time and was not analyzed within 90 days.
			HI	Result indicated that a high concentration (i.e., outside calibration range)
			J	Concentration above detection but below reporting limit.
			QCF	QC failure
			Q	Other quality concerns, not identified above
	LAB COMMENTS	text	Explanation for data flag(s) (if needed) or other comments.	

4.6.4   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.

4.7   Quality Measures

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

4.7.1   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

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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.

4.7.2 QC Samples

Once or twice during the survey, sets of five identical Performance Evaluation (PE) samples will be provided to all participating laboratories by an External QC Coordinator. As determine by the External QC Coordinator, the PE samples may be synthetic; aliquots of additional samples collected at NRSA sites; or reference samples obtained from an organization such as the National Institute of Standards and Technology or Eurofins Technologies (formerly Abraxis). Each laboratory shall analyze the PE samples following the same procedures used for the other samples analyzed. The External QC Coordinator will compare the results to the expected value. The results of the comparisons shall be made available to the EPA QC Coordinator for review. Based upon the evaluation, the External QC Coordinator may request additional information from one or more laboratories about any unique laboratory practices that might account for differences and may convene a conference call with all participating labs to identify causes of and if possible, reconcile those differences. With this additional information, the External QC Coordinator shall determine an appropriate course of action, including no action, re-running samples, flagging the data, or excluding some or all the laboratory's data.

4.7.3 Summary of QA/QC Requirements

Table 4.3 provides a summary of the quality control requirements described in **Sections 4.5** and **4.6**.

Table 4.3 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: ○ 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: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5$	If any requirement fails: • Results from the analytical run are flagged QCF 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 µg/L. That is, results must be between 0.565 and 0.935.	If either requirement fails: • Results from the analytical run are not reported

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Quality Control Activity	Description and Requirements	Corrective Action
Negative Control	The values for the negative control replicates must meet the following requirements: ○ All concentration values must be < 0.15 µg/L (i.e., the reporting limit); and One or more concentration results must be nondetectable (i.e., <0.10 µg/L)	• 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≤15% between its absorbance values.	If %CV of the absorbances for the sample>15%, then: 10. Record the results for both duplicates. 11. Report the data for both duplicate results as Quality Control Failure "QCF"; and 12. Re-analyze the sample in a new analytical run. No samples are to be **run more than twice without prior approval from EPA.** 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%).
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 µg/L for undiluted samples), then the requirement is met.	If a result registers as "HIGH", then record the result with a data flag of "HI." If one or both duplicates register as 'HIGH,' then the sample must be diluted and re-run. **No samples are to be run more than twice without prior approval from EPA.** If samples are re-run, do not enter concentration information of the first run.
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

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Quality Control Activity	Description and Requirements	Corrective Action
		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.

4.8   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.

5.0   BENTHIC MACROINVERTEBRATES

5.1 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 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 5.1**, **5.2, 5.3**, and **5.4** summarize the procedure; health and safety concerns; and required resources. **Section 5.5** provides the steps for acknowledging sample receipt. **Section 5.6** and **Section 5.7** provide the steps for preparing and sorting the sample. **Sections 5.8** – **5.10** provide the steps for the taxonomy identification, data entry, and sample and record retention. **Section 5.11** describes EPA's external review of laboratory operations. **Section 5.12** identifies references used in developing the procedure. **Table 5.8** summarizes the quality control measures.

5.2 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.

During the identification step, a taxonomist identifies the picked organisms to the target taxonomic levels for the survey. 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.

5.3 Health and Safety Warnings

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

- 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 (benthic organisms and sediments) avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs remove clothing immediately and wash /

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rinse thoroughly. Wash the affected skin areas thoroughly with large amounts of soap and water.

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

This section provides definitions and required resources for using this procedure. **Section 5.4.1** defines the terms used throughout the procedure. **Section 5.4.2** describes the expertise required for each laboratory using the procedure. **Section 5.4.3** describes the roles and responsibilities of the personnel involved in the procedure. **Section 5.4.4** identifies the equipment necessary to apply the procedure in preparing, sorting, and identifying benthic macroinvertebrate organisms in samples.

5.4.1 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 5.8** provides the steps used to identify which taxa are flagged.

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.

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

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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.

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

(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 taxnomists. In the following equation, N is the total number of organisms in the larger of the two counts.

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

(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 5.4 and **Table 5.5** 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 5.4** and **Table 5.5.**

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.)

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5.4.2 Laboratory

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

3. 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.

5.4.3 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 ≥90% in 5 consecutive samples evaluated by an experienced sorter; and
- Maintains a sorting proficiency of ≥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.

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- Checks sorted grids of all inexperienced laboratory personnel (those who have not achieved a ≥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≤5% and PTD≤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. 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 QC contractor, and have demonstrated expertise and experience to be used as a quasi "gold standard" for taxonomic evaluations. A QC taxonomist may work for both a primary and secondary laboratory, but must be assigned to different samples so that the external QC review is independent of the original identification.

QC Contractor supports the External QC Coordinator and is responsible for obtaining the External QC Taxonomists. The QC contractor must not have previously been involved with field sampling and/or benthic macroinvertebrate analyses.

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 ≥90% sorting efficiency in 5 consecutive samples. An experienced sorter can serve as the Sorting QC Officer.

5.4.4 Equipment/Materials

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

5.4.4.1 Sample Preparation (Subsampling) and Sorting Equipment/Materials

1.) U.S. 35 sieve (500 µm)
2.) Round buckets

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3.) Standard gridded screen (370-µm mesh screen, 30 squares, each 6 cm^2) with white plastic holding tray (Caton tray, 30cm x 36cm, 4cm deep)

4.) 6-cm scoop

5.) 6-cm^2 metal dividing frame ("cookie cutter")

6.) White plastic or enamel pan (6" x 9") for sorting

7.) Scissors

8.) Teaspoon

9.) Permanent ink pen (e.g Pigma Micron® pen)

10.) Dropper

11.) Fine-tipped forceps (watchmaker type, straight and curved)

12.) Vials with caps or stoppers

13.) Sample labels for vials

14.) 70-80% ethanol (denatured or non-denatured)

15.) Sorting Bench Sheet or other Sheet or database approved by EPA's External QC Coordinator

16.) Stereo zoom microscope (6-10X magnification)

5.4.4.2 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

5.5 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.

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- 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.

4. 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.

5. After refilling the sample containers, store them at room temperature until sorting begins.

6. 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).

7. Verify that the login information includes the required data elements in **Table 5.1**. After completing all required elements, provide the information to the data entry personnel.

Table 5.1 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	

5.6 Subsampling

1. Remove the lid from the sample container(s) and pull out 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 sample collection information on a Benthic Macroinvertebrate Laboratory 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). Set the bench sheet aside.

2. Carefully decant the alcohol from the sample container by pouring the fluid through a sieve (U.S. 35) into a separate container (the alcohol is saved to preserve the archived portion of the sample that does not get processed). Inspect the mesh of the sieve for any organisms and return organisms found to the sample.

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3. Transfer the homogenized sample material to the gridded screen portion of the grid (use more than one subsampling device if necessary). Wash the sample thoroughly by running tap water over it to remove any fine material. If there is more than one jar for any particular sample, empty and wash each jar onto the Caton-type grid one at a time, making sure to spread each jar's contents evenly across the tray. Multiple jars from the same sample should all be emptied onto the same Caton grid (or suitable alternative subsampling tray). If the amount of leaf litter or other detrital material exceeds that which fills they tray to the level of the wall panels (if should be spread as evenly as possible), it can be divided among two or more trays.

- Elutriation of a sample is acceptable for samples with heavy amounts of inorganic substrate (e.g., sample that has 4 or 5 jars total and 2 or 3 with gravel or sand) once it has been delivered to the lab, before subsampling has begun on that particular sample. An example of an acceptable elutriation method is as follows:

i. Pour alcohol off of sample containers through sieve (at least 500 μm). Also deposit leaf litter and any other **organic material** (leaves, sticks, algae) onto sieve.

ii. 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.

iii. 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 the sieve.

iv. Repeat this until the water runs clear.

v. 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 w/ 3x magnification for any remaining organisms. Have another sorter double check for organisms.

vi. Once you are certain there are no organisms remaining in the plastic container, wash the water through the sieve and dump the inorganic material into a waste bucket.

vii. Repeat this process until all of the inorganic material has been elutriated and checked for heavier organisms, such as clams, mussles, or worms.

viii. Spread the sample now in the circular sieve over the 30-grid Caton tray.

1) Place the gridded screen into the larger white tray. (Note: Some Cooperators may not use the gridded screen and holding tray). 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.

2) 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.

3) Use a random number generator to select at least 10% of the grids, usually 3 grids (in a 30-grid tray) to process (select one letter and one number, e.g., A-5, F-2). Three grids are sorted from the sample to ensure that the subsample

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material is representative of the overall sample. Remove all the material using the following procedure from that grid and place the removed material into a separate holding container, such as a white plastic or enamel pan. If two trays are being used to hold a large sample, the same grid on the second pan will also be removed. Continue until the material from all 3 grids is removed. The material is removed as follows:

a. 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.

b. 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 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:

- An organism belongs to the grid containing its head.

- 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.

- 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.

c. Quarter the grid (if necessary, see **Section 5.7**, #1). Place the material from the selected grid(s) into a separate white plastic or enamel pan. Add the necessary amount of water to the pan to facilitate sorting.

d. Set the subsampling device aside in case more grids need to be retrieved later. Cover the sample with aluminum foil to prevent desiccation of the sample and damage to specimens (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 specimens, which could possibly change the probability of selection.

5.7 Sorting

1. At least 10% of the tray or three grids in the case of a Caton tray (assuming 30 grids) would be randomly selected.

a. If the number of organisms appears to exceed the target number (500 organisms) in the collective three grids, then each grid is quartered, and a quarter is randomly selected for initial sorting. The quarter volume of the first grid would be sorted, with the remaining two grids (quartered) being sorted in successive order (compositing of the first three grids is not done).

b. If the number of organisms is below the target, then another fraction of each grid would be processed until the target number of 500 and a maximum of 600 (500+20%) is reached. All organisms from the selected fraction, or grid, must be sorted to minimize remove bias.

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c. If the target is not reached when the three grids are completely picked fully processed (including organisms recovered during QC checks), subsequent grids would be randomly selected and each picked to completion until 500+20% organisms is reached. If the target number of organisms is reached within the fraction of the first or second grids, sorting is stopped for that sample, on completion of the sorting of the corresponding fraction (i.e., the third grid quarter would not be processed).

a) Remove the macroinvertebrates from the detritus with forceps. All samples will be sorted under a minimum of 6x (maximum of 10x) dissecting microscope. Quality control checks will also be performed using the same power microscope. Place picked organisms in an internally labeled vial (or larger container, if necessary) containing 70-80% denatured ethanol.

b) 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 Benthic Macroinvertebrate Laboratory Bench Sheet. Enter the sorter's initials in the appropriate column on the bench sheet for each grid sorted.

c) Do *not* remove or count empty snail or bivalve shells, specimens of surface-dwelling or strict water column[2] arthropod taxa (e.g., Collembola, Veliidae, Gerridae, Notonectidae, Corixidae, Cladocera, or Copepoda), or incidentally-collected terrestrial taxa. Also, *do not* count fragments such as legs, antennae, gills, or wings. 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. If a sorter is unsure as to whether a specimen should be counted or not, he or she should place the organism in the sort vial <u>without counting it</u> (the final count is made by the taxonomist).

Table 5.2 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	*Clitellata*	*Branchiobdellida*	
ARTHROPODA	*Cladocera and other Branchiopoda*		
	Collembola		
	Insecta	*Ephemeroptera (adults only)*	
		Diptera (larvae of listed families, plus all adults)	*Chaoboridae*
			Culicidae
		Coleoptera	*Dytiscidae (adult)*
			Gyrinidae (adult)
		Heteroptera	*Corixidae*
			Gerridae
			Mesoveliidae
			Notonectidae
			Pleidae
		Neuroptera (adults)	
		Odonata (adults)	
		Plecoptera (adults)	

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

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		Trichoptera (adults)	
	Ostracoda		
BRYOZOA			
NEMATODA			

d) Each sample, once it is picked by the initial sorter, must be checked for missed organisms before another sample is processed. This step is performed by an experienced, certified, laboratory QC Officer, as detailed below. Any missed organisms found by the QC Officer will be counted and placed into the sample vial, or other suitable sample vial, and the number of organisms missed will be noted on the Benthic Macroinvertebrate Laboratory Bench Sheet and added to the final count of the sample.

3. If the last grid (or quarter) being processed results in more than 600 organisms (i.e., > 20% above target number), evenly redistribute all of the organisms (without detritus) in a petri dish (or other small container, i.e., finger bowl, etc.) divided into pie slices (1-8) containing water to cover. Randomly choose slices and count organisms that are wholly contained within the slices. If an organism is lying between two slices, use the criteria in **Section 5.6** #6 (b) to determine which slice it belongs in. Choose slices until you reach the target number (500 +20%). As with picking grids and quarters, the sorter must pick an entire pie slice, even if it the sample goes over 500 organisms as long as it remains under 600 total organisms.

4. Once the QC check of the material in the pan has been completed, it is removed from the pan and placed in a separate container with preservative (70-80% ethanol). The container should be labeled "Sorted Residue", on both internal and external labels ("Sorted Residue" will include material from *all* grids processed for each sample). Internal sample labels should be made of cotton rag paper or an acceptable substitute, recording the same information as before.

5. After the QC check is completed, and the target number has been reached, search the entire tray for 5-10 minutes, looking for large/rare organisms (Vinson and Hawkins, 1996). Large/rare is defined as any organism larger than ½" long and found in less than 1/8 of the tray holding the entire sample. Place any organisms found into a vial labeled "L/R" for "Large/Rare".

6. All material not subsampled (remaining on the grid) must be returned to the original container with the preservative. This container should include the original sample labels and a separate label "Unsorted Sample Remains" should be placed inside the container and on the outside. The lid should be tightly closed and the container archived until **all** appropriate QC checks are completed (subsampling and taxonomy). The decision to discard <u>any</u> sample portion should be done only following joint approval of the QC officer and the Project Manager.

7. Record the sorting date each sample was completed near the top right corner of the bench sheet. Verify that the bench sheet includes the required data elements in **Table 5.3**.

Table 5.3 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

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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

5.8 Taxonomy Identification

1. On receipt of a set of sample vials from the project cooperator or contractor laboratory, remove the chain-of-custody form from the shipping container, sign and date it to verify that the samples were received (in the "received by" space). Compare all sample numbers on the form with those entered on the labels of samples that actually were in the shipment. If any vials were broken, notify the project facilitator immediately. Maintain the chain-of-custody form with the samples; it will be needed to return the samples.

2. Empty one sample vial at a time into a small petri dish. Add 80% denatured ethanol to keep the organisms covered. Remove the internal sample label and complete the top portion of a Benthic Macroinvertebrate Taxonomic Bench Sheet (**Appendix C; Sample Laboratory Forms**, or comparable system), using the information from the label or that provided by the project facilitator.

3. Begin by viewing the sample under the stereo dissecting microscope and removing similar

   organisms to other dishes (keep covered with 80% ethanol). Organisms should be identified to the correct taxonomic level for the project (usually genus, **Table 5.4** and **Table 5.5**). However, according to the laboratory manager's discretion, a taxonomist can identify any organism farther than the target level if they are confident in the identification. Record the identifications on the Benthic Macroinvertebrate Taxonomic Bench Sheet (under taxon). Enter the number of larvae, pupae, and adults of each taxon under those columns on the bench sheet. Also enter the Taxonomic Serial Number (TSN; found in ITIS). Use the following steps to compare the final taxa list for each site to that of the ITIS website (http://www.itis.usda.gov) Record the TSN from ITIS on the Electronic Bench Sheet (**Table 5.6**).

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8. Copy block of taxa names to a text file.

1.1 Save the text file

1.2 Go to the ITIS taxa match screen (http://www.itis.usda.gov/taxmatch_ftp.html)

1.3 Follow the onscreen instructions to upload the file. Use all of the current defaults.

2. Finish with two lists, one of matches with TSNs and one with non-matches. Check the non-matches for the following common problems.

   5   Abbreviations

   6   Extra information identifiers (e.g., sp., spp., , nr., cf., genus 1, w/ hair chaete)

   7   Extra character (e.g., "?", "Acentrella ?turbida", blank space)

   8   The word "probably" or "prob" (e.g., "Microcylloepus prob. similis")

   9   IDing to a lower level than is in ITIS (e.g, to species rather than genus)

   10   Double names (e.g., Callibaetis callibaetis)

   11   Common misspellings

   12   Tribes/Subfamilies/Subgenus sometimes do not appear in ITIS

   13   Species w/ incorrect Genus (Hydatopsyche betteni)

   14   Split level taxonomy (e.g., Cricotopus/Orthocladius)

   15   Invalid name (e.g., taxonomic change, synonym; Sphaeriidae vs. Pisiidae)

   16   Valid name, in scientific literature, but not in ITIS (e.g., appears in Merritt & Cummins (1996) or Epler (2001), but not listed in ITIS - will not have a TSN)

4. Prepare slide mounts of Chironomidae and Oligochaeta as needed using CMCP-10 (or CMC-9, CMC-10, or other media) and applying a coverslip. View these organisms under the compound microscope to ensure that all necessary diagnostic characters have been observed, according to the taxonomic key or other literature. Record the identifications on the bench sheet as above. The slides should be labeled with the same sample number or log-in number as the alcohol specimens.

5. 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 project QC officer.

6. If *damaged organisms* can be identified, they are counted ONLY if:

   (a) the fragment includes the head, and, in the case of

      • arthropods, the thorax
      • oligochaetes, a sufficient number of segments

   (b) the mollusk shell (bivalve or gastropod) is occupied by a specimen

   (c) the specimen is the sole representative of a taxon in the sample

7. If *early instar or juvenile* specimens can be identified, they are counted:

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    (a) as the same taxon, if with confidence, they can be associated with one or more mature specimens that have a more developed morphology.

    (b) as a separate taxon, if the specimen is the sole representative of a taxon in the sample.

8. Add the number of organisms from each developmental stage and enter the total on the bench sheet.

9. Complete the bench sheet by entering 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. Make a copy of the bench sheet for the project file.

10. Create a reference collection with at least one specimen from each genus (or lowest taxonomic level IDed). When a sample is chosen to be the source of specimen(s) to represent a name in the master taxa list, the appropriate specimen(s) in that sample representing the concept of that taxon to the taxonomist should be removed and placed in the reference collection. Labels will be placed in the primary sample container indicating the placement of any specimen(s) removed for the reference collection. Circle slide-mounted specimens with a grease pencil (or other appropriate mark) to indicate those which belong to the reference collection. For all slides containing reference and non-reference specimens, be sure to place a label in the sample container that **does not** contain the reference collection. Each laboratory should maintain a master list of taxa recorded. The project facilitator will coordinate any necessary inter-lab communication and produce and integrated master taxa list for the project.

11. Carefully return the rest of the organisms to the original sample vial, fill with 70-80% denatured ethanol, and cap tightly.

12. Re-package the samples and slide mounted specimens carefully, and sign and date the chain-of-custody form in the next "relinquished by" space. The samples should be shipped, properly packed in a box, by overnight carrier to the project facilitator, and receipt confirmed by the person doing the shipping. Each taxonomist should retain a full set of bench sheet copies, and ship the original bench sheets in an envelope to the project facilitator. Samples and bench sheets should be shipped separately.

Table 5.4 Benthic macroinvertebrate: target level of taxonomic identification – benthics commonly found in freshwater

TAXON				Target Level of Identification	
Phylum	Class (or Subclass)	Order	Family	Family	Genus
ANNELIDA	(any, except *Hirudinea*, *Polychaeta*)				X
	Hirudinea				X
	Oligochaeta	(any, including *Tubificinae*, except as noted below)			X
		Enchytraeidae		X	
		Lumbriculidae		X	

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TAXON				Target Level of Identification	
	Polychaeta			X	
ARTHROPODA	*Hydrachnidia*				X
	Insecta	(any, except as noted)			X
		Coleoptera			X
		Diptera (any, except as noted)			X
			Ceratopogoninae	subfamily	
			Chironomidae	see Table 4	
			Dolichopodidae	X	
			Empididae	X	
			Ephydiridae	X	
			Muscidae	X	
			Phoridae	X	
			Scathophagidae	X	
			Sciomyzidae	X	
			Stratiomyidae	X	
			Syrphidae	X	
			Tabanidae	X	
		Ephemeroptera			X
		Hemiptera (Heteroptera)			X
		Lepidoptera			X
		Megaloptera			X
		Odonata			X
		Plecoptera			X
		Trichoptera			X
	Malacostraca	(any)			X
		Amphipoda			X
		Decapoda			X
		Isopoda			X
		Mysidacea			X
COELENTERATA†					
MOLLUSCA	(any, except *Hydrobiidae*)				X
	Bivalvia				X
	Gastropoda	(any, except *Hydrobiidae*)			X
		Hydrobiidae		X	
NEMERTEA					X
PLATYHELMINTHES	*Turbellaria*	any‡			

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

Table 5.5 Benthic macroinvertebrate: target Level of taxonomic identification – chironomidae

FAMILY	GENUS	REQUIRED LEVEL OF IDENTIFICATION
***Chironomidae* (except as noted below)**		Genus

	Cricotopus	*Cricotopus/Orthocladius if a clear distinction cannot be made*
	Orthocladius	*Cricotopus/Orthocladius if a clear distinction cannot be made*
	Conchapelopia	*Thienemannimyia* genus group
	Hayesomyia	
	Helopelopia	
	Meropelopia	
	Rheopelopia	
	Telopelopia	
	Thienemannimyia	

Table 5.6 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., *Thienemannimyia*)	
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 5.8**)	
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)

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		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

5.9 Data Entry

Table 5.1, **Table 5.3** and **Table 5.6** 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.

5.10 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.

5.11 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:

5. The QC contractor 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 QC Contractor will randomly select three samples for the evaluation.

   b. If the laboratory is responsible for processing 100 samples or more for the survey, the QC contractor 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 contractor).

   c. To the extent practicable, the External QC Coordinator and QC contractor will schedule batch evaluations evenly throughout the project period.

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8. The QC contractor coordinates and pays for delivery of the selected samples, bench sheets, and chain-of-custody forms.

9. The primary laboratory packs the selected samples for shipping to the central holding facility designated by the QC contractor. Although the QC contractor 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 QC contractor. In the event that the reference specimens are the only organisms representing a given taxon for a given sample, then the primary laboratory must make these specimens available by providing a digital image and/or one specimen from the reference library. If there is only a small number of the organisms, the primary laboratory also shall provide the count of the organisms.

c. Shipment must contain chain-of-custody documentation.

