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Washington, DC
EPA 841-B-21-010

National Lakes Assessment 2022

Laboratory Operations Manual

Version 1.1, May 2022

Version History

Version	Date	Revisions or Comments
0.0	February 2022	Internal EPA version for project QAC review and comments
1.0	February 2022	Final approved document
1.1	May 2022	• Corrected the atrazine sample preservation so that samples should be kept chilled until analysis, not frozen (Section 8) • Corrected typos in Tables 5.3, 7.2 and 11.2

NOTICE

The intention of the National Lakes Assessment 2022 (NLA 2022) is to provide a comprehensive assessment for lakes, ponds, and reservoirs across the United States. The complete documentation of overall project management, design, methods, standards, and Quality Assurance/Quality Control measures, is contained in companion documents, including:

National Lakes Assessment 2022: Quality Assurance Project Plan (QAPP) (EPA 841-B-21-009)

National Lakes Assessment 2022: Site Evaluation Guidelines (SEG) (EPA 841-B-21-008)

National Lakes Assessment 2022: Field Operations Manual (FOM) (EPA 841-B-21-011)

This document (Laboratory Operations Manual) contains information on the methods for analyses of the samples for nine indicators: algal toxins (cylindrospermopsin and microcystins), atrazine screen, benthic macroinvertebrates, fecal indicator (enterococci), phytoplankton, water chemistry and chlorophyll *a*, and zooplankton to be collected during the project, quality assurance objectives, sample handling, and data reporting. Environmental DNA (eDNA) and fish fillet contaminant analysis are also included as part of the NLA 2022, and those methods are available separately. The NLA laboratory methods are based on guidelines developed by federal agencies and methods employed by several key states that were involved in the planning phase of this project. Methods described in this document are to be used specifically in work relating to the NLA 2022. All project cooperator laboratories must follow these guidelines. Mention of trade names or commercial products in this document does not constitute endorsement or recommendation for use. Details on specific methods for site evaluation and sampling can be found in the appropriate companion document. Revision history information is found in the associated QAPP document.

The suggested citation for this document is:

U.S. EPA. 2022. National Lakes Assessment 2022. Laboratory Operations Manual. V.1.1. EPA 841-B-21-010. U.S. Environmental Protection Agency, Washington, DC.

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LIST OF ACRONYMS and ABREVIATIONS

A	absorbance
ANOVA	analysis of variance
ANC	acid neutralizing capacity
AV	assistance visit
Ca	calcium
CH_4	methane
Cl	chloride
CO_2	carbon dioxide
COB	close of business
CRM	certified reference manual
CV	coefficient of variation
d	days
DI	deionized
DO	dissolved oxygen
DOC	dissolved organic carbon
ELISA	enzyme-linked immunosorbent assay
EMAP	Environmental Monitoring and Assessment Program
EtOH	ethyl alcohol
FOM	field operations manual
HDPE	high density polyethylene
H_2SO_4	sulfuric acid
HNO_3	nitric acid
HQ	Headquarters
IBD	ion balance difference
IM	information management
IT	information technology
K	potassium
LCS	laboratory control sample
LOM	laboratory operations manual
LRL	lower reporting limit
Mg	magnesium
MS	matrix spike
MSD	matrix spike duplicate
MDL	method detection limit
MQO	measurement quality objective
MPCA	Minnesota Pollution Control Agency
MPN	most probable number
MRL	Mercury Research Laboratory
N	Nitrogen
NARS	National Aquatic Resource Surveys
NLA	National Lakes Assessment
N_2O	nitrous oxide
Na	sodium
NH_3	ammonia
NIST	National Institute of Standards
NO_3	nitrate

NO_2	nitrite
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PD	percent difference
PDE	percent difference in enumeration
PESD	Pacific Ecological Systems Division
PSE	percent sorting efficiency
PT	proficiency tests
PTD	percent taxonomic disagreement
QA	quality assurance
QAPP	quality assurance project plan
QA/QC	quality assurance/quality control
QC	quality control
QCCS	quality control check solution
QMP	Quality Management Plans
RL	Reporting Limit
RMSE	root mean square error
RO	reverse osmosis
RPD	relative percent difference
RSD	relative standard deviation
S	standard deviation
SEG	site evaluation guidelines
SiO_2	silica
SO_4	sulfate
SOPs	standard operating procedures
SRM	standard reference material
TMB	tetramethylbenzidine
TN	total nitrogen
TOC	total organic carbon
TOCOR	Task Order Contracting Officer's Representative
TP	total phosphorus
USEPA	United States Environmental Protection Agency
USGS	United States Geological Survey
WRS	Willamette Research Station

