United States Environmental Protection Agency
Office of Water
Office of Environmental Information
Washington, DC
EPA-841-B-07-010

National Rivers and Streams Assessment

Laboratory Methods Manual

November 2009

NOTICE

The intention of the National Rivers and Streams Assessment is to provide a comprehensive "State of the 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 four companion documents:

- National Rivers and Streams Assessment*: Quality Assurance Project Plan (EPA-841-B-07-007)*
- National Rivers and Streams Assessment*: Site Evaluation Guidelines (EPA-841-B-07-008)*
- National Rivers and Streams Assessment*: Field Operations Manual (EPA-841-B-07-009)*
- National Rivers and Streams Assessment*: Laboratory Methods Manual (EPA-841-B-07-010)*

This document (*Laboratory Methods 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. All Project Cooperator laboratories should follow these guidelines. Mention of trade names or commercial products in this document does not constitute endorsement or recommendation for use. More details on specific methods for site evaluation, sampling, and sample processing can be found in the appropriate companion document.

The suggested citation for this document is:

> USEPA. 2008. National Rivers and Streams Assessment: Laboratory Methods Manual. EPA-841-B-07-010. U.S. Environmental Protection Agency, Office of Water and Office of Research and Development, Washington, DC.

TABLE OF CONTENTS

1.0 INTRODUCTION

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

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

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

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

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

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

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

2.0 WATER CHEMISTRY

2.1 Parameters for the NRSA

A total of 19 parameters will be measured from each water chemistry sample collected (Table 2-1). Additionally, chlorophyll-*a* will be measured from a separate, discrete sample collected at the same location as the water chemistry sample.

Table 2-1. Water chemistry parameters measured for the National Rivers and Streams Survey.

Analyte	Units	Example Method(s)[1]	Method Detection Limit Objective[2]
Conductivity	µS/cm at 25˚C	EPA 120.6 (conductivity cell)	NA
Turbidity	NTU	APHA 214 A, EPA 180.1 (nephelometer)	0.1
pH	pH units	EPA 150.6 (modified) (Collected and measured without exposure to atmosphere (Closed system)	NA
Acid Neutralizing Capacity (ANC)	µeq/L (20 µeq/L=1 mg as $CaCO_3$)	EPA 310.1 (modified): (Automated acidimetric titration to pH<3.5, with Modified Gran Analysis)	NA
Total and Dissolved Organic Carbon (TOC/DOC)	mg C/L	EPA 415.2 (UV-persulfate oxidation)	0.1
Ammonia (NH_3)	mg N/L	EPA 350.1, or modifications (e.g., Automated Colorimetric, or modified to use salicylate and dichloroisocyanurate with analysis by flow injection analyzer (FIA)	0.02
Nitrate-Nitrate (NO_3-NO_2)	mg N/L	EPA 353.2 (modified) (Automated colorimetric analysis with cadmium reduction by FIA)	0.02
Total Nitrogen (TN)	mg/L	EPA 353.2 (modified) (Persulfate Digestion; Automated colorimetric analysis with cadmium reduction; modified for analysis by FIA)	0.01
Total Phosphorous (TP) & ortho-Phosphate (SRP)	µg P/L	EPA 365.1 (modified) (Persulfate digestion (TP only); automated colorimetric (molybdate, ascorbic acid) analysis by FIA)	2
Sulfate (SO_4)	mg SO_4/L	EPA 300.1 (ion chromatography w/ suppressed conductivity detection) EPA 375.2	0.1

Table 2-1. Continued.

Analyte	Units	Example Method(s)[1]	Method Detection Limit Objective[2]
Chloride (Cl)	mg Cl/L	EPA 300.1 (ion chromatography w/ suppressed conductivity detection); EPA 325.1	0.1
Nitrate (NO_3)	mg N/L	EPA 300.1 (ion chromatography w/ suppressed conductivity detection) EPA 352.1 (automated colorimetric)	0.03
Calcium (Ca)	mg Ca/L	EPA 215.1 (flame atomic absorption spectrometry (FAAS)	0.02
Magnesium (Mg)	mg Mg/L	EPA 242.1 (flame atomic absorption spectrometry (FAAS)	0.01
Sodium (Na)	mg Na/L	EPA 273.1 (flame atomic absorption spectrometry (FAAS)	0.02
Potassium (K)	mg K/L	EPA 258.1 (flame atomic absorption spectrometry (FAAS)	0.04
Silica (SiO_2)	mg SiO_2/L	EPA 370.1 (Automated Colorimetric analysis (molybdate, stannous chloride); modified for analysis using FIA)	0.05
Total Suspended Solids (TSS)	mg/L	EPA 160.2, APHA (1989) (gravimetric)	0.1
True Color	PCU	APHA 204 A/B, EPA 110.2 (Colorimetric; visual comparison to color standards) Modified to use color disk in place of standard solutions	NA
Chlorophyll-*a*	μg/L (in extract)	APHA 10200 H (spectrophotometric) Welschmeyer, N.A. 1994. Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. Limnology and Oceanography 39:1985-1992	1.5

[1] Methods presented are those used for WSA analyses (and have met the performance characteristics listed). In some cases, a potential alternative method is also presented. Methods presented here should not be interpreted as the required method(s) to be used by all laboratories analyzing NRSA samples.
[2] The method detection limit is determined as a one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves.

2.2 Performance-based Methods

As an alternative to specifying laboratory methods for sample analysis, a performance-based approach is being utilized that defines a set of laboratory method performance requirements for data quality. Method performance requirements for this project identify detection limit, precision, and accuracy objectives for each parameter (Table 2-2). Table 2-2 also lists example methods that have been demonstrated to achieve the required objectives. However, it should be noted that they are not required methods to be used by all participating laboratories. Laboratories may choose to use other analytical methods for any target analyte as long as they are able to achieve the same performance requirements as listed in Table 2-2.

Table 2-2. Laboratory method performance requirements for water chemistry and chlorophyll-a sample analysis.

Analyte	Units	Potential Range of Samples[1]	Method Detection Limit Objective[2]	Concentration Range[3]	Precision Objective[4]	Accuracy Objective[5]
Conductivity	µS/cm at 25˚C	1 to 15,000	NA	≤ 40 > 40	± 2 ±3%	± 2 ±5%
Turbidity	NTU	0 to 44,000	0.1	≤ 20 > 20	± 2 ±5%	± 2 ±5%
pH	pH units	3.7 to 10	NA	≤ 5.75 > 5.75	± 0.07 ± 0.15	± 0.05 ± 0.10
Acid Neutralizing Capacity (ANC)	µeq/L (20 µeq/L=1 mg as $CaCO_3$)	-300 to +75,000 (-16 to 3750 mg as $CaCO_3$)	NA	≤|±100| > |±100|	± 5 ±5%	± 4 ±5%
Total & Dissolved Organic Carbon (TOC/DOC)	mg C/L	0.1 to 109 (as DOC)	0.1	≤ 1.5 > 1.5	± 0.1 ±5%	± 0.1 ±5%
Ammonia (NH_3)	mg N/L	0 to 17	0.02	≤ 0.2 > 0.2	± 0.02 ±10%	± 0.02 ±10%
Nitrate-Nitrate (NO_3-NO_2)	mg N/L		0.02	≤ 0.2 > 0.2	± 0.02 ±10%	± 0.02 ±10%
Total Nitrogen (TN)	mg/L	0.1 to 90	0.01	≤ 0.1 > 0.1	± 0.01 ±10%	± 0.01 ±10%
Total Phosphorous (TP) and ortho-Phosphate (SRP)	µg P/L	0 to 22,000 (as TP)	2	≤ 20 > 20	± 2 ±10%	± 2 ±10%
Sulfate (SO_4)	mg SO_4/L	0 to 5,000	0.1	≤ 2 > 2	± 0.1 ±5%	± 0.1 ±5%
Chloride (Cl)	mg Cl/L	0 to 5000	0.1	≤ 2 > 2	± 0.10 ±5%	± 0.10 ±5%
Nitrate (NO_3)	mg N/L	0. to 360	0.03	≤ 0.6 > 0.6	± 0.03 ±5%	± 0.03 ±5%
Calcium (Ca)	mg Ca/L	0.04 to 5,000	0.02	≤ 0.8 > 0.8	± 0.10 ±10%	± 0.10 ±10%
Magnesium (Mg)	mg Mg/L	0.1 to 350	0.01	≤ 0.8 > 0.8	± 0.10 ±10%	± 0.10 ±10%
Sodium (Na)	mg Na/L	0.08 to 3,500	0.02	≤ 0.8 > 0.8	± 0.10 ±10%	± 0.10 ±10%

Table 2-2. Continued.

Analyte	Units	Potential Range of Samples[1]	Method Detection Limit Objective[2]	Concentration Range[3]	Precision Objective[4]	Accuracy Objective[5]

Potassium (K)	mg K/L	0.01 to 120	0.04	≤ 0.8 > 0.8	± 0.10 ±10%	± 0.10 ±10%
Silica (SiO_2)	mg SiO_2/L	0.01 to 100	0.05	≤ 0.5 > 0.5	± 0.05 ±10%	± 0.05 ±10%
Total Suspended Solids (TSS)	mg/L	0 to 27,000	0.1	≤ 10 > 10	± 1 ±10%	± 1 ±10%
True Color	PCU	0 to 350	NA	≤ 100 > 100	±10 or ±10%	±10 or ±10%
Chlorophyll-*a*	μg/L (in extract)	0.7 to 11,000	1.5	≤ 15 > 15	± 1.5 ±10%	± 1.5 ±10%

[1] 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.

[2] The method detection limit is determined as a one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves.

[3] Range for which absolute (lower concentrations) vs. relative (higher concentrations) objectives for precision and accuracy are used. 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.

[4] 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, 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.

[5] Accuracy is estimated as the difference between the measured (across batches) and target values of performance evaluation and/or internal reference samples at the lower concentration range, and as the percent difference at the higher concentration range.

2.3 Sample Processing and Preservation

Upon receipt of samples, inspect each sample and complete tracking form. Store samples in refrigerator at 4°C until aliquots are ready to be prepared. Figure 2-1 illustrates the sample preparation procedures, including filtering and acidifying, for the various analytes.

Filter all filtered aliquots through 0.4μm pore size polycarbonate filters within 48 hours of arrival at the Laboratory. Rinse vacuum filter funnel units thoroughly with reverse-osmosis (RO) or de-ionized (DI) 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, then 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 sample filtration.

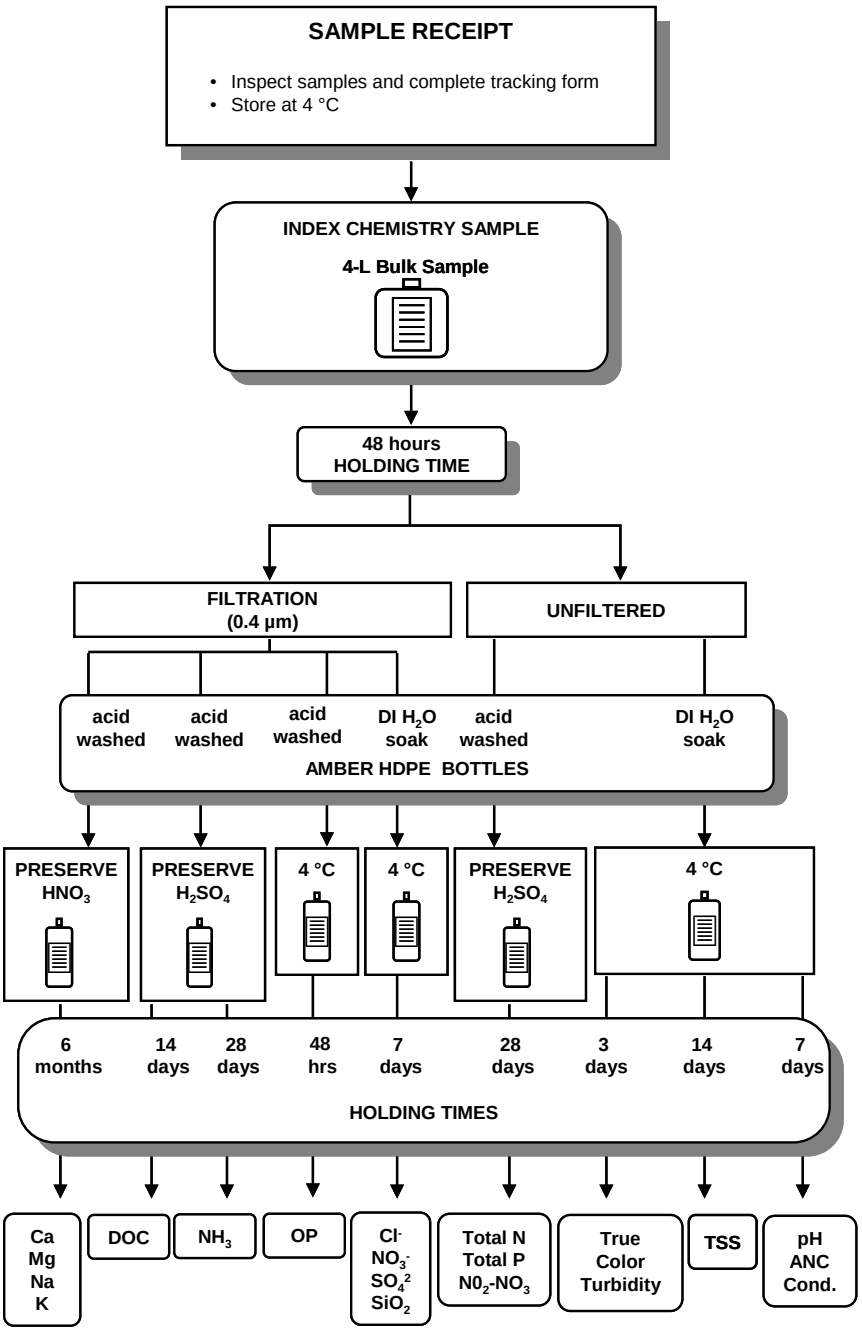


Figure 2-1. Water chemistry sample processing procedures.
After all filtered and unfiltered aliquots are collected, add ultra-pure acid (HNO_3 or H_2SO_4, depending on the analyte; Table 2-3) to the sample in the aliquot container. Store all aliquots except the cation aliquot (filtered, acidified with HNO_3) in a refrigerator at 4°C.

Table 2-3. Acid preservatives added for various analytes.

Preservative	
H_2SO_4	HNO_3

DOC	Ca
NH_3	Mg
Total N	Na
Total P	K
NO_2-NO_3	

Chlorophyll-*a* samples are usually filtered in the field, with the filter placed in a labeled centrifuge tube and stored on ice until arrival at the Laboratory. If unfiltered samples arrive at the Laboratory on the same day as collected, filter as soon as possible after arrival. Filter a known volume of sample through one Whatman 0.7 µm glass-fiber filter, keeping the vacuum pressure to 7 psi or less. Store the filter in the centrifuge tube in the freezer at –20°C ±2°C for up to 30 days before analysis.

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

Since multiple laboratories will be analyzing water chemistry samples, specific quality control procedures must be implemented to ensure that 1) the data quality objectives are being met, and 2) data is consistent and comparable among all participating labs. Specific QA/QC procedures will depend on whether or not the lab has been certified by the National Environmental Laboratory Accreditation Conference (NELAC). More stringent QA/QC requirements will be implemented for labs that are not NELAC certified.

2.4.1 Internal QA/QC Procedures

Prior to sample analysis, all laboratories will be required to provide internal QA documentation (e.g., Quality Management Plan, Quality Assurance Project Plan) for external review. QA documentation will be reviewed by Sarah Lehmann to ensure consistency among all participating labs.

Non-certified labs will need to consistently implement additional QA/QC procedures specific to the SNL. EPA will provide stock standards for use as Quality Control Check Samples (QCCS) in all labs. The QCCS can be used to estimate batch-to-batch precision and to track batch-to-batch comparability. Each lab will also prepare QCCS from stock standards and will analyze them with each batch of samples. For consistency, all labs should use the same concentrations for each analyte.

2.4.2 External QA/QC Procedures

2.4.2.1 Interlaboratory Proficiency Testing

As an external independent check on performance, each lab will be required to participate in an interlaboratory proficiency test (PT) study, which will be performed twice per year (December and May). The independent PT study will be performed by the Canadian National Water

Research Institute's (NWRI) National Laboratory for Environmental Testing (NLET). NLET will provide three sets of samples in each study. The rain and soft water set contains low ionic strength natural water samples with conductivity less than 100 µS/cm, the major ion and nutrient set contains higher ionic strength natural water samples with conductivity greater than 100 µS/cm, and the third set contains preserved, fortified natural samples for analysis of total phosphorus. The samples in each set cover a range of concentrations. Thirty to 50 laboratories participate in each study, and a median value is determined for each variable for each study. Flags, from extremely low to extremely high, are then assigned to each sample for each variable whose reported value is outside the acceptable limits for difference from the median value. Laboratory rankings of the results from the 10 samples in each study are used to identify bias for each variable for each laboratory. Bias classes (from slightly low to high) are assigned to a variable based on the procedure described by Youden (1969).

A summary sheet is prepared for each laboratory after a study, indicating the results (flags, and if ranking indicates a bias) for each variable. If a variable is flagged, or a bias is indicated, the first check is to confirm that the values were reported correctly, and that there were no transcription or unit conversion errors. Results are discussed with the analyst to identify the source of flagged results (e.g., calibration errors, pressure leaks, old electrodes, or errors in calibration standards).

2.4.2.2 "Internal" Performance Evaluation

All labs will be required to participate in an "internal" performance evaluation (PE) program. In the PE program, EPA or another centralized lab will provide single-blind samples with multiple concentrations of each analyte to all participating labs for analysis. The results of the program will be used to assess laboratory performance.

2.5 References

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

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

3.0 CHLOROPHYLL A

3.1 Scope of Application

This method details a procedure for measuring extracted chlorophyll *a* (chl-*a*) without acidification using a Turner Fluorometer equipped with a 13-mm cuvette holder and a Turner Optical Filter Kit (PN 10-040). The differences between this version (WRS 71A.3) and the previous version (WRS 71A.2) are primarily editorial.

3.2 Summary of Method

This method is based upon EPA and other published methods for the measurement of chl-*a* content in environmental samples (Arar and Collins 1997; Arar 1997; Welschmeyer 1994).

Chl-*a* containing phytoplankton in a measured volume of sample water are concentrated by filtering at low vacuum through a glass microfiber filter under low light conditions. The filter is then placed into a labeled centrifuge tube and frozen at -20° C. The pigments are extracted from the phytoplankton in 90% acetone with the aid of a mechanical tissue grinder. The filter slurry is allowed to steep for a minimum of two hours to ensure thorough extraction of the chl-*a*. The sample is centrifuged for 15 minutes at 675 x gravity. An aliquot of the supernatant is transferred to a glass cuvette and fluorescence is measured on the Turner Fluorometer. The concentration of chl-*a* in samples is determined from the calibrated instrument reading and from dilutions as necessary. The concentration of chl-*a* in the natural water sample is reported in μg/L. This SOP also includes chl-*a* standard preparation and calibration of the fluorometer.

3.3 Definitions

Laboratory Blank. An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, and surrogates that are used with samples. The laboratory blank is used to determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus.

Primary Calibration Standard. A solution containing an analyte that is prepared using a reference material traceable to EPA, the National Institute of Standards and Technology (NIST), or a source that will attest to the purity and authenticity of the reference material.

RO water. Water passed through a reverse osmosis system. See Section 7.1.3.

SSCS. Secondary Source Check Standard is a sample containing the analyte of interest at known concentrations. The SSCS is obtained from a source external to the laboratory or is prepared from standards obtained from a different source than the calibration standards. The purpose is to check laboratory performance using test materials prepared independently from the normal preparation process.

3.4 Interferences

After the filters are ground the samples are cloudy and filter particles are suspended in solution. The samples must be clarified by centrifugation prior to analysis to prevent interference.

Contamination that fluoresces in the red region of the light spectrum may interfere in the accurate measurement of chl-*a*. Interference by chlorophylls *b* and *c* are avoided by the use of the Turner Optical filter Kit 10-040. Thus, acidification of the sample followed by measuring fluorescence of the acidified sample is not necessary for this method.

Minimum sensitivity settings on the fluorometer should be avoided due to quenching effects in highly concentrated solutions. Dilutions should be performed instead. See also Section 9.6.

Fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. All standards and samples must be at the same relative temperature during analysis. Photosynthetic pigments are light and temperature sensitive. All work must be conducted in low light and samples must be stored in the dark at -20° C to prevent degradation.

3.5 Safety

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, the applicable Dynamac Hazardous Activity Safety Plans, and the Material Safety Data Sheets for the specific chemicals. Proper personal protection equipment (PPE) is worn to prevent exposure to organic solvents.

Measures need to be taken to minimize exposure to acetone fumes. Proper room ventilation and use of a chemical fume hood is required for all procedures involving acetone. Consult the MSDS for detailed safety and handling information for acetone

3.6 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.*

1. Model 10-AU Digital Turner Fluorometer equipped with: 1) 13-mm cuvette holder; 2) Optical Filter Kit PN 10-040, which includes: Blue lamp (PN 10-089); Emission Filter (PN 10-115); (436 nm) Excitation Filter (PN 10-113); (1 ND) Reference Filter (PN 10-032).
2. Centrifuge, capable of 675 x gravity
3. Tissue grinder, PowerGen 125
4. Whatman GF/F glass fiber filters
5. Centrifuge tubes, polypropylene, 50-mL capacity with screw caps
6. Tweezers or flat tipped forceps

7. Vacuum pump capable of maintaining a vacuum of 6 in. Hg (20 kPa)
8. Room thermometer
9. 13 x 100 mm Borosilicate glass culture tubes (10 mL)
10. 10-mL pipette and disposable tips
11. Graduated cylinders (various sizes as needed)
12. Safety glasses
13. Nitrile gloves
14. Lab coat
15. Laboratory exhaust fume hood
16. Analytical balance with resolution to 0.01 mg
17. Filter apparatus
18. 50-mL volumetric flasks
19. Explosion proof refrigerator
20. -20º C Freezer
21. Spectrophotometer , UV-visible

3.7 Reagents and Standards

3.7.1 Reagents

1. Acetone, reagent grade
2. Chlorophyll *a* free of chlorophyll *b*. (Sigma-Aldrich; P.O. Box 14508; St. Louis, MO 63178; 800-325-5832. C6144 from algae, C5753 from spinach. Purchased yearly.)
3. RO Water: Water passed through a reverse-osmosis system to produce water similar to ASTM Type I reagent with 16.7 megaohms resistivity (Reference 16.5).

3.7.2 Primary and Secondary Calibration Standard Preparation

> ***Caution**: Acetone is hazardous to your health and is a highly flammable material. Do not allow skin contact or inhale vapors. Acetone rapidly degrades PVC. Wear gloves constructed of resistant material and work in a chemical fume hood.*

1. Gently tap the contents of the chl-*a* standard ampoule/vial to the bottom of the ampoule/vial.
2. Break or open the ampoule/vial. Transfer 1-3 mL of 90% acetone to the ampoule/vial. Using a Pasteur pipette, carefully transfer the contents to a 50-mL volumetric flask. All glassware must be clean and acid-free before use. Thoroughly rinse the ampoule/vial (including sides) a second, then a third time with 90% acetone transferring the contents each time to the 50-ml volumetric flask.
3. Fill the volumetric flask to volume using 90% acetone.
4. Measure the chl-*a* primary standard using a UV-visible spectrophotometer. Verify the measured value of chl-*a* against its calculated concentration.