10. 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.

11. Upon receipt, the secondary laboratory sends a confirmation to the QC contractor that the samples have arrived. Within 24 hours, the secondary laboratory completes and returns the chain-of-custody forms to the QC contractor.

12. The QC taxonomist:

a. Performs whole-sample re-identifications, taking care to ensure inclusion of all slide-mounted organisms. The QC taxonomist follows the procedures in Taxonomy Identification **Section 5.8** with the following modifications:

      i. All *Chironomidae* and *Oligochaeta* organisms must be mounted on slides (i.e., the waiver is not an option). Before and after mounting, count the number of organisms. Compare the two numbers and attempt to resolve any discrepancies (e.g., check for organisms remaining on the forceps).

      ii. Step 5: Classifying organisms as "Distinct" is not relevant for the QC evaluations, and thus, not required.

      iii. Step 9: All organisms provided by the primary laboratory must be identified and counted (i.e., 500 count restriction does not apply).

      iv. Step 10 and 14: Internal QC taxonomist steps do not apply.

      v. Step 13: Physical and digital reference libraries are not required.

b. Completes a separate copy of the taxonomic bench sheet for each sample.

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c. Enters the data using EPA's template (Template found in **APPENDIX C: SAMPLE LABORATORY FORMS**, see **Section 5.9** for data entry instructions). The QC taxonomist shall label the bench sheet and database entries with the term "QC Re-ID."

13. 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 **Table 5.4** and **Table 5.5** or by nomenclature different than used by WQX or other acceptable references, the QC contractor 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 5.4.1**; and

b. Taxonomy as measured by PTD defined in **Section 5.4.1**. Table 3 of Stribling (2003) provides examples of what would be considered agreements.

14. If any sample has PDE>5% and/or PTD>15%, the QC contractor, with the External QC Coordinator's approval, will facilitate a reconciliation call with EPA and the laboratories. If the secondary laboratory has digital photography equipment, the QC Taxonomist shall provide digital photographs of organisms that will be discussed during the reconciliation call. The External QC Coordinator may decide that a reconciliation call (and the digital photographs) is unnecessary if there appears to be an obvious explanation for differences, few samples are affected or other reasons.

15. After completing the reconciliation calls and/or documenting decisions to skip them, the following occurs:

    a. 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.

    b. The QC contractor, or the secondary laboratory, will return the samples to the primary laboratory following appropriate tracking procedures.

16. 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 extreme discrepancy, the External QC Coordinator will arrange for another taxonomist to evaluate the specimen or its digital photograph.

17. 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:

28. Confirm the sorting and identification steps are properly implemented.

29. Assist with questions from laboratory personnel.

30. Suggest corrections if any errors are made.

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5.12 Quality Assurance/Quality Control (QA/QC)

Equation 5.1 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 5.2 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 5.3 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 ≤15%.

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

Table 5.7 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 5.8 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

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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 5.4.1 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

5.13 References

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Stribling, J.B., S.R. Moulton, and G.T. Lester. 2003. Determining the quality of taxonomic data. *Journal of the North American Benthological Society* 22(4):621-631.

Vinson, M.R. and C.P. Hawkins. 1996. Effects of sampling area and subsampling procedure on comparisons of taxa richness among streams. *Journal of the North American Benthological Society* 15(3): 392-3.

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6.0 FECAL INDICATOR: ENTEROCOCCI

6.1 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.

6.2 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 BeadsTM (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 6.4** of **Section 6.18**.

6.3 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, guanidine, 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.

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

6.5 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

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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.

6.6 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.

6.7 Equipment and Supplies

1) Clean powderless latex or vinyl gloves
2) Goggles or Face Shield
3) Roche MagNA Lyser
4) Roche MagNA Pure LC (automated nucleic acid isolation and purification platform)
5) High Speed Microfuge
6) Micropipettors
7) Semi-conical, screw cap microcentrifuge tubes (PGC, #506-636 or equivalent) pre-filled with 0.3 $\pm$ 0.02 g Acid-washed glass beads (Sigma, # G-1277 or equivalent). Filled tubes are autoclaved 15-min. Liquid Cycle (Slow Exhaust) OR
8) 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.
9) Roche MagNA Lyser Rotor Cooling Block
10) 2-mL tube racks
11) Permanent marking pens (fine point and regular point) for labeling tubes
12) Bench Sheets & Printouts of Computer Software Sampling Loading Screen

6.8 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

6.9 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.

6. 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.

18. **Preparation of stock Salmon Sperm (SS) DNA:** Dissolve Salmon DNA in PCR grade water at a concentration of ~10 μg/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 μg/mL depending on the GC content of the DNA's sequence(s).

19. Dilute Salmon testes DNA stock with AE buffer to make 0.2 μg/mL Salmon DNA Extraction Buffer (SAE). Extraction buffer may be prepared in advance and stored at 4 ºC 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 μL) 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 μL 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 μL. Hence for the SAE volume for 25 sample tubes is equal to 15,000 μL. Dilute the Salmon DNA working stock 1:50, for a total volume needed (15,000 μL) 50 = 300 μL of 10 μg/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 μL - 300 μL = 14,700 μL AE buffer needed.

20. 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.

21. *Enterococcus faecalis* DNA for Standards.

22. Frozen Reference Stock (20-μL) at 2.89 x 10^6 GEQs per μL.

23. Dilute 10-μL of the Frozen Reference stock 363-fold to a final volume of 3,630 μL AE buffer. Aliquot 20-μL volumes into many 200-μL microfuge tubes and store frozen at -20 ºC. The net concentration of *Enterococcus* GEQs is 8,000 / μL. Each week perform a series of 10-fold and 4-fold dilutions from one thawed tube of the 8,000 GEQ/μL standard solution to create 800 GEQ/μL, 80 GQ/μL and 20 GEQ/μL standard solutions. The analyst performs *Enterococcus* qPCR upon duplicate 5-μL 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:

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      i. Assemble calibrator positive control samples by thawing tubes of *E. faecalis* cell stocks, diluting their contents (10-μL) up to 1-mL AE buffer and spotting 10-μL 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 -20ºC freezer and thaw individual calibrators (three per week) for extraction with each week's batch of samples.

24. 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.

25. 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.

6.10 Procedures for Processing & qPCR Analysis of Sample Concentrates.
6.10.1 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 6.18.1**).

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-μL of SAE Extraction Buffer instead of the usual prescribed 600-μL 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-μL) of each sample filter concentrate added to the PCR reactions.

6. 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-μL AE buffer using a micropipettor.

26. To extract sample filters, uncap green bead tube (cold) and add 0.6-mL (600-μL) SAE Buffer (Qiagen AE Buffer spiked with Salmon DNA). Re-cap tubes tightly.

27. 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-μL of supernatant (sans debris) to new tube with a P-200 or P-1000 micropipettor.

28. Spin the supernatant tubes for 5-min at 14,000 rpm at Room Temperature. Recover >350-μL supernatant and transfer to new 1.7-mL tube. When all samples in a batch have been

extracted transfer dilute 20-µL of DNA extract (2nd supernatant) five-fold (5X) in 80-µL 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 6.18.2**).

6.10.2 Sample Analysis by *Enterococcus* qPCR

6.10.2.1 Preparation of qPCR assay mix

d. 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.

29. 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 µM primer and 100 µM probe and dissolve by extensive vortexing. Pulse centrifuge to coalesce droplets. Store stock solutions at -20ºC.

30. Prepare working stocks of *Enterococcus*, and Salmon DNA primer/probe mixes by adding 10 µL of each *Enterococcus* or Salmon DNA primer stock and 4 µL of respective probe stock to 676 µL 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 4ºC.

31. 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 6.1**.

32. 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.

33. 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.

34. Set 32 Smart tubes in Cepheid Racks in PCR cabinet along with micro-pippetors and expose to germicidal UV lamp for 15-min.

35. Pipette 20-uL of respective Master Mix into each labeled Smart tube. Transfer Smart tubes (racks) from PCR cabinet to disinfected Sample Loading Fume Hood.

36. Using P-10 or P-20 micro-pipettor load each Smart tube with 5-uL volume of respectively designated sample extract (dilution), standard, or buffer blank (SAE). Cap each sample's Smart tube after loading.

37. 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.

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Enterococcus (Ludwig) and Salmon (Sketa) qPCR assays (EPA Method 1606) will be performed upon 5-uL 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 it's 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 ($\geq$ 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 6.18.2**).

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 C*t* 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.

6.10.3 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 6.2** and **Table 6.3** of **Section 6.18**.

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.

6.11 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.

6.12 Chain of Custody

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

Field Sampling forms and NRSA 2023/24 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.

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

The Data Quality Objectives and the Laboratory QC Procedures are listed and summarized in **Table 6.5** and **Table 6.6** of **Section 6.18**.

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.

6.14 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.

6.15 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 2023/24 database stored on a computer server in Corvallis, Oregon.

6.16 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.

6.17 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)

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

Table 6.1 Enterococci: PCR assay mix composition (according to draft EPA method 1606)

Reagent	Volume/Sample (multiply by # samples to be analyzed per day)
Sterile H_2O	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 <u>strongly</u> recommended that preparation of assay mixes be performed each day <u>before</u> handling of DNA samples.

Table 6.2 Enterococci: batch calibrator & enterococcus standards PCR run – 7 samples

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

* Diluted equivalently to the water samples

Table 6.3 Enterococci: sub batch test sample PCR run – 26 samples & 1 method blank

Sample Description*	Quantity Samples	PCR Assay Master Mix	Quantity PCR Reactions
Water samples, (5-fold dilution)	26	*Enterococcus*	26
Method blank or Sample PCR Reaction Duplicate, (1- or 5-fold dilution)	1	*Enterococcus*	1
Non-diluted SAE Buffer	1	*Enterococcus*	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 6.4 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	-40Cto+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 *Enterococcus* qPCR
Method 1606 (*Enterococcus* & 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 *Enterococcus* qPCR NERL NRSA 2008 qPCR Analytical SOP

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

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Table 6.5 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 *Enterococcus* qPCR
***Enterococcus* & 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 *Enterococcus* qPCR; NERL NRSA 2008 2009 qPCR Analytical SOP (QAPP)
SPC & ENT DNA sequence numbers of Calibrators & Standards by AQM	RSD = 30%	80%	95%	
ENT CCEs by dC*t* RQM	RSD = 55%	40%	95%	
ENT CCEs by ddC*t* 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 6.6 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 *E. faecalis* calibrator	For all qPCR calibrator samples for quantitation	All calibrator sample C*p* (C*t*) must have an RSD $\leq$ 30%	If calibrator C*p* (C*t*) 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.

6.18.1 *Enterococcus* qPCR Analysis Decision Tree (ADT)
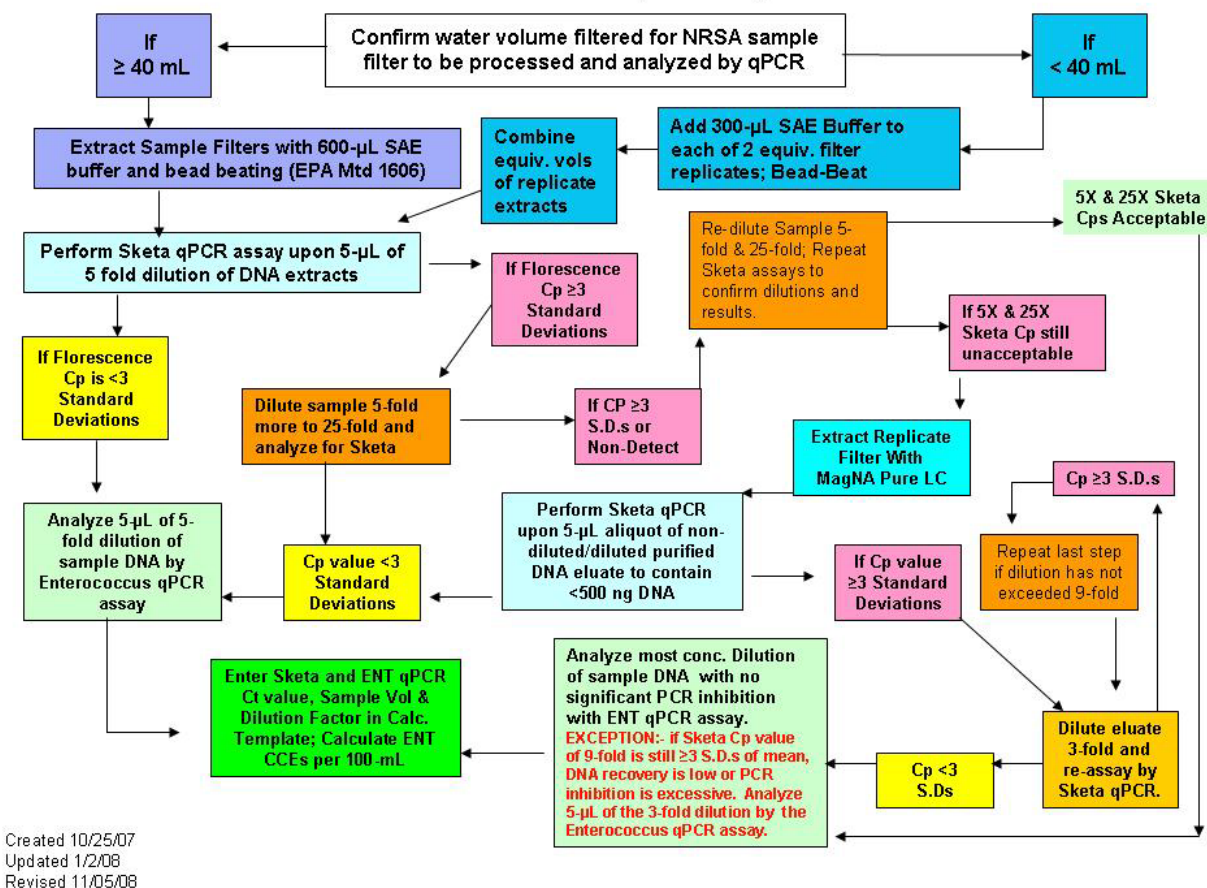
Figure 6.1 Enterococci: qPCR analysis decision tree (ADT)

6.18.2 "Modified" MagNA Pure LC DNA Purification Kit III Protocol

e. Pre-warm the MagNA Pure LC DNA Isolation Kit III Lysis Buffer to 65 ºC 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.

38. 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.

39. 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.

40. 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 4ºC. Press Start button. Centrifuge to collect drops and foam off of cap down into tube.

41. When centrifuge stops, open lid and remove tubes from rotor. Uncap tubes in order and add 40-µL 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.

42. Insert tubes into styrofoam floating rack. Incubate tubes 30-min at 65ºC 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 65ºC waterbath for 15-min. for total of 30-min.

43. 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.

44. After 30-min in 65 ºC 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.

45. Insert tubes in centrifuge rotor and spin 3-min at 12,000 rpm and 4 ºC 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.

46. Carefully remove rotor from centrifuge and set on bench. Remove tubes one at a time from rotor and use 200-µL pipettor and sterile aerosol-proof tips to transfer approximately 150µL lysate supernatant from tube to wells in MagNA Pure LC Sample Cartridge in pre-designated order.

47. 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 ºC.

48. 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

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microtiter plates in place. Insert the film-covered sample cartridge in MagNA Pure LC platform.

49. 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 1.34μL of 9.3μg/mL Salmon DNA Stock (10 μg/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-μL, dilute the Salmon DNA stock so that a volume $\geq$ 10-μL can be pipetted into the Lysis Binding Buffer. Rinse pipette tip up and down three times in Lysis Binding Buffer.

50. 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-μL Elution Buffer.

51. 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 ºC for long term preservation.

52. Prepare Elution Buffer Control from 9.3μg/mL Salmon DNA Stock by diluting a small volume to 37.2pg/1000μL (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.

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7.0 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 7.4** describes the sample receipt and login procedures. **Section 7.5** describes the identification steps. **Section 7.6** describes laboratory assistance visits. **Section 7.7** identifies the sample and record retention requirements. **Section 7.9** provides a summary of the quality control requirements. The final section provides references to aid in identifications.

7.1 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.

7.2 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 eyeware, 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.

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

This section provides definitions and required resources for using the procedure. **Section 7.3.1** defines the terms used throughout the procedure. **Section 7.3.2** describes the taxonomic expertise required for each QC taxonomist using the procedure. **Section 7.3.3** describes the roles and responsibilities of the personnel involved in the procedure. **Section 7.3.4** identifies the equipment necessary to apply the procedure in identifying fish voucher specimens in samples.

7.3.1 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 7.1** 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.

7.3.2 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.

53. 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.

54. 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).

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

7.3.3 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.

7.3.4 Equipment/Materials

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

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

7.4 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.

- Report receipt of QC voucher samples (including digital images) in the NARS IM sample tracking system (within 24 clock hours).
56. 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.

57. Store preserved samples at room temperature until identification begins.

58. Maintain the chain of custody or sample tracking forms with the samples.

59. 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.

60. 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 7.1 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

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7.5 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 7.1** 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 7.2**). The steps are described below:

- Process the QC voucher sample by voucher tag.

61. 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.

62. 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.

63. 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 7.9 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.

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64. 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.
65. 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.
66. 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.
67. 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.

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68. 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.

69. 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

70. Return the specimens to the bag along with the voucher tag. Place the bag back into the sample jar. See **Section 7.7** for retention requirements.

71. Select another bag and repeat Steps 1-10.

72. 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

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NRSA 2023/24/Data Templates folder. **Table 7.2** identifies the contents and formats used by EPA's file.

73. Save the submission worksheet file using the following convention: Taxonomist name+affliation-abbreviation+submission date (yyyymmdd).xlsx (e.g., JSmith_ABCfirm_20180615.xlsx).

74. 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 7.2 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 that 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**)		

7.6 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.

7.7 Sample and Record Retention

The laboratory shall retain:

- 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.
75. 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.

7.8 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 ≤15%.

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

Table 7.3 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 7.4 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 7.5 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.

7.9 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 7.1 Fish voucher: example QC fish taxonomy bench sheet (optional)

Additional Comments

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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 G: EXAMPLE SOPS FOR MERCURY IN FISH TISSUE PLUG ANALYSIS**.

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 -20º 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.

9.0 DIATOMS

This procedure is applicable to the analysis of the diatom samples collected for the National Rivers and Streams Assessment (NRSA) 2023/24. NRSA field crews collect composite samples of the algae present on benthic substrate; 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 diatoms benthic substrate. This procedure describes the steps for identifying and enumerating diatoms in the samples.

9.1 Summary of Procedure

The procedure describes the extraction and separate analysis of the diatom samples. Diatom samples are digested using concentrated nitric acid to remove organic matter. After digestion samples are centrifuged and supernatant decanted, and then refilled with distilled water. The rinsing process is performed a minimum of six times. Cleaned material is place on a glass microscope slide and a cover slip is mounted on to the cleaned material using Naphrax^{TM}. Once permanent slide mounts have been created, analysts can then begin to identify and enumerate diatom taxa for a given sample. To ensure transparent taxonomic consistency between analysts, both regionally and nationally, samples will be randomly distributed among analysts; and analysts will use pre-count voucher flora to assign operational taxonomic units (OTUs) to diatoms identified in individual samples. As new taxa are encountered, analysts can add new OTU codes to the pre-count voucher flora. In additional to the use of the pre-count voucher flora, 10% of samples are re-analyzed by the same analysts, and 10% of samples are re-analyzed by a second analyst. Both the 10% same analyst re-analyzed and 10% second analyst re-analyzed samples are randomly assigned to each analyst.

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 ^{TM}) 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 Required Equipment
9.3.1 Laboratory

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

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

76. Memorandum that identifies the relevant services that the laboratory provided for the National Aquatic Resource Surveys in the past five years.

77. Memorandum that describes the laboratory's participation in relevant round robin studies and/or performance studies.

78. Report of relevant findings from an on-site technical assessment or audit.

79. Quality procedures.

80. 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).

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

82. Reporting standardized data.

83. 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.

84. 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.

9.3.2 Equipment/Materials

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

9.3.2.1 Subsampling Equipment/Materials

- 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

9.3.2.2 Diatoms

9.3.2.2.1 Preparation of Diatom Slides

1. Corning ceramic-top hot plate with temperature control
2. Positive-draw chemical hood

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3. 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)

4. Glass microscope slides (1 x 3 inches; 2.5 x 7.5 cm)

5. 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.

6. Mounting medium with a high refractive (1.7) index (e.g., Naphrax^{TM})

7. Diamond scribe

8. Disposable plastic pipettes

9. Adjustable pipettor (0 - 250µl); adjustable pipettor (200 - 1000µl)

10. Pipette tips for adjustable pipettors

11. Round-style tooth picks

12. Forceps

13. Polished, rounded wooden splints

14. Wash bottle filled with distilled (DW) or reverse osmosis (RO) water

15. Single-edged razor blades

16. Ethanol, 70%

17. Acetone

18. Tissues (e.g., Kimwipe®)

19. Slide labels

20. Wax (the kind commonly used for candle making and canning foods)

21. 10% HCl

9.3.2.2.2 Analysis of Diatoms

- 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

9.4 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.

4. 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.

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85. 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**).

86. 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).

87. 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 Required data elements for diatom sample 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.	

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9.5 Sample Preparation

Before the sample is digested, the volume received is measure in a 100 mL graduated cylinder. The volume is recored to the nearest 1 mL. Once the total volume is recorded on the **Table 9.2** after completing all required elements, provide the information to the data entry personnel.

Table 9.2 Required data elements for diatom 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
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

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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:

*   Abide by the following **Safety Precautions**:
*   Wear safety glasses, protective gloves and lab coats at all times when handling liquid and/or solidified mounting medium. For example, Naphrax[d] is a hazardous substance because it contains toluene, which volatilizes readily when heated.
*   Use a positive-draw fume hood at all times for heating of the mounting medium.
*   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.
*   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:
*   Keep the laboratory room and bench surfaces as clean as possible and free of debris.
*   Use disposable pipettes, stirrers, etc. where feasible.
*   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.)
*   Dry all equipment before storing it. This step prevents growth of algae and fungi.
*   Verify that all necessary equipment is available. See **Section 9.3.2** for recommended supplies.
*   Determine if the vial's contents will produce satisfactory slides. Consider the following:
*   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).
*   Generally, between 10 and 20 diatom valves or frustules should be present in a single high power microscope field (1000X).
*   The number of specimens per field will need to be reduced if samples contain considerable amounts of silt.