(now known as Pacific Ecology Studies Division in EPA's Office of Research and Development)

NATIONAL LAKES ASSESSMENT 2022 LABORATORY OPERATIONS MANUAL

The U.S. Environmental Protection Agency (USEPA), in partnership with state and tribes, has designed the National Lakes Assessment (NLA) 2022 to assess the condition of lakes, ponds and reservoirs (referred to collectively as lakes throughout the document) in the United States. The NLA is one in a series of National Aquatic Resource Surveys (NARS) conducted to provide the public with a comprehensive assessment of the condition of waters in the U.S.

This manual contains procedures for laboratory analysis of samples collected from lakes throughout the lower 48 states of the United States. The purposes of this manual are to:

1) document the standardized sample processing and analysis procedures used in the various laboratories for the NLA 2022; and
2) provide guidance for data quality and a performance-based method approach to obtain comparable results across all participating laboratories.

Detailed laboratory procedures are described for the following indicators: algal toxins, benthic macroinvertebrates, fecal indicator (enterococci), phytoplankton, atrazine pesticide screen, water chemistry, chlorophyll *a*, and zooplankton. In addition to the indicators listed above, samples for two research indicators (i.e., environmental DNA (eDNA) and fish fillet contaminants) will be collected. These will be done in collaboration with the USEPA's Office of Research and Development (ORD) and Office of Science and Technology (OST), respectively. Methods for the fish fillet contaminants indicator are maintained by OST.

Specific laboratory analysis procedures for water chemistry samples are not presented here. A list of parameters to be analyzed as well as the performance-based methods and pertinent quality assurance/quality control (QA/QC) procedures are outlined as requirements for laboratories to follow. Alternative analytical methods for water chemistry are acceptable if they meet all specified performance requirements described in this document. Acceptability is determined by the NLA project management team (USEPA Office of Water).

1.0 GENERAL LABORATORY GUIDELINES

1.1 Responsibility and Personnel Qualifications

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

1.2 Roles and Contact Information

Table 1.1 presents contact information for the key personnel associated with NLA 2022. The **USEPA Headquarters Project Management Team** consists of the Project Leader, Alternate Project Leaders, and Project QA Lead. The Team is responsible for overseeing all aspects of the project and ensuring technical and quality assurance requirements are properly carried out. The Team is the final authority on all decisions regarding laboratory analysis.

The **Contractor Logistics Coordinator** and the **NARS Information Management (IM) Coordinator** track the location of each NLA 2022 sample that involves laboratory processing. These coordinators will be the laboratories main point of contact in regard to sample tracking.

Table 1.1 Contact information.

Title	Name	Contact Information
USEPA HQ Project Lead	Lareina Guenzel, OW	guenzel.lareina@epa.gov 202-566-0455
USEPA HQ Project QA Coordinator	Sarah Lehmann, OW	lehmann.sarah@epa.gov 202-566-1379
USEPA HQ Logistics Lead	Brian Hasty, OW	hasty.brain@epa.gov 202- 564-2236
USEPA HQ Laboratory Review Coordinator	Kendra Forde, OW	forde.kendra@epa.gov 202-566-0417
Information Management (IM) Center Coordinator	Michelle Gover, GDIT	gover.michelle@epa.gov 541-754-4793
Contractor Logistics Coordinator	Chris Turner, GLEC	cturner@glec.com 715-829-3737

1.3 Sample Tracking

Samples are collected by a large number of field crews during the index period (June through September). The actual number of lakes sampled on a given day will vary widely during this time. Field crews submit electronic forms when they have shipped samples and the NARS IM Center inputs each sample into the NARS IM database. Processing laboratories can track sample shipment from field crews by accessing the information uploaded in the NARS IM database by way of the NARS SharePoint site. Participating laboratories and all pertinent personnel will be given access to the NARS SharePoint site, where they can acquire site and sample status information. This will include check-in and batching

information on samples that have been shipped to the batch laboratory by field crews (either by overnight shipment for perishable samples or batch shipments for preserved samples). The NARS IM Center provides laboratories with spreadsheets of samples that the batch lab or field crews (as appropriate) have sent to them. Upon sample receipt, the analysis laboratory must immediately complete and email the sample tracking spreadsheet (containing the sample login and sample condition information) to the IM Center Coordinator for confirmation of sample receipt. Each laboratory will make arrangements with the USEPA HQ Laboratory Review Coordinator for access to the NARS SharePoint site and the NARS IM Center Coordinator, both listed above, to ensure the process of sample check-in has been organized before samples begin to arrive.