   After measuring absorbance at 750, 664, 647, and 630 nm, the concentration of chl-*a* in the 90% acetone standard may be calculated by the following equation, which corrects for absorbance at 750 nm (simplified from Reference 16.2):

   chl-*a* (mg/L) = 11.85 (Abs 664) – 1.54 (Abs 647) – 0.08 (Abs 630) – 13.31 (Abs 750)

   Remake the standard if the chl-a concentration is outside 5% of expected value. If this is not feasible, perform repeat measurements until a stock standard concentration can be confidently assigned.
5. Prepare the secondary source check standard (SSCS) in the same manner as above using an alternate source of chl-*a* (see Section 7.1.2).
6. Store all stock solutions in an explosion proof refrigerator at 4° C in a dark container.

3.7.3 Preparation of Working Standards

1. Prepare working standards of chl-*a* using the primary standard solution. The dilutions are prepared in 90% acetone to the final concentrations of 0, 2, 20, 50, 100 and 200 µL chl *a*/L.
2. Working standards are stored in an explosion proof refrigerator at 4° C in a dark container.

3.8 Sample collection, preservation and storage

1. Filtering should be performed in subdued light as soon as possible after sampling. The applied vacuum should be monitored with a gauge or manometer to ensure filtration pressure does not exceed 6 in. Hg (20 kPa). Higher filtration pressures may damage cells and result in a loss of chl-*a*.
2. Thoroughly shake the container to suspend particulates before the sample is poured for filtering. Accurately measure 500 mL of sample. Remove the filter from the base with tweezers, fold once and insert inside screw cap centrifuge tube. Cover the outside of the tube with foil for added protection from light.
3. Sample filters are stored frozen (-20° C) in the dark until extraction.
4. Samples can be stored up to 4 weeks before extracting (Arar and Collins, 1997).

3.9 Quality Control

1. Analytical Duplicate is a separate analysis from the same sample aliquot, run a minimum of once every 10 samples. The analytical duplicate is the same aliquot run near the end of the run.
2. Blank is a reagent blank taken from the 90% acetone solution and measured as a sample. A blank should be analyzed every 10 samples.
3. SSCS is mid-range in calibration and is measured every 10 samples. Recalibrate if outside 10% of the expected value.
4. Room temperature should be monitored and the instrument recalibrated if the room temperature varies ±3° C of the initial calibration.
5. Personnel performing this procedure are trained and must demonstrate their ability to handle standards in normal laboratory conditions without significant photodegradation. Stock solutions and working standards must routinely be transported through lighted hallways; during these times they should be well protected against the fluorescent lighting, which is particularly harmful to chlorophyll pigments. New analysts will demonstrate the ability to adequately light-proof standards before transporting them through lighted areas.

3.10 Calibration and Standardization

3.10.1 Calibration of the Fluorometer

1. Allow the fluorometer to warm up for at least 15 minutes.
2. Calibrate the fluorometer using the 50 µg chl-*a*/L primary calibration standard. Make sure to note the room temperature when recording calibration data.
3. Measure the fluorescence of each standard at the sensitivity setting that provides a midscale reading.
4. Record all measurements and analyze the linearity of the curve to ensure $r \geq 0.999$.
5. Calibration is performed before each analysis, or when there has been an adjustment made to the instrument such as replacement of lamps, filters or the photomultiplier. The

instrument should also be recalibrated if the room temperature fluctuates ±3° C from the initial calibration temperature.

3.10.2 Calibration Verification

1. Measure the 50 µL chl-*a*/L SSCS after calibration.
2. Record the SSCS measurement on the datasheet (Attachment 2).
3. Continue to measure the SSCS and a laboratory blank every 10 samples. If SSCS drifts outside ±10% recovery, recalibrate.

3.11 Procedure

3.11.1 Sample Preparation

1. Set up the filtration apparatus in the lowest light possible to prevent degradation of chl-*a* in the samples. The filter apparatus should be clean and acid-free.
2. Set-up a standard laboratory vacuum apparatus using a trap to collect excess/overflow water preventing aspiration into the vacuum system/pump.
3. Remove the samples from low-light containers and shake thoroughly to suspend the particulates. Carefully measure a 500-mL aliquot of the sample, and filter it through a Whatman GF/F glass microfiber filter. Vacuum filtration must not exceed 6 in. Hg (20 kPa). Higher vacuums may damage cells and result in loss of chl-*a*. **Important note:** Don't allow the filter to stay on the filter apparatus too long. The vacuum should be turned off at the valve when the sample is filtered to protect the chl-*a* in the sample.
4. Remove the filter using forceps, fold it in half, and place into a centrifuge tube labeled with an identification number. Centrifuge tubes should be placed in a lightproof secondary container before storage.
5. Store the filter in a freezer at -20° C for at least 24 hours, to lyse the cells and release the chl-*a* contained within them. The samples should be removed from the freezer and analyzed within approximately 25 days.

3.11.2 Extraction of Filter Samples

1. Remove the samples from the freezer. Maintain low light throughout the extraction.

> ***Caution****: Acetone is hazardous to your health and is a highly flammable material. Do not allow skin contact or inhale vapors. Acetone rapidly degrades PVC. Wear gloves constructed of resistant material and work in a chemical fume hood.*

2. Pipette 40 mL of 90% acetone into each centrifuge tube.
3. The filters are ground completely using the PowerGen 125 tissue homogenizer. Rinse the homogenizer thoroughly with RO water between samples to avoid cross-contamination. Small pieces of filter remaining on the homogenizer need not be transferred into the sample solution.
4. Vortex each tube to resuspend particulates within the solution.

5. Samples should be allowed to steep between 2-24 hours in an explosion proof refrigerator at 4° C.
6. After steeping is complete, vortex the sample again to resuspend particulates.
7. Centrifuge samples for 15 mins at 675 x gravity. Allow samples to come to ambient temperature before analysis. Check and record room temperature before analysis.

3.11.3 Sample Analysis

1. Allow the fluorometer to warm up for at least 15 minutes.
2. Use a 90% acetone solution to zero the instrument on the sensitivity setting that will be used for sample analysis.
3. Read SSCS in the mid-range of the calibration that is pertinent to the sample range.
4. Transfer 8 mL of the sample into a borosilicate culture tube. Care should be used not to disturb the solids at the bottom of the tube during the transfer.
5. Choose a sensitivity setting that yields a midscale reading when possible and avoid the minimum sensitivity setting. If the concentration of chl-*a* in the sample is too high to be measured, then dilute the sample to the appropriate midscale range using 90% acetone.
6. Record the fluorescence measurement and dilution data if applicable on the data sheet (Attachment 2).

3.12 Data Analysis and Calculations

1. To calculate the concentration factor for chl-*a*, enter measurements into spreadsheet template, which uses the equation:

   Concentration factor = extraction solution volume (mL)/sample volume (mL)

   Raw data are reported by electronic spreadsheet in µg/L, and this data is then used to calculate chl-*a* using the following calculations:

   (1) Cf/a = (extraction solution vol/filtered vol)*(total vol/area scraped)

   (2) Chl-*a* = raw data (µg/L) * Cf/a (mL/cm^2) * (1L/1000mL)

   Where: Extraction solution volume = 40 mL (unless noted)

   Filtered volume = volume of sample filtered (usually 25 mL)

   Total volume = volume of sample collected (usually 500 mL)

   Area scraped = (# of transects)×(area delimiter (cm^2))

   Cf/a = concentration factor per area (see above)
2. Duplicate precision is determined from analytical results.
3. Report results to three significant figures.

3.13 Method Performance

1. Method performance is measured through analysis of blank measurements and duplicate precision. Blank measurements should be $<$0.015 µg/L. These measurements

ensure accuracy at low-level samples. Duplicate precision must be within 10%. On occasion duplicates will fall outside this range; this variance is most likely a result of lack of homogeneity in the prefiltered sample solution. The possible reasons for imprecise duplicate measurements include lack of homogeneity, evaporation of acetone, and instrument drift, and should be noted on the datasheet. If duplicates are not in good agreement and there is no apparent reason or observable differences, the instrument should be recalibrated and samples reanalyzed.

3.14 Pollution Prevention

1. The chemicals used in this method pose little threat to the environment when properly managed.
2. All standards and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of waste. Wastes are collected for recycling or appropriate disposal.

3.15 Waste Management

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.
2. Contact the Dynamac Program 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.

3.16 References

Arar, E.J. and Collins, G.B. "Method 445.0: *In Vitro* Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Phytoplankton by Fluorescence", U.S. EPA methods for the determination of Chemical Substances in Marine and Estuarine Environmental Samples. 1997.

Arar, E. J. "Method 446.0, rev 1.2: *In Vitro* Determination of Chlorophylls a, b, $c_1 + c_2$ and Pheopigments in Marine and Freshwater Algae by Visible Spectrophotometry". U.S. Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory, Cincinnati, OH, 45268. 1997.

Welschmeyer, N.A. 1994. "Fluorometric Analysis of Chlorophyll *a* in the Presence of Chlorophyll *b* and Pheopigments", Limnology and Oceanography, 39:1985-1992.

"A Procedure for Measuring Extracted Chlorophyll *a* Free from the Errors Associated with Chlorophyll *b* and Pheopigments", Turner Designs Method. 2006.

ASTM. American Society for Testing and Materials. Standard Specifications for Reagent Water. D1193-77 (Reapproved 1983). Annual book of ASTM Standards, Vol. 11.01. ASTM: Philadelphia, Pennsylvania, 1991.

4.0 FISH COMMUNITY VOUCHER SPECIMENS

4.1 Scope of Application

This procedure is to be used to facilitate taxonomic identification of fish voucher specimens collected in rivers and streams as part of the EPA's National Rivers and Streams Assessment.

4.2 Summary of Method

Fish voucher specimens are submitted (after being preserved in formalin) for 10% of the sites for each field taxonomist. Identifications will be made using optical equipment where necessary to observe diagnostic characters. Dissection may be necessary to observe some characters (removal of pharyngeal teeth, abdominal incision to observe peritoneum or viscera, etc.).

Identification of a specimen as belonging to a taxon should be done by determination of the occurrence of characteristics diagnostic of that taxon. The definition of these diagnostic characteristics should be done by reference to the taxonomic literature. Taxonomic identification without use of keys or thorough comparative work may be done only by workers thoroughly familiar with all members of the group in the region under study.

4.3 Health and Safety Warnings

Standard laboratory protective clothing (lab coat, gloves) and eye covering is required. Refer to the chemical MSDS sheets for formalin and ethanol. When working with these potentially hazardous chemicals, avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs remove clothing immediately and wash the affected skin areas thoroughly with large amounts of soap and water. If inhalation, eye contact or ingestion occurs, consult the appropriate MSDS sheet for prompt action, and in all cases seek medical attention immediately.

4.4 Personnel Qualifications

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.

The instrument manager should be consulted for all instrument uses and procedures. Upon samples receipt, the laboratory must contact Marlys Cappaert at the Information Management Center by phone (541-754-4467) or fax to confirm samples have arrived. If expected samples do not arrive, labs must notify Ellen Tarquinio at EPA (202-566-2267).

4.5 Equipment and Supplies

- Dissecting microscope (magnification 3.5X to 12X)
- Keys or descriptions in up-to-date, regional faunal works (including online resources)
- Keys or descriptions in the most recent, accepted taxonomic revisions of individual groups
- Descriptions in other taxonomic studies, especially original descriptions.

4.6 Procedures

Specimens may be identified in any of several preservation media, including formalin, alcohol, and water (e.g., during water rinse between formalin and alcohol preservation).

Identifications will be made under well-lighted conditions, using optical equipment where necessary to observe diagnostic characters. For larger fish, magnification may not be needed. Smaller fish will usually require examination under a dissecting microscope (magnification 3.5X to 12X). Lighting conditions (reflected or transmitted) and specimen conditions (wet, dry) may be varied during identification to maximize the ability to observe different characters. Dissection may be necessary to observe some characters (removal of pharyngeal teeth, abdominal incision to observe peritoneum or viscera, etc.). Dissection should be done in such a way as to minimize damage to the specimen and loss of ability to determine other diagnostic characters. Field notes on color or other characters may be used to assist in identification.

Identification of a specimen as belonging to a taxon should be done by determination of the occurrence of characteristics diagnostic of that taxon. The definition of these diagnostic characteristics should be done by reference to the taxonomic literature, including one or more of the following sources:

- Keys or descriptions in up-to-date, regional faunal works (including online resources);
- Keys or descriptions in the most recent, accepted taxonomic revisions of individual groups;
- Descriptions in other taxonomic studies, especially original descriptions.

A summary of important faunal works for North American fishes is presented in Section 4.7. This list does not include all references which may be useful for identification. In particular, the list does not include journal publications or gray literature with species descriptions, distributional information or group revisions. Comparison with museum collections may be used, with the understanding that identifications may not be accurate in all cases.

Taxonomic identification without use of keys or thorough comparative work may be done only by workers thoroughly familiar with all members of the group in the region under study.

Identification of specimens should be confirmed by examination of a number of diagnostic characters and comparison of other characters noted in illustrations and published descriptions of a taxon with those of the specimen at hand. It should also be determined whether the collecting locality falls within the known range of the taxon, whether its size is within known size ranges, and whether it was collected from habitats appropriate to the species.

In some cases, specific identification may be possible only with mature males in breeding condition (e.g., on the basis of colors or tuberculation). Specific identification of other specimens on the basis of range or not fully diagnostic characters may be done where the ranges of the possible species are well-studied and determined to be allopatric.

<u>Difficult Determinations</u>

If any inconsistencies are noted in the confirmation of identification (conflicting diagnostic characters, deviation from illustration or descriptions, specimen out of geographical range, size range or in inappropriate habitat), the identification should be considered tentative until further work is done. The level of further work will depend on the requirements of the project plan and project resources. Further work may include:

- Reference to additional faunistic or taxonomic works, especially primary taxonomic descriptions and revisions;
- Reference to museum specimens;
- Reference to specialists in the group.

Unless explicitly specified otherwise in the project plan, characters will be those evident by examination using optical equipment of external characters and internal characters evident by partial dissection.

Factors influencing specific identification

Unless otherwise specified in the project plan, identifications will be to the lowest practical level. In most cases, this will be to the species. The following conditions will prevent a specific determination:

- State of preservation preventing observation of diagnostic characters;
- Loss of diagnostic characters prior to preservation;
- Inconsistencies among diagnostic characters or between diagnostic characters and other aspects of the specimen (other characters, size, range, habitat);
- Specimen of a size at which known diagnostic characters are not developed;
- Confusion of a taxon with hybrids of that taxon with others;
- Incomplete taxonomic or faunal knowledge about a group.

Reduction of Identification Effort

In some cases, a specific determination may be possible, but a higher level may be chosen to reduce identification time. These conditions should be specified in the project plan and noted in any reports of the identifications. These conditions may occur when:

- Large numbers of specimens are collected; either random subsampling or identification of larger specimens may be done, as noted in the project plan and/or project reports.
- Sampling is done in regions or of groups for which no comprehensive faunal or taxonomic works are available, so that identification would require major taxonomic or faunistic work.

Identification of subspecies

Where a specific identification is not possible, the identification may take several forms:

a) The specimen may be identified to the next higher taxonomic group (subgenus, genus, subfamily, family) to which it can be definitely identified. This is the usual form.

b) The specimen may be identified as belonging to one of a small number of species.

c) The specimen may be tentatively identified to a species; this is designated by using cf. before the specific name. This form may be used in the cases where an identification cannot be confirmed (see above) or where the taxa as currently defined may contain closely related undescribed species. It may also be used where a specimen fits the available descriptions of a species, but there is incomplete information on the existence or descriptions of other species.

d) The specimen may be identified as being "near to" some species. This may be used where a specimen is consistent with the description of one species within a group which is incompletely known.

e) Even though a specific determination cannot be made, it may be apparent that a collection or set of collections contains more than one species. These may be given arbitrary letter names to distinguish them (e.g., species A and B). Unless specifically noted otherwise, these distinctions are assumed to apply only to the specimens described within a single project report (e.g., if two reports from collections from two different years both contain identifications of species A and B from some genus, it is not assumed that species A is the same species in both reports, unless specifically stated).

f) Even if a positive determination cannot be made, the specimen may be identified to a species when that species is well represented by other individuals from the same collection or collecting areas, when no other similar species which are likely to be caught in the area. This type of identification should be clearly noted in project reports.

Reporting identifications and calculating numbers of species

In counting the number of species in a collection, a taxon higher than species may be counted as an additional species when no other member of that taxon is present within that collection. If more than one species are noted but not positively identified (see "e" above), each distinct species may be counted.

Identification will be of the following forms:

a) Scientific names (binomial, genus name, etc.); the name of the describer of the species is not typically used;

b) Common names which are of standard and unambiguous usage, e.g.; as defined by the latest version of the Checklist of common and scientific names of the American Fisheries Society and American Society of Ichthyologists and Herpetologists. Names in standard use for artificially-produced hybrids or strains may also be used; in any publications or technical reports, the scientific names associated with common names should be included at least once for each taxon (e.g., on first mention or in an accompanying table)

c) If names of local or specialized usage (e.g., regional hatchery strains) are used, field notes should contain information on the taxonomic identities of the groups, their diagnostic characters, and authorities for identification; publications should cite the source of information as a publication or personal communication.

Hybrids

Special care should be made to identify hybrids in groups known to hybridize frequently in nature (e.g., sunfishes) or for which artificial hybrids are stocked. Hybrids are typically detected by presence of diagnostic characters of two closely related species. Commonly-stocked hybrids, such as muskellunge x northern pike, and striped bass x white bass, are diagnosed in many keys. It should be recognized that some hybrids such as backcrosses (progeny of crosses of hybrids and a parent species) may not be distinguishable from the parent species on the basis of visual characters and would be identified as the parent species. Tentative identifications of ancestral species (e.g.; parents for F-1 crosses) may be made on the basis of characters present in different species or intermediate characters. Hybrids may be designated as "hybrid" or by listing the putative parental cross. In counting species richness in a collection, hybrids may be counted as additional species when neither of the presumed parental species (parental identification possible) or when none of the potential parental species (parental identification not possible) are also present in the collection.

Taxonomic controversy

There may be controversy in the scientific literature about the status of some taxa. These may include specific distinction or inclusion of allopatric forms or treatment of hybridizing forms as distinct or single species. For North American fishes, identification will be consistent with the most recent version of Common and Scientific Names of Species or a more recent publication. In the latter case, the citation should be noted in project reports.

4.7 Literature Cited

GENERAL REFERENCES AND TAXONOMIC GROUPS

Behnke, R.J. 1992. Native Trout of Western North America.American Fisheries Society Monograph 6. Bethesda. 275 pp.

Blair, W.R., A.P. Blair, P. Brodkorb, F.R. Cagle, and G.A. Moore. 1968. Vertebrates of the United States. Second Edition. McGraw-Hill. New York. 616 pp.

Hocutt, C.H., and E.O. Wiley (eds.). 1986. The zoogeography of North American Fishes. New York: John Wiley and Sons.

Kuehne, R.A., and R.W. Barbour. 1983. The American Darters. Lexington: Univ. Press of Kentucky. 177 pp.

Lee, D.S., C.R. Gilbert, C.H. Hocutt, R.E. Jenkins, D.E. McAllister and J.R. Stauffer, Jr. 1980. Atlas of North American freshwater fishes. North Carolina State Museum of Natural Hist. x + 854 pp.

Mayden, R.L. 1989. Phylogenetic Studies of North American Minnows, with Emphasis on the Genus *Cyrpinella* (Teleostei: Cypriniformes). Misc. Publ. 80. Univ. Kansas Museum of Natural History.

Nelson, J. S., E. J. Crossman, H. Espinosa-Perez, L. T. Findley, C. R. Gilbert, R.N. Lea, and J. D. Williams. 2004. Common and Scientific Names of Fishes from the United States, Canada, and Mexico. 6th ed. American Fisheries Special Publication No. 29. 386 pp.

Page. L.M., and B.M. Burr. 1991. A Field Guide to Freshwater Fishes. A Peterson Field Guide to fishes of North America north of Mexico. 432 pp.

Scott, W.B., and E.J. Crossman. 1973. Freshwater fishes of Canada. Bull. Fish. Res. Bd. Can. 184. 966 pp.

Taylor, W.R. 1969. A revision of the catfish genus *Noturus* Rafinesque with an analysis of higher groups of Ictaluridae. Bull 282. Smithsonian Institution, U.S. National Museum.

FISH IDS EAST

Cooper, E. L. 1983. Fishes of Pennsylvania and the Northeastern United States. Pennsylvania State Univ. Press. 243 pp.

Everhart, W.H. Fishes of Maine. Maine Dept Inland Fisheries and Wildlife. Augusta. 96 pp.

Loos, J.J., W.S. Woolcott , N. R. Foster. An Ecologist=s Guide to the Minnows of the Freshwater Drainage Systems of the Chesapeake Bay Area. ASB Bulletin Vol. 19, No.3, July 1972

Raasch, M.S. 2007. Delaware's Freshwater and Brackish Water Fishes 179 pp.

Rohde, F.C., F.G. Arndt, D.G. Lindquist, and J.F. Parnell. 1994. Freshwater fishes of the Carolinas, Virginia, Maryland and Delaware. University of North Carolina Press. Chapel Hill. 222 pp.

Scarola, John F., 1973. Freshwater Fishes of New Hampshire. New Hampshire Fish and Game Department. 131 pp.

Scott, W.G. 1968. Freshwater Fishes of Eastern Canada. 2nd ed. Toronto: Univ. of Toronto Press. 137 pp.

Smith, C.L. 1985. The Inland Fishes of New York. New York State Dept. of Environmental Conservation. 552 pp.

Stauffer Jr., Jay R., J. M. Boltz and L. R. White. The Fishes of West Virginia. 1995.The Proceedings of the Academy of Natural Sciences of Philadelphia 146: 1-389.

Trautman, M.B. 1981. The Fishes of Ohio with Illustrated Keys. Revised edition. Ohio: Ohio State Univ. Press. 782 pp.

Whitworth, W.R., P.L. Berrian, and W.T. Keller. 1968. Freshwater Fishes of Connecticut. State Geol. and Nat. Hist. Surv. of Conn. Bull. 101. 134 pp.

FISH IDS SOUTHEAST AND CENTRAL

Bennett, D.H., and R.W. McFarlane. 1983. Fishes of the Savannah River Plant. Savannah River Ecological Laboratory Publication. 152 pp.

Clay, W.M. 1975. The Fishes of Kentucky. Kentucky Dept. Fish and Wildlife Resources. Frankfort, KY. 416 pp.

Etnier, David A. and W. C. Starnes. 1993. The Fishes of Tennessee. The University of Tennessee Press. Knoxville. 681 pp.

Gilbert C.R.1992. Rare and Endangered Biota of Florida, Volume II. Fishes. University Press of Florida. 247pp.

Marcey, Barton C. et. al. 2005. Fishes of the Middle Savannah River Basin: with emphasis on the Savannah River site. The University of Georgia Press. 460 pp.

McEachran, J. D., and J.D. Fechhelm.1998. Fishes of The Gulf of Mexico, Vol.1. Univ. of Texas Press. 1112pp.

Menhinick, Edward F., 1991. The Freshwater Fishes of North Carolina. North Carolina Wildlife Resources Commission. Raleigh, North Carolina. 227 pp.

Stevenson, H.M. 1976. Vertebrates of Florida. University Presses of Florida. Gainesville. 607 pp.