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

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- 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).
- 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:

1. 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:
   1. Allow the vial to settle for approximately 4 hours.
   2. 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.
   3. Allow the vial to settle for approximately 4 more hours (total of approximately 8 hours)
   4. Use a micropipettor to remove the required amount of water from the vial of material. Keep track of volume removed from the vial.
   5. Record the SUBSAMPLE_DCF (i.e, subsample dilution/concentration factor).
2. 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).
3. 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.
4. 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:
   1. Scan the slide in its entirety under 1000x magnification, and estimate the total number of individuals on the slide.
   2. Determine if the slide is countable (i.e., 100 specimens or more can be counted within 4 hours), considering factors such as:

6.0   Density of diatoms.

7.0   Evidence of dissolution

8.0   Amount of debris (silt, clay, broken remains of diatoms and other siliceous organisms) that would make it difficult to identify specimens accurately.

3. 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:

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1) Drip a known portion of the untreated sample onto a coverslip.
2) Dry the coverslip at room temperature.
3) When the sample is dry, place the coverslip onto a hot plate for about 30 min at approximately 570°C.
    - Deposit cleaned material on coverslip as follows:
        1. Use forceps to remove single coverslips from the storage container.
        2. Carefully clean each by wiping with a tissue (e.g., Kimwipe®e).
        3. Place each coverslip on a marked space. (Be sure this space is clean and dry to avoid cross-contamination.)
        4. 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:
    - 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:
        - 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.
        - Agitate the sample vial to homogenize the diatom suspension.
        - Using an adjustable pipettor, quickly withdraw the required amount from near the central portion of the sample.
        - Eject this material smoothly and carefully into the layer of distilled water already on the coverslip.
        - Repeat until achieving a homogeneous suspension on the coverslip.
        - Record the subsample amount ejected.
    - In the case where more than1.0 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).
    - If the coverslip overflows, discard the coverslip, wipe the drying area, and repeat the procedure with a freshly cleaned coverslip.
    - Discard the pipette tip when finished with each vial.
        5. Once the marked space is loaded with coverslip preparations, do not disturb until the coverslips are dry.
        6. Dry the coverslips as follows:
    - Leave them undisturbed at room temperature for several hours;
             OR
    - Use gentle heat (warm to the touch only) to hasten evaporation using a:
    - Crook-neck lamp with incandescent light bulb placed 15 - 30 cm over the coverslips;

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e Kimwipe® is a commercially-available product.

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- Slide maker that provides heat;
- Or some other device that will <u>gently</u> dry the slides. Avoid any device that will rapidly evaporate the suspension. Rapid evaporation could produce strong patterns of diatoms settling on coverslips.
- Once completely dry, put the coverslips on the hot plate preheated to 250 to 300ºF.
- Leave coverslips on hot plate for 3 - 5 minutes. Verify that:
- Heat has driven nearly all water from the material on the coverslips; and
- Diatom frustules adhere to the surface of the glass.
- Remove the coverslips from the hotplate and inspect them. Consider:
- If the pattern of diatoms distributed on any coverslip is not even and smooth, discard and prepare a new coverslip.
- If coverslip distributions are still unsatisfactory after repeated attempts, consult an algal analyst.
- Mount coverslip on slide in a **POSITIVE-DRAW FUME HOOD** using the following steps.
- 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.
- Remove the appropriate coverslip from the aluminum plate with forceps, being careful to handle the coverslip only at the extreme corners.
- 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.
- 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.
- Remove the slide from the hot plate.
- Gently position the coverslip using the rounded toothpicks.
- Bring the edges of the coverslip parallel to the edges of the microscope slide.
- 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.
- Set aside the mount to finish cooling.
- 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.
- 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.

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- Wipe the mount clean with a tissue (e.g., Kimwipe®).
- Add a paper label to slides before analyzing the slides.
- Mark the sample ID number on the side of the slide without a label with a diamond pencil.
- 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.
- Preserve and store cleaned material.
- Record the data elements described in **Table 9.3**. Comments should include information such as:
2. Observations about any interfering materials (sand, silt, etc.)
3. Rationale for using a sparsely populated slide.
4. Information on all burn mount attempts, successful or unsuccessful.

Table 9.3 Required data elements for 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., Naphrax^{TM})
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

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9.7 Analysis of Diatoms

The taxonomist should count 600 valves for any properly concentrated and prepared sample as described in Section 9.6. If a taxonomist is unable to count 600 values after analyzing a complete slide, then the taxonomist should make note of this in the sample analysis data file comments section.

9.7.1 Creation of Pre-Count Regional Voucher Flora and Develop Diatom Analyst Schedule

The following section explains the process for creating a pre-count regional voucher flora. These regional flora were developed during NRSA 2018-2019 and will be used for NRSA 2023/24 and future NRSA surveys. This section can help understand how those voucher flora were developed and how new taxa could be added to the existing regional voucher flora. New regional voucher flora do not need to be Created for NRSA. This section also contains information on how to develop sample analysis schedules for diatom analysts.

For analyses of diatoms NRSA, EPA requires that all laboratories to develop pre-count regional voucher flora. The regional voucher flora documents must be completed before counting of diatom samples begins. The following section is an overview of how the voucher floras are created:

5. The project lead taxonomist obtains light micrographs (LM) images from a minimum of 80% of the project prepared slides.

For common taxa images should be limited to 1-2, for example, *Navicula rhynchocephala, Cocconeis placentula*.

The goal of the regional voucher flora document is to capture images taxa that analysts are most likely to encounter during the counting process. A target effort is for the taxonomist to spend 15 minutes per slide searching for species that need to be documented.

To save time, use the microscope camera in its fixed position (that is, it does not need to be rotated to orient cells vertically or horizontally), as image post-processing is more efficient.

Image filename should include slide number, date, and sequential number (e.g. "15565a_12092016_01").

6. Once all images are captured, process images as a group using GIMP, Photoshop or other image software. Rotate, crop and add a 10 μm scale bar to each raw image, then export as a jpeg file (Fig. 9.1)

It is important that these images are of optimal resolution and contrast to capture the morphological features of each taxon. These images will be used in project voucher flora publications, as well as in Diatoms of the US taxon pages.

7. Create image "plates" for each genus, or other grouping (Fig. 9.2), by gathering jpeg images into Photoshop (.psd) or gimp (.xcf) plates. Scale all images equally to a standard 100x, which allows users to measure cells directly on the digital plate.

Use a template with the dimensions 17x25cm, 600ppi, greyscale, white background.

Note that it is easiest to import all the jpeg images at once, each as a layer labelled with their sample identification information. Layers can be immediately locked together and scaled to 100x. Large cells may need to be scaled separately, for example, we capture some valves using the 60x objective.

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8. Sort images into their "best" morphological groups, or genera.
9. Provide a list of additional sequential provisional names on the plate (Fig. 9.2)
10. Once the regional voucher flora documents have been developed, then the project lead taxonomist will develop an analysts schedule.

Samples will be randomized across analysts, and laboratories if more than one, to ensure that samples are distributed randomly across geographic areas (i.e. avoiding one analyst getting all or most samples from one ecoregion or state).

The project lead taxonomist will also determine which samples are re-counted by randomly selecting 10% of analyst's processed samples to be re-counted by the sample analyst and 10% of an analyst's processed samples to be re-counted by a second analyst involved in the project.
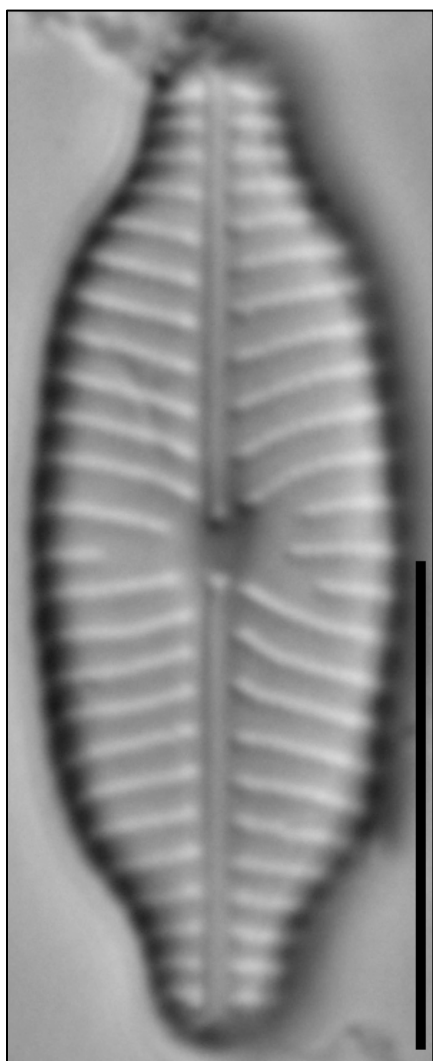
Figure 9.1 Example of light micrograph image after rotation, histogram, and scale bar processing.
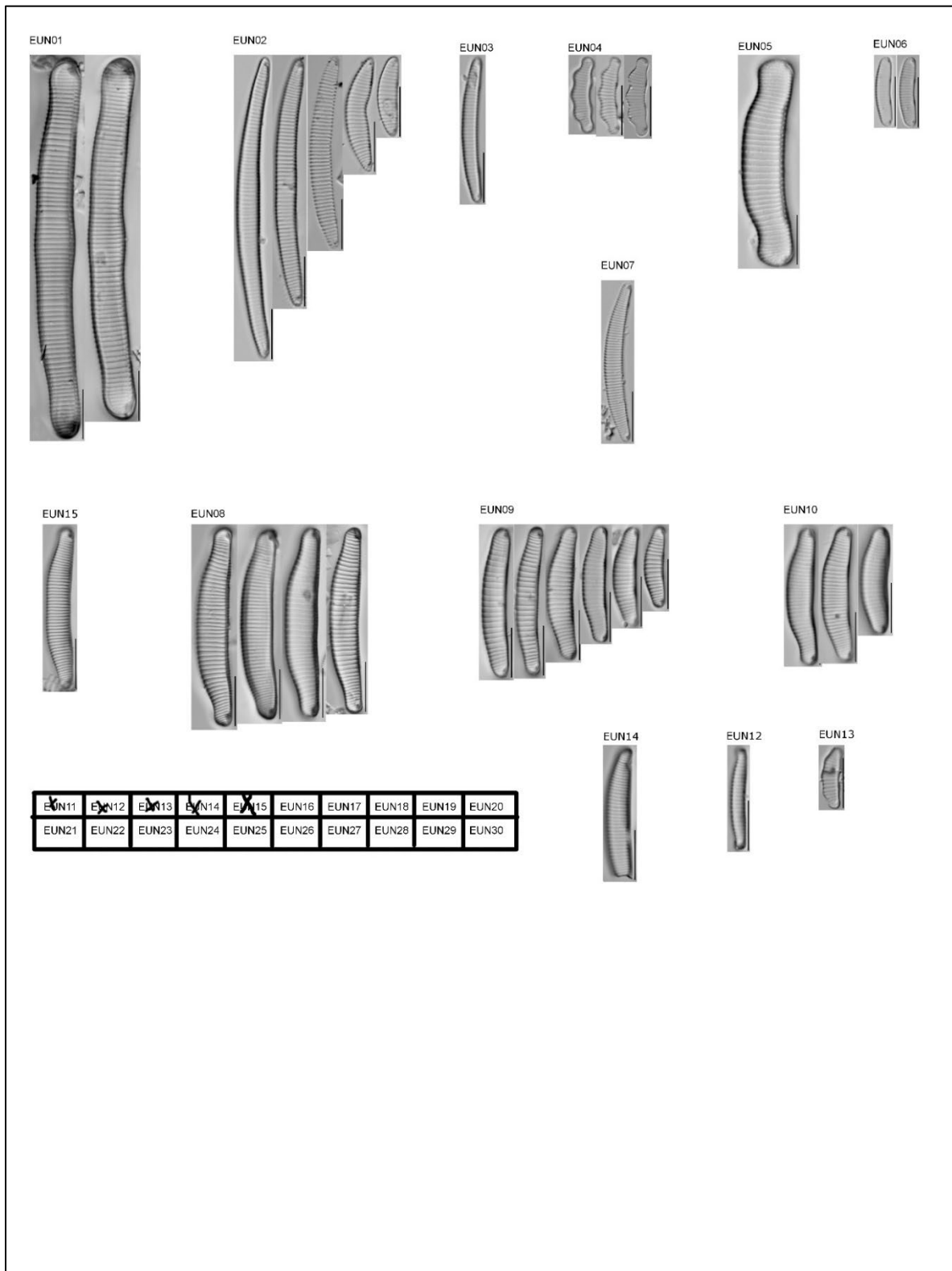
Figure 9.2 Example plate of a regional voucher flora collected before diatom sampling counting has begun.

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9.7.2 Diatom Sample Count

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 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:

9. Verify that all necessary equipment is available. See **Section 9.3.2** for recommended supplies.
10. Verify that the slides can be used for diatom analysis:

- Review comments from the subsampling, cleaning, and mounting steps for any deviations that may affect the outcome of diatom analysis.
- Scan slides at low to medium magnification (100x to 450x) to confirm that diatoms are:
  o Evenly distributed on the coverslip.
  o Present at a density appropriate for efficient counting. At high magnification (1000x), each field should have 5-10 diatoms.
  o If diatoms on the slides are very sparse, refer to procedures in **Section 9.6** for handling low-density samples.
- 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).

11. Create the transect and count the diatoms as follows:

- 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. Alternatively, maintain a spreadsheet that identifies the starting location and type of coordinates used in specifying the starting point. Either etching or documenting the starting location is an important step because another taxonomist may need to recount the slide for QA/QC purposes.
    e. If etching the slide before counting:
        c. Etch at least three lines on the diatom slide.
        d. 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.
    f. If etching during the count:
- 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).
- Etch a circle around the last field counted in the first row and at the start and end of all other rows.
        g. Verify that the etching is clearly visible so that another taxonomist can easily locate the circles and lines.

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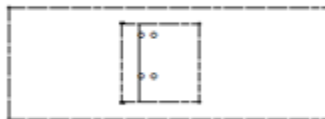
- Focus the first field under oil immersion.
- On a bench sheet and the upper right-hand corner of the coverslip, record in mm the transect start coordinates from the microscope stage. Transects are narrow rectangular areas (strips) of the slide with width equal to the field of view. The transect width should be the maximum width common to all participating taxonomists (at least 90 microns).
- For each species/taxon, count diatom valves along the transect using the following rules:
- One valve is:
- A complete valve; or
- 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).
  - Avoid counting valves in any disrupted areas of the mount, particularly edges that have optical aberrations.
  - 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).
  - Record the level of magnification used to view the diatoms.
  - Verify that photographs of the valves for the species/taxon exist in the regional voucher flora documents. If the valve is the first occurrence of the species/taxon, take a photograph following the specifications in **Section 9.7.1**.
  - On a bench sheet, record the transect end coordinates from the microscope stage.
12. Calculate the total transect length and width (units are mm) evaluated for the sample. Record as TRANS_LENGTH and TRANS_WIDTH in the database.
13. Verify that the SUBSAMPLE_DCF value reflects all dilution and concentration of the subsample. Make any necessary corrections to the value.
14. Record the required data elements in described in **Section 9.7.3** (**Table 9.4**).
15. Once all samples have been counted and any new OTU taxa have been added to the regional voucher flora by analysts, the lead taxonomist will develop a project species list that will coordinate the project OTU list with valid scientific names from Biodata. The taxa list created from this final process will become the final project taxa list for NRSA 2023/24. A current list of diatom taxa can be found at the biodata website: http://aquatic.biodata.usgs.gov

9.7.3 Internal Quality Control

For each sample, the laboratory must perform internal quality control evaluations. The lead taxonomist must randomly select 10% of each analyst's processed samples to be re-counted by the same analyst, and randomly select 10% of each analyst's processed samples to be re-count by a secondary (internal)

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analyst. Calculate the PDE, and PTD listed below. If any samples do not meet the QA requirements listed in the Attachment, perform a third re-count for the sample.

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.

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

Table 9.4 Diatom sample analysis required data elements

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 10.5.
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.
DATE_ANALYSIS	date	Date when the analysis was completed
OTUCODE	numeric or text	An identifier assigned by the laboratory to provide a unique number for each taxon identified for NRSA.
LAB_TAXON_NAME	text	Taxon name. If BiodataTaxonName is available, this field is optional.
SPECIES	text	Genus and species
TRANSECTS_COUNTED	numeric	Number of transects counted
TRANS_LENGTH	numeric	Length (mm) of transects counted

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TRANS_WIDTH	numeric	Width (mm) of transects counted	
COUNT	numeric	Number of diatom valves	
COUNT_CENSORED	text	Code for a sample for which the taxonomist counted fewer than 600 valves	
		CODE	**Definition**
		SS	Sparse sample
		OT	Other. Identify other reason in COMMENT_ANALYSIS
COMMENTS_ ANALYSIS	text	any comments about the analysis	

9.8 Data Entry

Table 9.2, and **Table 9.3** identify the required data elements that the laboratories must provide to EPA. **Table 9.4** provides a list of required data elements for the diatom count data. 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.

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:

11. Work under a fume hood to add two to four drops of 100% buffered formalin to each vial.

88. Add glass beads to the vials to help preserve the diatoms.

89. Tightly cap the vials and seal them by immersing the top 1/3 of the vial in melted wax.

90. Transfer the vials to long-term storage.

The laboratory shall retain:

4   The sample materials, including vials and slides, for a minimum of 1 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.

91. 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 deposit sample material in a public herbarium, such as the Academy of Natural Sciences of Drexel University.

9.0 DIATOMS

10.0 PERIPHYTON BIOMASS

Samples for periphyton biomass are processed alongside the periphyton Chloryphyll *a* and water column Chloryphyll *a* samples as Ash Free Dry Mass (AFDM). This procedure is applicable to the analysis of periphyton biomass from the filtered, composite periphyton sample collected in the field. The method is performance based (**Table 10.1**). Laboratories may use any method that meets the requirements below to analyze the samples. An example SOP is provided in **APPENDIX F: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS.**

Table 10.1 Quality assurance measures for AFDM

Activity	Evaluation/Acceptance Criteria	Corrective Action
Demonstrate competency for analyzing AFDM to meet the performance measures	Demonstration of past experience with AFDM 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 **Table 10.2**.
Store sample appropriately. While stored at the laboratory, the sample must be kept at a maximum temperature of -20º 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. 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 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.

Table 10.2 Data field requirements from labs for AFDM

FIELD	FORMAT	DESCRIPTION		
LABORATORY ID	text	Name or abbreviation for QC laboratory		
DATE RECEIVED	MMDDYY	Date sample was received by 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)		
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 not frozen
			Q	Other quality concerns, not identified above
CONDITION COMMENT	text	Comments about the condition of the sample.		

11.0 PERIPHYTON META-GENOMICS AND ANTIMICROIBAL ANALYSIS (RESEARCH EFFORT)

Laboratory methods incorporated in an ORD Quality Assurance Project Plan.

12.0 WATER CHEMISTRY AND CHLOROPHYLL A

12.1 Analytical Parameters

A total of 19 parameters are determined from each bulk water chemistry sample collected (**Table 12.1**). 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 12.1 Water chemistry: NRSA 2023 - 2024 parameters

Analyte	Units	Comments
Conductivity	µS/cm at 25˚C	
pH (laboratory)	Standard (Std) Units	
Turbidity	Nephelometric Turbidity Units (NTU)	
Acid Neutralizing Capacity (ANC)	µeq/L (20 µeq/L-1 mg as CACO$_3$)	
Dissolved Organic Carbon (DOC)	mg C/L	
Ammonia-N (NH$_3$-N)	mg N/L	The method measures ammonia and ammonium; the relative proportion between these two analytes depends on pH. Typically, NRSA (and other NARS) samples consist mostly of ammonium.
Nitrate-Nitrite (NO$_3$-NO$_2$)	mg N/L	Note different preservation methods and holding times depending on whether the lab is using ion chromatography (IC) or flow injection analysis (FIA).
Total Nitrogen (TN)	mg/L	
Total Phosphorus (TP)	µg/L	
Sulfate (SO$_4$)	mg/L	
Chloride (Cl)	mg/L	
Nitrate (NO$_3$)	mg/L	May be obtained as part of nitrate-nitrite determination (use FIA to obtain nitrate-nitrite separately, then calculate difference for nitrate), or as a direct measurement (e.g., IC)
Calcium (Ca)	Mg/L	
Magnesium (Mg)	Mg/L	
Sodium (Na)	mg/L	
Potassium (K)	mg/L	
Silica (SiO$_2$)	mg/L	
Total Suspended Solids (TSS)	mg/L	
True Color	PCU	Performance objective based on use of visual estimation method
Chlorophyll a	µg/L (in extract)	

12.0 WATER CHEMISTRY and CHLOROPHYLL A

12.2 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 **Table 2.1**).

Upon receipt of samples, the assigned lab personnel inspect 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 decide whether EPA will use the data in the NRSA assessment.

12.2.1 Water Chemistry Samples

Figure 12. 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 12.2**) 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 12.2 Water chemistry: acid preservatives added for various analytes

Preservatives	
H_2SO_4	**HNO_3**
DOC	Ca
NH_3-N	Mg
Total N	Na
Total P	K
NO_2-NO_3	

```
                        ┌─────────────────────────┐
                        │     Sample Receipt      │
                        │     4 L Bulk Sample     │
                        │ Inspect samples and     │
                        │ complete tracking form  │
                        └─────────────────────────┘
                                    │
                        ┌─────────────────────────┐
                        │     Process Sample      │
                        │     Within 24 hours     │
                        └─────────────────────────┘
```

Filtration

HDPE bottle Acid washed Preserve with HNO$_3$

HDPE bottle Not acid washed Store at 4 °C in darkness

HDPE bottle Acid washed Preserve with H$_2$SO$_4$

Not Filtered

HDPE bottle Acid washed Preserve with H$_2$SO$_4$

HDPE bottle Not acid washed Store at 4 °C in darkness

Analyses
Calcium (180 days)
Magnesium (180 days)
Sodium (180 days)
Potassium (180 days)

Analyses
Chloride (28 days)
Nitrate (7 days)
Sulfate (28 days)
Silica (28 days)
Nitrate-Nitrite (IC) (7 days)
True Color (3 days)

Analyses
Ammonia-N (28 days)
Dissolved Organic Carbon (28 days)
Nitrate-Nitrite (with FIA) (28 days)

Analyses
Total Phosphorus (28 days)
Total Nitrogen (28 days)

Analyses
pH (3 days)
ANC (7 days)
Conductivity (7 days)
TSS (7 days)
Turbidity (3 days)

Figure 12.1 Water chemistry: sample processing procedures

12.2.2 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 ± 2°C for no more than thirty days before analysis.