When the samples arrive from the field crews, laboratories also receive tracking forms in the shipment (refer to the NLA 2022 FOM). These forms list the samples included in the shipment. Laboratory personnel must cross check the forms with the samples received to verify that there are not any inconsistencies. If any sample is missing or damaged, contact the IM Center Coordinator and/or Contractor Logistics Coordinator immediately. For state laboratories conducting analyses in their own laboratories, a state sample tracking spreadsheet is available from EPA.

1.4 Reporting

All laboratories must provide data analysis information to the HQ Project Management Team and the NARS IM Center by March 1, 2023 or earlier as stipulated in contractual agreements. These reports must include the following information and be reported in the data templates available separately from EPA.

- Sample Type (indicator)
- Site ID (ex: NLA22_AL-10007)
- Sample ID (ex: 999000)
- Pertinent information to the indicator
- Metadata for all fields

The submitted file name must state the following:

- Indicator name (ex: microcystins)
- Date of files submission by year, month, and day (ex: 2022_11_01)
- Laboratory name (ex: MyLaboratory)

Combined, the file name would look as follows: Microcystin_2022_11_01_MyLaboratory.xlsx

As specified in the QAPP, remaining sample material and specimens must be maintained by the USEPA's designated laboratory or facilities as directed by the NLA 2022 Project Lead. All samples and raw data files (including logbooks, bench sheets, and instrument tracings) are to be retained by the laboratory for 3 years (or as outlined in EPA contract documents) or until authorized for disposal, in writing, by the USEPA Project Leader. Deliverables from contractors and cooperators, including raw data, are permanent as per USEPA Record Schedule 258. USEPA's project records are scheduled 501 and are also permanent.

2.0 LABORATORY QUALITY CONTROL

As part of the NLA 2022, field samples will be collected at each assessment site unless otherwise specified. These samples will be sent to laboratories cooperating in the assessment. To ensure quality, each Project Cooperator laboratory analyzing samples from the NLA 2022 will participate in a laboratory verification process. All Project Cooperator laboratories will follow these guidelines.

No national program of accreditation for laboratory processing for most of our indicators currently exists. For this reason, a rigorous program of laboratory evaluation and verification has been developed to support the NLA 2022.

Given the large number of laboratories participating in the NLA 2022, it is not feasible to perform an assistance visit[a] (AV) on each of these laboratories. An AV would include an on-site visit to the laboratory lasting at least a day. As a result, the USEPA Headquarters Project Management Team will conduct remote review of laboratory certifications and accreditations of all laboratories and an inter-laboratory comparison will be performed between some laboratories (mainly for biological indicators). This process is called laboratory verification and is conducted before sample processing and analysis begins. If issues arise from the remote review or inter-laboratory comparison that cannot be resolved remotely then an on-site visit to the laboratory will be performed. The NLA 2022 Project Management Team believes this approach meets the needs of this assessment and can ensure quality control on data generated by the participating laboratories. General information is provided here and more specifics are provided in **Sections 2.1** and **2.2**.

Competency

To demonstrate its competency, the laboratory shall provide analyte and matrix specific information to the USEPA; or information specific to the relevant biological indicator. The USEPA will accept one or more of the following as a demonstration of competency:

- Memorandum that identifies the relevant services that the laboratory provided for the National Aquatic Resource Surveys in the past five years.
- Documentation detailing the competency of the organization, including professional certifications for water-related analyses, membership in professional societies, a curriculum vita for taxonomists, and experience with analyses that are the same or similar to the requirements of this method.
- Demonstration of competency with sediment and water chemistry samples in achieving the method detection limits, accuracy, and precision targets.

Quality assurance and quality control requirements

To demonstrate its competency in quality assurance and quality control procedures, each laboratory shall provide the USEPA with copies of the quality-related documents relevant to the procedure. Examples include Quality Management Plans (QMP), QAPPs, and applicable SOPs.