FISH IDS MID-WEST

Bailey, Reeve M., and M. O. Allum.1962. Fishes of South Dakota. Museum of Zoology, Univeristy of Michigan, No. 119.

Becker, G,C. 1983. Fishes of Wisconsin. Univ. Wisconsin Press. 1058 pp.

Clay, W.M. 1975.The Fishes of Kentucky. Kentucky Dept.of Fish and Wildlife Resources. 416 pp.

Cross, F.B. 1967. Handbook of Fishes of Kansas. Misc. Publ. Univ. Kansas Mus. Nat. Hist. 45. 357 pp.

Harlan, James R., and E. B. Speaker. 1951. Iowa Fish and Fishing. State of Iowa. 2nd ed. 238 pp.

Hubbs, C.L., and K.F. Lagler. 1964. Fishes of the Great Lakes Region, with a new Preface. Univ. Mich. Press, Ann Arbor. 213 pp.

Phillips, Gary L., W. D. Schmid and J. C. Underhill. Fishes of the Minnesota Region. University of Minnesota Press. Minneapolis. 1982.248 pp.

Phlieger, W.L. 1975. The Fishes of Missouri. Missouri Department of Conservation. 343 pp.

Smith, P.W. 1979. The fishes of Illinois. Univ. Ill. Press. Urbana. 314 pp.

FISH IDS SOUTH

Boschung Jr., Herbert T., and R. L. Mayden. 2004. Fishes of Alabama. Smithsonian Institution. 736 pp.

Douglas, N.H. 1974. Freshwater fishes of Louisiana. Claitor=s Publishing Division. Baton Rouge, La. 443pp.

Hoese, H.D., and R.H. Moore 1977. Fishes of the Gulf of Mexico, Texas, Louisiana, and adjacent waters. Texas A. and M. University Press. College Station. 327 pp.

Hubbs, Clark., R. J. Edwards and G. P. Garrett. An Annotated Checklist of the Freshwater Fishes of Texas, with Keys to Identification of Species. The Texas Journal of Science, Suppl., Vol. 43, No. 4, 1991.

Mettee, Maurice F., P. E. O'Neil and J. M. Pierson. 1996. Fishes of Alabama and the Mobile Basin. Osmoor House, Inc. Alabama. 820 pp.

Miller, R.J., and H.W. Robison. 1973. The Fishes of Oklahoma. Oklahoma State Univ. Press. Stillwater. 246 pp.

Robinson, H.W., and T.M. Buchanan. 1988. Fishes of Arkansas. Fayetteville: Univ. Arkansas Press. 536 pp.

Ross, Stephen T. 2001.Inland Fishes of Mississippi. University Press of Mississippi.. 624 pp.

Smith-Vaniz, W.F. 1968. Freshwater fishes of Alabama. Auburn University, The Paragon Press. 211pp.

FISH IDS WEST

Baxter George T., and J. R. Simon. 1970. Wyoming Fishes. Wyoming Game and Fish Department. Cheyenne, Wyoming. Bulletin No. 4. 168 pp.

Brown, C. J. D. 1971. Fishes of Montana. Montana State University. Bozeman, Montana. 207 pp.

Koster, William J. 1957. Guide to the Fishes of New Mexico. University of New Mexico Press. 116 pp.

Minckley, W. L. 1973. Fishes of Arizona. Arizona Game and Fish Department. 293 pp.

Rivers, I.L. 1994. Fishes and Fisheries of Nevada. University of Nevada Press. Reno. 782 pp.

Sigler, William F., and R. R. Miller. 1963. Fishes of Utah. Utah State Department of Fish and Game. 263 pp.

Simpson, J. and R. Wallace. 1978. Fishes of Idaho. University Press of Idaho. Moscow. 237 pp.

Wydoski, R.S. and R.R. Whitney.1979. Inland Fishes of Washington. University of Washington Press. Seattle. 220 pp.

5.0 FISH TISSUE PREPARATION

5.1 Scope and Applicability

This Standard Operating Procedure (SOP) must be followed by all fish tissue sample preparation labs involved with the USEPA Office of Water's (OW) National Rivers and Stream Assessment (NRSA). Two laboratories have been designated to prepare fish tissue samples for the NRSA: Great Lakes Environmental Center (GLEC) in Traverse City, MI and EPA's National Exposure Research Laboratory in Cincinnati, OH (NERL-Cin). Adherence to this SOP by both labs will ensure that fish tissue sample preparation, homogenization, and distribution activities are standardized and reproducible.

5.2 Summary of Method

Two fish tissue sample preparation procedures will be detailed. The first procedure is for the preparation of fish fillet homogenates in which samples of 3-5 specimens of a single species of predator/gamefish are filleted and homogenized into fillet composite samples. The second procedure is for the preparation of whole fish homogenates in which composite samples of either small, short-lived species or large, longer-lived species are homogenized as whole fish into whole fish composite samples.

Portioning and distribution for both types of resulting homogenates (fillet and whole), as well as disposal of the offal from the fillet procedure, will also be detailed.

A quality assurance pilot study is described in Attachment 3. Completion of this study is required for all labs processing fish tissue for the NRSA.

5.3 Definitions/Acronyms

ECO:	composite samples for ecological applications which are homogenized as whole fish; sample being composites of either small, short-lived species or larger, long-lived species; composites consist of specimens of a single species
EMAP:	Environmental Monitoring and Assessment Program
GLEC:	Great Lakes Environmental Center, Traverse City, MI
LM:	lower Mississippi
NERL-Cin:	National Exposure Research Lab in Cincinnati, OH (division: EERD, branch: MIRB)
NRSA:	National Rivers and Streams Assessment
OW:	Office of Water
PBDEs:	polybrominated diphenyl ethers
RSD:	relative standard deviation, expressed as a percent value and calculated as: (standard deviation/average value) x 100

5.4 Health and Safety Warnings

Lab personnel must be trained in and follow standard laboratory safety practices such as wearing personal protective equipment (e.g., lab coats, safety glasses), safe handling of solvents and chemicals, and proper waste disposal. Fish tissue filleting and grinding equipment present additional physical hazards not normally encountered in labs. Therefore, lab personnel performing the procedures in this SOP must have additional training in the proper use of this processing equipment. A detailed description of laboratory safety equipment and procedures is beyond the scope of this SOP.

5.5 Cautions/Interferences

Care must be taken to thoroughly clean filleting and grinding equipment in between composite samples. Care must also be taken to thoroughly homogenize the fish tissue to ensure accurate analysis of the homogenates. Completion of the quality assurance pilot study detailed in Attachment 3 will be required for any lab processing fish samples for the NRSA prior to processing field samples. The pilot study was designed to demonstrate a lab's capability to prevent cross contamination during sample preparation and to prepare tissue samples with uniform homogenization.

5.6 Personnel Qualifications and Responsibilities; Contact List

This SOP may be used by any laboratory authorized by the USEPA to process fish tissue for the NRSA. Personnel listed in Table 5-1 are responsible for coordinating tissue/extract shipment and receiving.

Table 5-1: Project Contact List

Fish Tissue Sample Managers	
EPA Fish Tissue Sample Manager Leanne Stahl OW/Office of Science and Technology USEPA (4305T) 1200 Pennsylvania Ave., N.W. Washington, DC 20460 202-566-0404 stahl.leanne@epa.gov Leanne Stahl is the contact for any questions on fish composite samples from all sites that will be processed as fillets. Questions about the samples (shipping conditions, whether or not a sample is "routine" as defined later in this SOP, etc.) should be directed to her.	NHEERL-Dul NRSA Fish Tissue Manager Terri Jicha NHEERL/Mid-Continent Ecology Division USEPA Office of Research and Development 6201 Congdon Blvd. Duluth, MN 55804 218-529-5153 jicha.terri@epa.gov Terri Jicha is the contact for any questions on whole fish composite samples from the LM sites. Questions about the samples (shipping conditions, whether or not a sample is "routine" as defined later in this SOP, etc.) should be addressed to her.
NERL-Cin Lab Fish Tissue Coordinators	
Gerilyn Ahlers, Dynamac Corp. 513-569-7011 ahlers.gerilyn@epa.gov Gerilyn Ahlers coordinates sample receipt and preservation for the NERL-Cin lab. Address for NERL-Cin lab: NERL/Ecological Exposure Research Division USEPA Office of Research and Development 26 W. ML King Dr. Cincinnati, OH 45268	Jim Lazorchak 513-569-7076 lazorchak.jim@epa.gov Jim Lazorchak is the Branch Chief for the NERL-Cin lab. Dan Bender 513-569-7351 bender.dan@epa.gov Dan Bender is the Work Assignment Manager for the Dynamac contract performing the sample preparation and analysis at NERL-Cin.
GLEC NRSA Fish Tissue Coordinator	***Fish Tissue Contract Coordinator***
Dennis McCauley Great Lakes Environmental Center 739 Hastings Street Traverse City, MI 49686 231-941-2230 dmccauley@glec-tc.com	Blaine Snyder Tetra Tech, Inc. Center for Ecological Sciences 400 Red Brook Boulevard, Suite 200Owings Mills, MD 21117 410-356-8993 blaine.snyder@tetratech.com

5.7 Equipment and Supplies

- Laboratory logsheet (or equivalent)
- Top-loading balances (gram scale for weighing whole fish and 0.1 g scale for weighing tissue aliquots)
- Clean, powder-free nitrile gloves
- Reagent water (contaminant-free, distilled, deionized)
- Detergent solution (phosphate-free and scent-free)
- HDPE wash bottles (do not use Teflon® wash bottles)
- HNO3 (5% solution)

- Fillet knives (stainless steel)
- Glass cutting boards
- Stainless steel bowls (for tissue homogenization)
- High-speed, size-appropriate blender(s) (glass/stainless container and stainless steel blades)
- Hobart® food grinder
- Jar labels
- Black ballpoint pens and/or waterproof markers
- Wide clear tape (for securing jar labels)
- Laboratory freezer (capable of maintaining -20° C temperature)
- Acetone, Optima grade
- Hexane, Optima grade
- Methanol, Optima grade
- Food-grade plastic bags
- Aluminum foil
- Sample containers:
- Trace organics clean glass containers (assorted sizes with Teflon or foil-lined lids, detailed in Section 5.12 tables)

5.8 Sample Collection and Shipment

5.8.1 Sampling sites

Fish composite samples will be taken from several types of NRSA fish tissue indicator sites: nationally representative non-urban sites (~750, called “NRSA non-urban” sites in this SOP), , targeted reference sites (~200), and Lower Mississippi (LM) statistically selected non-urban sites (~44, called “LM non-urban” sites in this SOP).

5.8.2 Sample types: fillet composites

Field crews will collect one predator/gamefish (e.g., bass, walleye, pike, crappie, perch, or trout) composite from each of the NRSA fish tissue indicator sites. These fish composites are called “fillet composites” elsewhere in this SOP because they must be filleted prior to homogenization using the procedure in Section 5.10, in contrast to the ECO samples (Section 5.8.3) which are homogenized whole.

Each set of fish in the predator/gamefish composites consist of five individual adult fish of a single species. The length of the smallest specimen in the composite should be at least 75% of the length of the largest specimen in the composite (the “75% rule”). Paperwork that identifies the species, number, and length of fish in each composite should accompany the samples. NOTE that some of the fillet composite samples shipped to the labs may contain greater or less

than five individuals. They may also not meet the EPA's length requirements (the "75% rule"). These non-routine samples should be stored as intact whole fish until receipt of EPA/OW processing instructions from the EPA Fish Tissue Manager.

5.8.3 Sample types: ECO samples

Field teams also collected two additional composite samples from all of the LM River sampling locations which will be analyzed for ecological (ECO) applications. These "ECO samples" consist of a set of small-sized, short-lived species (i.e., small adults with a life span of 1 or 2 years) and a large-sized, longer-lived species (i.e., large adult fish with a life span of 3 to 5 years). ECO samples will be homogenized whole using the procedure in Section 5.11.

Each set of fish in the ECO composites, in both short-and long-lived species sets, will consist of five individual adult fish of a single species. These ECO composite samples are also subject to the 75% rule as defined in the section above. Paperwork that identifies the species, number, and length of fish in each composite should accompany the samples. Individual specimens should be <2 kg in weight. Samples that do not appear as expected should be stored as intact whole fish until receipt of processing instructions from the NHEERL-Dul NRSA Fish Tissue Manager. EPA's National Health and Environmental Effect Research Laboratory in Duluth, MN (NHEERL-Dul) is responsible for coordination of the LM River ECO sample preparation and analysis with NERL-Cin.

5.8.4 Sample Shipment and Analyte List

Samples will be shipped from the field for processing to either NERL-Cin or GLEC. The approximate number of samples and the analyte list for the types of samples can be seen in Table 5-2.

Table 5-2: Approximate Number of Samples, Destination, Analyte List

	Shipped from the field to NERL-Cin				Shipped from the field to GLEC		
Approximate # of sites and samples:				LM Non-urban **ECO** (44x2)*	NRSA Non-urban (~673)	LM Non-urban (~44)	Reference (200)
EMAP analyte list, extracted and analyzed by NERL-Cin: 1. Mercury 2. Selenium (OW request) 3. (22) Organochloride pesticides 4. (21) PCB congeners 5. (8) PBDE congeners 6. %Lipids 7. %Moisture				X	X	X	X
*ECO samples come in pairs from each site, thus the multiplication of number of sites by 2. Samples not labeled "ECO" are whole fish composites that need to be filleted prior to homogenization and come one set per site.							

5.9 Sample Handling and Preservation

Each sample preparation lab (prep lab) is responsible for receiving, temporarily storing (at < minus 20°C), preparing, homogenizing, and distributing the fish tissue samples. As fish composite samples are received, have the sample custodian:

1. Check that each shipping container has arrived undamaged and verify that samples are still frozen and in good condition. If fish are not able to be processed immediately upon receipt, they must be stored frozen at < -20°C until they are processed. Place a list on the outside of the storage container or freezer with the sample identification numbers to facilitate retrieval of fish composite samples for future sample preparation. Non-routine fish composite samples (i.e., samples containing specimens that do not meet the length criteria or samples with fish numbers different from five) must also be stored frozen at -20°C pending receipt of EPA/OW instructions for processing. Samples should be shipped on dry ice; however, it is possible that one or more shipments will be received on wet or blue ice. If this occurs, the sample custodian must contact one of the fish tissue managers as specified below.
2. Verify that all associated paperwork is complete, legible, and accurate. Paperwork that identifies the species, number, and length of fish in each composite should accompany the fish samples.
3. Verify that all specimens listed on the sample tracking form for each composite were included in the shipment and are properly wrapped and labeled.
4. The sample custodian must notify one of the fish tissue sample managers for guidance if there are problems with the samples. Samples must be kept frozen at <-20°C while awaiting guidance. Notification must be done in writing (e.g., by e-mail) and as soon as possible following sample receipt and inspection. The EPA Fish Tissue Sample Manager will use these reports to initiate corrective actions by survey participants to

prevent future problems, so it is important that reports about problems be thorough and provided in a timely manner.

Problems involving sample integrity, conformity, or custody inconsistencies for all fish tissue samples which would require notification and further guidance include:

1. Sample integrity: improperly wrapped, samples shipped in anything other than dry ice, not frozen solidly, not matching information on paperwork, etc.
2. Incomplete or illegible paperwork: paperwork should have all the information needed to determine if the samples follow criteria set in Sections 5.8.2 and 5.8.3, such as species, number and length of fish, etc. Paperwork should also include site information.
3. Samples with any deviation from sample criteria as defined in Sections 5.8.2 and 5.8.3: more than one species, more or less than 5 fish, violations of the 75% rule, etc.

If there are any problems or irregularities associated with fillet composite samples, the EPA Fish Tissue Sample Manager in OW must be notified, see Table 5-1 for the contact information. For samples that deviate from the sample criteria, thus would be considered non-routine, instructions to the lab from the EPA Fish Tissue Manager may direct the lab to process all of the specimens in the non-routine composite (e.g., samples containing fewer than 5 fish, but all specimens meet the 75% rule); to process a subset of the specimens in the non-routine composite (e.g., specimens that do not meet the 75% length rule may be eliminated); or to discard the entire non-routine composite (e.g., fields teams collected an inappropriate species).

When the field data forms for the fish tissue samples become available, the EPA Fish Tissue Sample Manager will determine whether each composite is routine or non-routine. Any composite with deviations in species, number, or length criteria are considered non-routine. The EPA Fish Tissue Sample Manager will develop and distribute spreadsheets that identify each fish composite as routine or non-routine. Use the site identification number to check whether a composite is listed as routine or non-routine in these spreadsheets. Routine composites can be processed immediately. Hold all non-routine composites in the freezer at -20°C pending receipt of processing instructions from the EPA Fish Tissue Sample Manager.

If there are any problems or irregularities associated with the ECO samples, the NHEERL-Dul NRSA Fish Tissue Manager must be notified, see Table 5-1 for contact information.

For bulk homogenates received from GLEC, NERL-Cin will:

1. Check that containers are undamaged and that each sample is still frozen and in good condition.
2. Verify that information on the chain-of-custody form and sample containers labels is complete, legible, and accurate; and that all sample containers listed on the chain-of-custody form are included in the shipment. Document receipt of sample containers on the laboratory project logsheets.
3. Notify the EPA Fish Tissue Manager in OW (see Table 5-1 for contact information) immediately of any problems encountered with sample shipment or with accompanying paperwork.

All homogenates must be kept frozen, at <-20°C, until they are extracted or analyzed. Bulk homogenates that are shipped to another lab must be shipped frozen.

For Rinsate samples received from GLEC, NERL-Cin will:

1. Check that containers are undamaged and that each sample is in good condition.
2. Verify that information on the chain-of-custody form and sample containers labels is complete, legible, and accurate; and that all sample containers listed on the chain-of-custody form are included in the shipment. Document receipt of sample containers on the laboratory project logsheets.
3. Notify the EPA Fish Tissue Manager in OW (see Table 5-1 for contact information) immediately of any problems encountered with sample shipment or with accompanying paperwork.

5.10 Fish Tissue Preparation: Fillet Procedure

All fillet samples, collected at NRSA sites as well as LM River sites, must be prepared using this procedure. For non-routine composites, only designated specimens (identified by specimen number) will be filleted before homogenization.

To control contamination, separate sets of utensils and cutting boards should be used for scaling fish and for filleting fish. Prior to preparing each composite sample, thoroughly clean utensils and cutting boards using the following series of procedures:

1. Wash with a detergent solution (phosphate- and scent-free) and warm tap water
2. Rinse three times with warm tap water
3. Rinse three times with deionized (DI) water
4. Rinse with acetone
5. Rinse three times with DI water
6. Rinse with (not soak in) 5% nitric acid
7. Rinse three times with DI water

Put on powder-free nitrile gloves before unpacking individual fish specimens for filleting and tissue homogenization. As samples are unpacked and unwrapped, inspect each fish carefully to verify that it has not been damaged during collection or shipment. If damage (e.g., tearing of the skin or puncturing of the gut) is observed, document it in the laboratory logsheet and notify the EPA Fish Tissue Manager in OW.

Weigh each fish to the nearest gram prior to any sample processing. Enter weight information for each individual fish into a laboratory logsheet. Individual specimen weights eventually will be transferred to spreadsheets for submission to EPA/OW and the EPA data repository in Corvallis, OR.

Rinse each fish with DI water as a precautionary measure to treat for possible contamination from sample handling in the field. Use HPDE wash bottles for rinsing fish and for cleaning homogenization equipment and utensils. Do not use Teflon® wash bottles for these procedures.

Fish with scales must be scaled (and any adhering slime should be removed) prior to filleting. Wearing powder-free nitrile gloves, scale each fish by laying it flat (on a clean glass cutting board) and scraping from the tail to the head using the blade edge of a clean stainless steel knife. Control cross-contamination by rinsing the cutting board and knife with deionized water between fish. Filleting can proceed after all the scales have been removed from the skin and a separate clean cutting board and fillet knife are prepared or available.

Place each fish on a glass cutting board in preparation for the filleting process. Note that filleting should be conducted under the supervision of an experienced fisheries biologist, if possible. Ideally, fish should be filleted while ice crystals are still present in the muscle tissue. Fish should be thawed only to the point where it becomes possible to make an incision into the flesh. Remove both fillets (lateral muscle with skin attached) from each fish specimen using clean, high-quality stainless steel knives. Include the belly flap (ventral muscle and skin) with each fillet. Avoid contaminating fillet tissues with material released from inadvertent puncture of internal organs. In the event that an internal organ is punctured, rinse the fillet with DI water immediately after filleting. Note the puncturing on the lab logsheet. Bones still present in the tissue after filleting should be carefully removed.

Determine the collective weights of the following tissue fractions prior to any further sample processing:

1. Measure the collective weight of the fillets in each composite to the nearest gram (wet weight) and record the fillet composite weight on a laboratory project logsheet.
2. Measure the collective weight of the carcasses or offal to the nearest gram (wet weight) and record the carcass composite weight on a laboratory project logsheet. Wrap composited carcasses in aluminum foil and place in food-grade plastic bags, seal and store at <-20°C for long-term storage.
3. Fillet composite weights and carcass composite weights will be transferred to spreadsheets for submission to EPA/OW and the EPA data repository in Corvallis, Oregon. Recording these weights will facilitate future comparisons of contaminant levels measured in both tissue fractions.

Samples should be homogenized partially frozen for ease of handling. Composite fillets using the “batch” method, in which all of the individual specimens that compromise the sample are homogenized together, regardless of each individual specimen’s proportion to one another (as opposed to the “individual” method, in which equal weights of each specimen are added together).

Process each sample using a size-appropriate homogenization apparatus (e.g., automatic grinder or high-speed blender). Entire fillets (with skin and belly flap) from both sides of each fish must be homogenized and the entire homogenized volume of all fish fillets from the composite must be used to prepare the homogenate composite. Mix the tissues thoroughly until they are completely homogenized as evidenced by a final composite sample that consists of a fine paste of uniform color and texture. Chunks of skin or tissue will hinder extraction and digestion and, consequently, are not acceptable. Grinding of tissue may be easier when tissues are partly frozen. Chilling the grinder briefly with a few chips of dry ice may also help keep the tissue from sticking to the equipment. Once the whole sample is initially blended, the accumulated mass of tissue must be mixed together thoroughly to provide one completely

homogenous sample. Be sure that the bowl is an adequate size to hold the full volume of homogenized tissue and to allow space for final mixing of the tissue.

Verify the uniformity of homogenization and the continued absence of equipment contamination as specified in Section 5.13 of this SOP.

Once the tissue is homogenized and composited, measure and record the final weight to the nearest gram (wet weight) and record the composite weight on a laboratory project logsheet.

Proceed to Section 5.12 for tissue aliquoting requirements.

5.11 Fish Tissue Preparation: Whole Fish Homogenization

Two types of whole fish (ECO) samples will be received by NERL-Cin from all LM River sites:

- one comprised of small-sized, short-lived species (i.e., small adults with a 1-2 year life span), and
- one comprised of a large-sized, longer-lived species (i.e., larger adults with a 3-5 year life span

For each of these composites, the entire fish (head, tail, and skin) will be prepared as a whole fish composite.