12.3 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 13.4**). The LRL is the lowest value that needs to be quantified (as opposed to just detected), and is equal to or above 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 (≤ transition value) to relative (> transition value). For pH, the objectives are established for samples with lower H^+ (or OH^-) concentrations (pH between 5.75 and 8.25) and higher H^+ (or OH^-) concentrations (<5.75 or >8.25).

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 in past surveys (EPA ORD-Corvallis) are summarized in **Table 12.3**. Participating laboratories may use alternative analytical methods for each target analyte if they can satisfactorily demonstrate the alternative method can achieve the performance requirements as listed in **Table 12.4**. 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 2023 - 2024 QA files by the EPA Laboratory Review Coordinator.

Table 12.3 Water chemistry: NRSA 2023 - 2024 analytical methods (PESD Analytical Laboratory, EPA ORD-Corvallis)

Analyte	Summary of Method[f]	References[g]	PESD-AL SOP[h]
pH (lab)	Automated, using ManSci PC-Titrate w/ Titra-Sip autotitrator and Ross combination pH electrode. Initial pH determination for ANC titration	EPA 150.1 (modified), APHA 4500H	PESD-AL 16A.2
Specific conductance (conductivity) @ 25°C	Electrolytic, Man-Tech TitraSip automated analysis OR manual analysis, electrolytic	EPA 120.1, APHA 2510	PESD-AL 16A.2 PESD-AL 11A.5
Acid neutralizing capacity (ANC)	Automated acidimetric titration to pH≤3.5, with modified Gran plot analysis	EPA 310.1, APHA 2320	PESD-AL 16A.2
Turbidity	Nephelometric; Man-Tech TitraSip automated analysis, OR Manual analysis using Hach turbidimeter (high turbidity samples)	EPA 180, APHA 2130	PESD-AL 16A.2 PESD-AL 13A.4
Total suspended solids (TSS)	Gravimetric, dried at 104 °C	EPA 160.2; APHA 2540D	PESD-AL 14B.4
True color (Hach Kit)	Visual comparison to calibrated glass color disk.	EPA 110.2 (modified), APHA 2120	PESD-AL 15A.4

[f] FIA=Flow injection analysis. AAS=Atomic Absorption Spectrometry

[g] 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.

[h] PESD-AL = Pacific Ecological System Division – Analytical Laboratory. References are to laboratory SOP being used at central laboratory. Available upon request. (contact the Project Lead)

Analyte	Summary of Method[f]	References[g]	PESD-AL SOP[h]
Dissolved Organic Carbon (DOC)[i]	UV promoted persulfate oxidation to CO_2 with infrared detection	APHA 5310-C, EPA 415.3	PESD-AL 21A.5
Nitrate+Nitrite, as N (fresh waters)	Ion Chromatography OR FIA automated colorimetric (cadmium reduction)	EPA 300.1; SW-846 9056A; APHA 4110B EPA 353.2 APHA 4500-NO_3-N-E Lachat 10-107-04-1-C	PESD-AL 36A.2 PESD-AL 40A.7
Nitrate	Measured as part of Nitrate-Nitrite when using IC or calculated after measuring Nitrate+Nitrite using FIA.	See above	See above
Ammonia, as N (fresh waters)	FIA automated colorimetric (salicylate, dichloroisocyanurate)	Lachat 10-107-06-3-D	PESD-AL 30A.5
Silica, dissolved (SiO_2) Fresh waters	FIA automated colorimetric (molybdate, stannous chloride)	EPA366.0, APHA 4500-SiO2 F Lachat 10-114-27-1-B	PESD-AL 32A.6
Total nitrogen (TN)	Persulfate Digestion; FIA Automated Colorimetric Analysis (Cadmium Reduction, sulfanilamide)	EPA353.2 (modified) APHA 4500-N-C (modified) ASTM WK31786	PESD-AL 34A.6
Total phosphorus (TP)	Persulfate Digestion; Automated Colorimetric Analysis (molybdate, ascorbic acid)	EPA 365.1 (modified), APHA 4500-P-E USGS I-4650-03 Lachat 115-01-1-B (modified)	PESD-AL 34A.6
Major anions, dissolved chloride, nitrate, nitrite, sulfate	Ion Chromatography	EPA 300.1; SW-846 9056A; APHA 4110B	PESD-AL 40A.7
Major cations, dissolved calcium, sodium, potassium, magnesium	Inductively-coupled Plasma Atomic Emission Spectroscopy (ICP-AES) OR Flame AAS	EPA 200.7; EPA 6010D	PESD-AL 52A.1
Chlorophyll-a (Chl-a)	Extraction 90% acetone analysis by fluorometry	EPA 445.0, EPA 446.0	PESD-AL 71A.5

12.4 Pertinent QA/QC Procedures

A single analytical 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:

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

The analytical laboratory demonstrated in previous studies that it can meet the required LRL (USEPA 2004). QA/QC procedures outlined in this manual and the NRSA 2023 - 2024 QAPP will be followed to ensure these LRLs are met for the NRSA 2023 - 2024.

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[i] 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).

12.0 WATER CHEMISTRY and CHLOROPHYLL A

12.4.1 Laboratory Performance Requirements

Table 12.4 summarizes the pertinent laboratory performance requirements for the water chemistry and chlorophyll-a indicators.

12.4.2 Laboratory Quality Control Samples

Table 12.5 summarizes the pertinent laboratory quality control samples for the water chemistry and chlorophyll-a indicators.

12.0 WATER CHEMISTRY and CHLOROPHYLL A

Table 12.4 Water chemistry & chlorophyll-a: laboratory method performance requirements

Analyte	Units	Potential Range of Samples[j]	Lower Reporting Limit[k]	Transition Value[l]	Precision Objective[m]	Bias Objective[n]
Conductivity	mS/cm at 25˚C	1 to 77,000	2.0	20	± 1 or ±10%	± 1 or 5%
pH (laboratory)	Std Units	3.5 to 10	N/A	5.75, 8.25	>5.75 or <8.25= ±0.15 ≤5.75 or >8.25=±0.07	>5.75 and <8.25 = ±0.05 ≤5.75 or >8.25 = ±0.15
Turbidity	Nephelometric Turbidity Units (NTU)	0 to 44,000	2.0	10	± 1 or ±10%	± 1 or ±10%
Acid Neutralizing Capacity (ANC)	µeq/L (1 mg/L as $CaCO_3$=20 µeq/L)	-300 to +75,000 (-16 to 3,750 mg as CaCO3)	N/A	50	± 5 or ±10%	± 5 or ±10%
Dissolved Organic Carbon (DOC)	mg/L	0.1 to 109	0.20	2	± 0.10 or ±10%	± 0.10 or ±10%

[j] 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.

[k] 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.*

[l] 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.

[m] 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 range. 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 apply to more highly alkaline samples. For NRSA, that is less of a concern than the ability to measure acidic samples accurately and precisely.

[n] 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.

12.0 WATER CHEMISTRY and CHLOROPHYLL A

Analyte	Units	Potential Range of Samples[j]	Lower Reporting Limit[k]	Transition Value[l]	Precision Objective[m]	Bias Objective[n]
Ammonia as N(NH_3-N)	mg/L	0 to 17	0.02	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Nitrate-Nitrite (NO_3-NO_2)	mg/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/L	0 to 22,000	4	20	± 2 or ±10%	± 2 or ±10%
Sulfate (SO_4)	mg/L	0 to 33,000	0.50	2.5	± 0.25 or ±10%	± 0.25 or ±10%
Chloride (Cl)	mg/L	0 to 19,000	0.20	1	± 0.10 or ±10%	± 0.10 or ±10%
Nitrate (NO_3)	mg/L	0 to 360	0.02	0.1	± 0.01 or ±10%	± 0.01 ±10%
Calcium (Ca)	mg/L	0.04 to 5,000	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Magnesium (Mg)	mg/L	0-5000	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Sodium (Na)	mg/L	0-22,500	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Potassium (K)	mgL	0-170	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Silica (SiO_2)	mg/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 1000	5	50	±5 or ±10%	±5 or ±10%
Chlorophyll a	mg/L (in extract)	0.7 to 11,000	0.5	15	± 1.5 or ±10%	± 1.5 or ±10%

Table 12.5 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 analytical baatch prior to sample analysis	Control limits ≤ 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 <LRL	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.
Initial and Continuing Calibration Verification			Analyze ICV after calibration. Analyze CCV after every 10 samples and at the end of analytical batch.	±10% or method criteria	Perform corrective action and repeat all associated samples since last successful CCV. Alternatively, recalibrate and re-analyze all samples since last successful CCV.

12.0 WATER CHEMISTRY and CHLOROPHYLL A

QC Sample Type and Description	Analytes	Description	Frequency	Acceptance Criteria	Corrective Action
Analytical Duplicate Sample	All analyses		One per 10 samples	Within precision objective	If results are below LRL: Prepare and analyze duplicate from different sample (volume permitting). Review precision of batch. Check preparation of duplicate sample.
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).

12.4.3 Data Reporting, Review, and Management

Checks made of the data in the process of review and verification are summarized in **Table 12.6**. Data reporting units and significant figures are given in **Table 12.7**. The NRSA 2023 - 2024 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 12.6 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 (see equation 11.1).
	Requirements:
	1. If total ionic strength ≤100 µeq/L
	   a. %IBD ≤ ±25%.
	2. If total ionic strength > 100 µeq/L
	   a. %IBD ≤±10%.
	Corrective actions:
	1. Determine which analytes, if any, are the largest contributors to the ion imbalance. Review suspect analytes for analytical error and reanalyze.
	   a. Flag = unacceptable %IBD
	2. If analytical error is not indicated, qualify sample to attribute imbalance to unmeasured ions. Reanalysis is not required.
	   a. 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).
	Requirements:
	1. If measured conductivity ≤ 25 µS/cm,
	   a. ([measured - calculated] ÷ measured) ≤ ±25%.
	2. If measured conductivity > 25 µS/cm,
	   a. ([measured - calculated] ÷ measured) ≤ ±15%.
	Corrective Actions:
	1. Determine which analytes, if any, are the largest contributors to the difference between calculated and measured conductivity.
	2. Review suspect analytes for analytical error and reanalyze.
	3. 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 12.7 Water chemistry: data reporting criteria

Measurement	Units	No. Significant Figures	Minimum No. Decimal Places
DO	mg/L	2	1
Temperature	°C	2	1
pH	pH units	3	2

Measurement	Units	No. Significant Figures	Minimum No. Decimal Places
Carbon, total & dissolved organic	mg/L	3	1
ANC	µeq/L	3	1
Conductivity	µS/cm at 25 °C	3	1
Calcium, magnesium, sodium, potassium, 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
Ammonia-N	mg/L	3	2
Turbidity	NTU	3	0
True color	PCU	2	0
TSS	mg/L	3	1
Chlorophyli-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 13.1 Percent ion difference (%IBD)

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

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 12.8**. For the conductivity check, equivalent conductivities for major ions are presented in **Table 12.9**.

Table 12.8 Water chemistry: constants for converting major ion concentration from mg/L to µeq/L

Analyte	Conversion from mg/L to µeq/Lº
Calcium	49.9
Magnesium	82.3
Potassium	25.6
Sodium	43.5
Ammonia-N	71.39

º Measured values are multiplied by the conversion factor. For ammonia and nitrate, two factors are provided, one if results are reported as mg N/L, the other if the ion is reported directly.

Ammonium	55.4
Chloride	28.2
Nitrate-N	71.39
Nitrate	16.1
Sulfate	20.8

Table 12.9 Water chemistry: factors to calculate equivalent conductivities of major ions. [p]

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	3.5×10^5 [q]
Sodium	2.13	Hydroxide	1.92×10^5
Ammonium	4.13	Bicarbonate	0.715
Chloride	2.14	Carbonate	2.82

12.5 Literature Cited

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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.

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[p] From Hillman et al. (1987).

[q] Specific conductance per mole/L, rather than per mg/L.

12.0 WATER CHEMISTRY and CHLOROPHYLL A

APPENDIX A: LABORATORY REMOTE EVALUATION FORMS

APPENDIX A: LABORATORY REMOTE EVALUATION FORMS

NRSA 2023/24 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 NRSA 2023/24, 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 NRSA 2023/24.

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 NRSA 2023/24**:**

☐ 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 NRSA 2023/24.

☐ 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 NRSA 2023/24 LOM).

APPENDIX A: LABORATORY REMOTE EVALUATION FORMS

☐ Documentation attesting to experience running all analytes for the NRSA 2023/24, including chloraphyll 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 mitchell.richard@epa.gov and lehmann.sarah@epa.gov. Questions concerning this request can be submitted forde.kendra@epa.gov (202-566-0417) or mitchell.richard@epa.gov (202-564-0644).

APPENDIX A: LABORATORY REMOTE EVALUATION FORMS

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 National Rivers and Streams Assessment (NRSA) 2023/24.

This applies to the _____ chemistry indicator.

1. Utilize procedures identified in the NRSA 2023/24 Lab Operations Manual (or equivalent). If using equivalent procedures, please provide procedures manual and obtain approval from EPA.
2. Read and abide by the NRSA 2023/24 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 Sharepoint.
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 March 1, 2024 (for samples collected in 2023) and March 1, 2025 (for samples collected in 2024) 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.

Signature _____ Date _____

NRSA 2023/24 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 NRSA 2023/24, 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 NRSA 2023/24.

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 NRSA 2023/24:

☐ Fish Voucher

☐ Benthic Macroinvertabrates

☐ 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 NRSA 2023/24.

☐ 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 NRSA 2023/24 LOM).

☐ Resume of independent fish taxonomist who will verify fish vouchers (if applicable)

APPENDIX A: LABORATORY REMOTE EVALUATION FORMS

This documentation may be submitted electronically via e-mail to forde.kendra@epa.gov with a cc: to mitchell.richard@epa.gov and lehmann.sarah@epa.gov. Questions concerning this request can be submitted forde.kendra@epa.gov (202-566-0417) or mitchell.richard@epa.gov (202-566-0644).

APPENDIX A: LABORATORY REMOTE EVALUATION FORMS

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 National Rivers and Streams Assessment (NRSA) 2023/24.

This applies to the _____ biological indicator.

9. Utilize procedures identified in the NRSA 2023/24 Lab Operations Manual (or equivalent). If using equivalent procedures, please provide procedures manual and obtain approval from EPA.

10. Read and abide by the NRSA 2023/24 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 NRSA 2023/24 Laboratory Operations 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 Sharepoint.

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 March 1, 2024 (for samples collected in 2023) and March 1, 2025 (for samples collected in 2024) 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 2023/24.

Signature _____ Date _____

APPENDIX A: LABORATORY REMOTE EVALUATION FORMS

APPENDIX B: 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 UniqueIdentifier)	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	Adults		

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

Comments:

Enterrococci (EPA Method 1606) Laboratory Bench Sheet

Site ID _____   Sample ID_____   Date_____

Purified DNA Extracts

Batch Sample #	Sample ID #	QA/QC Qual Code	Sample Vol (mL) Filtered	Vol. SAE Buffer Added (µL)	Color of Filter	25X Dilution Needed?	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 C: OTHER PERTINENT ATTACHMENTS

APPENDIX c: 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	*Hemichromis letourneuxi*	african jewelfish
2	Percopsiformes	Amblyopsidae	*Speoplatyrhinus poulsoni*	alabama cavefish
3	Perciformes	Percidae	*Etheostoma ramseyi*	alabama darter
4	Cypriniformes	Catostomidae	*Hypentelium etowanum*	alabama hog sucker
5	Clupeiformes	Clupeidae	*Alosa alabamae*	alabama shad
6	Cypriniformes	Cyprinidae	*Cyprinella callistia*	alabama shiner
7	Acipenseriformes	Acipenseridae	*Scaphirhynchus suttkusi*	alabama sturgeon
8	Esociformes	Umbridae	*Dallia pectoralis*	alaska blackfish
9	Clupeiformes	Clupeidae	*Alosa pseudoharengus*	alewife
10	Lepisosteiformes	Lepisosteidae	*Atractosteus spatula*	alligator gar
11	Cypriniformes	Cyprinidae	*Cyprinella xaenura*	altamaha shiner
12	Cypriniformes	Cyprinidae	*Gila alvordensis*	alvord chub
13	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon nevadensis*	amargosa pupfish
14	Cyprinodontiformes	Poeciliidae	*Poecilia formosa*	amazon molly
15	Siluriformes	Loricariidae	*Pterygoplichthys pardalis*	amazon sailfin catfish
16	Perciformes	Percidae	*Percina antesella*	amber darter
17	Petromyzontiformes	Petromyzontidae	*Lampetra appendix*	american brook lamprey
18	Anguilliformes	Anguillidae	*Anguilla rostrata*	american eel
19	Clupeiformes	Clupeidae	*Alosa sapidissima*	american shad
20	Cyprinodontiformes	Poeciliidae	*Gambusia amistadensis*	amistad gambusia
21	Cypriniformes	Cyprinidae	*Pteronotropis grandipinnis*	apalachee shiner
22	Perciformes	Percidae	*Percina gymnocephala*	appalachia darter
23	Salmoniformes	Salmonidae	*Salvelinus alpinus*	arctic char
24	Salmoniformes	Salmonidae	*Coregonus autumnalis*	arctic cisco
25	Salmoniformes	Salmonidae	*Thymallus arcticus*	arctic grayling
26	Petromyzontiformes	Petromyzontidae	*Lampetra camtschatica*	arctic lamprey
27	Perciformes	Percidae	*Etheostoma cragini*	arkansas darter
28	Cypriniformes	Cyprinidae	*Notropis girardi*	arkansas river shiner
29	Perciformes	Percidae	*Etheostoma euzonum*	arkansas saddled darter
30	Perciformes	Percidae	*Etheostoma sagitta*	arrow darter
31	Perciformes	Gobiidae	*Clevelandia ios*	arrow goby
32	Cypriniformes	Cyprinidae	*Gila orcuttii*	arroyo chub
33	Cyprinodontiformes	Goodeidae	*Empetrichthys merriami*	ash meadows poolfish
34	Perciformes	Percidae	*Etheostoma cinereum*	ashy darter
35	Synbranchiformes	Synbranchidae	*Monopterus albus*	asian swamp eel
36	Perciformes	Sciaenidae	*Micropogonias undulatus*	atlantic croaker
37	Beloniformes	Belonidae	*Strongylura marina*	atlantic needlefish

APPENDIX c: OTHER PERTINENT ATTACHMENTS

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

APPENDIX c: OTHER PERTINENT ATTACHMENTS

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

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
114	Siluriformes	Ictaluridae	*Ictalurus furcatus*	blue catfish
115	Cypriniformes	Cyprinidae	*Gila coerulea*	blue chub
116	Scorpaeniformes	Cottidae	*Cottus caeruleomentum*	blue ridge sculpin
117	Cypriniformes	Cyprinidae	*Cyprinella caerulea*	blue shiner
118	Cypriniformes	Catostomidae	*Cycleptus elongatus*	blue sucker
119	Perciformes	Cichlidae	*Oreochromis aureus*	blue tilapia
120	Clupeiformes	Clupeidae	*Alosa aestivalis*	blueback herring
121	Perciformes	Elassomatidae	*Elassoma okatie*	bluebarred pygmy sunfish
122	Perciformes	Percidae	*Etheostoma camurum*	bluebreast darter
123	Cyprinodontiformes	Fundulidae	*Lucania goodei*	bluefin killifish
124	Cypriniformes	Cyprinidae	*Campostoma pauciradii*	bluefin stoneroller
125	Perciformes	Centrarchidae	*Lepomis macrochirus*	bluegill
126	Cypriniformes	Cyprinidae	*Nocomis leptocephalus*	bluehead chub
127	Cypriniformes	Cyprinidae	*Pteronotropis hubbsi*	bluehead shiner
128	Cypriniformes	Catostomidae	*Catostomus discobolus*	bluehead sucker
129	Cypriniformes	Cyprinidae	*Pteronotropis welaka*	bluenose shiner
130	Perciformes	Percidae	*Etheostoma jessiae*	blueside darter
131	Perciformes	Centrarchidae	*Enneacanthus gloriosus*	bluespotted sunfish
132	Perciformes	Percidae	*Percina cymatotaenia*	bluestripe darter
133	Cypriniformes	Cyprinidae	*Cyprinella callitaenia*	bluestripe shiner
134	Cypriniformes	Cyprinidae	*Cyprinella camura*	bluntface shiner
135	Perciformes	Percidae	*Etheostoma chlorosoma*	bluntnose darter
136	Cypriniformes	Cyprinidae	*Pimephales notatus*	bluntnose minnow
137	Cypriniformes	Cyprinidae	*Notropis simus*	bluntnose shiner
138	Salmoniformes	Salmonidae	*Prosopium gemmifer*	bonneville cisco
139	Salmoniformes	Salmonidae	*Prosopium spilonotus*	bonneville whitefish
140	Cypriniformes	Cyprinidae	*Gila elegans*	bonytail
141	Cypriniformes	Cyprinidae	*Gila boraxobius*	borax lake chub
142	Perciformes	Percidae	*Etheostoma wapiti*	boulder darter
143	Amiiformes	Amiidae	*Amia calva*	bowfin
144	Cypriniformes	Cyprinidae	*Hybognathus hankinsoni*	brassy minnow
145	Cypriniformes	Catostomidae	*Catostomus columbianus*	bridgelip sucker
146	Cypriniformes	Cyprinidae	*Notropis bifrenatus*	bridle shiner
147	Perciformes	Percidae	*Etheostoma lynceum*	brighteye darter
148	Siluriformes	Ictaluridae	*Noturus miurus*	brindled madtom
149	Salmoniformes	Salmonidae	*Coregonus nasus*	broad whitefish
150	Cypriniformes	Cyprinidae	*Pteronotropis euryzonus*	broadstripe shiner
151	Cyprinodontiformes	Fundulidae	*Fundulus euryzonus*	broadstripe topminnow