[a] The evaluation and verification of the laboratories is being considered as a form of an AV rather than an audit because the evaluation and verification phase is designed for the laboratories to demonstrate competency of performance and for the EPA HQ Project Management Team to provide guidance to the laboratories rather than as "inspection" as in a traditional audit.

To demonstrate its ongoing commitment, the person in charge of quality issues for the participating laboratory shall sign the NLA QAPP Certification Page, which will be maintained at the USEPA in a quality assurance file.

2.1 Laboratory Verification Process/Technical Assessment

Procedural review and assistance personnel are trained to the specific implementation and data collection methods detailed in this NLA 2022 LOM. Laboratory evaluation and verification reinforces the specific techniques and procedures for both field and laboratory applications. A remote evaluation and verification procedure has been developed for performing assessment of all laboratories.

Laboratory evaluation and verification process will be conducted prior to data analysis to ensure that specific laboratories are qualified and that techniques are implemented consistently across the multiple laboratories generating data for the program. Laboratory evaluation and verification plans have been developed to ensure uniform interpretation and guidance in the procedural reviews.

The procedure being utilized involves requesting the laboratory to provide documentation of its policies and procedures. For the NLA 2022 project, we have requested that each participating laboratory provide the following documentation:

- The laboratory's Quality Manual, (QMP) or similar document.
- SOPs for each analysis to be performed.
- Long term Method Detection Limits (MDLs) for each instrument used and Demonstration of Capability for each analysis to be performed.
- A list of the laboratory's accreditations and certifications (e.g. NELAP, ISO, etc.), if any.
- Results from Proficiency Tests (PT) for each analyte to be analyzed under the NLA project.
- Relevant curriculum vitae and documents demonstrating previous survey participation.

If a laboratory has clearly documented procedures for sample receiving, storage, preservation, preparation, analysis, and data reporting; has successfully analyzed PT samples (if required by the USEPA, the USEPA will provide the PT samples); has a Quality Manual that thoroughly addresses laboratory quality including standard and sample preparation, record keeping and QA non-conformance; participates in a nationally recognized or state certification program; and has demonstrated the ability to perform the testing for which program/project the audit is intended, then the length of an on-site visit will be minimum, if not waived entirely. A final decision on the need for an actual on-site visit should be made after the review and evaluation of the documentation requested.

If a laboratory meets or exceeds all of the major requirements and is deficient in an area that can be corrected remotely, suggestions will be offered, and the laboratory will be given an opportunity to correct the issue. A correction of the deficiency will then be verified remotely. The on-site visit should only be necessary if the laboratory fails to meet the major requirements and is in need of assistance or fails to produce the requested documentation.

All laboratory personnel responsible for quality must sign the NLA 2022 QAPP signature page.

In addition, all laboratories must sign a Laboratory Signature Form (in **APPENDIX A: LABORATORY REMOTE EVALUATION AND VERIFICATION FORMS**) indicating that they will abide by the following:

1. Utilize procedures identified in the NLA 2022 Laboratory Operations Manual (or equivalent). If using equivalent procedures, please provide procedural manual to demonstrate ability to meet the required MQOs.

2. Read and abide by the NLA 2022 Quality Assurance Project Plan (QAPP) and related SOPs.
3. Have an organized IT system in place for recording sample tracking and data analysis.
4. Provide data to the USEPA using the template provided in the Laboratory Operations Manual.
5. Provide data results in a timely manner. This will vary with the type of analysis and the number of samples to be processed. Sample data must be received no later than May 1, 2018 or as otherwise negotiated with the USEPA.
6. Participate in a laboratory technical assessment or audit if requested by the USEPA NLA staff (this may be a conference call or on-site audit).

If a laboratory is participating in biological analyses, they must, in addition, abide by the following:

1. Use taxonomic standards outlined in the NLA 2022 Laboratory Manual.
2. Participate in taxonomic reconciliation exercises during the field and data analysis season, which include conference calls and other laboratory reviews (see more below on Inter-laboratory comparison).

2.2 Inter-laboratory Comparison

An inter-laboratory investigation is being implemented for the laboratories performing analysis on benthic macroinvertebrates and zooplankton data for the NLA 2022. This process is defined as an inter-laboratory comparison since the same protocols and method will be used by all participating laboratories as described in this manual. No on-site assistance visit is envisioned for these laboratories unless the data submitted and reviewed by the USEPA does not meet the requirements of the inter-laboratory comparison described.