Prior to preparing each composite sample, thoroughly clean utensils and cutting boards using the following series of procedures:

1. Wash with a detergent solution and rinse three times with warm tap water
2. Rinse three times with deionized (DI) water
3. Rinse with acetone
4. Rinse three times with DI water
5. Rinse with (not soak in) 5% nitric acid
6. Rinse three times with DI water

Put on powder-free nitrile gloves before unpacking individual fish specimens for filleting and tissue homogenization. As samples are unpacked and unwrapped, inspect each fish to verify that it has not been damaged during collection or shipment. Note major damage, such as missing chunks of tissue, on the laboratory logsheet and notify the NHEER-Dul NRSA Fish Tissue Manager (see Table 5-1 for contact info).

Collectively weigh all fish in the composite to the nearest gram (wet weight) prior to homogenization. Enter the total composite weight into a laboratory logsheet.

Rinse each fish with DI water as a precautionary measure to treat for possible contamination from sample handling in the field. Use HPDE wash bottles for rinsing fish and for cleaning homogenization equipment and utensils. Do not use Teflon® wash bottles for these procedures.

Chopping/grinding of small fish: Very small fish (e.g., minnows) may be effectively homogenized without using a blender. Place the fish on a cutting board and chop them using a meat cleaver. The small fish do not need to be separated before chopping. Chop in both horizontal and vertical directions until there are no visible eyeballs, skin or bones. Flip the sample over using the meat cleaver and chop the same way on the other side. Continue until the sample resembles a paste. If the sample does not appear to be completely homogenized, place it in a small blender to finish processing. Mix the tissues thoroughly until completely homogenized as evidenced by a final composite that consists of a fine paste of uniform color and texture.

Chopping/grinding of medium to large fish: Medium and large fish need to be separated and cut into chunks for processing. For samples containing a lot of bones, serrated knives work best for cutting. Choose the appropriate blender for homogenization based on specimen size. For excessively large fish that need to be homogenized with the Hobart® food grinder, be sure the

chunks are small enough to fit through the grinder opening. Assemble the food grinder (refer to manual) and slowly add fish chunks until the sample becomes visible from the entry point at the top of the grinder. At this point, the machine needs to be stopped so the homogenate can be emptied into the sample containers. The skin and bones which build up on the filter will need to be scraped off and rerun though the grinder. Mix the tissues thoroughly until they are completely homogenized as evidenced by a final composite sample that consists of a find paste of uniform color and texture. Chunks of skin or tissue will hinder extraction and digestion and consequently are not acceptable. Grinding of tissue may be easier when tissues are partially frozen. Chilling the grinder briefly with a few chips of dry ice may also keep the tissue from sticking to the equipment.

Verify the uniformity of homogenization and the continued absence of equipment contamination as specified in Sections 5.13 of this SOP.

Once the tissue is homogenized and composited, measure and record the final weight of the composite.

Proceed to Section 5.12 for tissue aliquoting requirements.

5.12 Aliquoting and Distributing Homogenates

Aliquots are to be stored in trace organics clean glassware. Prep labs will use foil-lined lids for jars containing archive tissue aliquots. The labs can use Teflon®-lined lids for jars containing all other types of tissue aliquots. Size and lid specifications are detailed below in the tables in this section. When filling jars, leave sufficient space to allow for expansion of the tissue when it is frozen. In no case should jars be filled beyond 80% capacity as this may result in breakage on freezing. Wipe off the outside of the jars to remove any tissue residue or moisture. Pre-print or fill out a label for each container using a waterproof marker. Include the site identification number (e.g., OH038), sample identification number (e.g., 528799), aliquot type (e.g., bulk homogenate, archived sample, , etc.), collection date (e.g., mm/dd/yyyy), and lab name (i.e., GLEC or NERL-Cin) on each label. Affix the label to the jar using clear, wide tape if not using a label designed for freezers. Place each jar inside two food-grade plastic bags to avoid sample loss due to breakage. Freeze the tissue aliquots at -20^{o}C before shipping the samples. NERL-Cin has been designated as the sample repository for the archive tissue storage and will freeze these tissue aliquots at -20°C for long-term storage.

Table 5-3: Fillet Composite Aliquot Requirements for GLEC Prep Lab (non-urban sites)

Site Type and Approx. Number of Samples	Analyte Group	Minimum Tissue Mass Requirements	Container Specifications	Sample Recipient
NRSA non-urban (~750) LM non-urban (44) Reference (200)	Bulk homogenate for EMAP analyte list*	100 to 300g	16 oz straight-sided amber glass jar with Teflon®-lined lid	NERL-Cin
	Archive tissue sample	300g to all remaining mass	32 oz straight-sided clear glass jar with foil-lined lid#	
*Detailed analyte lists can be found in Table 5-2 # Archive samples may require more than one container and should be labeled with the container number and total number of containers (e.g., 1 of 3, 2 of 3, etc.).				

GLEC fillet composites:
GLEC will prepare two aliquots per homogenized fillet composite (one for sample analysis and one for archiving) for samples from each of the NRSA non-urban, LM River non-urban, and reference sites. See Table 5-3 for aliquot requirements. GLEC will ship the aliquoted, homogenized samples following the shipping instructions in Section 5.12.

In the event that insufficient tissue mass exists to prepare the required number of aliquots, NERL-Cin will use the following order of priority for preparation of fillet composite tissue aliquots:

1. From the non-urban and reference sites (tissues received from GLEC), NERL-Cin will analyze the fish tissue for the EMAP analyte list in the following priority order: mercury, selenium, PBDEs, PCBs, and pesticides. NERL-Cin will communicate these decisions to the EPA Fish Tissue Sample Manager.

NERL-Cin whole fish (ECO) composites:
ECO samples will be processed by NERL-Cin and aliquoted as per the requirements in the table below. Two samples per site (one each of short-lived and longer-lived species) will result in double the number of samples per site as compared to the fillet samples.

Table 5-5: ECO Composite Aliquot Requirements for NERL-CI

Table 12-3: ECO Composite Aliquot Requirements for NERL-CI				
Site Type and Approx. Number of Samples	**Analyte Group**	**Minimum Tissue Mass Requirements**	**Container Specifications**	**Sample Recipient**
LM non-urban (~44 sites x 2) LM urban (~15 x 2)	Bulk homogenate for EMAP analyte list*	100 to 300g	16 oz straight-sided amber glass jar with Teflon®-lined lid	NERL-Cin will process and analyze these samples
	Archive tissue sample	300g to all remaining mass	32 oz straight-sided clear glass jar with foil-lined lid#	
*Detailed analyte lists can be found in Table 8-1 # Archive samples may require more than one container and should be labeled with the container number and total number of containers (e.g., 1 of 3, 2 of 3, etc.).				

Homogenate aliquot shipment:
Keep individual jars double-bagged in the food-grade plastic bags. Place these bags in a cooler with adequate space for the tissue containers, packing materials, and dry ice. Secure each of the tissue containers with packing materials (e.g., foam or bubble wrap) before adding the dry ice. The amount of dry ice required for shipping will depend on the number of tissue samples in the cooler and needs to be enough to keep the tissue samples frozen for 48 hours (e.g., 15 pounds of dry ice for 2 pounds of tissue samples and 25 pounds of dry ice for 10 pounds of tissue samples). Additional guidance for shipping perishables on dry ice is available online from the University of Tennessee Agricultural Extension Service at http://cpa.utk.edu/pdffiles/cpa81.pdf. Document the samples contained in the cooler on a sample tracking form and include the form in the cooler. Secure the outside of the cooler with a chain-of-custody seal and address it to the sample recipient identified in the tables above. Ship the cooler via an overnight express on a date that will allow delivery of the cooler to the recipient on a normal business day.

Sample prep progress reports:
Each prep lab will prepare a weekly progress report to document the status of fish preparation activities and forward the report electronically to the EPA Fish Tissue Manager in OW and copy the Fish Tissue Contract Coordinator (Blaine Snyder, Tetra Tech). For each composite processed during that period, include the following information in the report: the site identification number, the specimen numbers of the fish homogenized for the composite, and the date the composite was homogenized.

5.13 Quality Assurance/Quality Control

Pilot Study
Prior to processing tissue for the NRSA, prep labs must complete the QA pilot study detailed in Attachment 3. Completion of the QA pilot study will demonstrate a lab's capability to thoroughly homogenize samples and to prevent cross contamination between samples.

Ongoing verification of fillet composite homogenization during NRSA fillet composite fish tissue preparation will be done by analyzing one set of triplicate lipid aliquots at a minimum 5% frequency (i.e., once every group of 20 samples which may be called a homogenization batch). If the relative standard deviation (RSD) of the triplicate lipid measurements for each homogenate sample is <20%, then the homogenization procedures are judged to be effective. Each prep lab will conduct the triplicate lipid analysis for its own homogenization verification. Corrective action for homogenization results that do not meet the QC criterion may involve re-homogenization of the fillet composite samples. The EPA Fish Tissue Sample Manager should be consulted to explore additional options. Any solution will attempt to avoid data qualification.

GLEC may not ship any samples in a homogenization batch until the triplicate lipid analyses for the batch have been performed and evaluated. If the RSD for the three QC samples exceeds the acceptance criterion, then GLEC must re-homogenize all of the samples in that batch, test one sample in triplicate for lipids, and meet the same acceptance criterion for the re-homogenized batch.

NERL-Cin may not begin analysis of any samples in a homogenization batch until the triplicate lipid analyses for the batch have been completed and evaluated. If the RSD for the three QC samples exceeds the acceptance criterion, then NERL-Cin must re-homogenize all of the samples in that batch, test one sample in triplicate for lipids, and meet the same acceptance criterion for the re-homogenized batch.

Ongoing verification of the prevention of fillet composite contamination will be done by the analysis of equipment rinsates prepared as described in the QA pilot study (Appendix A). Rinsates will be performed to continually demonstrate that cleaning procedures effectively remove contamination from filleting and homogenization activities above method detection limits. Rinsates will be generated at a minimum 5% frequency (for samples from all sampling sites) during fillet composite sample preparation. The type of rinsate needed will depend on the sampling site, and therefore the analyte list, for the samples being processed. Table 13-1 specifies the type of rinsates needed per field site and analyte list. Only one type of rinsate should be done after a sample homogenization. For example, the methanol rinse would be done after the 18th sample and the hexane rinse after the 19th sample, and the aqueous rinse after the 20th. The next round would be the 38th, 39th, and 40th. Each time a rinsate is collected a sample of the solvent or DI water used for the rinsate shall also be collected and submitted to the lab. GLEC will ship its rinsate samples to NERL-Cin for analysis.

NERL-Cin will prepare and analyze the rinsates as follows:

1. The GLEC 100ml hexane rinse and hexane blank will be surrogate fortified, and concentrated by evaporation on an N-EVAP to 1ml.
2. The 1ml is cleaned through 3g of Alumina-N that has been muffled for 4 hours at 400°C and deactivated to level III two hours prior to use, and packed into a column with a small amount of glass wool in the bottom and approximately 0.5cm of sodium sulfate on the top. Pre-elute the column with 5mL of hexane and then with 5mL of the 20%/80% methylene chloride/hexane mix.
3. Elute rinsate with 14mL of the methylene chloride/hexane mix.
4. Concentrate the rinsates to 0.5mL and add 25µL of the IS solution used for SOP MIRB 046.
5. Adjust the volume to 1mL with hexane. Rinsates will be quantitated via SOP MIRB 046.

The GLEC 600ml water rinsate and water blank will be analyzed for Mercury via SOP MIRB 033 and for Selenium without digestion via MIRB 040.

The NERL-Cin 100ml hexane rinse and hexane blank will be split and a 50ml aliquot will be analyzed for EMAP organics. The 50ml aliquot will be surrogate fortified, N-EVAPed and treated as in Section 13.3.1.
The NERL-Cin 600ml water rinsate and water blank will be split A 100 ml aliquot is preserved to pH < 2 with HNO3 and analyzed for Mercury via SOP MIRB 033 and for Selenium without digestion via MIRB 040.

All results will be reported to the EPA Fish Tissue Sample Manager in mass units. Because the rinsate samples are liquids (rather than tissue), and the initial sample volumes differ between the two types of rinsates (DI water, methanol, and hexane), the results of these contamination checks will be evaluated on the basis of the mass of any contaminants found. NERL-Cin will convert the concentrations in the rinsate samples to results mass units (e.g., ng or µg, as appropriate in the total volume of rinsate). Rinsates results will be reported to MDLs. If levels are found above the MDLs, a comparison of the mass of contaminant in the rinsate to the mass of the smallest tissue composite in the last 20 samples will be done. If this comparison yields a value above the fish tissue MDL's corrective action is needed.

Corrective action for rinsates that exhibit contamination must be coordinated immediately with the EPA Fish Tissue Sample Manager. Depending on the nature and severity of the potential contamination, the EPA Fish Tissue Sample Manager may require the laboratory responsible for homogenizing the batch of potentially contaminated samples to suspend homogenization of tissue samples until the adequacy of equipment cleaning procedures had been demonstrated.

Suspension of processing: NERL-Cin has been instructed that it may proceed with homogenizing a second batch of samples before the rinsate analyses are completed for the first batch of samples. However, they may not proceed with the homogenization of a third batch of samples until the rinsates for the first batch of samples has been analyzed and found to demonstrate that the equipment cleaning procedures are adequate. Similarly, GLEC will need

to hold processing of samples until the rinsates have been analyzed and found to demonstrate that the equipment cleaning procedures are adequate.

Reporting QC results: For both homogenization and contamination verification, QC results will be reported in electronic spreadsheets to the EPA Fish Tissue Manager as soon as they are available and kept on file at the respective laboratories.

Table 5-6: Ongoing Equipment Rinsates Generated by GLEC: Type and Analyte List*

Sites and approximate # of fillet composite samples:	Generated by GLEC		
	NRSA Non-urban (~717)	LM Non-urban (~44)	Reference (200)
****100mL hexane rinsate *Shipped in* pesticide clean 4 oz amber narrow mouth bottle, Teflon lid**	**X** *EMAP organics, ~36 rinsates*	**X** *EMAP organics ~2 rinsates*	**X** *EMAP organics ~10 rinsates*
*****600 mL water rinsate *Shipped in* pre-cleaned HDPE bottle, preserved with HNO_3 to pH<2**	**X** *Hg, Selenium ~36 rinsates*	**X** *Hg, Selenium ~2 rinsates*	**X** *Hg, Selenium ~10 rinsates*
* See Table 8-1 for more analyte information ** A 100mL hexane blank must be provided with each set of 100mL hexane rinsates *** A 600mL water blank (preserved with HNO_3) must be provided with each set of 600mL rinsates			

5.14 References

U.S. Environmental Protection Agency (USEPA). 2007. Guidance for Preparing Standard Operating Procedures (QA/G-6). USEPA Office of Environmental Information, Quality Staff, Washington, DC. EPA/600/B-07/001.

U.S. Environmental Protection Agency (USEPA). 2000. Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories. Volume 1: Fish Sampling and Analysis. Third Edition. USEPA Office of Water, Washington, DC. EPA 823-R-05-005.

U.S. Environmental Protection Agency (USEPA). 2005. Quality Assurance Report for the National Study of Chemical Residues in Lake Fish Tissue: Analytical Data for Years 1 through 4. USEPA Office of Water, Washington, DC. EPA 823-R-05-005.

7.0 PERIPHYTON

7.1 Scope of Application

The protocols described here are to be used in preparation and analysis of the diatom and soft algal components of all samples collected for the National Rivers and Streams Assessment project 2008-2010.

- The diatom analysis protocol follows procedures outlined for NAWQA in the assessment of slides for Richest Targeted Habitat (RTH) and Depositional Targeted Habitat (DTH).
- The soft algal analysis protocol describes a quantitative procedure for analyzing the soft-algal component of samples collected for the National Rivers and Streams Assessment project. A two-count procedure, with extended soft algae assessment, will be used to insure that fuller representation of the soft algae present is reached. Used in conjunction with the diatom analysis, results of this procedure should provide a clear picture of the algal community as a whole.

7.2 Summary of Method

Algal samples are examined and the algal species encountered are identified (to lowest possible taxon level), enumerated and recorded. Estimates of the biovolume of dominant species are made using existing parameters, or those found in the literature, and used to determine the biovolume of the sample.

This procedure is applicable to the analysis of the diatom and soft algal components of samples collected for the National Rivers and Streams Assessment (NRSA). Personnel responsible for these procedures include diatom and soft algal analysts, and data entry personnel.

7.3 Health and Safety Warnings

Always turn instrument off before repairing or troubleshooting. Use the instrument manual to identify potential hazards, and take appropriate precautions while using or repairing the instrument.

Standard laboratory protective clothing (lab coat, gloves) and eye covering is required. Refer to chemical MSDS sheets (in labeled binders) of chemicals used for reagent preparation.

7.4 Responsibility and Personnel Qualifications

The instrument manager should be consulted for all instrument uses and procedures. Upon samples receipt, the laboratory must contact Marlys Cappaert at the Information Management Center by phone (541-754-4467) or fax to confirm samples have arrived. If expected samples do not arrive, labs must notify Ellen Tarquinio at EPA (202-566-2267).

7.5 Preparation Protocols

7.5.1 Preservation

On receipt of samples add 3% (1.5 ml per 50 ml volume) formalin.

7.5.2 Protocols

Protocols for the preparation of soft algal sub-samples and diatom slides can be obtained from the ANSP - PCER – Phycology Section

http://diatom.ansp.org/nawqa/protocols.asp

Documents: P-13-48: Subsample Procedures for USGS NAWQA Program Periphyton Samples
P-13-42: Diatom Cleaning by Nitric Acid Digestion with a Microwave Apparatus
P-13-49: Preparation of Diatom Slides Using Naphrax Mounting Medium

7.5.3 Sample loss

When <20 ml of the collected sample remains the samples is considered lost. Record of the loss should be made on the tracking website.

https://emapsw.cor.epa.gov/sec-htdocs/NRSATracker3.php3

7.5.4 Sample Leakage:

When a sample has leaked in transit i.e. <50 ml, but >20 ml remains, reduce the volume sub-sampled for soft algal analysis as required (e.g., 45 ml remains therefore soft algae sub-sample = 15 ml). Advise the primary soft algae analyst of any reductions in sample volume on handover of the samples. When necessary the amount of sub-sample taken for diatom slide preparation can be reduced. The minimum for any sub-sample, either for diatom side preparation of soft algae analysis, is 10 ml.

7.6 Diatom Analysis

7.6.1 Methods

Diatom analysis will be completed following the NAWQA protocols, for RTH and DTH samples, with a 600 valve count completed for each sample. The time limit for analysis per sample is 4 hours. The full protocol can be obtained from the ANSP - PCER – Phycology Section.

http://diatom.ansp.org/nawqa/protocols.asp

Document: P-13-39: Analysis of Diatoms on Microscope Slides Prepared From USGS NAWQA Program Algae Samples.

The taxa names to be used for this project, when identifying the diatom forms, are listed in the NRSA taxa list 2008 list for diatoms. Amendments will be adopted following the 2009 diatom workshop. This is to aid with taxonomic harmonization.

Digital images are required for all identified taxa occupying 5% or more of the count (Section 7.3).

Biovolume measurements of any taxa are to be made if

(a) None are already held (Appendix 1)
and
(b) no values can be obtained from the literature.

It is advised that measurements and/or literature searches are made at the time of counting.

7.7 Soft Algae Analysis

7.7.1 Definitions

Natural counting unit. To facilitate the counting of soft algal forms that have linked cells that may be hard to differentiate, each natural grouping of algae (i.e., each individual unicell, filament or colony) is defined as a single counting unit. Diatoms are an exception; each diatom cell is considered a natural counting unit.

Multicellular natural unit. A natural counting unit of soft algae consisting of multiple cells e.g. *Nostoc* colony, *Stigeoclonium* branched filament.

Macro-algal material. This term refers to filamentous or large algal material visible to the naked eye e.g. *Cladophora*, when completing an initial assessment of a sample to ascertain the necessity for a Sedgewick-Rafter count.

Palmer-Maloney Fraction. A diluted or concentrated sample derived from the original sub-sample taken from the total collected material.

7.7.2 Count Criteria

For the NRSA project a two-part, extended soft algae assessment, count protocol has been adopted. The Sedgewick-Rafter count will assess the macro-algal material within a sample and the extended Palmer-Maloney count provides more comprehensive data with regard the soft algal component of the algal community as a whole.

7.7.2.1 Sedgewick-Rafter Count (S-R)

This count type is to be completed, at 20x magnification on samples when a visual assessment reveals the presence of macro-algal material. This count will ensure that larger, often under-recorded or missed, soft algal taxa are sufficiently represented within the final sample count.

7.7.2.2 Palmer-Maloney Count (P-M)

This count type is to be completed on all samples collected for analysis in the NRSA project. The count consists of three parts. A maximum volume of 0.05 ml of the soft algal sub-sample, viewed in two, half, Palmer-Maloney cells, will be assessed. Using a transect approach, 300 natural counting units, including live diatoms, are enumerated. Dead diatoms are also enumerated during this time. On reaching 300 natural counting units the enumeration of diatoms (both live and dead) ceases. The enumeration of the soft algae only, continues for the

remaining portion of the total transect length (118 mm for a field of view measuring 0.54 mm – total transect length formula πr2/field of view) of the first, half, Palmer-Maloney cell or until 300 soft algae natural counting units is reached. A second full length transect (117.5 mm) is also enumerated for soft algae only, or until 300 soft algae natural counting units is reached, using a separate, second, half Palmer-Maloney cell. This three part approach allows for the soft algal portion of any sample to be better represented.

7.7.3 Count Methods

7.7.3.1 Sedgewick-Rafter count (S-R). To be completed first when required

EQUIPMENT:

1. Compound microscope with 20x objective for a total system magnification of 200x.
2. Sedgewick-Rafter Cell 50 mm long, 20 mm wide, 1 mm deep. Total area 1000 mm^2 with a total volume of 1000 mm^3 (1 ml).
3. Glass microscope cover slips, rectangular, 22 x 50 mm, #1 thickness.
4. Glass, wide-bore pipettes, > 1 mm inside diameter, or eye-dropper.

METHOD:

1. The coverslip for the S-R should be placed diagonally across the slide.
2. Shake each sample before using a wide-bore pipette to deliver some of the sample into the Sedgewick-Rafter Cell (S-R). Use scissors or a razor blade to cut up larger pieces prior to extraction if necessary. Add the sample into one of the open corners until the cell is full and no bubbles exist. The coverslip should slide into position itself. The cell must be full to insure that the area analyzed is equal for all samples.
3. Allow the sample to stand for 15 minutes prior to analysis to allow settling. It may be necessary to add a few drops of distilled water to the edges during this time to prevent air bubbles occurring.
4. Using a compound microscope (20x objective, 200x total system magnification), scan the entire S-R cell.
5. Identify and enumerate all filamentous and branching Chlorophyte and Rhodophyte soft algal forms possible at this magnification within the S-R cell. (These forms include, but are not exclusive to, species belonging to the genera *Cladophora*, *Ulothrix, Microspora, Mougeotia, Oedogonium, Stigeoclonium, Batrachospermum* and also unknown Rhodophyte species in chantrasia phase). Algal forms are enumerated using natural counting units. Natural counting units are as defined above. Algal forms are identified to the lowest possible taxonomic level. Diatoms are not included in this count.
6. The taxa names to be used for this project, when identifying soft algal forms, should be consistent with the NRSA taxa list 2008 for soft algae, amended at the 2008 soft algal workshop. This is to aid with taxonomic harmonization.
7. Count the number of algal cells for each multicellular natural unit. The number of cells for multicellular natural units are recorded in parenthesis beside the tally of natural counting units.