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
152	Perciformes	Percidae	*Percina palmaris*	bronze darter
153	Perciformes	Percidae	*Etheostoma burri*	brook darter
154	Atheriniformes	Atherinopsidae	*Labidesthes sicculus*	brook silverside
155	Gasterosteiformes	Gasterosteidae	*Culaea inconstans*	brook stickleback
156	Salmoniformes	Salmonidae	*Salvelinus fontinalis*	brook trout
157	Siluriformes	Ictaluridae	*Ameiurus nebulosus*	brown bullhead
158	Perciformes	Percidae	*Etheostoma edwini*	brown darter
159	Siluriformes	Callichthyidae	*Hoplosternum littorale*	brown hoplo
160	Siluriformes	Ictaluridae	*Noturus phaeus*	brown madtom
161	Salmoniformes	Salmonidae	*Salmo trutta*	brown trout
162	Perciformes	Percidae	*Etheostoma bison*	buffalo darter
163	Cypriniformes	Cyprinidae	*Nocomis raneyi*	bull chub
164	Carcharhiniformes	Carcharhinidae	*Carcharhinus leucas*	bull shark
165	Salmoniformes	Salmonidae	*Salvelinus confluentus*	bull trout
166	Cypriniformes	Cyprinidae	*Pimephales vigilax*	bullhead minnow
167	Perciformes	Channidae	*Channa marulius*	bullseye snakehead
168	Gadiformes	Gadidae	*Lota lota*	burbot
169	Cypriniformes	Cyprinidae	*Macrhybopsis marconis*	burrhead chub
170	Cypriniformes	Cyprinidae	*Notropis asperifrons*	burrhead shiner
171	Perciformes	Cichlidae	*Cichla ocellaris*	butterfly peacock bass
172	Siluriformes	Ictaluridae	*Noturus taylori*	caddo madtom
173	Cypriniformes	Cyprinidae	*Notropis cahabae*	cahaba shiner
174	Cypriniformes	Cyprinidae	*Hesperoleucus symmetricus*	california roach
175	Perciformes	Percidae	*Etheostoma osburni*	candy darter
176	Cypriniformes	Cyprinidae	*Notropis mekistocholas*	cape fear shiner
177	Cypriniformes	Cyprinidae	*Luxilus cardinalis*	cardinal shiner
178	Cypriniformes	Cyprinidae	*Notropis percobromus*	carmine shiner
179	Perciformes	Percidae	*Etheostoma collis*	carolina darter
180	Siluriformes	Ictaluridae	*Noturus furiosus*	carolina madtom
181	Perciformes	Elassomatidae	*Elassoma boehlkei*	carolina pygmy sunfish
182	Esociformes	Umbridae	*Umbra limi*	central mudminnow
183	Cypriniformes	Cyprinidae	*Campostoma anomalum*	central stoneroller
184	Esociformes	Esocidae	*Esox niger*	chain pickerel
185	Perciformes	Percidae	*Percina nevisense*	chainback darter
186	Siluriformes	Ictaluridae	*Ictalurus punctatus*	channel catfish
187	Perciformes	Percidae	*Percina copelandi*	channel darter
188	Cypriniformes	Cyprinidae	*Notropis wickliffi*	channel shiner
189	Siluriformes	Ictaluridae	*Noturus flavater*	checkered madtom

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
190	Perciformes	Percidae	*Etheostoma scotti*	cherokee darter
191	Perciformes	Percidae	*Etheostoma etnieri*	cherry darter
192	Cypriniformes	Cyprinidae	*Lythrurus roseipinnis*	cherryfin shiner
193	Petromyzontiformes	Petromyzontidae	*Ichthyomyzon castaneus*	chestnut lamprey
194	Perciformes	Percidae	*Etheostoma cervus*	chickasaw darter
195	Cypriniformes	Cyprinidae	*Gila nigrescens*	chihuahua chub
196	Cypriniformes	Cyprinidae	*Notropis chihuahua*	chihuahua shiner
197	Salmoniformes	Salmonidae	*Oncorhynchus tshawytscha*	chinook salmon
198	Cypriniformes	Cyprinidae	*Acrocheilus alutaceus*	chiselmouth
199	Perciformes	Percidae	*Etheostoma davisoni*	choctawhatchee darter
200	Perciformes	Percidae	*Etheostoma hopkinsi*	christmas darter
201	Cypriniformes	Cyprinidae	*Notropis potteri*	chub shiner
202	Salmoniformes	Salmonidae	*Oncorhynchus keta*	chum salmon
203	Salmoniformes	Salmonidae	*Coregonus artedi*	cisco
204	Cypriniformes	Cyprinidae	*Hybopsis winchelli*	clear chub
205	Cyprinodontiformes	Poeciliidae	*Gambusia heterochir*	clear creek gambusia
206	Cypriniformes	Cyprinidae	*Pogonichthys ciscoides*	clear lake splittail
207	Perciformes	Gobiidae	*Microgobius gulosus*	clown goby
208	Osteoglossiformes	Notopteridae	*Chitala ornata*	clown knifefish
209	Perciformes	Percidae	*Percina brevicauda*	coal darter
210	Perciformes	Percidae	*Etheostoma colorosum*	coastal darter
211	Cypriniformes	Cyprinidae	*Notropis petersoni*	coastal shiner
212	Scorpaeniformes	Cottidae	*Cottus aleuticus*	coastrange sculpin
213	Salmoniformes	Salmonidae	*Oncorhynchus kisutch*	coho salmon
214	Perciformes	Percidae	*Etheostoma ditrema*	coldwater darter
215	Cypriniformes	Cyprinidae	*Ptychocheilus lucius*	colorado pikeminnow
216	Scorpaeniformes	Cottidae	*Cottus hubbsi*	columbia sculpin
217	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon elegans*	comanche springs pupfish
218	Cypriniformes	Cyprinidae	*Notropis amoenus*	comely shiner
219	Cypriniformes	Cyprinidae	*Cyprinus carpio*	common carp
220	Cypriniformes	Cyprinidae	*Luxilus cornutus*	common shiner
221	Perciformes	Centropomidae	*Centropomus undecimalis*	common snook
222	Perciformes	Percidae	*Percina jenkinsi*	conasauga logperch
223	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon eximius*	conchos pupfish
224	Perciformes	Cichlidae	*Cichlasoma nigrofasciatum*	convict cichlid
225	Perciformes	Percidae	*Etheostoma coosae*	coosa darter
226	Cypriniformes	Cyprinidae	*Notropis xaenocephalus*	coosa shiner
227	Perciformes	Percidae	*Etheostoma aquali*	coppercheek darter

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
228	Perciformes	Percidae	*Etheostoma basilare*	corrugated darter
229	Cypriniformes	Cyprinidae	*Semotilus atromaculatus*	creek chub
230	Cypriniformes	Catostomidae	*Erimyzon oblongus*	creek chubsucker
231	Perciformes	Percidae	*Etheostoma collettei*	creole darter
232	Cypriniformes	Cyprinidae	*Luxilus cerasinus*	crescent shiner
233	Perciformes	Gobiidae	*Lophogobius cyprinoides*	crested goby
234	Perciformes	Belontiidae	*Trichopsis vittata*	croaking gourami
235	Perciformes	Percidae	*Etheostoma corona*	crown darter
236	Perciformes	Percidae	*Crystallaria asprella*	crystal darter
237	Cypriniformes	Catostomidae	*Chasmistes cujus*	cui-ui
238	Perciformes	Percidae	*Etheostoma susanae*	cumberland darter
239	Perciformes	Percidae	*Etheostoma uniporum*	current darter
240	Cypriniformes	Cyprinidae	*Exoglossum maxillingua*	cutlip minnow
241	Salmoniformes	Salmonidae	*Oncorhynchus clarkii*	cutthroat trout
242	Perciformes	Percidae	*Etheostoma proeliare*	cypress darter
243	Cypriniformes	Cyprinidae	*Hybognathus hayi*	cypress minnow
244	Perciformes	Gobiidae	*Ctenogobius boleosoma*	darter goby
245	Salmoniformes	Salmonidae	*Coregonus johannae*	deepwater cisco
246	Scorpaeniformes	Cottidae	*Myoxocephalus thompsonii*	deepwater sculpin
247	Salmoniformes	Osmeridae	*Hypomesus transpacificus*	delta smelt
248	Cypriniformes	Cyprinidae	*Eremichthys acros*	desert dace
249	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon macularius*	desert pupfish
250	Cypriniformes	Catostomidae	*Catostomus clarkii*	desert sucker
251	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon diabolis*	devils hole pupfish
252	Cypriniformes	Cyprinidae	*Dionda diaboli*	devils river minnow
253	Cypriniformes	Cyprinidae	*Semotilus thoreauianus*	dixie chub
254	Perciformes	Centrarchidae	*Lepomis marginatus*	dollar sunfish
255	Salmoniformes	Salmonidae	*Salvelinus malma*	dolly varden
256	Perciformes	Percidae	*Percina sciera*	dusky darter
257	Cypriniformes	Cyprinidae	*Notropis cummingsae*	dusky shiner
258	Cypriniformes	Cyprinidae	*Luxilus pilsbryi*	duskystripe shiner
259	Perciformes	Percidae	*Etheostoma percnurum*	duskytail darter
260	Cypriniformes	Cyprinidae	*Rhinichthys atratulus*	eastern blacknose dace
261	Cyprinodontiformes	Poeciliidae	*Gambusia holbrooki*	eastern mosquitofish
262	Esociformes	Umbridae	*Umbra pygmaea*	eastern mudminnow
263	Perciformes	Percidae	*Ammocrypta pellucida*	eastern sand darter
264	Cypriniformes	Cyprinidae	*Hybognathus regius*	eastern silvery minnow
265	Perciformes	Percidae	*Etheostoma pseudovulatum*	egg-mimic darter

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
266	Siluriformes	Ictaluridae	*Noturus elegans*	elegant madtom
267	Perciformes	Percidae	*Etheostoma baileyi*	emerald darter
268	Cypriniformes	Cyprinidae	*Notropis atherinoides*	emerald shiner
269	Gasterosteiformes	Gasterosteidae	*Gasterosteus aculeatus*	espinocho
270	Perciformes	Percidae	*Etheostoma etowahae*	etowah darter
271	Salmoniformes	Osmeridae	*Thaleichthys pacificus*	eulachon
272	Perciformes	Elassomatidae	*Elassoma evergladei*	everglades pygmy sunfish
273	Cypriniformes	Cyprinidae	*Semotilus corporalis*	fallfish
274	Perciformes	Percidae	*Etheostoma flabellare*	fantail darter
275	Perciformes	Eleotridae	*Dormitator maculatus*	fat sleeper
276	Cypriniformes	Cyprinidae	*Pimephales promelas*	fathead minnow
277	Cypriniformes	Cyprinidae	*Phenacobius crassilabrum*	fatlips minnow
278	Cypriniformes	Cyprinidae	*Cyprinella pyrrhomelas*	fieryblack shiner
279	Cypriniformes	Cyprinidae	*Phoxinus neogaeus*	finescale dace
280	Perciformes	Percidae	*Etheostoma pyrrhogaster*	firebelly darter
281	Perciformes	Cichlidae	*Cichlasoma meeki*	firemouth cichlid
282	Cypriniformes	Cyprinidae	*Pteronotropis signipinnis*	flagfin shiner
283	Cyprinodontiformes	Cyprinodontidae	*Jordanella floridae*	flagfish
284	Cypriniformes	Cyprinidae	*Hemitremia flammea*	flame chub
285	Cypriniformes	Catostomidae	*Catostomus latipinnis*	flannelmouth sucker
286	Siluriformes	Ictaluridae	*Ameiurus platycephalus*	flat bullhead
287	Siluriformes	Ictaluridae	*Pylodictis olivaris*	flathead catfish
288	Cypriniformes	Cyprinidae	*Platygobio gracilis*	flathead chub
289	Perciformes	Centrarchidae	*Centrarchus macropterus*	flier
290	Lepisosteiformes	Lepisosteidae	*Lepisosteus platyrhincus*	florida gar
291	Perciformes	Percidae	*Ammocrypta bifascia*	florida sand darter
292	Cypriniformes	Cyprinidae	*Notropis edwardraneyi*	fluvial shiner
293	Perciformes	Percidae	*Etheostoma fonticola*	fountain darter
294	Scorpaeniformes	Cottidae	*Myoxocephalus quadricornis*	fourhorn sculpin
295	Gasterosteiformes	Gasterosteidae	*Apeltes quadracus*	fourspine stickleback
296	Perciformes	Percidae	*Percina stictogaster*	frecklebelly darter
297	Siluriformes	Ictaluridae	*Noturus munitus*	frecklebelly madtom
298	Perciformes	Percidae	*Percina lenticula*	freckled darter
299	Siluriformes	Ictaluridae	*Noturus nocturnus*	freckled madtom
300	Perciformes	Sciaenidae	*Aplodinotus grunniens*	freshwater drum
301	Perciformes	Gobiidae	*Ctenogobius shufeldti*	freshwater goby
302	Perciformes	Percidae	*Etheostoma crossopterum*	fringed darter
303	Cypriniformes	Cyprinidae	*Notropis buchanani*	ghost shiner

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
304	Cyprinodontiformes	Aplocheilidae	*Rivulus hartii*	giant rivulus
305	Cypriniformes	Cyprinidae	*Gila intermedia*	gila chub
306	Cyprinodontiformes	Poeciliidae	*Poeciliopsis occidentalis*	gila topminnow
307	Salmoniformes	Salmonidae	*Oncorhynchus gilae*	gila trout
308	Perciformes	Percidae	*Percina evides*	gilt darter
309	Clupeiformes	Clupeidae	*Dorosoma cepedianum*	gizzard shad
310	Perciformes	Percidae	*Etheostoma vitreum*	glassy darter
311	Perciformes	Percidae	*Etheostoma denoncourti*	golden darter
312	Cypriniformes	Catostomidae	*Moxostoma erythrurum*	golden redhorse
313	Cypriniformes	Cyprinidae	*Notemigonus crysoleucas*	golden shiner
314	Cyprinodontiformes	Fundulidae	*Fundulus chrysotus*	golden topminnow
315	Hiodontiformes	Hiodontidae	*Hiodon alosoides*	goldeye
316	Cypriniformes	Cyprinidae	*Carassius auratus*	goldfish
317	Perciformes	Percidae	*Percina aurolineata*	goldline darter
318	Perciformes	Percidae	*Etheostoma parvipinne*	goldstripe darter
319	Cypriniformes	Cyprinidae	*Ctenopharyngodon idella*	grass carp
320	Cypriniformes	Cyprinidae	*Erimystax x-punctatus*	gravel chub
321	Cypriniformes	Catostomidae	*Moxostoma congestum*	gray redhorse
322	Perciformes	Lutjanidae	*Lutjanus griseus*	gray snapper
323	Cypriniformes	Catostomidae	*Moxostoma lachneri*	greater jumprock
324	Cypriniformes	Catostomidae	*Moxostoma valenciennesi*	greater redhorse
325	Acipenseriformes	Acipenseridae	*Acipenser medirostris*	green sturgeon
326	Perciformes	Centrarchidae	*Lepomis cyanellus*	green sunfish
327	Cyprinodontiformes	Poeciliidae	*Xiphophorus hellerii*	green swordtail
328	Perciformes	Percidae	*Etheostoma jordani*	greenbreast darter
329	Perciformes	Percidae	*Etheostoma chlorobranchium*	greenfin darter
330	Cypriniformes	Cyprinidae	*Cyprinella chloristia*	greenfin shiner
331	Cypriniformes	Cyprinidae	*Notropis chlorocephalus*	greenhead shiner
332	Perciformes	Percidae	*Etheostoma blennioides*	greenside darter
333	Perciformes	Percidae	*Etheostoma lepidum*	greenthroat darter
334	Perciformes	Centrarchidae	*Micropterus treculii*	guadalupe bass
335	Cyprinodontiformes	Fundulidae	*Fundulus parvipinnis*	guadalupe cardinalfish
336	Cypriniformes	Cyprinidae	*Dionda nigrotaeniata*	guadalupe roundnose minnow
337	Perciformes	Percidae	*Etheostoma oophylax*	guardian darter
338	Perciformes	Eleotridae	*Guavina guavina*	guavina
339	Perciformes	Percidae	*Etheostoma swaini*	gulf darter
340	Cyprinodontiformes	Fundulidae	*Fundulus grandis*	gulf killifish
341	Perciformes	Percidae	*Percina suttkusi*	gulf logperch

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
342	Gasterosteiformes	Syngnathidae	*Syngnathus scovelli*	gulf pipefish
343	Cyprinodontiformes	Poeciliidae	*Poecilia reticulata*	guppy
344	Cypriniformes	Cyprinidae	*Mylopharodon conocephalus*	hardhead
345	Siluriformes	Ariidae	*Ariopsis felis*	hardhead catfish
346	Cypriniformes	Catostomidae	*Moxostoma lacerum*	harelip sucker
347	Perciformes	Percidae	*Etheostoma histrio*	harlequin darter
348	Siluriformes	Ictaluridae	*Ictalurus lupus*	headwater catfish
349	Cypriniformes	Cyprinidae	*Gila nigra*	headwater chub
350	Perciformes	Percidae	*Etheostoma lawrencei*	headwater darter
351	Clupeiformes	Clupeidae	*Alosa mediocris*	hickory shad
352	Cypriniformes	Cyprinidae	*Hybopsis hypsinotus*	highback chub
353	Cypriniformes	Catostomidae	*Carpiodes velifer*	highfin carpsucker
354	Cypriniformes	Cyprinidae	*Notropis altipinnis*	highfin shiner
355	Perciformes	Percidae	*Etheostoma kantuckeense*	highland rim darter
356	Cypriniformes	Cyprinidae	*Notropis micropteryx*	highland shiner
357	Cypriniformes	Cyprinidae	*Notropis hypsilepis*	highscale shiner
358	Cypriniformes	Cyprinidae	*Lavinia exilicauda*	hitch
359	Pleuronectiformes	Achiridae	*Trinectes maculatus*	hogchoker
360	Perciformes	Percidae	*Etheostoma brevirostrum*	holiday darter
361	Cypriniformes	Cyprinidae	*Nocomis biguttatus*	hornyhead chub
362	Cypriniformes	Cyprinidae	*Gila cypha*	humpback chub
363	Salmoniformes	Salmonidae	*Coregonus pidschian*	humpback whitefish
364	Cypriniformes	Cyprinidae	*Leuciscus idus*	ide
365	Salmoniformes	Salmonidae	*Stenodus leucichthys*	inconnu
366	Atheriniformes	Atherinopsidae	*Menidia beryllina*	inland silverside
367	Perciformes	Percidae	*Etheostoma exile*	iowa darter
368	Perciformes	Gerreidae	*Diapterus auratus*	irish pompano
369	Cypriniformes	Cyprinidae	*Notropis chalybaeus*	ironcolor shiner
370	Perciformes	Cichlidae	*Cichlasoma octofasciatum*	jack dempsey
371	Perciformes	Cichlidae	*Cichlasoma managuense*	jaguar guapote
372	Perciformes	Percidae	*Etheostoma nigrum*	johnny darter
373	Cypriniformes	Catostomidae	*Chasmistes liorus*	june sucker
374	Perciformes	Percidae	*Etheostoma kanawhae*	kanawha darter
375	Cypriniformes	Cyprinidae	*Phenacobius teretulus*	kanawha minnow
376	Perciformes	Percidae	*Etheostoma rafinesquei*	kentucky darter
377	Petromyzontiformes	Petromyzontidae	*Lampetra hubbsi*	kern brook lamprey
378	Cypriniformes	Cyprinidae	*Notropis ortenburgeri*	kiamichi shiner
379	Salmoniformes	Salmonidae	*Coregonus kiyi*	kiyi

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
380	Scorpaeniformes	Cottidae	*Cottus princeps*	klamath lake sculpin
381	Petromyzontiformes	Petromyzontidae	*Lampetra similis*	klamath lamprey
382	Cypriniformes	Catostomidae	*Catostomus snyderi*	klamath largescale sucker
383	Cypriniformes	Catostomidae	*Catostomus rimiculus*	klamath smallscale sucker
384	Elopiformes	Elopidae	*Elops saurus*	ladyfish
385	Cypriniformes	Cyprinidae	*Richardsonius egregius*	lahontan redside
386	Cypriniformes	Cyprinidae	*Couesius plumbeus*	lake chub
387	Cypriniformes	Catostomidae	*Erimyzon sucetta*	lake chubsucker
388	Acipenseriformes	Acipenseridae	*Acipenser fulvescens*	lake sturgeon
389	Salmoniformes	Salmonidae	*Salvelinus namaycush*	lake trout
390	Salmoniformes	Salmonidae	*Coregonus clupeaformis*	lake whitefish
391	Perciformes	Centrarchidae	*Micropterus salmoides*	largemouth bass
392	Cypriniformes	Cyprinidae	*Campostoma oligolepis*	largescale stoneroller
393	Cypriniformes	Catostomidae	*Catostomus macrocheilus*	largescale sucker
394	Perciformes	Eleotridae	*Eleotris amblyopsis*	largescaled spinycheek
395	Cyprinodontiformes	Poeciliidae	*Gambusia geiseri*	largespring gambusia
396	Cypriniformes	Cyprinidae	*Rhinichthys deaconi*	las vegas dace
397	Cypriniformes	Cyprinidae	*Phoxinus saylori*	laurel dace
398	Petromyzontiformes	Petromyzontidae	*Lampetra aepyptera*	least brook lamprey
399	Cypriniformes	Cyprinidae	*Iotichthys phlegethontis*	least chub
400	Salmoniformes	Salmonidae	*Coregonus sardinella*	least cisco
401	Perciformes	Percidae	*Etheostoma microperca*	least darter
402	Cyprinodontiformes	Poeciliidae	*Heterandria formosa*	least killifish
403	Siluriformes	Ictaluridae	*Noturus hildebrandi*	least madtom
404	Cypriniformes	Cyprinidae	*Snyderichthys copei*	leatherside chub
405	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon bovinus*	leon springs pupfish
406	Cypriniformes	Cyprinidae	*Rhinichthys falcatus*	leopard dace
407	Perciformes	Percidae	*Percina pantherina*	leopard darter
408	Cypriniformes	Cyprinidae	*Hybopsis lineapunctata*	lined chub
409	Cyprinodontiformes	Fundulidae	*Fundulus lineolatus*	lined topminnow
410	Perciformes	Percidae	*Etheostoma chuckwachatte*	lipstick darter
411	Cypriniformes	Cyprinidae	*Lepidomeda vittata*	little colorado spinedace
412	Cypriniformes	Cyprinidae	*Rhinichthys cobitis*	loach minnow
413	Perciformes	Percidae	*Percina caprodes*	logperch
414	Perciformes	Percidae	*Etheostoma neopterum*	lollypop darter
415	Perciformes	Centrarchidae	*Lepomis megalotis*	longear sunfish
416	Cypriniformes	Cyprinidae	*Agosia chrysogaster*	longfin dace
417	Perciformes	Percidae	*Etheostoma longimanum*	longfin darter