3.0 ALGAL TOXIN IMMUNOASSAY PROCEDURE: CYLINDROSPERMOPSIN

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

3.1 Summary of Method

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

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

3.2 Health and Safety Warnings

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

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

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

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

3.3.1 Definitions

The following terms are used throughout the procedure:

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

Calibration Range is the assay range for which analysis results can be reported with confidence. For undiluted samples, it ranges from the reporting limit of 0.1 µg/L to a maximum value of 2.0 µg/L. Please note, NARS IM cannot accept characters within numeric fields. Values outside the range are handled as follows. If the value is:

- < 0.05 µg/L, then the laboratory reports the result as is (without characters) and flags the sample as a non-detect (i.e. DATA_FLAG=ND).
- Between 0.05 µg/L and the reporting limit of 0.1 µg/L (i.e., >0.05 µg/L and <0.1 µg/L), the laboratory should record the value, but assign a Quality Control (QC) code to the value indicating that the result is between the detection limit and the reporting limit (i.e., DATA_FLAG=J).
- >2.0 µg/L, the result indicates that the sample value is outside of the calibration range and must be diluted and re-run using another analytical run. Leave the CONC column blank and record 'HI' in the DATA FLAG column.

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

Equation 3.1 Standard deviation

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

where *n* is the number of replicate samples, A_i, is the absorbance measured for the i^{th} replicate. Per **Section** Error! Reference source not found., samples are evaluated in duplicate (i=1 or 2); controls are either evaluated in duplicate or triplicate (i=1, 2, 3). $\bar{A}$ is the average absorbance of the replicates. Then, calculate %CV as:

Equation 3.2 Percent (%) coefficient of variation

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

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

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

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

limit of 0.1 µg/L, at which the *measured* value of the analyte can be reported with confidence. Also see "Sample-Specific Detection Limit" below.

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

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

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

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

Standard Deviation (S) shows variation from the average.

3.4 General Requirements for Laboratories

3.4.1 Expertise

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

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

3.4.2 Quality assurance and quality control requirements

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

To demonstrate its ongoing commitment, the person in charge of quality issues for the organization shall sign the NLA 2022 QAPP Certification Page.

3.4.3 Personnel

Laboratory Technician: This procedure may be used by any laboratory technician who is familiar with the NLA 2022 QAPP, and this procedure in the NLA 2022 LOM. The laboratory technician also must be familiar with the use of a multichannel pipette and plate readers.

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

3.5 Equipment/Materials

The procedures require the following equipment and information:

- Eurofins Technologies Cylindrospermospin ELISA (Microtiter) Test Kit, Product # 522011 (see items in **Section** Error! Reference source not found.).
- Adhesive Sealing Film (Parafilm) for Micro Plates: Used to cover plates during incubation.
- Data Template – See **Figure 3.1**
- Distilled or Deionized Water: For diluting samples when necessary.
- ELISA evaluation software.
- 2 glass scintillation vials (20 mL).
- Multichannel Pipette & Tips: An 8-channel pipette is used for this method. Proficient use of the multichannel pipette is necessary to achieve reliable results. Practice with water if you have never used this before.
- Norm-ject syringes (or equivalent).
- Paper Towels: For blotting the microtiter plates dry after washing.
- Permanent Marker (Sharpie Fine Point): For labeling samples, bottles, plates and covers.
- Plate Reader (such as Metertech, Model M965 AccuReader): Complete with Metertech PC Mate software for operation of machine. This machine reads the microtiter plates.
- Project Quality Control Samples.
- Reagent Reservoirs (Costar Cat Number 4870): Plain plastic reservoir for reagents that accommodate the use of a multi-channel pipette.
- Test tubes: For dilutions, if needed.
- Timer: For measuring incubation times.
- Vortex Genie: For mixing dilutions.
- Whatman Glass fiber syringe filter (25mm, GF 0.45 µm filter).

3.6 Sample Receipt

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

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

1. Report receipt of samples to the NARS IM Team by completing and emailing the sample tracking spreadsheet with the sample login and sample condition information. (See **Section** Error! Reference source not found. of the manual for contact information).
2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
   - Verify that the sample IDs in the shipment match those recorded on the:
     - Chain of custody forms when the batching laboratory sends the samples to the cylindrospermopsin laboratory; or
     - Sample tracking form if the field crew sends the shipment directly to the state.
   - For each sample, record the date received and lab comment (including Condition Code as described below) in the sample tracking spreadsheet with the appropriate Site ID/ Sample ID for the NARS IM Team.
     i. *OK*: Sample is in good condition
     ii. *C*: Sample container was cracked
     iii. *L*: Sample container is leaking
     iv. *ML*: Sample label is missing
     v. *NF*: Sample is not frozen
   - If any sample is damaged or missing, contact the USEPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed.
3. Store samples in the freezer until sample preparation begins.
4. Maintain the chain of custody or sample tracking forms with the samples.