8. Tally data onto a bench sheet or into a computer based program. Notes with regards difficulties encountered in taxa identification due to the use of Lugol's should be made during the count and logged with the count data.
9. Digital images are required for all identified taxa occupying 5 % or more of the count (section 6.3).
10. Biovolume measurements of any taxa are to be made if

    (a) None are already held (Appendix 1)

    and

    (b) no values can be obtained from the literature.

    It is advised that measurements and/or literature searches are made at the time of counting.

7.7.3.2 Palmer-Maloney count (P-M)

To be completed for **every** soft algal sample.

The following is an expansion of the protocol outlined for NAWQA. There are three parts to the count. The total sample volume scanned for this count is 0.1 ml.

EQUIPMENT:

1. Compound microscope; 40-45x objectives for a total system magnification of 400-450x.
2. Two Palmer-Maloney Counting Cells with ceramic chamber; chamber depth of 0.4 mm; volume of 0.1 ml.
3. Glass microscope cover slips, rectangular, 22 x 50 mm, #1 thickness.
4. Glass, pasteur pipettes, 5.25 inch, ≤ 1 mm diameter.

METHOD:

1. Place a rectangular cover slip (#1 thickness, 22 x 50 mm) at 45^{o} to the counting cell, covering about 1/3rd of the chamber, but not across the center of the cell.
2. Thoroughly mix the Palmer-Maloney fraction and draw into an elongated Pasteur pipette (5.25 inch). Quickly add the fraction drop-wise, into the center of the chamber. When the surface tension starts to draw the cover slip across the chamber, adjust the sides of the cover slip so that ends of the chamber are covered and the cover slip hangs over both sides of the ceramic portion of the counting cell.
3. Add glycerin to the area where the cover slip extends past the ceramic portion. This seals the cover slip to the counting cell temporarily (without excess heat or vibration, the counting cell can be used for a week or more).
4. **Palmer-Maloney Fractions**.
    a. Dilutions or concentration of samples with extremely high or extremely low diatom abundance may be necessary. This must be considered on a case by case basis. In general 15 – 30 natural counting units (both diatom and soft algae) should be visible per field of view. Assessment of 5 fields of view is advised. For samples were the number exceeds 30 natural counting units then dilution is recommended. If the number is < 15 then consider concentration. Silt/detritus levels are also a factor. In samples with high silt/detritus levels, even if there are < 15 natural units per field of view, consider dilution. Avoid concentrating samples were the silt/detritus level would be raised to a level too high to complete a successful count. Record should be made on the count sheet when silt/detritus levels prohibit concentration or lead to a dilution. Both Palmer-Maloney cells should be prepared using the same Palmer-Maloney fraction. The S-R count should be completed on the original sub-sample taken from the total collected material. Only concentrate/dilute a portion (10 ml or less) of the original sub-sample. No sample should be diluted or concentrated to more than 20% of its original volume.
    b. Palmer-Maloney fraction data should be recorded on a separate log and the sheet included with the deliverables.
5. **Part 1: Enumerate 300 natural counting units.**
    a. Position the microscope stage so that the first field of view is in the top left hand area of the Palmer-Maloney cell at, but not overlapping, the cell edge. Using transects, of varying length, with a width of 0.54 mm (or width of the field of view) scan up to, but not exceeding, a total transect length of 118 mm (or equivalent) i.e. one half of the Palmer-Maloney cell. Every other transect, moving down the Palmer-Maloney cell, is to be enumerated, i.e. each transect enumerated should be separated by a width of one field of view. This will prevent field of view overlap. For microscopes where the 40x field of view differs from 0.54 mm, recalculation of the maximum transect length is required. The full length of each transect should be scanned for algal enumeration. Record the start and end coordinates for each transect for the purpose of total transect length calculation.

b. Identify and enumerate all soft algal forms within the field of view. Algal forms are enumerated using algal natural units and identified to the lowest possible taxonomic level.

c. The taxa names to be used for this project, when identifying the soft algal forms, are listed in the NRSA start list for soft algae, amended at the 2008 soft algal workshop. This is to aid with taxonomic harmonization.

d. Diatoms are differentiated as to "living" or "dead" at the time of collection. If there is any protoplast material in the frustules the diatom is considered to have been living when collected.

e. Count the number of algal cells for each multicellular natural unit. When cell length within a filament cannot be determined then a standard length of 10 µm per cell should be used. Note should be made on the count report when this standard length is employed.

f. The number of cells for multicellular natural units are recorded in parentheses beside the tally of natural counting units. All diatoms are grouped into one category – undifferentiated diatoms.

g. Tally data onto a bench sheet or into a computer based program. Notes with regards to difficulties encountered in taxa identification due to the use of Lugol's should be made during the count and logged with the count data.

h. Repeat procedures until 300 natural counting units have been enumerated. Only "living" diatoms are counted against the needed 300 natural algal units.

i. Digital images are required for all taxa with 5% or more of the total 300 count (see 7.3 for full criteria).

j. Biovolume measurements of any taxa are to be made if

(a) None are already held (Attachment ?)

and

(b) no values can be obtained from the literature.

It is advised that measurements and/or literature searches are made at the time of counting

k. Once 300 natural counting units have been enumerated, record the total length of transects scanned.

l. In the event that a total of 300 natural counting units is not reached during the first full length transect (118 mm (or equivalent)/one half P-M cell). Continue the count, using the above protocol, in the second, half, P-M cell. Record the additional transect length(s) required. Once the 300 natural counting units is reached, calculate the total transect length (118 mm (or equivalent) + additional transects) and record this with the count. The remainder of the P-M half cell should then be enumerated for soft algal taxa only as is required for Parts 2 and 3.

6. **Part 2: Analysis continuation for the first, half, Palmer-Maloney cell.**

For samples were 300 natural counting units are enumerated in less than the total 118 mm transect length (or under half a Palmer-Maloney cell) a count continuation is required.

a. For the remainder of the full transect length, or until 300 soft algae natural counting units (i.e. natural counting units excluding both live and dead diatoms) are reached, identify and enumerate natural counting units of soft algal taxa only. Record the start and end co-ordinates for each transect for the purpose of total transect length calculation.

b. Count the number of algal cells for each multicellular natural unit. When cell length within a filament cannot be determined then a standard length of 10 µm per cell should be used. Notes should be made on the count report when this standard length is employed.

c. The number of cells for multicellular natural units are recorded in parentheses beside the tally of natural counting units.

d. Do not record live and dead diatom accuracies.

e. As before, tally data onto a bench sheet or into a computer based program and record problems with taxa identification due to the use of Lugol's.

f. Digital images are required for all taxa with 5% or more of the total count (see 7.3 for full criteria).

g. Biovolume measurements of any taxa are to be made if

(a) None are already held (Attachment ?)

and

(b) no values can be obtained from the literature

It is advised that measurements and/or literature searches are made at the time of counting.

h. Once the total transect length of 118 mm (or equivalent) is reached, cease counting this, half, Palmer-Maloney cell.

7. **Part 3: Count of the second Palmer-Maloney half cell**.

a. Setup a second P-M cell following steps 1 – 3 of the P-M protocol.

b. As outlined previously, position the stage so that the first field of view is in the top left portion of the Palmer-Maloney cell.

c. Identify and enumerate natural counting units for soft algal taxa only for a total transect length of 117.5 mm (or equivalent), or until 300 soft algae natural counting units (i.e. natural counting units excluding both live and dead diatoms) are reached. Record the start and end co-ordinates for each transect for the purpose of total transect length calculation.

d. Count the number of algal cells for each multicellular natural unit. The number of cells for multicellular natural units are recorded in parentheses beside the tally of natural counting units.

e. As before, tally data onto a bench sheet or into a computer based program and record problems with taxa identification due to the use of Lugol's.

f. Digital images are required for all taxa with 5% or more of the total count (see 7.3 for full criteria).

g. Biovolume measurements of any taxa are to be made if

(a) None are already held (Attachment ?)

and

(b) no values can be obtained from the literature.

It is advised that measurements and/or literature searches are made at the time of counting.

h. Once the total transect length of 235.5 mm (or equivalent) is reached, or 300 soft algae natural counting units (i.e. natural counting units excluding both live and dead diatoms) have been enumerated, cease counting and record the total transect length analyzed on the count sheet.

7.8 Images

1. Images are to be supplied for any taxon that occupies 5% or great of either count.
2. One (to several) images are required per taxon, that meet this criteria, for the project. Several images are required when a taxon demonstrates significant differences in morphologies between samples.
3. Unknown taxon should be imaged and share with the rest of the project group. This may lead to identification but is also to insure that the same NADID and name is used for commonly occurring 'unknowns' throughout the project.
4. The naming convention for images is:

Taxa name_magnification (if applicable)_sample ID.xxx
e.g. Homoeothrix janthina_40x1.5x_NRSA0001.jpg

7.9 QA/QC

7.9.1 Diatoms

Section to be added following conference call in January 2009

7.9.2 Soft Algae

Section to be added following conference call in January 2009

7.10 References

United States Geological Survey, National Water-Quality Assessment Program. 1997. Procedures for Processing NAWQA Algal Samples. Draft Manuscript. February 1997.

Palmer, C.M. and T.E. Maloney. 1954. A new counting slide for nannoplankton. American Society of Limnology and Oceanography, Special Publication No. 21. 6pp.

Weber, C.I. 1973. Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents. EPA-670/4-73-001. National Environmental Research Center. Office of Research & Development, U.S. Environmental Protection Agency. Cincinnati, Ohio.

8.0 PERIPHYTON ENZYMES

8.1 Scope of Application

Periphyton samples are collected, preserved and analyzed to determine extracellular enzyme activity using the Bio-tek microplate reader of fluorescence/luminescence.

8.2 Summary of Method

Periphyton samples are collected in clean 50-60ml polypropylene test tubes and frozen until analysis. Thawed samples are quantitatively transferred to a 50 ml sterile wide mouth glass jar. Prepared samples are stored in the refrigerator, and stirred with stir bar during sample pipetting. Samples are run (or diluted and run) on the Bio-tek fluorescence detector.

8.2.1 Definitions

DIW- deionized water

8.2.2 Interferences

Minimal quenching effects (5-10%)

8.3 Health and Safety Warnings

Always turn instrument off before repairing or troubleshooting. Use the instrument manual to identify potential hazards, and take appropriate precautions while using or repairing the instrument.

Standard laboratory protective clothing (lab coat, gloves) and eye covering is required. Refer to chemical MSDS sheets (in labeled binders) of chemicals used for reagent preparation.

8.4 Personnel Qualifications

This SOP provides the steps necessary for assays and enzyme activity determinations by technicians with basic training in laboratory procedures; however, proper training in the use of the instrument and assay principles is necessary.

The instrument manager should be consulted for all instrument uses and procedures. Upon samples receipt, the laboratory must contact Marlys Cappaert at the Information Management Center by phone (541-754-4467) or fax to confirm samples have arrived. If expected samples do not arrive, labs must notify Ellen Tarquinio at EPA (202-566-2267).

8.5 Equipment and Supplies

- Bio-tek fluorescence detector with emission wavelength set at 450nm; and excitation wavelength at 365nm.
- Incubator or oven set at 30˚C.
- Sterilized volumetric flasks and deionized waters for enzyme substrates, disposable trays for 8 channel pipetors, disposable 96 well black plates, various volume Eppendorf pipets and disposable tips.

8.6 Reagents and Standards

1. Standards: Prepare 100 µm solutions of 4-methylumbelliferone and 7-amino-4-methylcoumarin in sterile water. Dilute to 10 µm.
2. Prepare substrate stock solutions in sterile water. Amount needed to make 100 ml of 200 um stock solutions in sterile water (*unless indicated differently)

"AlaMCM"	L-Alanine-7-amido-4-methylcoumarin 7.21mg
	4-MUB-a-L-Arabinopyranoside 6.17 mg
"ArgMCM"	L-Arginine-7-amido-4-methylcoumarin 6.63 mg
	Asparagine-7-amido-4-methylcoumarin 8.07 mg
	Aspartic acid-7-amido-4-methylcoumarin 5.81 mg
	4-MUB-b-D-cellobioside 10.00 mg
"A-gala"	4-MUB-a-D-galactoside 6.77 mg
"B-gala"	4-MUB-b-D-galactoside 6.77 mg
"A-gluc"	4-MUB-a-D-glucoside 6.77 mg
"B-gluc"	4-MUB-b-D-glucoside 6.77 mg
	4-MUB-b-D-glucuronide 7.05 mg
	4-MUB-b-D-mannopyranoside 6.77 mg
	L-glutamic acid g-7-amido-4 methylcoumarin(100um) 3.05 mg*
"GlyMCM"	Glycine-7-amido-4-methylcoumarin 6.26 mg
	4-MUB-p-guanidinobenzoate 7.48 mg
"LeuMCM"	L-Leucine 7-amido-4-methylcoumarin 6.50 mg
"N-ace"	4-MUB-N-acetyl-b-glucosaminide 7.59 mg
"Phos"	4-MUB-phosphate 5.12 mg
	L-proline-7-amido-4-methylcoumarin 7.06 mg
	L-pyroglutamic acid-7-amido-4-methylcoumarin 5.73 mg
	L-serine-7-amido-4-methylcoumarin 5.97 mg
"Sulf"	4-MUB-sulfate 5.89 mg
	L-tyrosine-7-amido-4-methylcoumarin (100um) 3.38 mg*
"Xylo"	4-MUB-b-D-xyloside 6.17 mg

Note: *Once prepared in sterile water, substrate solutions appear to be stable for weeks if not contaminated. Some substrates, notably esterase substrates like MUB acetate, are*

highly sensitive to contamination. Punctilious care is needed to prevent spurious results. Use gloves, sterile tips, etc. It can be difficult to measure fatty acid esterase activity in some systems because it is so high.

3. Acetate buffer: Make a 50 mM pH 5 acetate buffer solution by dissolving 6.805 g Trihydrate NaAcetate in 1.00 L of milli-Q water, adjust pH to 5 with 1mL glacial acetic acid.

8.7 Procedure

Warm up microplate fluorometer for 30 minutes. Set/check to make sure excitation wavelength at 365 nm and emission wavelength at 450 nm.

8.7.1 Sample Preparation

1. Samples should be frozen if not to be analyzed immediately.
2. One or two days before analysis, thaw samples in tubes, and transfer sample to sterile, 50ml wide mouth glass. Prepared samples should be stored in refrigerator and analyzed within 1-2 weeks.
3. When ready to analyze, add stir bar to sample and stir hard to maintain a uniform suspension. Use an 8-channel pipetter to withdraw 50, 100, or 200 µL aliquots of the sample suspension and dispense them into prepared microplates. Sample volume will depend on expected concentration of enzyme for reaction.

***Note**: Strong mixing and good pipetting technique will minimize well to well variation. Use wide mouth pipette tips (in RM 117) or snip off the ends of standard tips. A larger diameter opening will improve uniformity of dispensing.*

8.7.2 Microplate Preparation

Assay wells: add 50 µL of substrate solution to an eight well column containing 200 µL of sample suspension.

Sample control wells get 50 µL of acetate buffer + 200 µL of sample suspension.

Substrate control wells get 50 µL substrate solution plus 200 µL of acetate buffer.

Quench standard wells get 50 µL of standard (4-methylumbelliferone or 7-amino-4-methyl coumarin) + 200 µL sample suspension.

Reference standard wells get 50 µL of standard (4-methylumbelliferone or 7-amino-4-methyl coumarin) + 200 µL acetate buffer.

***Note**: In this design, there are four replicate wells for assays, standards, controls and blanks. Even with good technique, the well to well variation may be 20% or more because of differences in the amount of material hitting each well and because you are measuring changes in fluorescence against a high quench background. When scaling assays down from test tube to microplate, you sacrifice precision for economy. Depending on the nature of your samples and the magnitude of difference you consider significant, you may want to increase the number of replicate wells to 8 or 16. In our "standard" design, we use one column of wells for each standard curve, quench*

(reference) standard, and substrate; and four wells for each sample and quench (spike) assay, and for sample controls.

***Note**: As an organizational hint, it is generally easier to do only 2 assays with 6 samples on each plate. The best strategy is to set up all the plates first by dispensing everything except samples to assay wells. Then start the incubations by adding sample slurry to assay wells. Substrates are divided according to historical reaction rates.*

Incubate plates at room temperature. Depending on your question, you may choose to incubate at ambient temperature or at an elevated temperature (currently the MCM linked substrates at 30C).

8.8 Analysis and Calculation

1. To measure fluorescence it is necessary to raise the pH above at least 7.5. Raise pH by adding a 10 µL aliquot of 0.5 N NaOH to each well (assays, standards, negative controls, blanks) at the termination of incubation.
2. Insert plate into reader and run protocol "enzyme" on Bio-tek fluorometer. Sample files should be named so as to allow for easy reference of which sample and substrate are being analyzed. Analysis takes about 1 minute.

***Note**: In this situation, each well can only be read one time so preliminary trials may be required to determine appropriate incubation intervals or else a series of wells will have to set up for series of destructive fluorescence readings at appropriate time intervals.*

3. Some assays such as N-acetylglucosaminidase, B glucosidase and phosphatase may be rapid; results may be obtained within a few minutes. Activity against other substrates will be weak; the plates will need to incubate for several hours to get detectable activity.

***Note**: In this protocol, the assays are incubated at something close to the ambient pH of most soils. Buffer composition can be changed for a better match. Glycosidases have pH optima in the 4-6 range, so the acetate buffer "optimizes" those assays. Peptidases generally have pH optima around 8, so these activities are discriminated against in this procedure. Phosphatases can have acid or alkaline pH optima, so profiles generally show high activity across a broad range of pH. If desired, a separate buffer system can be used for each type of enzyme to facilitate detection. A tris-hydroxymethyl aminomethane buffer will work at pH 8. If you run assays at pH 8 or above, it will be possible to read fluorescence directly and repeatedly without the addition of NaOH.*

4. Calculate activity as nmol substrate converted per hour per mL of sample.

   Activity (nmol h-1 g-1) = (mean Fluorescence of assay wells - mean Fluorescence of negative control wells - mean Fluorescence of blank wells) /(emission coefficient)(quench coefficient)(0.05, 0.1 or0.2 mL)(incubation interval, hr)(dry wt in gms of sample)

   Emission coefficient (fluor/nmole) = (mean fluorescence of reference standard)/0.5 nmole

Quench coefficient = (mean fluorescence of quenched standard)/mean fluorescence of reference standard)

Note: *0.05, 0.1, or 0.2 is the volume of sample suspension in each well. If these volumes are changed during sample and plate preparation, you must change them for the final calculations.*

8.9 Quality Control and Quality Assurance

Duplicate field samples should be collected on 10% of total number of samples

Replicate lab samples should be analyzed on at least 10% of total number of samples analyzed.

Replicate lab samples should agree within 20-30% of each determination.

8.10 References

Center for Dead Plant Studies, University of Toledo. Nov. 14, 2000. **NOT IN TEXT**

Burns, A. and D. S. Ryder. 2001. Response of bacterial extracellular enzymes to inundation of floodplain sediments. Freshwater Biology 46:1299-1307. **NOT IN TEXT**

Marxsen, J. and K-P Witzel. 1991. Significance of extracellular enzymes for organic matter degradation and nutrient regeneration in small streams. Reprinted from Microbial Enzymes in Aquatic Environments, ed. R. J. Chrost.

9.0 SEDIMENT ENZYMES

9.1 Scope of Application

Sediment samples are collected, preserved and analyzed to determine extracellular enzyme activity using the Bio-tek microplate reader of fluorescence/luminescence.

9.2 Summary of Method

Sediment samples are collected in clean ziplock bags and frozen until analysis. The subsamples are weighed (0.5-2.0g wet weight) into 125mL Nalgene bottles and either refrozen until analysis, or used immediately. Seventy-five (75) ml acetate buffer is added to sample, homogenized, then quantitatively transferred to a 300 ml sterile wide mouth glass jar. An additional125ml of buffer is added, and re-homogenized if necessary. Prepared samples are stored in the refrigerator, and stirred with stir bar during sample pipetting. Samples are run (or diluted and run) on the Bio-tek fluorescence detector.

9.2.1 Definitions

DIW- deionized water

9.2.2 Interferences

A soil suspension of around 1 g/100 mL quenches fluorescence by 20-40%.

9.3 Health and Safety Warnings

Always turn instrument off before repairing or troubleshooting. Use the instrument manual to identify potential hazards, and take appropriate precautions while using or repairing the instrument.

9.4 Personnel Qualifications

This SOP provides the steps necessary for assays and enzyme activity determinations by technicians with basic training in laboratory procedures; however, proper training in the use of the instrument and assay principles is necessary.

The instrument manager should be consulted for all instrument uses and procedures. Upon samples receipt, the laboratory must contact Marlys Cappaert at the Information Management Center by phone (541-754-4467) or fax to confirm samples have arrived. If expected samples do not arrive, labs must notify Ellen Tarquinio at EPA (202-566-2267).

9.5 Equipment and Supplies

- Bio-tek fluorescence detector with emission wavelength set at 450nm; and excitation wavelength at 365nm.
- Incubator or oven set at 30˚C.

- Sterilized volumetric flasks and deionized waters for enzyme substrates, disposable trays for 8 channel pipetors, disposable 96 well black plates, various volume Eppendorf pipets and disposable tips.

9.6 Reagents and Standards

1. Standards: Prepare 100 µm solutions of 4-methylumbelliferone and 7-amino-4-methylcoumarin in sterile water. Dilute to 10 µm.

2. Prepare substrate stock solutions in sterile water. Amount needed to make 100 ml of 200 um stock solutions in sterile water (*unless indicated differently)

"AlaMCM"	L-Alanine-7-amido-4-methylcoumarin 7.21mg
	4-MUB-a-L-Arabinopyranoside 6.17 mg
"ArgMCM"	L-Arginine-7-amido-4-methylcoumarin 6.63 mg
	Asparagine-7-amido-4-methylcoumarin 8.07 mg
	Aspartic acid-7-amido-4-methylcoumarin 5.81 mg
	4-MUB-b-D-cellobioside 10.00 mg
"A-gala"	4-MUB-a-D-galactoside 6.77 mg
"B-gala"	4-MUB-b-D-galactoside 6.77 mg
"A-gluc"	4-MUB-a-D-glucoside 6.77 mg
"B-gluc"	4-MUB-b-D-glucoside 6.77 mg
	4-MUB-b-D-glucuronide 7.05 mg
	4-MUB-b-D-mannopyranoside 6.77 mg
	L-glutamic acid g-7-amido-4 methylcoumarin(100um) 3.05 mg*
"GlyMCM"	Glycine-7-amido-4-methylcoumarin 6.26 mg
	4-MUB-p-guanidinobenzoate 7.48 mg
"LeuMCM"	L-Leucine 7-amido-4-methylcoumarin 6.50 mg
"N-ace"	4-MUB-N-acetyl-b-glucosaminide 7.59 mg
"Phos"	4-MUB-phosphate 5.12 mg
	L-proline-7-amido-4-methylcoumarin 7.06 mg
	L-pyroglutamic acid-7-amido-4-methylcoumarin 5.73 mg
	L-serine-7-amido-4-methylcoumarin 5.97 mg
"Sulf"	4-MUB-sulfate 5.89 mg
	L-tyrosine-7-amido-4-methylcoumarin (100um) 3.38 mg*
"Xylo"	4-MUB-b-D-xyloside 6.17 mg

Note: *Once prepared in sterile water, substrate solutions appear to be stable for weeks if not contaminated. Some substrates, notably esterase substrates like MUB acetate, are highly sensitive to contamination. Punctilious care is needed to prevent spurious results. Use gloves, sterile tips, etc. It can be difficult to measure fatty acid esterase activity in some systems because it is so high.*

3. Acetate buffer: Make a 50 mM pH 5 acetate buffer solution by dissolving 6.805 g Trihydrate NaAcetate in 1.00 L of milli-Q water, adjust pH to 5 with 1mL glacial acetic acid.