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
418	Salmoniformes	Osmeridae	*Spirinchus thaleichthys*	longfin smelt
419	Perciformes	Percidae	*Percina macrocephala*	longhead darter
420	Perciformes	Gobiidae	*Gillichthys mirabilis*	longjaw mudsucker
421	Cypriniformes	Cyprinidae	*Rhinichthys cataractae*	longnose dace
422	Perciformes	Percidae	*Percina nasuta*	longnose darter
423	Lepisosteiformes	Lepisosteidae	*Lepisosteus osseus*	longnose gar
424	Cypriniformes	Cyprinidae	*Notropis longirostris*	longnose shiner
425	Cypriniformes	Catostomidae	*Catostomus catostomus*	longnose sucker
426	Cypriniformes	Catostomidae	*Deltistes luxatus*	lost river sucker
427	Elopiformes	Elopidae	*Elops affinis*	machete
428	Scorpaeniformes	Cottidae	*Cottus bendirei*	malheur sculpin
429	Cypriniformes	Cyprinidae	*Dionda argentosa*	manantial roundnose minnow
430	Cyprinodontiformes	Poeciliidae	*Gambusia rhizophorae*	mangrove gambusia
431	Cyprinodontiformes	Aplocheilidae	*Rivulus marmoratus*	mangrove rivulus
432	Scorpaeniformes	Cottidae	*Cottus klamathensis*	marbled sculpin
433	Siluriformes	Ictaluridae	*Noturus insignis*	margined madtom
434	Scorpaeniformes	Cottidae	*Cottus marginatus*	margined sculpin
435	Cyprinodontiformes	Fundulidae	*Fundulus confluentus*	marsh killifish
436	Perciformes	Percidae	*Etheostoma sellare*	maryland darter
437	Perciformes	Cichlidae	*Cichlasoma urophthalmus*	mayan cichlid
438	Perciformes	Gobiidae	*Ctenogobius claytonii*	mexican goby
439	Cyprinodontiformes	Poeciliidae	*Poecilia sphenops*	mexican molly
440	Cypriniformes	Catostomidae	*Moxostoma austrinum*	mexican redhorse
441	Cypriniformes	Cyprinidae	*Campostoma ornatum*	mexican stoneroller
442	Characiformes	Characidae	*Astyanax mexicanus*	mexican tetra
443	Perciformes	Cichlidae	*Cichlasoma citrinellum*	midas cichlid
444	Petromyzontiformes	Petromyzontidae	*Lampetra minima*	miller lake lamprey
445	Cypriniformes	Cyprinidae	*Notropis volucellus*	mimic shiner
446	Cypriniformes	Cyprinidae	*Notropis spectrunculus*	mirror shiner
447	Atheriniformes	Atherinopsidae	*Menidia audens*	mississippi silverside
448	Cypriniformes	Cyprinidae	*Hybognathus nuchalis*	mississippi silvery minnow
449	Perciformes	Percidae	*Etheostoma tetrazonum*	missouri saddled darter
450	Cypriniformes	Cyprinidae	*Moapa coriacea*	moapa dace
451	Perciformes	Percidae	*Percina kathae*	mobile logperch
452	Cypriniformes	Catostomidae	*Catostomus microps*	modoc sucker
453	Hiodontiformes	Hiodontidae	*Hiodon tergisus*	mooneye
454	Scorpaeniformes	Cottidae	*Cottus bairdii*	mottled sculpin
455	Petromyzontiformes	Petromyzontidae	*Ichthyomyzon greeleyi*	mountain brook lamprey

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
456	Siluriformes	Ictaluridae	*Noturus eleutherus*	mountain madtom
457	Mugiliformes	Mugilidae	*Agonostomus monticola*	mountain mullet
458	Cypriniformes	Cyprinidae	*Phoxinus oreas*	mountain redbelly dace
459	Cypriniformes	Cyprinidae	*Lythrurus lirus*	mountain shiner
460	Cypriniformes	Catostomidae	*Catostomus platyrhynchus*	mountain sucker
461	Salmoniformes	Salmonidae	*Prosopium williamsoni*	mountain whitefish
462	Perciformes	Cichlidae	*Oreochromis mossambicus*	mozambique tilapia
463	Perciformes	Percidae	*Etheostoma asprigene*	mud darter
464	Perciformes	Centrarchidae	*Acantharchus pomotis*	mud sunfish
465	Cyprinodontiformes	Fundulidae	*Fundulus heteroclitus*	mummichog
466	Esociformes	Esocidae	*Esox masquinongy*	muskellunge
467	Perciformes	Gobiidae	*Gobiosoma bosc*	naked goby
468	Perciformes	Percidae	*Ammocrypta beanii*	naked sand darter
469	Siluriformes	Ictaluridae	*Noturus placidus*	neosho madtom
470	Cypriniformes	Cyprinidae	*Notropis scabriceps*	new river shiner
471	Perciformes	Percidae	*Etheostoma nianguae*	niangua darter
472	Perciformes	Cichlidae	*Oreochromis niloticus*	nile tilapia
473	Gasterosteiformes	Gasterosteidae	*Pungitius pungitius*	ninespine stickleback
474	Petromyzontiformes	Petromyzontidae	*Ichthyomyzon fossor*	northern brook lamprey
475	Percopsiformes	Amblyopsidae	*Amblyopsis spelaea*	northern cavefish
476	Cypriniformes	Catostomidae	*Hypentelium nigricans*	northern hog sucker
477	Siluriformes	Ictaluridae	*Noturus stigmosus*	northern madtom
478	Esociformes	Esocidae	*Esox lucius*	northern pike
479	Cypriniformes	Cyprinidae	*Ptychocheilus oregonensis*	northern pikeminnow
480	Cyprinodontiformes	Fundulidae	*Fundulus kansae*	northern plains killifish
481	Cypriniformes	Cyprinidae	*Phoxinus eos*	northern redbelly dace
482	Cyprinodontiformes	Fundulidae	*Fundulus catenatus*	northern studfish
483	Cypriniformes	Catostomidae	*Moxostoma collapsum*	notchlip redhorse
484	Cypriniformes	Cyprinidae	*Dionda serena*	nueces roundnose minnow
485	Cypriniformes	Cyprinidae	*Cyprinella callisema*	ocmulgee shiner
486	Petromyzontiformes	Petromyzontidae	*Ichthyomyzon bdellium*	ohio lamprey
487	Perciformes	Percidae	*Etheostoma okaloosae*	okaloosa darter
488	Perciformes	Elassomatidae	*Elassoma okefenokee*	okefenokee pygmy sunfish
489	Perciformes	Percidae	*Percina squamata*	olive darter
490	Esociformes	Umbridae	*Novumbra hubbsi*	olympic mudminnow
491	Gasterosteiformes	Syngnathidae	*Microphis brachyurus*	opossum pipefish
492	Perciformes	Percidae	*Etheostoma radiosum*	orangebelly darter
493	Perciformes	Percidae	*Etheostoma bellum*	orangefin darter

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
494	Siluriformes	Ictaluridae	*Noturus gilberti*	orangefin madtom
495	Cypriniformes	Cyprinidae	*Notropis ammophilus*	orangefin shiner
496	Perciformes	Sciaenidae	*Cynoscion xanthulus*	orangemouth corvina
497	Perciformes	Centrarchidae	*Lepomis humilis*	orangespotted sunfish
498	Cypriniformes	Cyprinidae	*Pteronotropis merlini*	orangetail shiner
499	Perciformes	Percidae	*Etheostoma spectabile*	orangethroat darter
500	Cypriniformes	Cyprinidae	*Oregonichthys crameri*	oregon chub
501	Cypriniformes	Cobitidae	*Misgurnus anguillicaudatus*	oriental weatherfish
502	Siluriformes	Loricariidae	*Pterygoplichthys multiradiatus*	orinoco sailfin catfish
503	Perciformes	Cichlidae	*Astronotus ocellatus*	oscar
504	Siluriformes	Ictaluridae	*Noturus lachneri*	ouachita madtom
505	Cypriniformes	Cyprinidae	*Lythrurus snelsoni*	ouachita shiner
506	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon radiosus*	owens pupfish
507	Cypriniformes	Catostomidae	*Catostomus fumeiventris*	owens sucker
508	Perciformes	Centrarchidae	*Ambloplites constellatus*	ozark bass
509	Percopsiformes	Amblyopsidae	*Amblyopsis rosae*	ozark cavefish
510	Cypriniformes	Cyprinidae	*Erimystax harryi*	ozark chub
511	Perciformes	Percidae	*Percina fulvitaenia*	ozark logperch
512	Siluriformes	Ictaluridae	*Noturus albater*	ozark madtom
513	Cypriniformes	Cyprinidae	*Notropis nubilus*	ozark minnow
514	Scorpaeniformes	Cottidae	*Cottus hypselurus*	ozark sculpin
515	Cypriniformes	Cyprinidae	*Notropis ozarcanus*	ozark shiner
516	Petromyzontiformes	Petromyzontidae	*Lampetra tridentata*	pacific lamprey
517	Scorpaeniformes	Cottidae	*Leptocottus armatus*	pacific staghorn sculpin
518	Acipenseriformes	Polyodontidae	*Polyodon spathula*	paddlefish
519	Cypriniformes	Cyprinidae	*Lepidomeda altivelis*	pahranagat spinedace
520	Cyprinodontiformes	Goodeidae	*Empetrichthys latos*	pahrump poolfish
521	Scorpaeniformes	Cottidae	*Cottus beldingii*	paiute sculpin
522	Perciformes	Percidae	*Etheostoma pallididorsum*	paleback darter
523	Cypriniformes	Cyprinidae	*Notropis albizonatus*	palezone shiner
524	Cypriniformes	Cyprinidae	*Hybopsis amnis*	pallid shiner
525	Acipenseriformes	Acipenseridae	*Scaphirhynchus albus*	pallid sturgeon
526	Cypriniformes	Catostomidae	*Moxostoma pisolabrum*	pealip redhorse
527	Cypriniformes	Cyprinidae	*Mylocheilus caurinus*	peamouth
528	Cypriniformes	Cyprinidae	*Margariscus margarita*	pearl dace
529	Perciformes	Percidae	*Percina aurora*	pearl darter
530	Cyprinodontiformes	Poeciliidae	*Gambusia nobilis*	pecos gambusia
531	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon pecosensis*	pecos pupfish

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
532	Cypriniformes	Cyprinidae	*Macrhybopsis tetranema*	peppered chub
533	Cypriniformes	Cyprinidae	*Notropis perpallidus*	peppered shiner
534	Cypriniformes	Cyprinidae	*Notropis orca*	phantom shiner
535	Perciformes	Percidae	*Percina crassa*	piedmont darter
536	Perciformes	Haemulidae	*Orthopristis chrysoptera*	pigfish
537	Cyprinodontiformes	Poeciliidae	*Belonesox belizanus*	pike killifish
538	Perciformes	Percidae	*Etheostoma mariae*	pinewoods darter
539	Cypriniformes	Cyprinidae	*Lythrurus matutinus*	pinewoods shiner
540	Perciformes	Sparidae	*Lagodon rhomboides*	pinfish
541	Salmoniformes	Salmonidae	*Oncorhynchus gorbuscha*	pink salmon
542	Percopsiformes	Aphredoderidae	*Aphredoderus sayanus*	pirate perch
543	Scorpaeniformes	Cottidae	*Cottus pitensis*	pit sculpin
544	Petromyzontiformes	Petromyzontidae	*Lampetra lethophaga*	pit-klamath brook lamprey
545	Cyprinodontiformes	Fundulidae	*Fundulus zebrinus*	plains killifish
546	Cypriniformes	Cyprinidae	*Hybognathus placitus*	plains minnow
547	Cyprinodontiformes	Fundulidae	*Fundulus sciadicus*	plains topminnow
548	Cypriniformes	Cyprinidae	*Cyprinella lepida*	plateau shiner
549	Salmoniformes	Osmeridae	*Hypomesus olidus*	pond smelt
550	Cypriniformes	Cyprinidae	*Notropis ariommus*	popeye shiner
551	Cyprinodontiformes	Poeciliidae	*Poeciliopsis gracilis*	porthole livebearer
552	Scorpaeniformes	Cottidae	*Cottus girardi*	potomac sculpin
553	Cypriniformes	Cyprinidae	*Macrhybopsis australis*	prairie chub
554	Cypriniformes	Cyprinidae	*Lythrurus bellus*	pretty shiner
555	Scorpaeniformes	Cottidae	*Cottus asper*	prickly sculpin
556	Cypriniformes	Cyprinidae	*Cyprinella proserpina*	proserpine shiner
557	Cypriniformes	Cyprinidae	*Opsopoeodus emiliae*	pugnose minnow
558	Cypriniformes	Cyprinidae	*Notropis anogenus*	pugnose shiner
559	Perciformes	Centrarchidae	*Lepomis gibbosus*	pumpkinseed
560	Cyprinodontiformes	Fundulidae	*Leptolucania ommata*	pygmy killifish
561	Siluriformes	Ictaluridae	*Noturus stanauli*	pygmy madtom
562	Scorpaeniformes	Cottidae	*Cottus paulus*	pygmy sculpin
563	Salmoniformes	Salmonidae	*Prosopium coulterii*	pygmy whitefish
564	Cypriniformes	Catostomidae	*Carpiodes cyprinus*	quillback
565	Cyprinodontiformes	Goodeidae	*Crenichthys nevadae*	railroad valley springfish
566	Perciformes	Percidae	*Etheostoma caeruleum*	rainbow darter
567	Cypriniformes	Cyprinidae	*Notropis chrosomus*	rainbow shiner
568	Salmoniformes	Osmeridae	*Osmerus mordax*	rainbow smelt
569	Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	rainbow trout

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
570	Cyprinodontiformes	Fundulidae	*Lucania parva*	rainwater killifish
571	Cypriniformes	Catostomidae	*Xyrauchen texanus*	razorback sucker
572	Perciformes	Sciaenidae	*Sciaenops ocellatus*	red drum
573	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon rubrofluviatilis*	red river pupfish
574	Cypriniformes	Cyprinidae	*Notropis bairdi*	red river shiner
575	Cypriniformes	Cyprinidae	*Cyprinella lutrensis*	red shiner
576	Perciformes	Percidae	*Etheostoma luteovinctum*	redband darter
577	Perciformes	Cichlidae	*Tilapia zillii*	redbelly tilapia
578	Perciformes	Centrarchidae	*Lepomis auritus*	redbreast sunfish
579	Perciformes	Centrarchidae	*Lepomis microlophus*	redear sunfish
580	Perciformes	Centrarchidae	*Micropterus coosae*	redeye bass
581	Cypriniformes	Cyprinidae	*Notropis harperi*	redeye chub
582	Cyprinodontiformes	Fundulidae	*Fundulus rubrifrons*	redface topminnow
583	Perciformes	Percidae	*Etheostoma whipplei*	redfin darter
584	Esociformes	Esocidae	*Esox americanus*	redfin pickerel
585	Cypriniformes	Cyprinidae	*Lythrurus umbratilis*	redfin shiner
586	Perciformes	Percidae	*Etheostoma rufilineatum*	redline darter
587	Cypriniformes	Cyprinidae	*Notropis chiliticus*	redlip shiner
588	Cypriniformes	Cyprinidae	*Clinostomus elongatus*	redside dace
589	Cypriniformes	Cyprinidae	*Richardsonius balteatus*	redside shiner
590	Cypriniformes	Cyprinidae	*Nocomis asper*	redspot chub
591	Perciformes	Percidae	*Etheostoma artesiae*	redspot darter
592	Perciformes	Centrarchidae	*Lepomis miniatus*	redspotted sunfish
593	Perciformes	Cichlidae	*Geophagus surinamensis*	redstriped eartheater
594	Cypriniformes	Cyprinidae	*Nocomis effusus*	redtail chub
595	Cypriniformes	Cyprinidae	*Relictus solitarius*	relict dace
596	Perciformes	Percidae	*Etheostoma chienense*	relict darter
597	Scorpaeniformes	Cottidae	*Cottus perplexus*	reticulate sculpin
598	Cypriniformes	Cyprinidae	*Lythrurus fumeus*	ribbon shiner
599	Cypriniformes	Cyprinidae	*Phenacobius catostomus*	riffle minnow
600	Scorpaeniformes	Cottidae	*Cottus gulosus*	riffle sculpin
601	Cypriniformes	Cyprinidae	*Gila pandora*	rio grande chub
602	Perciformes	Cichlidae	*Cichlasoma cyanoguttatum*	rio grande cichlid
603	Perciformes	Percidae	*Etheostoma grahami*	rio grande darter
604	Cypriniformes	Cyprinidae	*Notropis jemezanus*	rio grande shiner
605	Cypriniformes	Cyprinidae	*Hybognathus amarus*	rio grande silvery minnow
606	Cypriniformes	Catostomidae	*Catostomus plebeius*	rio grande sucker
607	Cypriniformes	Catostomidae	*Carpiodes carpio*	river carpsucker

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
608	Cypriniformes	Cyprinidae	*Nocomis micropogon*	river chub
609	Perciformes	Percidae	*Percina shumardi*	river darter
610	Perciformes	Gobiidae	*Awaous banana*	river goby
611	Petromyzontiformes	Petromyzontidae	*Lampetra ayresii*	river lamprey
612	Cypriniformes	Catostomidae	*Moxostoma carinatum*	river redhorse
613	Cypriniformes	Cyprinidae	*Notropis blennius*	river shiner
614	Perciformes	Percidae	*Etheostoma podostemone*	riverweed darter
615	Perciformes	Centrarchidae	*Ambloplites cavifrons*	roanoke bass
616	Perciformes	Percidae	*Percina roanoka*	roanoke darter
617	Cypriniformes	Catostomidae	*Hypentelium roanokense*	roanoke hog sucker
618	Perciformes	Percidae	*Percina rex*	roanoke logperch
619	Cypriniformes	Catostomidae	*Moxostoma robustum*	robust redhorse
620	Perciformes	Centrarchidae	*Ambloplites rupestris*	rock bass
621	Perciformes	Percidae	*Etheostoma rupestre*	rock darter
622	Cypriniformes	Cyprinidae	*Notropis suttkusi*	rocky shiner
623	Cypriniformes	Cyprinidae	*Lythrurus ardens*	rosefin shiner
624	Cypriniformes	Cyprinidae	*Hybopsis rubrifrons*	rosyface chub
625	Cypriniformes	Cyprinidae	*Notropis rubellus*	rosyface shiner
626	Cypriniformes	Cyprinidae	*Clinostomus funduloides*	rosyside dace
627	Scorpaeniformes	Cottidae	*Cottus asperrimus*	rough sculpin
628	Cypriniformes	Cyprinidae	*Notropis baileyi*	rough shiner
629	Atheriniformes	Atherinopsidae	*Membras martinica*	rough silverside
630	Cypriniformes	Cyprinidae	*Notropis semperasper*	roughhead shiner
631	Perciformes	Gobiidae	*Neogobius melanostomus*	round goby
632	Salmoniformes	Salmonidae	*Prosopium cylindraceum*	round whitefish
633	Cypriniformes	Cyprinidae	*Dionda episcopa*	roundnose minnow
634	Cypriniformes	Cyprinidae	*Gila robusta*	roundtail chub
635	Cypriniformes	Cyprinidae	*Scardinius erythrophthalmus*	rudd
636	Perciformes	Percidae	*Gymnocephalus cernuus*	ruffe
637	Perciformes	Percidae	*Etheostoma phytophilum*	rush darter
638	Cyprinodontiformes	Fundulidae	*Fundulus escambiae*	russetfin topminnow
639	Cypriniformes	Catostomidae	*Thoburnia hamiltoni*	rustyside sucker
640	Cypriniformes	Cyprinidae	*Notropis sabinae*	sabine shiner
641	Cypriniformes	Cyprinidae	*Orthodon microlepidotus*	sacramento blackfish
642	Perciformes	Centrarchidae	*Archoplites interruptus*	sacramento perch
643	Cypriniformes	Cyprinidae	*Ptychocheilus grandis*	sacramento pikeminnow
644	Cypriniformes	Catostomidae	*Catostomus occidentalis*	sacramento sucker
645	Perciformes	Percidae	*Percina vigil*	saddleback darter

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
646	Perciformes	Percidae	*Etheostoma flavum*	saffron darter
647	Cypriniformes	Cyprinidae	*Notropis rubricroceus*	saffron shiner
648	Cyprinodontiformes	Poeciliidae	*Poecilia latipinna*	sailfin molly
649	Cypriniformes	Cyprinidae	*Pteronotropis hypselopterus*	sailfin shiner
650	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon salinus*	salt creek pupfish
651	Cyprinodontiformes	Fundulidae	*Fundulus jenkinsi*	saltmarsh topminnow
652	Cyprinodontiformes	Poeciliidae	*Gambusia georgei*	san marcos gambusia
653	Percopsiformes	Percopsidae	*Percopsis transmontana*	sand roller
654	Cypriniformes	Cyprinidae	*Notropis stramineus*	sand shiner
655	Cypriniformes	Cyprinidae	*Notropis scepticus*	sandbar shiner
656	Cypriniformes	Cyprinidae	*Semotilus lumbee*	sandhills chub
657	Cypriniformes	Catostomidae	*Catostomus santaanae*	santa ana sucker
658	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon arcuatus*	santa cruz pupfish
659	Cypriniformes	Cyprinidae	*Cyprinella zanema*	santee chub
660	Cypriniformes	Cyprinidae	*Cyprinella analostana*	satinfin shiner
661	Perciformes	Percidae	*Sander canadensis*	sauger
662	Perciformes	Percidae	*Etheostoma fricksium*	savannah darter
663	Perciformes	Percidae	*Etheostoma serrifer*	sawcheek darter
664	Clupeiformes	Clupeidae	*Harengula jaguana*	scaled sardine
665	Perciformes	Percidae	*Ammocrypta vivax*	scaly sand darter
666	Cypriniformes	Cyprinidae	*Lythrurus fasciolaris*	scarlet shiner
667	Siluriformes	Ictaluridae	*Noturus trautmani*	scioto madtom
668	Petromyzontiformes	Petromyzontidae	*Petromyzon marinus*	sea lamprey
669	Perciformes	Percidae	*Etheostoma thalassinum*	seagreen darter
670	Cyprinodontiformes	Fundulidae	*Fundulus seminolis*	seminole killifish
671	Perciformes	Centrarchidae	*Ambloplites ariommus*	shadow bass
672	Cypriniformes	Catostomidae	*Erimyzon tenuis*	sharpfin chubsucker
673	Perciformes	Percidae	*Etheostoma acuticeps*	sharphead darter
674	Perciformes	Percidae	*Percina oxyrhynchus*	sharpnose darter
675	Scorpaeniformes	Cottidae	*Clinocottus acuticeps*	sharpnose sculpin
676	Cypriniformes	Cyprinidae	*Notropis oxyrhynchus*	sharpnose shiner
677	Perciformes	Percidae	*Etheostoma tecumsehi*	shawnee darter
678	Perciformes	Sparidae	*Archosargus probatocephalus*	sheepshead
679	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon variegatus*	sheepshead minnow
680	Perciformes	Percidae	*Percina peltata*	shield darter
681	Perciformes	Gobiidae	*Tridentiger bifasciatus*	shimofuri goby
682	Perciformes	Embiotocidae	*Cymatogaster aggregata*	shiner perch
683	Perciformes	Centrarchidae	*Micropterus cataractae*	shoal bass