Table 3.1 Cylindrospermopsin login: required data elements

FIELD	FORMAT	DESCRIPTION	
LAB_NAME	Text	Name or abbreviation for QC laboratory	
DATE_RECEIVED	MMDDYY	Date sample was received by laboratory	
SITE_ID	text	NLA site ID as used on sample label	
VISIT_NO	numeric	Sequential visits to site (1 or 2)	
SAMPLE_ID	numeric	Sample ID as used on field sheet (on sample label)	
DATE_COL	MMDDYY	Date sample was collected	
CONDITION_CODE	text	Condition codes describing the condition of the sample upon arrival at the laboratory.	
		Flag	Definition
		OK	Sample is in good condition
		C	Sample container is cracked
		L	Sample or container is leaking
		ML	Sample label is missing
		NF	Sample is not frozen
		Q	Other quality concerns, not identified above
COND_COMMENT	text	Comments about the condition of the sample.	

3.7 Procedure

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

3.7.1 Sample Preparation

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

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

3.7.2 Kit Preparation

The technician prepares the kits using the following instructions:

Check the expiration date on the kit box and verify that it has not expired. If the kit has expired, discard and select a kit that is still within its marked shelf life. (Optional: Instead of discarding the kit clearly mark all expired components as expired and consider keeping it for training activities.)

1. Verify that each kit contains all the required contents:

   - Microtiter plate
   - Standards (7) referenced in this procedure as follows with the associated concentration:
     - S0: 0 µg/L
     - S1: 0.05 µg/L
     - S2: 0.1 µg/L,
     - S3: 0.25 µg/L
     - S4: 0.5 µg/L

  - S5: 1.0 µg/L
  - S6: 2.0 µg/L
- Kit Control (KC): 0.75 µg/L
- Cylindrospermospin-HRP conjugate Solution (vortex before use)
- Antibody solution (rabbit anti-Cylindrospermopsin)
- Wash Solution 5X Concentrate
- Substrate (Color) Solution
- Stop Solution
- Dilutant
- Foil bag with 12 microtiter plate strips

2. If any bottles are missing or damaged, discard the kit. This step is important because Abraxis has calibrated the standards and reagents separately for each kit.
3. Adjust the microtiter plate, samples, standards, and the reagents to room temperature.
4. Remove 12 microtiter plate strips (each for 8 wells) from the foil bag for each kit. The plates contain 12 strips of 8 wells. If running less than a whole plate, remove unneeded strips from the strip holder and store in the foil bag, ziplocked closed, and store in the refrigerator (4-8 °C).
5. Prepare a negative control (NC) using distilled water.
6. The standards, controls, antibody solution, enzyme conjugate, color solution, and stop solutions are ready to use and do not require any further dilutions.
7. Dilute the wash solution with deionized water. (The wash solution is a 5X concentrated solution.) In a 1L container, dilute the 5X solution 1:5 (i.e., 100 mL of the 5X wash solution plus 400 mL of deionized water). Mix thoroughly. Set aside the diluted solution to wash the microtiter wells later.
8. Handle the stop solution containing diluted H_2SO_4 with care.

3.7.3 Insertion of Contents into Wells

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

9. While preparing the samples and kit, turn the plate reader on so it can warm up. The plate reader needs a minimum of 30 minutes to warm up.
10. Turn on the computer so that it can control and access the plate reader.
11. Print the template (**Figure 3.1**) to use as reference when loading the standards, controls, and samples as described in the next step. Templates contain rows, labeled with a marking pen, of strips of 8 wells that snap into the blank frame. If the laboratory wishes to use a different template, provide a copy to the USEPA HQ Laboratory Review Coordinator for approval prior to first use.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S4	NC	P4	P8	P12	P16	P20	P24	P28	P32	P36
B	S0	S4	NC	D4	D8	D12	D16	D20	D24	D28	D32	D36
C	S1	S5	P1	P5	P9	P13	P17	P21	P25	P29	P33	P37
D	S1	S5	D1	D5	D9	D13	D17	D21	D25	D29	D33	D37
E	S2	S6	P2	P6	P10	P14	P18	P22	P26	P30	P34	P38
F	S2	S6	D2	D6	D10	D14	D18	D22	D26	D30	D34	D38
G	S3	KC	P3	P7	P11	P15	P19	P23	P27	P31	P35	P39
H	S3	KC	D3	D7	D11	D15	D19	D23	D27	D31	D35	D39