9.7 Procedure

Warm up microplate fluorometer for 30min. Set/check to make sure excitation wavelength at 365 nm and emission wavelength at 450 nm.

9.7.1 Sample Preparation

1. Place the equivalent of 1.0 g dry mass of soil into a 125 mL screw-cap Nalgene bottle (about 1.5-2gms wet weight). Samples should be frozen if not to be analyzed immediately.
2. When ready to analyze, add about 75 mL of 50 mM pH 5 acetate buffer to the bottle. Homogenize the soil sample using a polytron. Quantitatively transfer slurry to a 300 ml sterile wide mouth glass jar using additional aliquots of buffer, but not more than 125ml. Re-homogenize with the polytron if necessary. Prepared samples are stored in CT3.
3. Add a stir bar and stir hard to maintain a uniform suspension. Use an 8-channel pipetter to withdraw 200 µL aliquots of the sample suspension and dispense them into prepared microplates. If samples are too concentrated, a 50 or 100uL sample volume may be used. Prepare plates with buffer accordingly.

***Note**: Strong mixing and good pipetting technique will minimize well to well variation. Use wide mouth pipette or snip off the ends of standard tips. A larger diameter opening will improve uniformity of dispensing.*

9.7.2 Microplate Preparation

Assay wells: add 50 µL of substrate solution to an eight well column containing 200 µL of sample suspension.

Sample control wells get 50 µL of acetate buffer + 200 µL of sample suspension.

Substrate control wells get 50 µL substrate solution plus 200 µL of acetate buffer.

Quench standard wells get 50 µL of standard (4-methylumbelliferone or 7-amino-4-methyl coumarin) + 200 µL sample suspension.

Reference standard wells get 50 µL of standard (4-methylumbelliferone or 7-amino-4-methyl coumarin) + 200 µL acetate buffer.

***Note**: In this design, there are four replicate wells for assays, standards, controls and blanks. Even with good technique, the well to well variation may be 20% or more because of differences in the amount of material hitting each well and because you are measuring changes in fluorescence against a high quench background. When scaling assays down from test tube to microplate, you sacrifice precision for economy. Depending on the nature of your samples and the magnitude of difference you consider significant, you may want to increase the number of replicate wells to 8 or 16. In our "standard" design, we use one column of wells for each standard curve, quench*

(reference) standard, and substrate; and four wells for each sample and quench (spike) assay, and for sample controls.

***Note**: As an organizational hint, it is generally easier to do only 2 assays with 6 samples on each plate. The best strategy is to set up all the plates first by dispensing everything except samples to assay wells. Then start the incubations by adding sample slurry to assay wells. Substrates are divided according to historical reaction rates.*

Incubate plates at room temperature. Depending on your question, you may choose to incubate at ambient temperature or at an elevated temperature (currently the MCM linked substrates at 30C).

9.8 Analysis and Calculation

1. To measure fluorescence it is necessary to raise the pH above at least 7.5. Raise pH by add a 10 µL aliquot of 0.5 N NaOH to each well (assays, standards, negative controls, blanks) at the termination of incubation.
2. Insert plate into reader and run protocol “enzyme” on Bio-tek fluorometer. Sample files should be named so as to allow for easy reference of which sample and substrate are being analyzed. Analysis takes about 1 minute.

***Note**: In this situation, each well can only be read one time so preliminary trials may be required to determine appropriate incubation intervals or else a series of wells will have to set up for series of destructive fluorescence readings at appropriate time intervals.*

3. Some assays such as N-acetylglucosaminidase, B glucosidase and phosphatase may be rapid; results may be obtained within a few minutes. Activity against other substrates will be weak; the plates will need to incubate for several hours to get detectable activity.

***Note**: In this protocol, the assays are incubated at something close to the ambient pH of most soils. Buffer composition can be changed for a better match. Glycosidases have pH optima in the 4-6 range, so the acetate buffer “optimizes” those assays. Peptidases generally have pH optima around 8, so these activities are discriminated against in this procedure. Phosphatases can have acid or alkaline pH optima, so profiles generally show high activity across a broad range of pH. If desired, a separate buffer system can be used for each type of enzyme to facilitate detection. A tris-hydroxymethyl aminomethane buffer will work at pH 8. If you run assays at pH 8 or above, it will be possible to read fluorescence directly and repeatedly without the addition of NaOH.*

4. Calculate activity as nmol substrate converted per hour per mL of sample.

Activity (nmol h-1 g-1) = (mean Fluorescence of assay wells - mean Fluorescence of negative control wells - mean Fluorescence of blank wells) (200 mL)/(emission coefficient)(quench coefficient)(0.2 mL)(incubation interval, hr)(g soil used to make suspension)

Emission coefficient (fluor/nmole) = (mean fluorescence of reference standard)/0.5 nmole

Quench coefficient = (mean fluorescence of quenched standard)/mean fluorescence of reference standard)

Note: *200 mL is the total volume of sample suspension and 0.2 is the volume of sample suspension in each well. If these volumes are changed during sample and plate preparation, you must change them for the final calculations.*

9.9 Quality Control and Quality Assurance

Duplicate field samples should be collected on 10% of total number of samples

Replicate lab samples should be analyzed on at least 10% of total number of samples analyzed.

Replicate lab samples should agree within 20-30% of each determination.

9.10 References

Center for Dead Plant Studies, University of Toledo. Nov. 14, 2000.

Burns, A. and D. S. Ryder. 2001. Response of bacterial extracellular enzymes to inundation of floodplain sediments. Freshwater Biology 46:1299-1307.

Marxsen, J. and K-P Witzel. 1991. Significance of extracellular enzymes for organic matter degradation and nutrient regeneration in small streams. Reprinted from Microbial Enzymes in Aquatic Environments, ed. R. J. Chrost.

10.0 FECAL INDICATOR

10.1 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 (NRSA 2008) 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.

10.2 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 4 of Section 10.18

10.3 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 "Unkown" test sample extracts by Absolute Quantitation Method.

10.4 Interferences

a. Low pH (acidic) water

b. Humic and fulvic acid content

c. Suspended solids (e.g. fecal matter) and particulates (sand, dirt)

d. Excessive algal growth

10.5 Health & Safety Warnings

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

When working with potential hazardous chemicals (e.g. 95% ethanol) or biological agents (fecally-contaminated water) avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs remove clothing immediately and wash / rinse thoroughly. Wash the affected skin areas thoroughly with large amounts of soap and water. If available consult the MSDS for prompt action, and in all cases seek medical attention immediately. If inhalation, eye contact or ingestion occurs, consult the MSDS for prompt action, and in all cases seek medical attention immediately.

10.6 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.

10.7 Equipment and Supplies

- Clean powderless latex or vinyl gloves
- Goggles or Face Shield
- Roche MagNA Lyser
- Roche MagNA Pure LC (automated nucleic acid isolation and purification platform)
- High Speed Microfuge
- Micropipettors
- Semi-conical, screw cap microcentrifuge tubes (PGC, #506-636 or equivalent) pre-filled with 0.3 $\pm$ 0.02 g Acid-washed glass beads (Sigma, # G-1277 or equivalent). Filled tubes are autoclaved 15-min. Liquid Cycle (Slow Exhaust).

  Or
- Roche MagNA Lyser Green Bead tubes (Roche Applied Science, #03-358-941-001) sterile, siliconized 3-mm diameter ceramic beads in a siliconized 2-mL microfuge tube.
- Roche MagNA Lyser Rotor Cooling Block
- 2-mL tube racks
- Permanent marking pens (fine point and regular point) for labeling tubes

- Bench Sheets & Printouts of Computer Software Sampling Loading Screen

10.8 Reagents & Standards

a Qiagen AE buffer (Qiagen 19077)

b. Salmon DNA (Sigma D1626)

c. Frozen tubes of *Enterococcus faecalis* (ATCC #29212) calibrator cell stock

d. Purified *Enterococcus* faecalis (ATCC #29212d) genomic DNA

e. ABI TaqMan® Universal PCR Master Mix (ABI #4304437)

f. *Enterococcus* PCR primers and TaqMan® probe

g. Sketa PCR primers and TaqMan® probe

h. Bovine Serum Albumen (BSA) Sigma Cat. #B-4287)

i. Roche MagNA Pure LC DNA Isolation Kit III for Fungi & Bacteria

10.9 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. Fill out a batch sample analysis bench sheet. (See Attachment ?).

1. Micropipettors are calibrated annually and tested for accuracy on a weekly basis. Follow manufacturer instructions for calibration check. Measure three replicate volumes per pipettor and keep log book of their weights on a calibrated balance scale.
2. Preparation of stock Salmon Sperm (SS) DNA: Dissolve Salmon DNA in PCR grade water at a concentration of ~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).
3. 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.

4. Make Dilution Series of *Enterococcus faecalis* purified genomic DNA for use as internal standards in individual qPCR runs and to generate the weekly *Enterococcus* qPCR Standard Curve for quantitation purposes.
5. *Enterococcus faecalis* DNA for Standards
6. Frozen Reference Stock (20-µL) at 2.89 x 10^6 GEQs per µL
7. 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.
8. Make *Enterococcus faecalis* calibrator filter samples:
    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.
    iii. The calibrator sample filters are spotted with 10^4 or 10^5 *Enterococcus faecalis* cells and this number is incorporated into the Relative Quantitation EXCEL spreadsheet.
9. Prior to and after conducting work with cells and / or genomic DNA standards, disinfect and inactivate (render non-amplifiable) DNA in the Sample Extraction Hood, the qPCR Cabinet, and the qPCR Sample Loading Hood with 10% bleach and $\geq$ 15-min. exposure to high intensity germicidal (254 nm) ultraviolet light.

10.10 Procedures for Processing & qPCR Analysis of Sample Concentrates.

10.10.1 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 10.18.7).

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.

1. Pre-chill MagNA Lyser Rotor Cooling Block in -20˚C freezer. Label 1.7-mL sterile microfuge tubes with sample ID number to match them with Green Bead Tubes. Two supernatant recovery tubes and one "5-fold" dilution tube is needed per sample and should be labeled accordingly. The dilution tube shall be filled with 80-µL AE buffer using a micropipettor.
2. 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.
3. Insert Green Bead tubes of samples into MagNA Lyser and bead-beat for 60-sec (1-min) at 5,000 rpm at Room Temperature. Transfer sample tubes to microfuge. Spin tubes at 12,000 rpm for 2-min. Being careful to move filter aside, recover and transfer up to 400-µL of supernatant (sans debris) to new tube with a P-200 or P-1000 micropipettor.
4. Spin the supernatant tubes for 5-min at 14,000 rpm at Room Temperature. Recover >350-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 10.18.8).

10.10.2 Sample Analysis by *Enterococcus* qPCR

10.10.2.1 Preparation of qPCR assay mix

1. To minimize environmental DNA contamination, routinely treat all work surfaces with a 10% bleach solution, allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. If available, turn on UV light for 15 minutes.
2. Using a micropipettor with aerosol barrier tips, add PCR grade water to the lyophilized primers and probe from the vendor to create stock solutions of 500 µM primer and 100 µM probe and dissolve by extensive vortexing. Pulse centrifuge to coalesce droplets. Store stock solutions at -20ºC.
3. 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.
4. Using a micropipettor, prepare assay mix of the *Enterococcus*, and Salmon DNA reactions in separate, sterile, labeled 1.7 mL microcentrifuge tubes as described in
   a. Table 1.
5. Finger vortex the assay mix working stocks; then pulse microcentrifuge to coalesce droplets. Return the primer/probe working stocks and other reagents to the refrigerator.
6. Thaw and finger vortex sample extract (dilution) tubes that will be assayed in PCR run. Microfuge a few seconds to coalesce droplets. Finger mix and spin the standards and calibrator samples (dilutions). Temporarily store all samples in 4˚C refrigerators until use in assay or return to long term storage at -20˚C. Discard disposable gloves and put on a new pair.
7. Set 32 Smart tubes in Cepheid Racks in PCR cabinet along with micro-pippetors and expose to germicidal UV lamp for 15-min.
8. 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.
9. 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.
10. Check to make sure each Smart tube is properly labeled and identifiable by sample number or I-core position (e.g. A4). Insert loaded Smart tubes into Smart Tube microfuge. Close lid and spin 5-sec. Pop lid to stop. Remove Smart Tubes from microfuge and insert into proper position in SmartCycler.

Enterococcus (Ludwig) and Salmon (Sketa) qPCR assays (EPA Method 1606) will be performed upon 5-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 10.18.8).

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.

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 2 and Table 3 of Section 10.18 (below).

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.

10.11 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.

10.12 Chain of Custody

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

Field Sampling forms and NRSA 2008-2009 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.

10.13 Quality Control / Quality Assurance

The Data Quality Objectives and the Laboratory QC Procedures are listed and summarized in Tables 5 and 6 of section 10.18 below.

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.

10.14 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.

10.15 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 2008-2009 database stored on a computer server in Corvallis, Oregon.

10.16 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.

10.17 References

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)

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

10.18.1 Table 1. 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 strongly recommended that preparation of assay mixes be performed each day before handling of DNA samples.

10.18.2 Table 2. 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

10.18.3 Table 3. 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.

10.18.4 Table 4. Laboratory Methods: Fecal Indicator (Enterococci)

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.

10.18.5 Table 5. Parameter Measurement Data Quality Objectives

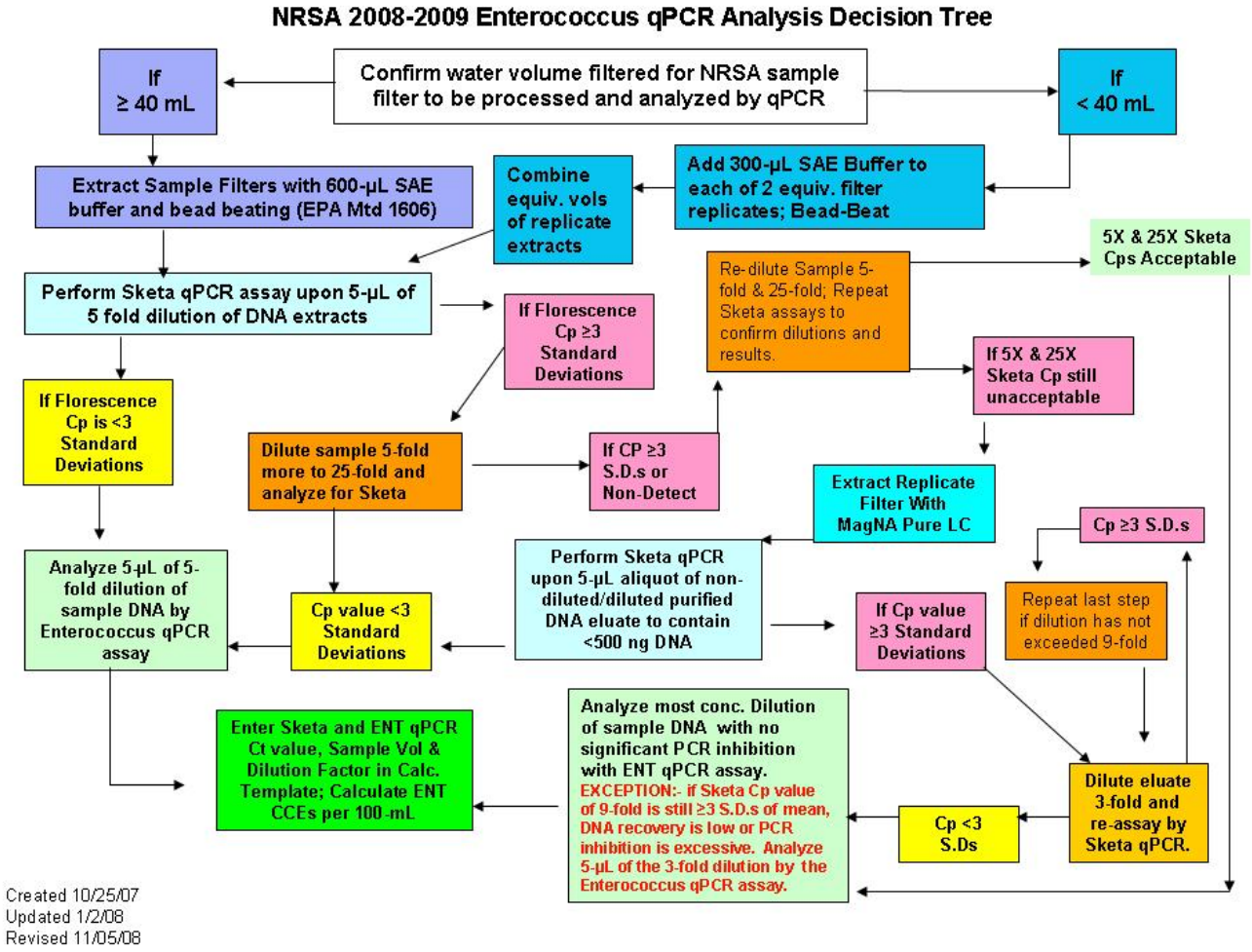
Variable or Measurement	QA Class	Expected Range and/ or Units	Summary of Method	References
DNA Extraction (Recovery)	C	10-141%	Bead-beating of filter in buffer containing Extraction Control (SPC) DNA. DNA recovery measured	EPA Draft Method 1606 *Enterococcus* qPCR
Enterococcus & SPC qPCR	C	<60 to >10,000 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

10.18.6 Table 6. 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 ≤ 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.

10.18.7 *Enterococcus* qPCR Analysis Decision Tree (ADT)



10.18.8 SOP for "Modified" MagNA Pure LC DNA Purification Kit III Protocol

1. 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 5-10 sec to mix buffer with beads and filter. Let stand at RT until batch of 16 samples (including positive control LFB or LFM and negative control LB samples) have all had Lysis Buffer and had their caps sealed tight. Leave water bath on to use during 30-minute Proteinase K treatment period.
2. Load the 16 samples into MagNA Lyser Rotor Plate and insert into MagNA Lyser. Tighten the three handscrews of the locking mechanism. Close the lid tightly. Set controls to shake for 60-sec at 5,000 rpm. Press the start button.
3. When the shake cycle has ended press the Open Lid Button. Open the lid and unlock the locking mechanism screws. Remove tube plate and set on bench top MagNA Lyser tube ring hub. Remove tubes, insert into tube styrofoam water bath float and cool tubes in ice water for 2-min. or place directly into 24-place microfuge rotor, pre-chilled in freezer.
4. Insert tubes into centrifuge rotor symmetrically in order to balance rotor. Close lid of centrifuge. Set spin parameters for 3,000 rpm for 1-min at 4ºC. Press Start button. Centrifuge to collect drops and foam off of cap down into tube.
5. When centrifuge stops, open lid and remove tubes from rotor. Uncap tubes in order and add 40-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.
6. 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.
7. Repeat steps 3 to 8 to process 16 more samples in parallel for loading MagNA Pure LC sample cartridge with 32 DNA extracts for downstream processing in the robotic platform.
8. After 30-min in 65 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.
9. 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.
10. 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.
11. When all 16 sample supernatants transferred to sample cartridge put adhesive film over cartridge to prevent contamination and evaporation. Put sample cartridge in ice water bath or fridge to maintain 4 ºC.

12. Repeat steps 9 to 13 for second batch of 16 samples (lysates). Re-cover sample cartridge with adhesive film for storage. Centrifuge sample cartridge opposite a balance cartridge for 75-sec (1-min, 15-sec) at 2800 rpm in IEC centrifuge (or equivalent) with rotor adaptors for microtiter plates in place. Insert the film-covered sample cartridge in MagNA Pure LC platform.

13. Load the MagNa Pure LC platform with volumes of extraction kit reagents prescribed by MagNA Pure LC computer software for the number of samples being extracted. Before closing the platform' lid and starting the extraction process add 1.34µL 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.

14. Remove film from top of sample cartridge and re-insert in Roche MagNA Pure LC platform set up with DNA Purification Kit III (Fungi; Bacteria) reagents in tubs, tips, tip holders, and processing / elution cartridges. Close platform lid and after checking off checklist of loaded items (e.g. reagents, tips) lock the lid and start the automated DNA III Extraction Protocol which purifies each sample's DNA and elutes it into 100-µL Elution Buffer.

15. When extraction process is complete, unlock the MagNA Pure LC platform lid and remove the sample eluate cartridge. Cover the cartridge with adhesive film and store at 4 C until qPCR analysis. Store cartridge at $\leq$ -20 ºC for long term preservation.

16. 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.

11.0 BENTHIC MACROINVERTEBRATES

11.1 Scope of Application

This procedure is to be used to facilitate processing and identification of benthic organisms collected in the littoral zone of rivers and streams.

11.2 Summary of Method

Samples are preserved with 95% ethanol and stored in ethanol until sorting begins. The lab technician will sort and preserve a randomized 500-organism subsample separately from the rest of the sample using a gridded screen. At least 10% of the grids would be randomly selected. All material not subsampled (remaining on the grid) must be returned to the original container with the preservative.

A qualified taxonomist will identify the organisms to the correct taxonomic level for the project (usually genus, Attachment 4). The taxonomist will create a reference collection with at least one specimen from each genus (or lowest taxonomic level identified).

11.2.1 Definitions

Caton grid: Subsampling grid that consists of a solid outer tray, a mesh-bottomed inner tray, a square "cookie cutter" and a scoop.

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

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

Inorganic material: 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

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 the original sorter compared to the number of total recoveries

Percent disagreement in enumeration (PDE): measure of taxonomic precision comparing the number of specimens counted in a sample by the first taxonomist with the number of specimens counted by the QC taxonomist.

Percent taxonomic disagreement (PTD): measure of taxonomic precision comparing the number of agreements (positive comparisons) and *N* is the total number of specimens in the larger of the two counts.

Pickate: sort residue from all grids originally sorted.

Subsampling: a portion of the sample obtained by random selection and division

Taxonomic Serial Number (TSN): stable and unique identifier that ITIS couples with each scientific name to serve as the "common denominator" for accessing information.

11.3 Health and Safety Warnings

All proper personal protection clothing and equipment (e.g. lab coat, protective eyeware / 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 / 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.

11.4 Responsibility and Personnel Qualifications

This SOP provides the steps necessary for sorting and subsampling by technicians with basic training in laboratory procedures; however, proper training in the use of the caton tray is necessary.

The instrument manager should be consulted for all instrument uses and procedures. Upon samples receipt, the laboratory must contact Marlys Cappaert at the Information Management Center by phone (541-754-4467) or fax to confirm samples have arrived. If expected samples do not arrive, labs must notify Ellen Tarquinio at EPA (202-566-2267).

11.4.1 Sorting and Subsampling Qualifications

This procedure may be used by any person who has received training in processing and identification of benthic macroinvertebrates. A laboratory staff member qualified to perform quality control (QC) checks must be present when samples are processed by an inexperienced individual, or when QC checks are needed for 10% of an experienced sorter's samples. The qualifications of this individual include achieving 90% sorting efficiency. The roles and responsibilities of the QC Officer are described below.

- Provides oversight of daily operations and sample processing, monitors QC activities to determine conformance, and conducts performance and systems audits of the procedures.
- Verifies the completeness of every Benthic Macroinvertebrate Laboratory Bench Sheet (Attachment 1) to ensure header information is correctly entered.
- 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 blank of the Benthic Macroinvertebrate Laboratory Bench Sheet.
- Checks 10% of an experienced individual's samples.
- Determines the sorting efficiency for each sample and sorter. The sorter's sorting efficiency is recorded on the bench sheet.