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
684	Cypriniformes	Cyprinidae	*Macrhybopsis hyostoma*	shoal chub
685	Perciformes	Gobiidae	*Tridentiger barbatus*	shokihaze goby
686	Cyprinodontiformes	Poeciliidae	*Poecilia mexicana*	shortfin molly
687	Cypriniformes	Catostomidae	*Moxostoma macrolepidotum*	shorthead redhorse
688	Scorpaeniformes	Cottidae	*Cottus confusus*	shorthead sculpin
689	Salmoniformes	Salmonidae	*Coregonus zenithicus*	shortjaw cisco
690	Salmoniformes	Salmonidae	*Coregonus reighardi*	shortnose cisco
691	Lepisosteiformes	Lepisosteidae	*Lepisosteus platostomus*	shortnose gar
692	Acipenseriformes	Acipenseridae	*Acipenser brevirostrum*	shortnose sturgeon
693	Cypriniformes	Catostomidae	*Chasmistes brevirostris*	shortnose sucker
694	Scorpaeniformes	Cottidae	*Cottus greenei*	shoshone sculpin
695	Acipenseriformes	Acipenseridae	*Scaphirhynchus platorynchus*	shovelnose sturgeon
696	Cypriniformes	Cyprinidae	*Macrhybopsis meeki*	sicklefin chub
697	Cypriniformes	Cyprinidae	*Hypophthalmichthys molitrix*	silver carp
698	Cypriniformes	Cyprinidae	*Macrhybopsis storeriana*	silver chub
699	Petromyzontiformes	Petromyzontidae	*Ichthyomyzon unicuspis*	silver lamprey
700	Perciformes	Sciaenidae	*Bairdiella chrysoura*	silver perch
701	Cypriniformes	Catostomidae	*Moxostoma anisurum*	silver redhorse
702	Cypriniformes	Cyprinidae	*Notropis photogenis*	silver shiner
703	Cypriniformes	Cyprinidae	*Notropis shumardi*	silverband shiner
704	Cypriniformes	Cyprinidae	*Notropis buccatus*	silverjaw minnow
705	Cypriniformes	Cyprinidae	*Notropis candidus*	silverside shiner
706	Cypriniformes	Cyprinidae	*Notropis stilbius*	silverstripe shiner
707	Clupeiformes	Clupeidae	*Alosa chrysochloris*	skipjack herring
708	Cypriniformes	Cyprinidae	*Notropis uranoscopus*	skygazer shiner
709	Perciformes	Percidae	*Etheostoma smithi*	slabrock darter
710	Perciformes	Percidae	*Etheostoma boschungi*	slackwater darter
711	Perciformes	Gobiidae	*Ctenogobius pseudofasciatus*	slashcheek goby
712	Cypriniformes	Cyprinidae	*Erimystax cahni*	slender chub
713	Siluriformes	Ictaluridae	*Noturus exilis*	slender madtom
714	Scorpaeniformes	Cottidae	*Cottus tenuis*	slender sculpin
715	Perciformes	Percidae	*Percina phoxocephala*	slenderhead darter
716	Cypriniformes	Cyprinidae	*Pimephales tenellus*	slim minnow
717	Scorpaeniformes	Cottidae	*Cottus cognatus*	slimy sculpin
718	Perciformes	Percidae	*Etheostoma gracile*	slough darter
719	Cypriniformes	Cyprinidae	*Notropis buccula*	smalleye shiner
720	Perciformes	Centrarchidae	*Micropterus dolomieu*	smallmouth bass
721	Cypriniformes	Catostomidae	*Ictiobus bubalus*	smallmouth buffalo

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
722	Cypriniformes	Catostomidae	*Moxostoma breviceps*	smallmouth redhorse
723	Perciformes	Percidae	*Etheostoma microlepidum*	smallscale darter
724	Perciformes	Centropomidae	*Centropomus parallelus*	smallscale fat snook
725	Perciformes	Eleotridae	*Eleotris perniger*	smallscaled spinycheek
726	Pristiformes	Pristidae	*Pristis pectinata*	smalltooth sawfish
727	Siluriformes	Ictaluridae	*Noturus baileyi*	smoky madtom
728	Siluriformes	Ictaluridae	*Ameiurus brunneus*	snail bullhead
729	Perciformes	Percidae	*Percina tanasi*	snail darter
730	Cypriniformes	Catostomidae	*Chasmistes muriei*	snake river sucker
731	Perciformes	Percidae	*Etheostoma simoterum*	snubnose darter
732	Salmoniformes	Salmonidae	*Oncorhynchus nerka*	sockeye salmon
733	Cypriniformes	Cyprinidae	*Gila ditaenia*	sonora chub
734	Cypriniformes	Catostomidae	*Catostomus insignis*	sonora sucker
735	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon eremus*	sonoyta pupfish
736	Perciformes	Percidae	*Etheostoma olivaceum*	sooty darter
737	Cypriniformes	Catostomidae	*Cycleptus meridionalis*	southeastern blue sucker
738	Petromyzontiformes	Petromyzontidae	*Ichthyomyzon gagei*	southern brook lamprey
739	Percopsiformes	Amblyopsidae	*Typhlichthys subterraneus*	southern cavefish
740	Pleuronectiformes	Paralichthyidae	*Paralichthys lethostigma*	southern flounder
741	Perciformes	Percidae	*Percina austroperca*	southern logperch
742	Cyprinodontiformes	Poeciliidae	*Xiphophorus maculatus*	southern platyfish
743	Cypriniformes	Cyprinidae	*Phoxinus erythrogaster*	southern redbelly dace
744	Siluriformes	Loricariidae	*Pterygoplichthys anisitsi*	southern sailfin catfish
745	Perciformes	Percidae	*Ammocrypta meridiana*	southern sand darter
746	Siluriformes	Doradidae	*Platydoras armatulus*	southern striped raphael
747	Cyprinodontiformes	Fundulidae	*Fundulus stellifer*	southern studfish
748	Cypriniformes	Cyprinidae	*Macrhybopsis aestivalis*	speckled chub
749	Cypriniformes	Cyprinidae	*Rhinichthys osculus*	speckled dace
750	Perciformes	Percidae	*Etheostoma stigmaeum*	speckled darter
751	Cyprinodontiformes	Fundulidae	*Fundulus rathbuni*	speckled killifish
752	Siluriformes	Ictaluridae	*Noturus leptacanthus*	speckled madtom
753	Cypriniformes	Cyprinidae	*Meda fulgida*	spikedace
754	Perciformes	Percidae	*Etheostoma barrenense*	splendid darter
755	Cypriniformes	Cyprinidae	*Pogonichthys macrolepidotus*	splittail
756	Scorpaeniformes	Cottidae	*Cottus ricei*	spoonhead sculpin
757	Perciformes	Sciaenidae	*Leiostomus xanthurus*	spot
758	Cypriniformes	Cyprinidae	*Erimonax monachus*	spotfin chub
759	Cyprinodontiformes	Fundulidae	*Fundulus luciae*	spotfin killifish

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
760	Cypriniformes	Cyprinidae	*Cyprinella spiloptera*	spotfin shiner
761	Perciformes	Percidae	*Etheostoma squamiceps*	spottail darter
762	Cypriniformes	Cyprinidae	*Notropis hudsonius*	spottail shiner
763	Perciformes	Centrarchidae	*Micropterus punctulatus*	spotted bass
764	Siluriformes	Ictaluridae	*Ameiurus serracanthus*	spotted bullhead
765	Perciformes	Percidae	*Etheostoma maculatum*	spotted darter
766	Lepisosteiformes	Lepisosteidae	*Lepisosteus oculatus*	spotted gar
767	Perciformes	Sciaenidae	*Cynoscion nebulosus*	spotted seatrout
768	Perciformes	Eleotridae	*Eleotris picta*	spotted sleeper
769	Cypriniformes	Catostomidae	*Minytrema melanops*	spotted sucker
770	Perciformes	Centrarchidae	*Lepomis punctatus*	spotted sunfish
771	Perciformes	Cichlidae	*Tilapia mariae*	spotted tilapia
772	Percopsiformes	Amblyopsidae	*Forbesichthys agassizii*	spring cavefish
773	Perciformes	Elassomatidae	*Elassoma alabamae*	spring pygmy sunfish
774	Perciformes	Percidae	*Percina uranidea*	stargazing darter
775	Cypriniformes	Cyprinidae	*Phenacobius uranops*	stargazing minnow
776	Cyprinodontiformes	Fundulidae	*Fundulus dispar*	starhead topminnow
777	Pleuronectiformes	Pleuronectidae	*Platichthys stellatus*	starry flounder
778	Cypriniformes	Cyprinidae	*Cyprinella whipplei*	steelcolor shiner
779	Perciformes	Percidae	*Etheostoma punctulatum*	stippled darter
780	Cyprinodontiformes	Fundulidae	*Fundulus bifax*	stippled studfish
781	Perciformes	Percidae	*Etheostoma derivativum*	stone darter
782	Siluriformes	Ictaluridae	*Noturus flavus*	stonecat
783	Perciformes	Percidae	*Etheostoma fragi*	strawberry darter
784	Cypriniformes	Cyprinidae	*Erimystax dissimilis*	streamline chub
785	Perciformes	Percidae	*Etheostoma striatulum*	striated darter
786	Perciformes	Percidae	*Percina notogramma*	stripeback darter
787	Perciformes	Moronidae	*Morone saxatilis*	striped bass
788	Perciformes	Percidae	*Etheostoma virgatum*	striped darter
789	Cypriniformes	Catostomidae	*Moxostoma rupiscartes*	striped jumprock
790	Perciformes	Gerreidae	*Eugerres plumieri*	striped mojarra
791	Mugiliformes	Mugilidae	*Mugil cephalus*	striped mullet
792	Cypriniformes	Cyprinidae	*Luxilus chrysocephalus*	striped shiner
793	Perciformes	Percidae	*Etheostoma kennicotti*	stripetail darter
794	Cypriniformes	Cyprinidae	*Macrhybopsis gelida*	sturgeon chub
795	Siluriformes	Loricariidae	*Hypostomus plecostomus*	suckermouth catfish
796	Cypriniformes	Cyprinidae	*Phenacobius mirabilis*	suckermouth minnow
797	Salmoniformes	Osmeridae	*Hypomesus pretiosus*	surf smelt

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
798	Perciformes	Centrarchidae	*Micropterus notius*	suwannee bass
799	Cypriniformes	Cyprinidae	*Notropis procne*	swallowtail shiner
800	Perciformes	Percidae	*Etheostoma fusiforme*	swamp darter
801	Percopsiformes	Amblyopsidae	*Chologaster cornuta*	swampfish
802	Perciformes	Percidae	*Etheostoma swannanoa*	swannanoa darter
803	Perciformes	Centropomidae	*Centropomus ensiferus*	swordspine snook
804	Siluriformes	Ictaluridae	*Noturus gyrinus*	tadpole madtom
805	Cypriniformes	Catostomidae	*Catostomus tahoensis*	tahoe sucker
806	Cypriniformes	Cyprinidae	*Notropis maculatus*	taillight shiner
807	Perciformes	Percidae	*Etheostoma tallapoosae*	tallapoosa darter
808	Cypriniformes	Cyprinidae	*Cyprinella gibbsi*	tallapoosa shiner
809	Cypriniformes	Cyprinidae	*Notropis braytoni*	tamaulipas shiner
810	Perciformes	Percidae	*Percina aurantiaca*	tangerine darter
811	Elopiformes	Megalopidae	*Megalops atlanticus*	tarpon
812	Perciformes	Centropomidae	*Centropomus pectinatus*	tarpon snook
813	Perciformes	Percidae	*Etheostoma barbouri*	teardrop darter
814	Cypriniformes	Cyprinidae	*Notropis telescopus*	telescope shiner
815	Cypriniformes	Cyprinidae	*Tinca tinca*	tench
816	Cypriniformes	Cyprinidae	*Phoxinus tennesseensis*	tennessee dace
817	Cypriniformes	Cyprinidae	*Notropis leuciodus*	tennessee shiner
818	Perciformes	Percidae	*Etheostoma olmstedi*	tessellated darter
819	Perciformes	Percidae	*Percina carbonaria*	texas logperch
820	Cypriniformes	Cyprinidae	*Notropis amabilis*	texas shiner
821	Cyprinodontiformes	Poeciliidae	*Gambusia speciosa*	tex-mex gambusia
822	Cypriniformes	Cyprinidae	*Cyprinella labrosa*	thicklip chub
823	Cypriniformes	Cyprinidae	*Gila crassicauda*	thicktail chub
824	Clupeiformes	Clupeidae	*Dorosoma petenense*	threadfin shad
825	Perciformes	Gobiidae	*Eucyclogobius newberryi*	tidewater goby
826	Perciformes	Gerreidae	*Eucinostomus harengulus*	tidewater mojarra
827	Perciformes	Percidae	*Etheostoma tippecanoe*	tippecanoe darter
828	Perciformes	Percidae	*Etheostoma lachneri*	tombigbee darter
829	Cypriniformes	Cyprinidae	*Exoglossum laurae*	tonguetied minnow
830	Siluriformes	Ictaluridae	*Trogloglanis pattersoni*	toothless blindcat
831	Cypriniformes	Cyprinidae	*Notropis topeka*	topeka shiner
832	Scorpaeniformes	Cottidae	*Cottus rhotheus*	torrent sculpin
833	Cypriniformes	Catostomidae	*Thoburnia rhothoeca*	torrent sucker
834	Cypriniformes	Cyprinidae	*Cyprinella trichroistia*	tricolor shiner
835	Perciformes	Percidae	*Etheostoma trisella*	trispot darter

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
836	Percopsiformes	Percopsidae	*Percopsis omiscomaycus*	trout-perch
837	Perciformes	Gobiidae	*Proterorhinus marmoratus*	tubenose goby
838	Perciformes	Percidae	*Etheostoma gutselli*	tuckasegee darter
839	Cypriniformes	Cyprinidae	*Gila bicolor*	tui chub
840	Perciformes	Embiotocidae	*Hysterocarpus traskii*	tule perch
841	Perciformes	Percidae	*Etheostoma inscriptum*	turquoise darter
842	Perciformes	Percidae	*Etheostoma tuscumbia*	tuscumbia darter
843	Perciformes	Percidae	*Etheostoma douglasi*	tuskaloosa darter
844	Cypriniformes	Cyprinidae	*Rhinichthys umatilla*	umatilla dace
845	Cypriniformes	Cyprinidae	*Oregonichthys kalawatseti*	umpqua chub
846	Cypriniformes	Cyprinidae	*Rhinichthys evermanni*	umpqua dace
847	Cypriniformes	Cyprinidae	*Ptychocheilus umpquae*	umpqua pikeminnow
848	Cypriniformes	Cyprinidae	*Gila atraria*	utah chub
849	Scorpaeniformes	Cottidae	*Cottus echinatus*	utah lake sculpin
850	Cypriniformes	Catostomidae	*Catostomus ardens*	utah sucker
851	Cyprinodontiformes	Poeciliidae	*Xiphophorus variatus*	variable platyfish
852	Perciformes	Percidae	*Etheostoma variatum*	variegate darter
853	Siluriformes	Loricariidae	*Pterygoplichthys disjunctivus*	vermiculated sailfin catfish
854	Perciformes	Percidae	*Etheostoma chermocki*	vermilion darter
855	Perciformes	Gobiidae	*Gobioides broussonetii*	violet goby
856	Cypriniformes	Cyprinidae	*Gila seminuda*	virgin chub
857	Cypriniformes	Cyprinidae	*Lepidomeda mollispinis*	virgin spinedace
858	Cypriniformes	Catostomidae	*Moxostoma pappillosum*	v-lip redhorse
859	Perciformes	Percidae	*Etheostoma perlongum*	waccamaw darter
860	Cyprinodontiformes	Fundulidae	*Fundulus waccamensis*	waccamaw killifish
861	Atheriniformes	Atherinopsidae	*Menidia extensa*	waccamaw silverside
862	Salmoniformes	Osmeridae	*Hypomesus nipponensis*	wakasagi
863	Siluriformes	Clariidae	*Clarias batrachus*	walking catfish
864	Perciformes	Percidae	*Sander vitreus*	walleye
865	Perciformes	Cichlidae	*Oreochromis urolepis*	wami tilapia
866	Perciformes	Centrarchidae	*Lepomis gulosus*	warmouth
867	Cypriniformes	Catostomidae	*Catostomus warnerensis*	warner sucker
868	Cypriniformes	Cyprinidae	*Luxilus coccogenis*	warpaint shiner
869	Perciformes	Percidae	*Etheostoma bellator*	warrior darter
870	Cypriniformes	Cyprinidae	*Lythrurus alegnotus*	warrior shiner
871	Perciformes	Percidae	*Etheostoma nuchale*	watercress darter
872	Cypriniformes	Cyprinidae	*Notropis greenei*	wedgespot shiner
873	Cypriniformes	Cyprinidae	*Notropis texanus*	weed shiner

APPENDIX C: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
874	Cypriniformes	Cyprinidae	*Rhinichthys obtusus*	western blacknose dace
875	Petromyzontiformes	Petromyzontidae	*Lampetra richardsoni*	western brook lamprey
876	Cyprinodontiformes	Poeciliidae	*Gambusia affinis*	western mosquitofish
877	Perciformes	Percidae	*Ammocrypta clara*	western sand darter
878	Cypriniformes	Cyprinidae	*Hybognathus argyritis*	western silvery minnow
879	Cyprinodontiformes	Fundulidae	*Fundulus blairae*	western starhead topminnow
880	Perciformes	Moronidae	*Morone chrysops*	white bass
881	Siluriformes	Ictaluridae	*Ameiurus catus*	white catfish
882	Perciformes	Centrarchidae	*Pomoxis annularis*	white crappie
883	Mugiliformes	Mugilidae	*Mugil curema*	white mullet
884	Perciformes	Moronidae	*Morone americana*	white perch
885	Cypriniformes	Cyprinidae	*Lepidomeda albivallis*	white river spinedace
886	Cyprinodontiformes	Goodeidae	*Crenichthys baileyi*	white river springfish
887	Cyprinodontiformes	Cyprinodontidae	*Cyprinodon tularosa*	white sands pupfish
888	Cypriniformes	Cyprinidae	*Luxilus albeolus*	white shiner
889	Acipenseriformes	Acipenseridae	*Acipenser transmontanus*	white sturgeon
890	Cypriniformes	Catostomidae	*Catostomus commersonii*	white sucker
891	Cypriniformes	Cyprinidae	*Cyprinella nivea*	whitefin shiner
892	Cyprinodontiformes	Fundulidae	*Fundulus albolineatus*	whiteline topminnow
893	Cypriniformes	Cyprinidae	*Notropis alborus*	whitemouth shiner
894	Cypriniformes	Cyprinidae	*Cyprinella galactura*	whitetail shiner
895	Siluriformes	Ictaluridae	*Satan eurystomus*	widemouth blindcat
896	Scorpaeniformes	Cottidae	*Cottus leiopomus*	wood river sculpin
897	Perciformes	Percidae	*Etheostoma vulneratum*	wounded darter
898	Cypriniformes	Cyprinidae	*Plagopterus argentissimus*	woundfin
899	Siluriformes	Ictaluridae	*Ictalurus pricei*	yaqui catfish
900	Cypriniformes	Cyprinidae	*Gila purpurea*	yaqui chub
901	Cypriniformes	Catostomidae	*Catostomus bernardini*	yaqui sucker
902	Perciformes	Percidae	*Etheostoma raneyi*	yazoo darter
903	Cypriniformes	Cyprinidae	*Notropis rafinesquei*	yazoo shiner
904	Perciformes	Moronidae	*Morone mississippiensis*	yellow bass
905	Siluriformes	Ictaluridae	*Ameiurus natalis*	yellow bullhead
906	Perciformes	Percidae	*Perca flavescens*	yellow perch
907	Perciformes	Cichlidae	*Cichlasoma salvini*	yellowbelly cichlid
908	Perciformes	Percidae	*Etheostoma moorei*	yellowcheek darter
909	Perciformes	Gobiidae	*Acanthogobius flavimanus*	yellowfin goby
910	Siluriformes	Ictaluridae	*Noturus flavipinnis*	yellowfin madtom
911	Cypriniformes	Cyprinidae	*Notropis lutipinnis*	yellowfin shiner

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
912	Perciformes	Percidae	*Etheostoma juliae*	yoke darter
913	Perciformes	Percidae	*Sander lucioperca*	zander
914	Cypriniformes	Catostomidae	*Moxostoma cf. poecilurum*	apalachicola redhorse
915	Salmoniformes	Salmonidae	*Salmo salar*	atlantic salmon juvenile
916	Atheriniformes	Atherinopsidae	*Menidia menidia*	atlantic silverside
917	Cypriniformes	Cyprinidae	*Hypophthalmichthys nobilis*	bighead carp
918	Cypriniformes	Cyprinidae	*Mylopharyngodon piceus*	black carp
919	Perciformes	Pomatomidae	*Pomatomus saltatrix*	bluefish
920	Perciformes	Percidae	*Etheostoma meadiae*	bluespar darter
921	Salmoniformes	Salmonidae	*Oncorhynchus clarkii utah*	bonneville cutthroat trout
922	Cypriniformes	Catostomidae	*Moxostoma cf. lachneri*	brassy jumprock
923	Anura	Ranidae	*Rana catesbeiana*	bullfrog
924	Anura	Ranidae	*Rana catesbeiana*	bullfrog tadpole
925	Scorpaeniformes	Cottidae	*Cottus chattahoochee*	chattahoochee sculpin
926	Salmoniformes	Salmonidae	*Oncorhynchus tshawytscha*	chinook salmon (yoy)
927	Scorpaeniformes	Cottidae	*Cottus cf. broadband sculpin*	clinch sculpin
928	Salmoniformes	Salmonidae	*Oncorhynchus clarkii clarkii*	coastal cutthroat trout
929	Salmoniformes	Salmonidae	*Oncorhynchus clarkii pleuriticus*	colorado river cutthroat trout
930	Anguilliformes	Congridae	*Conger oceanicus*	conger eel
931	Cypriniformes	Cyprinidae	*Macrhybopsis cf. aestivalis*	coosa chub
932	Cypriniformes	Cyprinidae	*Semotilus X Luxilus atromaculatus x chrysocephalus*	creek chub x striped shiner
933	Salmoniformes	Salmonidae	*Oncorhynchus clarkii x mykiss*	cutbow
934	Perciformes	Percidae	*Etheostoma planasaxatile*	duck darter
935	Perciformes	Percidae	*Etheostoma orientale*	eastrim darter
936	Perciformes	Centropomidae	*Centropomus parallelus*	fat snook
937	Esociformes	Esocidae	*Esox americanus vermiculatus*	grass pickerel
938	Clupeiformes	Clupeidae	*Brevoortia patronus*	gulf menhaden
939	Scorpaeniformes	Cottidae	*Cottus kanawhae*	kanawha sculpin
940	Salmoniformes	Salmonidae	*Oncorhynchus clarkii henshawi*	lahontan cutthroat trout
941	Cypriniformes	Catostomidae	*Pantosteus lahontan*	lahontan sucker
942	Perciformes	Centrarchidae	*Micropterus salmoides*	largemouth bass (yoy)
943	Perciformes	Eleotridae	*Eleotris amblyopsis*	largescaled spinycheek sleeper
944	Cypriniformes	Catostomidae	*Catostomus cf. latipinnis*	little colorado river sucker
945	Cypriniformes	Cyprinidae	*Notropis amplamala*	longjaw minnow
946	Cypriniformes	Cyprinidae	*Pteronotropis stonei*	lowland shiner
947	Cypriniformes	Cyprinidae	*Cyprinus carpio*	mirror carp
948	Caudata	Ambystomatidae	*Dicamptodon tenebrosus*	pacific giant salamander
949	Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	rainbow trout (steelhead)