Figure 3.1 Cylindrospermopsin: sample template

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

13. Add 50 µL of the conjugate solution to each well using the multi-channel pipettor and a reagent reservoir. Add 50 µL of the cylindrospermopsin antibody solution to each well using the multi-channel pipettor and a reagent reservoir. Use dedicated reagent reservoirs for each reagent to avoid contamination from one reagent to another.
14. Place the sealing Parafilm over the wells.
15. Manually mix the contents by moving the strip holder in a rapid circular motion on the benchtop for 30 seconds. Be careful not to spill the contents.
16. Place the plate in an area away from light for 45 minutes.
17. After 45 minutes, carefully remove the Parafilm.
18. Empty the contents of the plate into the sink, pat inverted plate dry on a stack of paper towels, and then wash the wells of the plate four times with 250 µL of washing solution using the multi-channel pipette. After adding the washing solution each time, empty the solution into the sink and use the paper towels as before.
19. Add 100 µL of substrate/ color solution to all wells using the multi-channel pipettor.
20. Cover the wells with Parafilm.
21. Manually mix the contents by moving the strip holder in a rapid circular motion on the benchtop for 30 seconds. Be careful not to spill the contents.

22. Place the strip holder in an area away from light for 30-45 minutes.
23. After 30-45 minutes, remove the Parafilm, add 100 µL of stop solution to the wells using the multi-channel pipette and reagent reservoir in the same sequence as the substrate solution.
24. Use a microplate ELISA photometer (plate reader) to determine the absorbance at 450 nm. The software (i.e., commercial ELISA evaluation program) calculates the absorbance and concentration values of the samples from the calibration curve and the average values for each pair. Use a 4-parameter standard curve fit to determine the concentrations.
25. Dispose of solution in plates in a laboratory sink. Rinse plates and sink with water to dilute the weak acid present.
26. Perform QC evaluations of the data as follows:
    a. If the following failures occur in the standards and controls, then the laboratory must reanalyze all samples in the analytical run:
        i. Standard curve with a correlation coefficient of less than 0.99 (i.e., R<0.99)
        ii. Standards S0-S6 must have decreasing absorbance values. First, calculate the average values for each standard. That is, if $\bar{A}_i$ is the absorbance average for S_i, then the absorbance averages must be: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5 > \bar{A}_6$
        iii. The average absorbance of the standard S0 less than 0.8 (i.e., $\bar{A}_0 < 0.8$).
        iv. Two or more negative control samples with detectable concentrations of Cylindrospermopsin (i.e., values > 0.1 µg/L). If this occurs, then evaluate possible causes (e.g., cross-contamination between samples), and if appropriate, modify laboratory processes before the next analytical run.
        v. Results for control samples of outside the acceptable range of 0.75 +/- 0.15 ppb. That is, results must be between 0.60 and 0.90.
    b. If either, or both, of the following failures occur for the sample, then the sample must be reanalyzed (maximum of two analyses, consisting of the original analysis and, if necessary, one reanalysis):
        i. The concentration value registers as HIGH (exceeds the calibration range). Dilute the sample for the reanalysis per **Section Error! Reference source not found.**.
        ii. The %CV > 15% between the duplicate absorbance values for a sample.
27. Record the results, even if the data failed the quality control requirements in #26b, for each well in the USEPA's data template (**Table 3.2**). The required entries are for the following columns:
    a. **SAM_CODE** should be one of the following codes: S0-S6 for standards; KC or NC, for controls; QC for quality control samples; P for primary run of unknown samples, D for duplicate/secondary run of unknown samples within a well plate.
    b. **CONC** contains the numeric concentration value. Two special cases:
        i. Non-detected concentrations: If the sample is non-detected, provide the result within CONC column, record the data as 'ND' in the DATA FLAG column and provide the sample-specific detection limit in the method detection limit column (MDL). See **Section Error! Reference source not found.** for calculating the sample-specific detection limit for a diluted sample.