- Performs evaluations to ensure that QC is maintained throughout the laboratory sorting and subsampling procedure. Evaluations include double-checking work as it is completed and providing written documentation of these reviews to ensure that the standards set forth in the QAPP are met or exceeded.

11.4.2 Taxonomy Qualifications

This procedure may be used by any person who has received training in identification of freshwater 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. A second taxonomist will reidentify 10% of the samples for quality control (QC), as noted below, to quantify the rate of error, help target corrective actions, and thus minimize problems during data analysis. Samples will be sent to the taxonomists on a regular basis during the project as subsampling of the field samples is completed to avoid delays in identifying the organisms.

11.4.3 Sorting and Subsampling Precautions

1. Because it can be difficult to detect the organisms in rivers and streams samples (due to inexperience, detritus, etc.), a QC check must be performed by a person who has received instruction by senior biology staff familiar with processing benthic samples.
2. The QC checks in the Pertinent QA and QC Procedures section must be performed only by qualified personnel (QC Officers). These QC checks must be performed immediately following sorting of each grid.
3. Be sure that all sorting equipment is thoroughly cleaned and free of organisms prior to sorting the next sample.

11.4.4 Taxonomy Precautions

1. Identifications should be based on current published taxonomic references.
2. If technical literature citations specifying nomenclatural validity are not available or otherwise are unknown, taxon names from the Integrated Taxonomic Information System (ITIS), available on the Web at http://www.itis.usda.gov/, are to be used.
3. A list of primary and secondary technical literature used in completing the identifications must be prepared and submitted to the Tetra Tech project facilitator when samples are returned (see below).

11.5 Equipment/Materials

Sorting and Subsampling Equipment/Materials

- U.S. 35 sieve (500 µm or smaller)
- Round buckets
- Standardized gridded screen (370-µm mesh screen, 30 squares, each 6 cm^2)[1]
- White plastic holding tray for gridded screen[1]
- cm scoop
- 6-cm^2 metal dividing frame (“cookie cutter”)
- White plastic or enamel pan (6" x 9") for sorting
- Scissors
- Teaspoon
- Sample labels
- 70-80% denatured ethanol
- Benthic Sample Log-In form
- India ink pens
- Dropper
- Benthic Macroinvertebrate Laboratory Bench Sheet
- Stereo zoom microscope (6-10x)

Taxonomy Equipment/Materials

- Stereo dissecting microscope with fiberoptics light source
- Compound microscope
- Petri dishes
- Microscope slides (1" x 3" flat, precleaned)
- Cover slips (appropriately sized)
- CMCP-10 (or other appropriate mounting medium)
- India ink pens
- Dropper
- Forceps
- Specimen vials, with caps or stoppers
- Sample labels
- 70-80% denatured ethanol in plastic wash bottle
- Benthic Macroinvertebrate Taxonomic Bench Sheet
- Hand tally counter

[1]Some Cooperators may choose not to use the gridded screen in a plastic holding tray

11.6 Procedures

1. Receipt of samples must be recorded in the laboratory on the Benthic Sample Log-In form (Attachment 2). Assign the appropriate chronological bench number to each sample. Store samples at room temperature until ready for processing. For low gradient stream samples, be sure to record whether the sample is the "primary" or "secondary" sample. These are two distinct samples and they need to be kept separate.
2. Sample container(s) will arrive with very little alcohol to expedite shipping times and to account for hazardous material handling requirements. Refill the sample container(s) with 70-80% ethanol on THE SAME DAY THEY ARE RECEIVED in the laboratory. After the additional alcohol is in the sample, store it until sorting begins.
3. Sort and preserve a randomized 500-organism subsample separately from the rest of the sample using a gridded screen.
4. Document the level of effort, or proportion of sample processed, on the Benthic Macroinvertebrate Laboratory Bench Sheet (Attachment 1) for each sample as it is subsampled and sorted. Again, for low gradient stream samples, be sure to record whether the sample is the "primary" or "secondary" sample. These are two distinct samples and they need to be kept separate.
5. Record the following information on internal sample labels used for vials of sorted material with India ink pen on cotton rag paper or an acceptable substitute.

Station Name	Date Sorted
Station Location	Sorter's Initials
Station Number	"1 of 2" or "2" if necessary

11.6.1 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.
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.

a. 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.

4. 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.

5. 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.

6. 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 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 1.4.3, #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.

11.6.2 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.

   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).

2. 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.

3. 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.

4. 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 without counting it (the final count is made by the taxonomist).

5. 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.

    a. 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 1.4.2 #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.

6. 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.

7. 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".

8. 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

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

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 any sample portion should be done only following joint approval of the QC officer and the Project Manager.

9. Record the sorting date each sample was completed near the top right corner of the bench sheet.

11.6.3 Taxonomy Procedures

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 (Attachment 3), 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, Attachment 4). 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 (Attachment 3).

    a. Copy block of taxa names to a text file.
    b. Save the text file
    c. Go to the ITIS taxa match screen (http://www.itis.usda.gov/taxmatch_ftp.html)
    d. Follow the onscreen instructions to upload the file. Use all of the current defaults.
    e. Finish with two lists, one of matches with TSNs and one with non-matches. Check the non-matches for the following common problems.
        i. Abbreviations
        ii. Extra information identifiers (e.g., sp., spp., , nr., cf., genus 1, w/ hair chaete)
        iii. Extra character (e.g., "?", "Acentrella ?turbida", blank space)
        iv. The word "probably" or "prob" (e.g., "Microcylloepus prob. similis")
        v. IDing to a lower level than is in ITIS (e.g, to species rather than genus)
        vi. Double names (e.g., Callibaetis callibaetis)

vii. Common misspellings

viii. Tribes/Subfamilies/Subgenus sometimes do not appear in ITIS

ix. Species w/ incorrect Genus (Hydatopsyche betteni)

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

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

xii. 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:

   (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. Enter a taxonomic certainty rating (from 1 to 5, most certain to least certain) for each taxon identified on the bench sheet (under the column "TCR"). Also enter the number of the reference collection specimen(s) used in the identification or prepared for this project under that column on the bench sheet.

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

10. 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.

11. 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.

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

13. 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.

11.7 QA and QC Procedures

11.7.1 Sorting and Subsampling QA/QC

1. Experienced QC Officers will use 6-10x microscopes to check **all** sorted grids from the first five samples processed by a sorter to ensure that each meets the 90% SE. This will not only apply to inexperienced sorters, but also to those initially deemed as "experienced." Qualification will only occur when sorters achieve ≥90% sorting efficiency for five samples consecutively.
2. The QC Officer will calculate percent sorting efficiency (PSE) for each sample as follows:

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

   where A = number of organisms found by the primary sorter, and B = number of recoveries (organisms missed by the primary sort and found by the QC check).

   If the sorting efficiency for each of these five consecutive samples is ≥90% for a particular individual, this individual is considered "experienced" and can serve as a QC Officer. In the event that an individual fails to achieve ≥90% sorting efficiency, they will be required to sort an additional five samples to continue to monitor their sorting efficiency. However, if they show marked improvement in their sorting efficiency prior to completion of the next five samples, whereby they acquire the ≥90% sorting efficiency, the QA Officer may, at his/her discretion, consider this individual to be "experienced." Sorting efficiency should not be calculated for samples processed by more than one individual.
3. After individuals qualify, 10% (1 out of 10, randomly selected) of their samples will be checked.
4. If an "experienced" individual fails to maintain a ≥90% sorting efficiency as determined by QC checks, QC checks will be performed on every grid of five consecutive samples until a ≥90% sorting efficiency is achieved on all five. During this time, that individual will not be able to perform QC checks.
5. Randomly select 10% of the sample pickates to be sent to an external lab for QC checks for missed specimans. Pickate will consist of sort residue from all grids originally sorted. If samples contain more than 10% of the original number of organisms found in the sample, a determination will be made as to whether more of the samples need to be resorted (upon closer examination of the data).

11.7.2 Corrective Actions

1. In the case that any specimens are recovered through pickate rechecks when the original sort FAILS the 90% sorting efficiency (SE), recoveries will be identified and added to the original sample. However, samples that PASS the SE will not have any recovered organisms added to the sampled. Recovered organisms will simply be placed in a labeled vial and stored with the original sample, but not identified at this time.

11.7.3 Taxonomy QA/QC

1. On receipt of the samples, the project facilitator will randomly select 10% of the samples to be sent to the QC taxonomist, another experienced taxonomist who did not participate in the original identifications. A chain-of-custody form will be completed and sent with the samples.
2. The QC taxonomist will perform whole-sample re-identifications, with care taken to ensure inclusion of all slide-mounted specimens, completing another copy of the Benthic Macroinvertebrate Taxonomic Bench Sheet for each sample. Each bench sheet should be labeled with the term "QC Re-ID." As each bench sheet is completed, it should be faxed to the project facilitator.
3. The project facilitator will compare the taxonomic results (counts AND identifications) generated by the primary and QC taxonomists for each sample and calculate percent disagreement in enumeration (PDE) and percent taxonomic disagreement (PTD) as measures of taxonomic precision (Stribling et al. 2003) as follows:

$$PDE = \frac{|n1 - n2|}{n1 + n2} \times 100$$

where $n1$ is the number of specimens counted in a sample by the first taxonomist and $n2$ is the number of specimens counted by the QC taxonomist.

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

where $comp_{pos}$ is the number of agreements (positive comparisons) and N is the total number of pecimens in the larger of the two counts.

4. Unless otherwise specified by project goals and objectives, the measurement quality objective for enumerations will be a mean PDE less than or equal to 5 and a mean PTD less than or equal to 15, calculated from all the samples in the 10% set sent to the QC taxonomist. Results greater than these values will be investigated and logged for indication of error patterns or trends, but all values will generally be considered acceptable for further analysis, unless the investigation reveals significant problems.
5. Corrective action will include determining problem areas (taxa) and consistent disagreements, addressing problems through taxonomist interactions. Disagreements resulting from identification to a specific taxonomic level, creating the possibility to double-count "unique" or "distinct" taxa will also be rectified through corrective actions.
6. A report or technical memorandum will be prepared by the project facilitator. This document will quantify both aspects of taxonomic precision, assess data acceptability, highlight taxonomic problem areas, and provide recommendations for improving precision. This report will be submitted to the project manager, with copies sent to the primary and QC taxonomists and another copy maintained in the project file.

11.8 References

Barbour, M.T., J. Gerritsen, B.D. Snyder, and J.B. Stribling. 1999. Rapid Bioassessment Protocols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish, Second Edition. EPA 841-B-99-002. U.S. Environmental Protection Agency, Office of Water, Washington, DC.

Cox, S. and G. Lester. 2004. EcoAnalysts, Inc. Standard Operating Procedures: Sorting. Moscow, ID.

Epler, J.H. 2001. Identification manual for the larval chironomidae (Diptera) of North and South Carolina. A guide to the taxonomy of the midges of the southeastern United States, including Florida. Special Publication SJ2001-SP13. North Carolina Department of Environment and Natural Resources, Raleigh, NC, and St. Johns River Water Management District, Palatka, FL. 526 pp.

Merritt, R.W. and K.W. Cummins (editors). 1996. An introduction to the aquatic insects of North America, 3rd edition. Kendall/Hunt Publishing Company, Dubuque, Iowa.

Peck, D.V., J.M. Lazorchak, and D.J. Klemm (editors). Unpublished draft. *Environmental Monitoring and Assessment Program - Surface Waters: Western Pilot Study Field Operations Manual for Wadeable Streams*. EPA/XXX/X-XX/XXXX. U.S. Environmental Protection Agency, Washington, D.C.

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

ATTACHMENT 1: WILLAMETTE RESEARCH STATION ANALYTICAL LABORATORY SAMPLE PROCESSING AND TRACKING INFORMATION

2009 Willamette Research Station Analytical Laboratory
NRSA Sample Processing and Tracking Information

Analyst / Filter Lot	Lab # (YPNNNN)	Site ID	CU ID	CHLA ID	PCHL ID	PBIO ID	Coll. Date	Rec. Date	Filter Date	Acid Date
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
		FW08								
	d	FW08								

ATTACHMENT 2: CHLOROPHYLL A LABORATORY RECORD

Tables, diagrams, flowcharts, and validation data

Chlorophyll *a*

WRS Lab #	Site ID	Bar-code	Collect Date	Analysis Date	Volume (mL) Complete	Volume (mL) Sample	Raw Data (ug/L)	10x	100x	Analytical Duplicate	Calibration Data	Comments
											0	
											20	
											50	
											100	
											200	
											100 Check	
											0 Check	
Cal Check												

Analyst Initials _____

ATTACHMENT 3: National Rivers and Streams Assessment Homogenization and Contaminant Carryover QA Pilot Study

A. SCOPE AND APPLICATION

The purpose of the QA study (a.k.a. "pilot study") is to assess a prep lab's ability to produce homogenous fish tissue samples and to assess the effectiveness of the lab's routine cleaning procedures at minimizing sample-to-sample contamination that may be introduced during the homogenization process. Two prep labs, NERL-Cin and GLEC, will be processing tissue samples for the NRSA. Both labs must successfully complete this QA study prior to processing NRSA fillet composite samples.

B. SUMMARY

Homogeneity of filleted, processed fish tissue composite samples will be tested using percent lipids as a surrogate measure. Triplicate measurements of percent lipids will be performed on 6 separate fillet composite samples in each laboratory. If the relative standard deviation (RSD) of the triplicate measurements for each homogenate is <20%, then the homogenization procedures in that laboratory are judged to be effective and those procedures may be used for processing samples from the NRSA. Triplicate lipid determinations will be performed at a 5% frequency during the NRSA as an ongoing check on the sample homogenization procedures of each laboratory.

In order to determine if the routine equipment cleaning procedures are adequate to prevent sample-to-sample contamination, the prep labs will prepare an equipment rinsate after processing each of the 6 samples in the QA study. Homogenization equipment will be cleaned using routine procedures. Equipment used and cleaned after the preparation of three of the composites will be rinsed with deionized water and each of the three rinsates will be analyzed for metals. Equipment used and cleaned after the preparation of the other three composites will be rinsed with hexane with each of the three rinsates analyzed for organic compounds (PCBs, PBDEs, pesticides). If the analysis of the rinsates prepared during this QA study demonstrates that there is no contamination from the equipment, then the lab's cleaning procedures are judged to be effective and those procedures may be used during the NRSA. If contaminants are detected in the rinsates, the corresponding fish tissues may need to be analyzed in order to determine the origin of the contamination.

C. EQUIPMENT AND MATERIALS

Equipment and materials listed in the Fish Tissue Preparation SOP will also be used for this procedure. Additional materials needed for this QA pilot study are listed below.

C.1. 4 oz pesticide clean amber glass narrow mouth bottles with Teflon® lids for storage and shipment of the 100mL hexane rinsates.

D. PROCEDURE FOR DETERMINATION OF HOMOGENEITY

D.1. Six fish composite samples composed of 3 fish each will be processed, with the equipment cleaned in between composite sample processing (see Section E below for instructions pertaining to cleaning and testing of equipment). Fillet the fish samples following the Fish Tissue Preparation SOP, Section 10, including removing scales, maintaining skin, and including the belly flap in the fillet and homogenizing until a fine paste of uniform color and texture is produced.

D.2. Each prep lab (NERL-Cin and GLEC) will remove 3 portions of tissue from each of the 6 QA composite samples and set them aside for lipid testing with portion sizes being at least 5 grams each for the GLEC lipid analysis (SOP #ORG 3023) and at least 8 grams each for the NERL-Cin lipid analysis (SOP #MIRB 011.1E). Labs will test each set of six QA study composite samples (in triplicate) and record the results electronically in a spreadsheet, see Section D.3 below for reporting requirements and Quality Control (QC) acceptance criteria.

D.3. Both labs will submit their QA study lipid analysis results to the EPA Fish Tissue Manager. In order for the homogenization processes to be considered acceptable, the RSD of the triplicate analysis results must be <20% for all 5 QA study composite samples. Each lab must achieve this QC acceptance criterion before processing any of the NRSA fish tissue samples. If this criterion is not met, the EPA Fish Tissue Manager will work with the lab management to determine what changes to the homogenization procedures are needed. Processing of NRSA tissue samples will not begin in that laboratory until the adequacy of the homogenization procedures has been demonstrated by meeting the QC criterion.

D.4. Once the NRSA fillet fish tissue preparation begins, each laboratory will perform similar triplicate lipid determinations for 1 sample out of every 20 samples homogenized at that laboratory as a routine QC check on homogeneity. The same acceptance criterion (RSD<20%) will apply to these determinations.

E. PROCEDURE FOR VERIFICATION OF EQUIPMENT CLEANING PROCEDURES

E.1. Fillet and homogenize the first composite sample as described in Section D.1 above. After collecting all of the homogenized tissue, clean all the equipment as described in Section 10.1 of the Fish Tissue Preparation SOP. Rinse all of the equipment with 600mL of reagent water and collect the rinsate in an organics clean straight-sided 32 oz (or 1L) amber glass narrow mouth bottle with a Teflon® lid. Label the container “DIrinse01.” Repeat after each of the next 2 QA study composites are processed and the equipment is cleaned, resulting in a total of 3 reagent water rinsates labeled DIrinse01, DIrinse02, and DIrinse03. Save and provide for analysis a clean 600 mL portion of the reagent water used for this process in the same type of bottle as the rinsates. Label the bottle “DIblank.” The 3 reagent water rinsates (and one reagent water blank) from both GLEC and NERL-Cin will be tested for mercury and selenium.

The order in which the rinsates are taken, whether starting with the water rinsates as written in this Section or starting with the hexane set of rinsates (Section E.2) is not important. Either set may be taken first.

E.2. Fillet and homogenize the fourth sample as described in Section D.1 above. After collecting all of the homogenized tissue, clean all the equipment as described in Section 10.1 of the Fish Tissue Preparation SOP. Rinse all of the equipment with 100 mL of hexane and collect the rinsate in an organics clean straight-sided 4 oz amber glass narrow mouth bottle with a Teflon® lid. Label the container “hexrinse01.” Repeat after each of the next 2 QA study composites are processed and the equipment is cleaned, resulting in a total of 3 hexane rinsates labeled hexrinse01, hexrinse02, and hexrinse03. Save and provide for analysis a clean 100 mL portion of the hexane used for this process in the same type of bottle as the rinsates. NERL-Cin will test the solvent to make sure any positive results are from the rinsed equipment, not the solvent itself. Label the bottle “hexane blk.” The 3 hexane rinsates (and one hexane

blank) from both GLEC and NERL-Cin will be tested for the EMAP list of pesticides, PCBs, and PBDEs at NERL-Cin.

E.3. Because the rinsate samples are liquid, in contrast to the tissue samples, contamination will be evaluated on the basis of the total mass of contamination. NERL-Cin will convert rinsate concentrations to mass units in total volume of rinsate and compare the mass of contaminant in the rinsate to the mass of contaminant in a nominal tissue sample size at the detection limit for the contaminant. If there are positive hits for the analytes, NERL-Cin will decide whether or not to then analyze the corresponding pilot study composite sample to help determine the origin of the contaminant, in consultation with the EPA Fish Tissue Sample Manager.

E.4. NERL-Cin will report results for all 6 rinsate samples (and 2 blanks) from each lab in electronic spreadsheets and submit them to the EPA Fish Tissue Sample Manager. The results will be forwarded to expert chemists for evaluation of all blank contamination results to determine the likelihood that sample-to-sample contamination might occur. All results will be reported down to the lab's method detection limit (MDL) for the analyte to minimize censoring of the data. The analytical lab needs to provide the MDLs for each target analyte. Results for the rinsate samples will be evaluated on the basis of the mass of the contaminant relative to the nominal tissue sample size as described in Section E.3 above.

E.5. If contamination is detected in one or more of the rinsate samples, the EPA Fish Tissue Sample Manager will work with the lab management to determine what changes to the equipment cleaning procedures are required. Processing of NRSA tissue samples will not begin in that laboratory until the adequacy of the cleaning procedures has been demonstrated. The cleaning procedure will be determined as adequate when the rinsate results demonstrate no detection of pesticides above the MDL.