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
950	Anura	Ranidae	*Rana aurora*	red-legged frog
951	Salmoniformes	Salmonidae	*Oncorhynchus mykiss gairdneri*	redband rainbow trout
952	Caudata	Salamandridae	*Taricha granulosa*	rough-skinned newt
953	Siluriformes	Ictaluridae	*Noturus fasciatus*	saddled madtom
954	Perciformes	Percidae	*Sander canadensis x vitreus*	saugeye
955	Cypriniformes	Cyprinidae	*Notropis cf. spectrunculus*	sawfin shiner
956	Cypriniformes	Cyprinidae	*Hypophthalmichthys molitrix*	silver carp
957	Cypriniformes	Catostomidae	*Moxostoma robustum*	smallfin redhorse
958	Perciformes	Channidae	*Channa argus*	snakehead
959	Anguilliformes	Ophichthidae	*Myrophis punctatus*	speckled worm eel
960	Anura	Ascaphidae	*Ascaphus truei*	tailed frog
961	Anura	Ascaphidae	*Ascaphus truei*	tailed frog (tadpole)
962	Scorpaeniformes	Cottidae	*Cottus tallapoosae*	tallapoosa sculpin
963	Perciformes	Percidae	*Etheostoma tennesseense*	tennessee darter
964	Cypriniformes	Cyprinidae	*Cyprinella cf. zanema*	thinlip chub
965	Gasterosteiformes	Gasterosteidae	*Gasterosteus aculeatus*	threespine stickleback
966	Esociformes	Esocidae	*Esox lucius x masquinongy*	tiger muskellunge
967	Salmoniformes	Salmonidae	*Salmo X Salvelinus trutta x fontinalis*	tiger trout
968	Anura	Bufonidae	*Bufo boreas*	western toad
969	Perciformes	Percidae	*Etheostoma occidentale*	westrim darter
970	Salmoniformes	Salmonidae	*Oncorhynchus clarkii lewisi*	westslope cutthroat trout
971	Perciformes	Moronidae	*Morone na*	wiper
972	Perciformes	Gerreidae	*Gerres cinereus*	yellowfin mojarra
973	Perciformes	Percidae	*Percina burtoni*	blotchside darter
974	Anura	Pipidae	*Xenopus laevis*	african clawed frog
975	Salmoniformes	Salmonidae	*Oncorhynchus apache x mykiss*	apache x rainbow trout
976	Anura	Bufonidae	*Bufo microscaphus*	arizona toad
977	Anura	Hylidae	*Pseudacris maculata*	boreal chorus frog
978	Caudata	Ambystomatidae	*Dicamptodon ensatus*	california giant salamander
979	Caudata	Salamandridae	*Taricha torosa*	california newt
980	Anura	Hylidae	*Pseudacris cadaverina*	california treefrog
981	Anura	Bufonidae	*Bufo hemiophrys*	canadian toad
982	Anura	Hylidae	*Hyla arenicolor*	canyon treefrog
983	Anura	Ranidae	*Rana cascadae*	cascade frog
984	Anura	Ranidae	*Rana luteiventris*	columbia spotted frog
985	Caudata	Rhyacotritonidae	*Rhyacotriton kezeri*	columbia torrent salamander
986	Anura	Ranidae	*Rana boylii*	foothill yellow-legged frog
987	Salmoniformes	Salmonidae	*Oncorhynchus aguabonita*	golden trout

APPENDIX c: OTHER PERTINENT ATTACHMENTS

LINE NO.	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME
988	Anura	Ranidae	*Rana clamitans*	green frog
989	Caudata	Ambystomatidae	*Dicamptodon aterrimus*	idaho giant salamander
990	Petromyzontiformes	Petromyzontidae	*Lampetra similis*	klamath river lamprey
991	Anura	Ranidae	*Lithobates pipiens*	leopard frog
992	Caudata	Ambystomatidae	*Ambystoma macrodactylum*	longtoed salamander
993	Anura	Ranidae	*Rana yavapaiensis*	lowland leopard frog
994	Anura	Ranidae	*Rana muscosa*	mountain yellow-legged frog
995	Anura	Hylidae	*Pseudacris regilla*	pacific tree frog
996	Anura	Ranidae	*Lithobates blairi*	plains leopard frog
997	Salmoniformes	Salmonidae	*Oncorhynchus mykiss x aguabonita*	rainbow x golden trout
998	Anura	Bufonidae	*Bufo punctatus*	red-spotted toad
999	Cypriniformes	Cyprinidae	*Richardsonius X Rhinichthys balteatus x osculus*	redside shiner x speckled dace
1000	Anura	Ranidae	*Rana pretiosa*	spotted frog
1001	Anura	Ranidae	*Rana sylvatica*	wood frog
1002	Anura	Bufonidae	*Bufo woodhousii*	woodhouse's toad
1003	Anura	Ranidae	*Lithobates chiricahuensis*	chiricahua leopard frog

APPENDIX c: OTHER PERTINENT ATTACHMENTS

APPENDIX D: REPORTING TEMPLATES

Templates will be provided on the NARS SharePoint.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

SOP

FOR
The Determination of Ash Free Dry Mass
WRS 73A.5

Prepared for:
Devin Peck, EPA

Technical Review/Contributors:

Dianne Meyer and Phil Monaco

Dynamac Corporation

Management Approval:
Signature indicates that this SOP is approved and will be implemented in conducting research.

Tony Olsen:

_____ _____
FEB Branch Chief   Signature   Date
                                             10/22/14

David Peck:

_____ _____
Principal Investigator   Signature   Date
                                             10/21/14

Quality Assurance:
Signature indicates that this SOP meets the quality requirements of WED

Peter Beedlow:

_____ _____
Quality Assurance Manager Signature   Date
                                             2-24-14

_____   _____
SAEM   6/10/15

SOP

FOR
The Determination of Ash Free Dry Mass
WRS 73A.5

Biennial Reviews*			
Date	EPA Reviewer (WA#, if WACOR)	Date entered by:	QA Manager

*Listed EPA reviewer has personally verified, or has been assured directly or indirectly by those currently using this SOP, that it is being used, essentially as written and that no revisions are necessary.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

Standard Operating Procedure for the Determination of Ash Free Dry Mass WRS 73A.5

Willamette Research Station
Analytical Laboratory
200 SW 35th Street
Corvallis, Oregon

Prepared by Karen Baxter
December 2002
Revised by Dianne Meyer
April 2012

Contract Ep-D-11-027
Dynamac Corporation

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

**Standard Operating Procedure for the
Determination of Ash Free Dry Mass
WRS 73A.5**

List of Authors Contributing
to this SOP

Originally prepared by Karen Baxter, 2002

Revisions:

Karen Baxter
Dianne Meyer
Phil Monaco

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

Standard Operating Procedure for the
Determination of Ash Free Dry Mass
WRS 73A.5

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APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

Standard Operating Procedure for the Determination of Ash Free Dry Mass WRS 73A.5

1.0 Scope and Application

1.1 This method is designed for the laboratory determination of ash free dry mass (AFDM) in periphyton. Samples were collected following the procedures described in *Field Operations Manual for Wadeable Streams* (Peck et al., 2005).

1.2 The differences between this version (WRS 73A.5) and the previous version (WRS 73A.4) include editorial changes as well as the addition of the personnel qualifications section.

2.0 Summary of Method

2.1 Sample filters are dried in a forced-air oven at a set temperature to evaporate all weight from water. The dry filter is weighed and the measurement recorded. The sample filters are then combusted (ashed) using a muffle furnace to decompose the organic matter. The filters are saturated with water to rehydrate the clays, and placed back in the forced-air oven. Dry filters are weighed and that measurement is recorded. Ash free dry mass is then calculated using weight measurements and field data (area scraped, number of transects, volume of sample collected and volume of sample filtered). The results are reported as grams of Total Ash Free Dry Mass.

2.2 This method is based upon EPA methods and associated methods for the determination of Ash Free Dry Mass (Biomass) or Ash Free Dry Weight (Peck, et al, 2005; APHA 1998, 10300 D; Chaloud and Peck, 1994). The adapted procedure may vary from the drying and/or ashing temperatures and/or times stated in the referenced procedures

3.0 Definitions

3.1 AFDM: Ash Free Dry Mass

3.2 Filter Blank: A GF/F glass fiber filter that is treated as a sample throughout the entire ashing procedure.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

3.3 Glass-fiber filter: Whatman GF/F glass-fiber filter discs (≈0.7 μm particle retention).

3.4 QCS: A Quality Control Sample is run at least once per run to demonstrate inter-run consistency and for duplicate precision. The QCS is an in house reference standard made by filtering periphyton rich water through a Whatman GF/F glass fiber filter. Treated filters are frozen until needed.

3.5 Reagent Water: Water passed through a reverse osmosis and a deionization system to produce ASTM Type 1 reagent water (≥18 megaohms resistivity) (Specification D-1193-99e1, ASTM 1999).

4.0 Health and Safety Warnings

4.1 Personal Protective Equipment (PPE) is advised for any laboratory procedures.

4.2 Use heat protection such as oven mitts and tongs to prevent contact with hot surfaces.

4.3 Allow muffle furnace to cool to 200°C before opening door to access samples.

4.4 This method does not address all safety issues associated with its use. The laboratory staff is responsible for safely conducting lab work and chemical analysis in accordance with the NHEERL-WED Health and Safety Handbook (U.S. EPA, 2010), , the applicable Dynamac Hazardous Activity Safety Plans, and the Material Safety Data Sheets for the specific chemicals.

5.0 Cautions

5.1 Filters placed in the forced-air oven should be covered with foil to prevent filter from being blown out of identifying aluminum weigh-boat.

6.0 Interferences

6.1 Samples must be stored in the desiccator after the drying process to ensure that there is no increase in weight derived from atmospheric moisture.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

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7.0 Equipment and Supplies

Note: *Trade names, suppliers and part numbers are for informational purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.*

7.1 Analytical balance with resolution to 0.1 mg

7.2 Glass fiber filters (GF/F or equivalent)

7.3 Forced-air oven, with over temperature control and set at a temperature of 60°C

7.4 Aluminum weigh-boats

7.5 Muffle furnace with temperature capacity of 550°C

7.6 Tongs

7.7 Safety glasses

7.8 Nitrile gloves

7.9 Lab coat

8.0 Reagents and Standards

8.1 Reagent Water

8.2 NIST certified weight set (0.0010 – 200 g) to check balance calibration.

8.3 QCS: A large volume (approximately 5 gallons bulk sample) of periphyton rich water is collected from a local stream. Batches of QCS samples (filters) are prepared at one time and frozen for future use in the AFDM analyses. To prepare the QCS samples, 50-mL aliquots are filtered through GF/F filters previously baked in a muffle furnace at 550°C for 30 minutes, and the filters are frozen (-20°C) in aluminum foil. Two previously frozen QCS samples (filter) are processed with each batch of AFDM sample filters.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

9.0 Sample Handling and Storage

9.1 Samples are received, logged into the laboratory data system, and stored in a deep freeze at -20°C.

9.2 Samples may be stored in the freezer for several months prior to analysis (APHA, 1998, Method 10300 D). .

10.0 Quality Control

10.1 QCS is an aliquot from a bulk sample that is filtered through a GF/F filter then stored frozen at -20°C until needed. Two filters are processed with each batch of AFDM filters to monitor inter-run consistency and precision.

10.2 A filter blank is processed each run as a background check.

11.0 Calibration and Standardization

11.1 Analytical balances: Calibration is checked to within 2% of the theoretical weight with certified standard weights. A weight with a mass similar to the weigh-boat+sample mass should be used to check the calibration.

12.0 Procedure

121 Remove the sample from freezer and record sample ID, sample volumes, and collection date on laboratory data sheets (see Section 19.0).

12.2 Remove filter from field packaging and place into an engraved (labeled) aluminum weigh-boat. The weigh-boat label should match up with the correct sample ID number found on the weigh sheet. If sample filter is folded within the centrifuge tube, unfold prior to drying. *Note: some of the centrifuge tubes may contain a small amount of water (less than 10 mL). This should be transferred to the weigh-boat with the sample. If the sample contains more than 10 mL of water, the sample is treated differently, see appendix A for procedure.*

12.3 Cover samples with foil to prevent filters from blowing out of weigh-boat and dry the sample in a forced-air oven at 60°C for 24 hours.

12.4 Place warm, dry samples in a desiccator to cool. Weigh samples in aluminum weigh-boats and record to the nearest 0.1 mg. Remove the

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

weigh-boat+sample from balance. Ensure the balance is zeroed. This is the initial sample weight.

12.5   Place the samples in a muffle furnace and bring the temperature to 550°C. Ash the samples in a muffle furnace by holding them at 550°C for 30 minutes.

12.6   Allow the furnace to cool to approximately 200°C. Remove the samples from the muffle furnace and place in a desiccator to finish cooling. After ashing, the precipitate should appear gray and crumbly.

12.7   Saturate the cooled samples with reagent water to rehydrate the clays.

12.8   Cover samples with foil to prevent filters from blowing out and dry the samples in a forced-air oven at 60°C for 24 hours.

12.9   Store the dry samples in a desiccator to cool. Calibrate balance and record. Weigh the samples in aluminum weigh-boats and record the weight on the datasheet (see Section 19.0) to the nearest 0.1 mg. Remove the sample and weigh-boat from balance and ensure the balance is zeroed. This is the final (ash free) sample weight.

13.0   Data Analysis and Calculations

13.1   The lab does not receive all of the required field data to standardize Ash Free Dry Mass results to a unit area. Therefore, initial and final weights (second value) are reported by electronic spreadsheet to EPA and the raw data is used to calculate the AFDM using the following calculation:

$\text{AFDM}_{m \text{ (mass)}} \text{ (g)} = \text{Initial wt (g)} - \text{Final wt (g)}$

$\text{AFDM}_{m/v \text{ (mass per unit volume)}} \text{ (g/mL)} = \text{AFDM}_m \text{ (g)} / \text{Filtered volume (mL)}$

$\text{AFDM}_{m/a \text{ (mass per unit area)}} \text{ (g/cm}^2\text{)} = \text{AFDM}_{m/v} * [\text{Total volume (mL)} / \text{area scraped (cm}^2\text{)}]$

Where:
Filtered volume = volume of sample filtered in the field (usually 25 mL)
Total volume = volume of sample collected (usually 500 mL)
Area scraped = (# of transects)*(area delimiter (cm^2)) (usually 12 cm^2 x 11 transects = 132 cm^2)

13.2   All results are recorded on a datasheet (see Section 19.0). Datasheets are filed in the AFDM binder created and maintained for that calendar year.

Results are entered into electronic format and entries are verified by a second person.

14.0 Method Performance

14.1 Method performance is measured through analysis of filter blanks, bulk collection QCS samples, and precision (Quality Assurance Plan, 2012). Blank measurements should not gain or lose any weight after complete ashing and drying process. Two filters prepared from a bulk sample are analyzed each analysis batch and results are monitored over time. Duplicate measurements are based on the QCS duplicates.

14.2 2009 QCS results had a mean AFDM of 7.3 mg.

15.0 Pollution Prevention

15.1 The chemicals used in this method pose little threat to the environment when properly managed.

15.2 Whenever possible waste is reduced at the source. When waste cannot be feasibly reduced the waste is collected for recycling.

16.0 Waste Management

16.1 It is the laboratory's responsibility to comply with all federal, state and local regulations governing waste management, and to protect the environment by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is required.

17.6 Contact the Dynamac Environmental, Health and Safety Manager for guidance on waste collection and disposal. For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel*, and *Less is Better: Laboratory Chemical Management for Waste Reduction*, both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, DC 20036.

17.0 Personnel Qualifications

It is required that qualified technicians with appropriate traing and background will conduct the procedures described in this document. Those using this SOP

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

will have demonstrated that they are able to perform all aspects of the SOP thoroughly and independently prior to collecting research data.

17.1   Training

17.1.1 Not required of anyone who prepared or significantly contributed to the preparation of this SOP.

17.1.2 Personnel assigned to implement the tasks in this SOP are required to read and understand the procedures herein. The EPA Principle Investigator or designee (or contractor Work Plan Manager), "trainers", will review the contents of this document with the EPA (or contractor) personnel assigned to this SOP and observe its implementation from start to finish. If hands-on training appears to be required, it will be provided.

If this SOP is for an instrument, proficiency is demonstrated following the applicable sections of the Demonstration of Analyst Proficiency paragraph of ORD policy PPM 13.4 Minimum QA/QC Procedures for the ORD Laboratories Conducting Research.

17.2   Training Documentation

17.2.1 EPA, non-contract staff: when the trainer has determined a trainee can successfully implement this SOP in the collection of research data, he/she will provide to the Division QA Manager within 30 days, the SOP name, trainee, trainer, and date of the determination. The QAM will add this information to the SOP table in WED's QA database.

17.2.2 Contract staff: successful training will be documented on the Employee Training Record form and signed by the supervisor. A copy of the contractor Employee Acceptance Form for this SOP signed by contract staff, will be forwarded to the contract Program Quality Assurance Manager. The Work Plan Manager is encouraged to inform the WACOR when staff is newly trained on this SOP, providing names of trainee and trainer, and applicable date. The WACOR will pass this information on to the WED QAM.

18.0   References

APHA. Standard Methods for the Examination of Water and Wastewater. 20th Edition. 1998. American Public Health

Association, 1015 Fifteenth Street, N.W., Washington DC, 20005, Method 10300 D.

ASTM. . American Society for Testing and Materials. Standard Specifications for Reagent Water. D1193-99e1 ASTM: Philadelphia, PA, 1999.

Chaloud, D.J. and D.V. Peck (Eds.). Environmental Monitoring and Assessment Program: Integrated Quality Assurance Project Plan for the SurfaceWaters Resource Group, 1994 Activities. EPA 600/X-91/080, Rev. 2.00. U.S. Environmental Protection Agency, Las Vegas, NV. 1994.

Peck, D.V., J.M. Lazorchak, and D.J. Klemm. In press. Western Pilot Study Field Operations Manual for Wadeable Streams. U.S. Environmental Protection Agency, Washington, DC. 2005.

Quality Assurance Plan. Willamette Research Station, Analytical Laboratory. Dynamac Corporation. Revision 4, May 2012. Corvallis, Oregon.

U.S. EPA. Western Ecology Division, Health and Safety Handbook, U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Corvallis, OR 97333, January 2010.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

19.0 Tables, Diagrams, Flowcharts, and Validation Data

19.1 Example of Data Form

WRS Analytical Laboratory
Ash Free Dry Mass

Analyst/Date _____

Drying/Ashing:	Date/Time In	Date/Time Out
60°C		
550°C		
60°C		

Filter Number	WRS Sample ID	Volume Filtered (mL)	Initial Weight (g) [After 24 hrs @60°C]	Final Weight (g) [After ashing @550°C, wetting, & 24 hrs @60°C]	Comments

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

20.0 Appendix A

Procedure for Ash Free Dry Mass Problem Samples

Samples received from the field that contain more than 10 mL of water are considered to be problem samples due to the water washing sample off of the filter used to determine ash free dry mass. Samples are subjected to extra filtration in order to retrieve the solid material from the water in the tube.

1. Remove the sample from freezer and record sample ID, sample volumes, and collection date on laboratory data sheets (see Section 19.0). In the comments, record the amount of water each problem sample contains.

2. Allow samples with more than 10 mL of water to fully defrost before beginning the sample preparation.

3. Assemble cleaned filter apparatus with new, previously baked , GF/F filter and apply vacuum.

4. Whenever possible, remove the sample filter from the centrifuge tube and place in an aluminum weigh boat. Empty the water from the centrifuge tube in to the filtration apparatus. Using reagent water, rinse the centrifuge tube to ensure all sample material is filtered.

5. Rinse the filter apparatus with reagent water to maximize the recovery of sample.

6. Continue suction for at least 30 seconds after all visible water passes through the filter.

7. Carefully remove the filter from the apparatus and place it in the same aluminum weigh boat as the original filter.

8. Cover samples with foil to prevent filters from blowing out and dry the sample in forced-air oven at 60°C for 24 hours.

9. Place warm, dry samples in a desiccator to cool. Weigh samples in aluminum weigh-boats and record to the nearest 0.1 mg). This is the initial sample weight.

10. Place the samples in a muffle furnace and bring the temperature to 550°C. Ash the samples in a muffle furnace by holding them at 550°C for 30 minutes.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

11. Allow the furnace to cool to approximately 200°C. Remove the samples from the muffle furnace and place in a desiccator to finish cooling. After ashing, the precipitate should appear gray and crumbly.

12. Saturate the cooled samples with reagent water to rehydrate the clays.

13. Cover samples with foil to prevent filters from blowing out and dry the samples in a forced-air oven at 60°C for 24 hours.

14. Store the dry samples in a desiccator to cool. Weigh the samples in aluminum weigh-boats and record the weight on the datasheet (see Section 19.0) to he nearest 0.1 mg. This is the final (ash free) sample weight.

APPENDIX E: EXAMPLE SOP FOR ASH FREE DRY MASS ANALYSIS OF PERIPHYTON BIOMASS

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

Please see separate attachement for Appendix G