ATTACHMENT 4: NRSA HOMOGENIZATION QC - RINSATES

NRSA Homogenization QC – Rinsates

Homogenization Batch #:_____ Rinsate Date:_____

Rinsates:

DI Rinsate ID:	**Volume (ml):**	**Analyst:**	**Sample # (before)**	
Hexane Rinsate ID:	**Volume (ml):**	**Analyst:**	**Sample # (before)**	
Solvent Blk ID:	**Volume (ml)**	**Analyst:**	**Lot #:**	**Manufacturer:**
DI:	**Lot #:**		**Manufacturer:**	

Hexane Rinsate (Split):

Musk Rinsate ID:_____ Volume (ml):_____ Legacy Rinsate ID:_____ Volume (ml):_____

Legacy Rinsate Cleanup:

Date: _____ Analyst:_____

Drying step

Na_2SO4	Amount (g):	Lot #:	Manufacturer:	Date Muffled:
Glasswool		Lot #:	Manufacturer:	
Hexane	Amount (ml):	Lot #:	Manufacturer:	

NRSA Homogenization QC – Rinsates

SPE Step

Alumina N-Super I	Amount (g):	Lot #:	Manufacturer:	Date Muffled:
Hexane/$MeCl_2$ (80/20)	Amount (ml):	Lot #:	Manufacturer:	
Acetonitrile	Amount (ml):	Lot #:	Manufacturer:	

Musk Rinsate Cleanup/Concentration:

Date:_____ Analyst:_____ Final volume (ml):_____ Ship date:_____

Na_2SO4	Amount (g):	Lot #:	Manufacturer:	Date Muffled:
Toluene	Amount (ml):	Lot #:	Manufacturer:	

Metals Rinsate:

Preservative Date:_____ Analyst:_____ Total Metals volume_____

Preservative type:_____ Volume (ml):_____

Selenium Analysis: Date:_____ Analyst:_____

Nitric Acid	Lot #:	Manufacturer:
Peroxides	Lot #:	Manufacturer:

LRB	Sample Volume (ml):	Final Volume (ml):		
Rinsate	Sample Volume (ml):	Final Volume (ml):		
LFM	Sample Volume (ml):	Final Volume (ml):	Spike Lot #:	Spike Concentration:
LFM Dup	Sample Volume (ml):	Final Volume (ml):	Spike Lot #:	Spike Concentration:

Hg Analysis

Date:_____ Analyst:_____ Analysis volume (ml):_____

NRSA Homogenization QC – PPCP Rinsates

PPCP Rinsate ID:_____ Total PPCP Rinsate Volume:_____

Perseveration (Ascorbic Acid Na_2EDTA): Date:_____ Volume (ml):_____2_____ Analyst:_____
Ascorbic Acid: Lot #:_____ Manufacturer:_____ Na_2EDTA: Lot #:_____
Manufacturer:_____

Internal Standard:
Concentration:_____ Volume (ml):_____ Added by:_____

Extraction Cartridge: Type:___Oasis MCX_____ Lot #:_____

Conditioning Solvents:

Acetonitrile	Volume (ml): 6	Lot #:	Manufacturer:
DI water	Volume (ml): 6	Lot #:	Manufacturer:

Sample Wash (2% Formic Acid):
Amount (ml):_____ Lot #:_____ Manufacturer:_____

Elution:

1. Acetonitrile	Volume (ml): 4 (twice)	Lot #:	Manufacturer:
2. Acetonitrile with 5% NH4OH	Volume (ml): 4 (twice)		
	Acetonitrile	Lot #:	Manufacturer:
	NH4OH	Lot #:	Manufacturer:

Extraction: Date:_____ Chemist:_____

Sample ID	Sample Date	Arrival Date	Filtered Date	Extract, Evaporation/ Analysis date Elute 1	Elute 1 evaporated by	Extract, Evaporation/ Analysis date Elute 2	Elute 2 evaporated by	Notes

NRSA Homogenization QC – Lipids

Homgenization Batch #:_____

Sample #:_____

ASE:

Date:_____ Analyst:_____

Na2SO4 Amount (g):	Lot #:	Manufacturer:	Date Muffled:
Hexane	Lot #:	Manufacturer:	
MeCl2	Lot #:	Manufacturer:	

Drying:

Date:_____ Analyst:_____

Na2SO4 amount (g):_____ Lot #:_____ Manufacturer:_____ Date Muffled:_____

Lipid Analysis:

Date:_____ Analyst:_____

Sample/Lab ID	Sample Weight (g)	ASE Cell #	Final Volume (ml)	Lipid Volume (g)	Empty pan weight (g)	3 hour pan weight (g)	4 hour pan weight (g)
LRB	NA						

ATTACHMENT 5: SAMPLE PROCESSING RECORD FOR NRSA

Sample Processing Record for NRSA

Site ID:_____ **Sample ID:**_____ **Sample Date:**_____ **Homogenization Batch #:**_____

Project Code: NRSA ☐ LM ☐ **Site Code:** Urban ☐ Non-urban ☐ **Sample type:** ECO ☐ Non-ECO ☐

Species name:_____ **Number of indiviuals:**_____ **Barcode:**

Fish	Pre Wt (g)	Scales removed		Fillet		Comments/Flags
***.1**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
***.2**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
***.3**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
***.4**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
***.5**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
***.6**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
***.7**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
***.8**		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	
Composite (sm. ECO only)		**YES** ☐	**No** ☐	**Yes** ☐	**No** ☐	

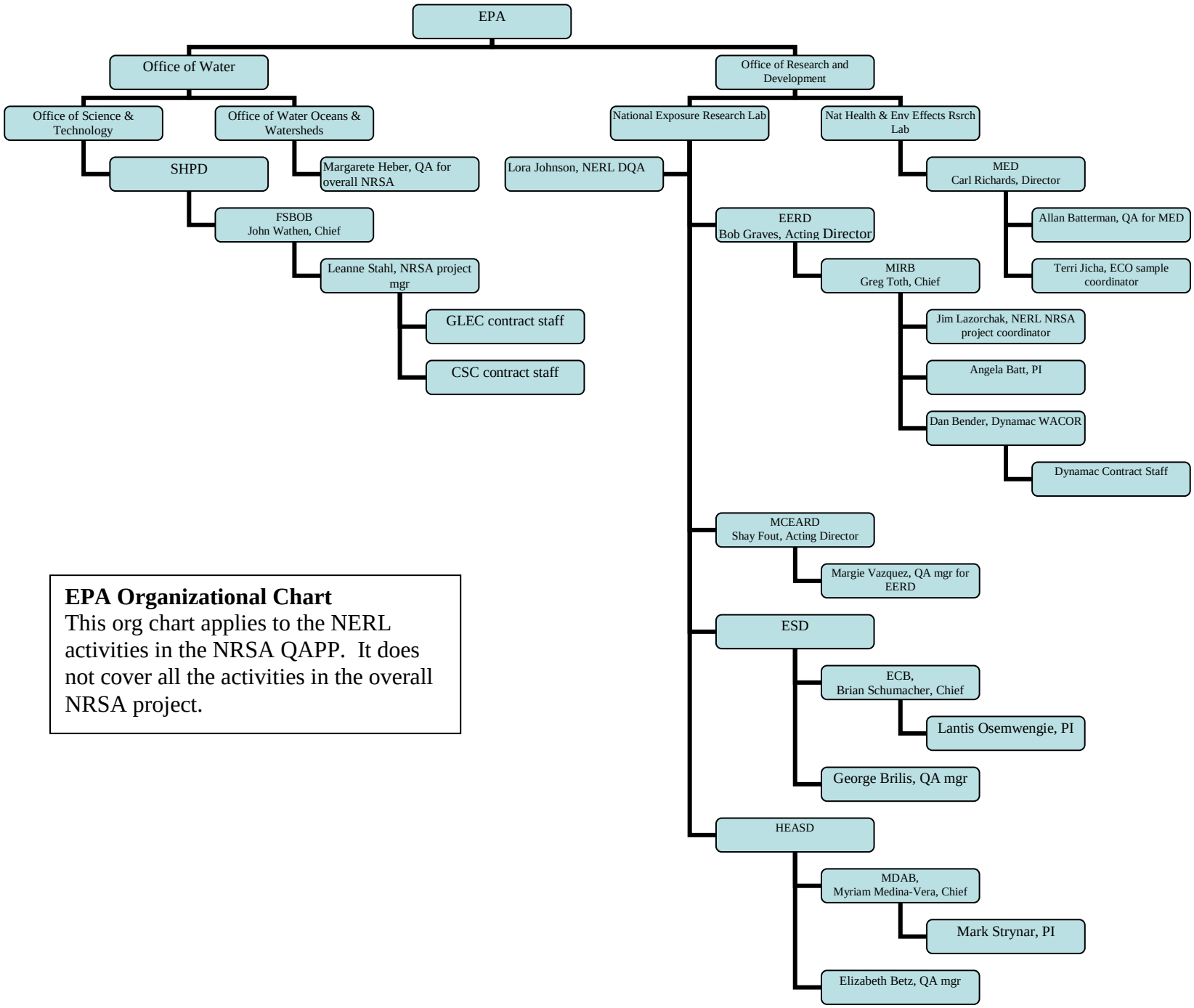
Analyst:_____ **Processing date:**_____

Total fillet wt (g):_____ **Total Carcass wt (g):**_____ **OR Total whole composite wt (g):**_____

Aliqouts

1. Bulk (100-300g)	Wt (g):		2. PPCP (8-20g)	Wt (g):		3. Musks (3-10g)	Wt (g):	
4. PFC (2-5g)	Wt (g):		5. Archive	Wt (g):				

ATTACHMENT 6: EPA ORGANIZATIONAL CHART



EPA Organizational Chart
This org chart applies to the NERL activities in the NRSA QAPP. It does not cover all the activities in the overall NRSA project.

ATTACHMENT 7: A LIST OF ANALYTES KNOWN IN THE NRSA QAPP AS THE EMAP LEGACY ANALYTES, PERFORMED ON THE FISH TISSUE FROM ALL SAMPLING LOCATIONS

A list of analytes known in the NRSA QAPP as the EMAP legacy analytes, performed on the fish tissue from all sampling locations

	Analyte	CAS#	MDL* (primary), ng/g	MDL* (confirmatory) ng/g	QL ng/g
1	Total Mercury	7783-34-8	1.47	NA	3.3
2	Total Selenium	7782-49-2	34	NA	200
3	Aldrin	309-00-2	0.20	0.18	0.67
4	α-Chlordane (cis)	5103-71-9	0.16	NA	0.63
5	α-BHC	319-84-6	NA	0.31	1.04
6	γ-Chlordane (trans)	5103-74-2	0.10	0.17	0.63
7	γ-BHC (Lindane)	58-89-9	0.16	NA	0.63
8	2,4'-DDD	53-19-0	0.22	0.15	0.72
9	4,4'-DDD	72-54-8	0.15	0.12	0.63
10	2,4'-DDE	3424-82-6	0.20	0.14	0.66
11	4,4'-DDE	72-55-9	0.13	0.24	0.8
12	2,4'-DDT	789-02-6	0.18	0.20	0.67
13	4,4'-DDT	55-29-3	0.11	NA	0.63
14	Dieldrin	60-57-1	0.20	0.15	0.66
15	Endosulfan I	959-98-8	0.32	NA	1.07
16	Endosulfan II	33213-65-9	0.29	0.46	1.53
17	Endrin	72-20-8	0.12	0.13	0.63
18	Endrin ketone	53494-70-5	0.26	0.17	0.88
19	Heptachlor	76-44-8	0.20	NA	0.67
20	Heptachlor epoxide	1024-57-3	0.13	0.18	0.63
21	Hexachlorobenzene	118-74-1	0.15	0.18	0.63
22	Mirex	2385-85-5	0.17	0.23	0.77
23	*cis*-Nonachlor	5103-73-1	0.12	0.13	0.63
24	*trans*-Nonachlor	39765-80-5	0.13	0.17	0.63
25	Oxychlordane	27304-13-8	0.12	0.15	0.63
29	2,4'-Dichlorobiphenyl	34883-43-7	NA	0.60	1.99
30	2,2',5-Trichlorbiphenyl	37680-65-2	0.35	NA	1.18
31	2,4,4'-Trichlorobiphenyl	7012-37-5	0.19	NA	0.64
32	2,2',3,5'-Tetrachlorobiphenyl	41464-39-5	0.23	0.26	0.87
33	2,2',5,5'-Tetrachlorobiphenyl	35693-99-3	0.29	0.29	0.98
34	2,3',4,4'-Tetrachlorobiphenyl	32598-10-0	0.11	0.24	0.79
35	3,3',4,4'-Tetrachlorobiphenyl	32598-13-3	0.21	0.23	0.76
37	2,2',4,5,5'-Pentachlorobiphenyl	37680-73-2	0.28	NA	0.95
39	2,3,3',4,4'-Pentachlorobiphenyl	32598-14-4	0.13	0.18	0.63
41	2,3',4,4',5-Pentachlorobiphenyl	31508-00-6	0.15	0.19	0.64
42	3,3',4,4',5-Pentachlorobiphenyl	57465-28-8	0.16	0.15	0.63
43	2,2',3,3',4,4'-Hexachlorobiphenyl	38380-07-03	0.20	0.16	0.68
44	2,2',3,4,4',5-Hexachlorobiphenyl	35065-28-2	0.54	NA	1.79
45	2,2',4,4',5,5'-Hexachlorobiphenyl	35065-27-1	0.15	0.14	0.63
47	3,3',4,4',5,5'-Hexachlorobiphenyl	32774-16-6	0.16	0.25	0.82
48	2,2',3,3',4,4',5-Heptachlorobiphenyl	35065-30-6	0.22	0.23	0.75
49	2,2',3,4,4',5,5'-Heptachlorobiphenyl	35065-29-3	NA	0.24	0.81
50	2,2',3,4',5,5',6-Heptachlorobiphenyl	52663-68-0	0.14	0.20	0.65
51	2,2',3,3',4,4',5,6-Octachlorobiphenyl	52663-78-2	0.14	0.20	0.65
52	2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl	40186-72-9	0.13	0.22	0.72
53	Decachlorobiphenyl	2051-24-3	0.15	0.24	0.79
54	2,2',4,4'-Tetrabromodiphenyl ether	5436-43-1	NA	0.37	1.23

	Analyte	CAS#	MDL* (primary), ng/g	MDL* (confirmatory) ng/g	QL ng/g
55	2,3',4,4'-Tetrabromodiphenyl ether	189084-61-5	0.13	0.26	0.86
56	2,2',4,4',5-Pentabromodiphenyl ether	60348-60-9	0.16	0.29	0.98
57	2,2',4,4',6-Pentabromodiphenyl ether	189084-64-8	0.39	0.43	1.44
58	2,2',4,4',5,5'-Hexabromodiphenyl ether	68631-49-2	0.59	0.34	1.97
59	2,2',4,4',5,6'-Hexabromodiphenyl ether	207122-15-4	0.58	0.37	1.17
60	2,2',3,4,4',5'-Hexabromodiphenyl ether	182677-30-1	0.36	0.55	1.84
61	2,2',3,4,4',5',6-Heptabromodiphenyl ether	207122-16-5	0.82	0.42	2.74
62	% lipids	NA	NA	NA	NA
63	% moisture	NA	NA	NA	NA

NA = not applicable

*MDLs may be periodically updated for these analyses, and therefore the MDLs may change throughout the course of the study.

ATTACHMENT 8: ADDITIONAL ANALYTES KNOWN IN THE NRSA QAPP AS CECS TO BE INCLUDED IN THE ANALYSIS OF FISH TISSUE COLLECTED FROM URBAN RIVER SITES

Additional analytes known in the NRSA QAPP as CECs to be included in the analysis of fish tissue collected from Urban River sites**

	Analyte	CAS#	MDL* g/g	QL ng/g
1	cimetidine	51481-61-9	n/a	n/a
2	ranitidine	66357-59-3	n/a	n/a
3	trimethoprim	738-70-5	n/a	n/a
4	sulfamethoxazole	723-46-6	n/a	n/a
5	10-hydroxy-amitriptyline	64520-05-4	n/a	n/a
6	promethazine	58-33-3	n/a	n/a
7	paroxetine	110429-35-1	n/a	n/a
8	alprazolam	28981-97-7	n/a	n/a
9	amitriptyline	549-18-8	n/a	n/a
10	benztropine	86-13-5	n/a	n/a
11	norfluoxetine	83891-03-6	n/a	n/a
12	fluoxetine	59333-67-4	n/a	n/a
13	desmethylsertraline	79902-63-9	n/a	n/a
14	sertraline	79559-97-0	n/a	n/a
15	albuterol	18559-94-9	n/a	n/a
16	atenolol	29122-68-7	n/a	n/a
17	clonidine	4205-91-8	n/a	n/a
18	oxycodone	124-90-3	n/a	n/a
19	amphetamine	51-63-8	n/a	n/a
20	hydrocodone	143-71-5	n/a	n/a
21	triamterene	396-01-0	n/a	n/a
22	metoprolol	56392-17-7	n/a	n/a
23	enalipril	76095-16-4	n/a	n/a
24	propranolol	318-98-9	n/a	n/a
25	desmethyldiltiazem	130606-60-9	n/a	n/a
26	diltiazem	33286-22-5	n/a	n/a
27	norverapamil	67814-42-4	n/a	n/a
28	verapamil	137862-53-4	n/a	n/a
29	propoxyphene	1639-60-7	n/a	n/a
30	amlodipine	111470-99-6	n/a	n/a
31	acetaminophen	103-90-2	n/a	n/a
32	prednisone	53-03-2	n/a	n/a
33	prednisolone	50-24-8	n/a	n/a
34	hydrocortisone	50-23-7	n/a	n/a
35	carbamazepine	298-46-4	n/a	n/a
36	betamethasone	378-44-9	n/a	n/a
37	methylprednisolone	83-43-2	n/a	n/a
38	norethindrone	68-22-4	n/a	n/a
39	testosterone	58-55-9	n/a	n/a
40	valsartan	396-01-0	n/a	n/a
41	fluocinonide	356-12-7	n/a	n/a
42	atorvastatin	134523-00-5	n/a	n/a
43	fluticasone	80474-14-2	n/a	n/a
44	progesterone	57-83-0	n/a	n/a
45	simvastatin	79902-63-9	n/a	n/a
46	theophylline	58-55-9	n/a	n/a
47	hydrochlorothiazide	58-93-5	n/a	n/a
48	2-hydroxy-ibuprofen	51146-55-5	n/a	n/a

	Analyte	CAS#	MDL* g/g	QL ng/g
49	furosemide	54-31-9	n/a	n/a
50	warfarin	81-81-2	n/a	n/a
51	glipizide	29094-61-9	n/a	n/a
52	ibuprofen	15687-27-1	n/a	n/a
53	gemfibrozil	25812-30-0	n/a	n/a
54	glyburide	10238-21-8	n/a	n/a
55	galaxolide	1222-05-5	17.8	53.4
56	tonalide	1506-02-1	9.5	28.5
57	musk xylene	81-15-2	5.2	15.6
58	musk ketone	81-14-1	14.0	42.0
59	amino musk ketone	not found	12.6	37.8
60	4-amino musk xylene	107342-55-2	12.2	36.6

n/a= not available, see ** below

*MDLs may be periodically updated for these analyses, and therefore the MDLs may change throughout the course of the study.

**Method development is not completed for the analysis of pharmaceuticals in fish tissue (compounds 1-54). Therefore MDLs and QLs are not available at the time of this writing.

ATTACHMENT 9: PHARMACEUTICAL ANALYTES IN WATER SAMPLES FROM URBAN RIVER SAMPLING LOCATIONS

Pharmaceutical analytes in water samples from Urban River sampling locations

	Analyte	CAS#	MDL ng/L	QL ng/L
1	cimetidine	51481-61-9	0.6	1.9
2	ranitidine	66357-59-3	3.5	11
3	trimethoprim	738-70-5	0.8	2.5
4	sulfamethoxazole	723-46-6	0.5	1.6
5	10-hydroxy-amitriptyline	64520-05-4	0.2	0.6
6	promethazine	58-33-3	0.4	1.3
7	paroxetine	110429-35-1	0.5	1.6
8	alprazolam	28981-97-7	2.9	9.1
9	amitriptyline	549-18-8	0.2	0.6
10	benztropine	86-13-5	0.5	1.6
11	norfluoxetine	83891-03-6	2.3	7.2
12	fluoxetine	59333-67-4	0.9	2.8
13	desmethylsertraline	79902-63-9	3	9.4
14	sertraline	79559-97-0	0.9	2.8
15	albuterol	18559-94-9	3.1	9.7
16	atenolol	29122-68-7	1.9	6.0
17	clonidine	4205-91-8	11	35
18	oxycodone	124-90-3	0.8	2.5
19	amphetamine	51-63-8	0.5	1.6
20	hydrocodone	143-71-5	1.2	3.8
21	triamterene	396-01-0	0.4	1.3
22	metoprolol	56392-17-7	4.3	14
23	enalipril	76095-16-4	0.3	0.9
24	propranolol	318-98-9	1.4	4.4
25	desmethyldiltiazem	130606-60-9	0.5	1.6
26	diltiazem	33286-22-5	0.9	2.8
27	norverapamil	67814-42-4	1.4	4.4
28	verapamil	137862-53-4	0.8	2.5
29	propoxyphene	1639-60-7	5.1	16
30	amlodipine	111470-99-6	0.4	1.3
31	acetaminophen	103-90-2	1.5	4.7
32	prednisone	53-03-2	9.5	30
33	prednisolone	50-24-8	3.4	11
34	hydrocortisone	50-23-7	4.6	14
35	carbamazepine	298-46-4	1.4	4.4
36	betamethasone	378-44-9	6	19
37	methylprednisolone	83-43-2	5.8	18
38	norethindrone	68-22-4	2.2	6.9
39	testosterone	58-55-9	1.1	3.5
40	valsartan	396-01-0	3.6	11
41	fluocinonide	356-12-7	2.8	8.8
42	atorvastatin	134523-00-5	12	38
43	fluticasone	80474-14-2	6.2	19
44	progesterone	57-83-0	60	188
45	simvastatin	79902-63-9	13	41
46	theophylline	58-55-9	28	88
47	hydrochlorothiazide	58-93-5	3.2	10
48	2-hydroxy-ibuprofen	51146-55-5	12	38
49	furosemide	54-31-9	12	38

	Analyte	CAS#	MDL ng/L	QL ng/L
50	warfarin	81-81-2	3.6	11
51	glipizide	29094-61-9	11	35
52	ibuprofen	15687-27-1	3.8	12
53	gemfibrozil	25812-30-0	1.2	3.8
54	glyburide	10238-21-8	51	160

ATTACHMENT 10: BATCH SAMPLE ANALYSIS BENCH SHEET FOR EPA METHOD 1606

BATCH SAMPLE ANALYSIS BENCH SHEET FOR EPA METHOD 1606

Purified DNA Extracts

NRSA Batch #_____ **Dates** _____

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

ATTACHMENT 11: BENTHIC MACROINVERTEBRATE LABORATORY BENCH SHEET

BENTHIC MACROINVERTEBRATE LABORATORY BENCH SHEET (FRONT)

Project Name/Number _____

Serial ID# _____ Waterbody Name _____

Sorter (initially spread sample) _____ Site ID

Sort Date _____ Collection Date _____

GRID ORDER	SORTER'S INITIALS	RANDOM NUMBER GRID ID	NUMBER OF INDIVIDUALS PER GRID	CUMULATIVE NUMBER OF ORGANISMS
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30				

Check off grids as selected:

	1	2	3	4	5	6
1						
2						
3						
4						
5						

ATTACHMENT 12: BENTHIC SAMPLE INFORMATION REPORT

SITE_ID	DATE_COL	VISIT_NO	SAMPTYPE	SAMP_ID	LAB_ID	JARS_COL	JARSRECD	DATE_REC	PCTCOUNT	COM_LAB
Site ID	Sample Collection	Within Year	Benth. Samp. Type	Sample Tracking Number	Lab Sample	Number of	Number of	Date Samples	Percent of Sample	Lab Personnel
	Date	Site Visit Number		from Jar Label	ID Number	Jars Collected	Jars Received	Received	Counted	Comments

ATTACHMENT 13: BENTHIC MACROINVERTEBRATE TAXONOMIC LEVEL OF EFFORT

Taxonomic Level of Effort

This is the Standard Taxonomic Effort list for benthic macroinvertebrates. It represents the minimum level needed for mature and well preserved specimens. The lowest targeted taxonomic level will be genus. Due to taxonomic limitations, some groups cannot be identified to the genus level and therefore should be taken to the level specified below. Foe all taxonomic groups, if the level can easily go lower, for example monotypic genera, or if only one genus or species is known to occur in a certain geographic area, then these specimens should be identified at the lowest possible taxonomic level (e.g., Ephemerellidae *Drunella doddsI*). If the minimum taxonomic level cannot be achieved due to immature, damaged, or pupal specimens this should be noted in the data file "flag" variable (e.g., IM = y, DD = y, PP = y). If a unique taxon is determined for which the appropriate taxonomic level is not available in the literature and there are other taxa in that taxonomic level, these specimens shall be given a code of UN = y (e.g., Ephemerellidae *Drunella doddsi* and *Drunella* sp. UN = y vs. *Drunella* sp. UN = n) so that these specimens can be distinguished from specimens that are NOT unique and are to be grouped at a higher taxonomic level due to imprecise identification.

PHYLUM ANNELIDA

Class Branchiobdellida	Identify to family
Class Hirudinea	Identify to genus
Class Oligochaeta	Identify to genus
Class Polychaeta	Identify to family

PHYLUM ARTHROPODA

Class Arachnoidea	
Acari	Identify to family
Class Insecta	
Coleoptera	Identify to genus
Diptera	Identify all to genus except in the following cases:
Chironomidae	Identify to genus (this may not be possible for some groups which should be identified to at least tribe or subfamily)
Dolichopodidae	Identify to family
Phoridae	Identify to family
Scathophagidae	Identify to family
Syrphidae	Identify to family
Ephemeroptera	Identify to genus
Hemiptera	Identify to genus
Lepidoptera	Identify to genus
Megaloptera	Identify to genus
Odonata	Identify to genus
Plecoptera	Identify to genus
Trichoptera	Identify to genus

Class Malacostraca	Identify to genus
Amphipoda	Identify to genus
Decapoda	Identify to genus
Isopoda	Identify to genus
Mysidacea	Identify to genus
PHYLUM COELENTERATA	
PHYLUM MOLLUSCA	
Class Bivalvia	Identify to genus
Class Gastropoda	Identify to genus except in the following cases: Hydrobiidae - Identify to family
PHYLUM NEMERTEA	Identify to genus