ii. If the result shows that it is "HIGH," this indicates that the sample value is outside of the calibration range and must be diluted and re-run using another analytical run. Leave the CONC column blank and record 'HI' in the DATA FLAG column.[b]

c. **DATA FLAGS** have codes for the following special cases:

i. **ND** if the sample was non-detected;
ii. **J** if the value is detected but at a level below the reporting limit of 0.1 µg/L (for undiluted samples);
iii. **H** if the sample did not meet the holding time and was not analyzed within 90 days.
iv. **HI** if the concentration value registers as HIGH (exceeds the calibration range).

d. **QUALITY FLAGS** have codes for the following special cases:

i. **QCF** if there is a QC failure per step 26 above. The QCF code must be used for all failures to facilitate data analysis.
ii. **Q** for any other quality issue (describe in **COMMENTS**)

e. **DILUTION FACTOR** is only required if the sample was diluted.

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

Table 3.2: Cylindrospermopsin: required data elements- data submission

FIELD	COLUMN HEADING	FORMAT	DESCRIPTION	
LABORATORY ID	LAB_ID	Text	Name or abbreviation for QC laboratory	
DATE RECEIVED	DATE_RECEIVED	MMDDYY	Date sample was received by lab	
SITE ID	SITE_ID	Text	NLA site ID code as recorded on sample label or tracking form (blank if standard or control)	
VISIT NUMBER	VISIT_NO	Numeric	Sequential visits to site (1 or 2) (blank if standard or control)	
SAMPLE ID	SAMPLE_ID	Numeric	6-digit Sample ID number as recorded on sample jar or tracking form (blank if standard or control)	
DATE COLLECTED	DATE_COL	MMDDYY	Date sample was collected (blank if standard or control)	
CONDITION CODE	CONDITION_CODE	Text	Sample condition upon arrival at the laboratory (blank if standard or control)	
			Flag	Definition
			Blank or N	Not a sample (blank, standard, or control)
			OK	Sample is in good condition
			C	Sample container is cracked

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

			L	Sample or container is leaking
			ML	Sample label is missing
			NF	Sample is not frozen
CONDITION COMMENT	COND_COMMENT	Text	Any comment based on the condition code flags	
BATCH IDENTIFICATION	BATCH_ID	Numeric	Batch identification code; assigned by lab	
TECHNICIAN	TECHNICIAN	Text	Name or initials of technician performing the procedure	
DATE ANALYZED	DATE_ANALYZED	MMDDYY	Date when samples are inserted into the wells	
KIT EXPIRE DATE	KIT_EXPIRE_DATE	MMDDYY	Expiration date on kit box	
KIT ID	KIT_ID	Text	Kit identification code. If one does not exist, assign a unique code to each kit.	
R2	R2	Numeric	R^2 from curve fit to the average absorbance values for the standards. Value is between 0 and 1.	
SAMPLE CODE	SAM_CODE	Text	Type of solution being tested in the well	
			Code	Definition
			KC	Kit Control
			NC	Negative Control
			S0, S1, S2, S3, S4, S5, S6	Standard
			QC	Quality Control
LOCATION	LOCATION	Text	Location of well in the kit (e.g., B5 would be the fifth well from the left in the second row B)	
PRIMARY OR DUPLICATE	PRIM_DUP	Text	Regular samples are listed as “P” for Primary/first run or “D” for second run (see Figure 3.1)	
CONCENTRATION	CONC	Numeric	Concentration or sample-specific detection limit of contents of well in µg/L. Sample-specific detection limit should be 0.1 µg/L if the sample hasn’t been diluted.	
UNITS	UNITS	Text	The units of the concentration of the CONC column	
MDL*	MDL	Numeric	Method detection limit of the machine in same units as the CONC column	
RL	RL	Numeric	Reporting limit in same units as the CONC column	
ABSORBANCE	ABSORBANCE	Numeric	Absorbance value	
DILUTION FACTOR	DILUTION_FACTOR	Numeric	10, 100, etc for number of times the sample was diluted. If not diluted, leave blank or record 1	
CV ABSORBANCE	CV_ABSORB	Numeric	Calculated %CV of duplicate values of absorbance for all runs. Enter %CV. Value is between 0 and 100%.	
AVERAGE ABSORBANCE	AVG_ABSORB	Numeric	Calculated average of absorbance values for all samples and standards. Average value of the original sample and its duplicate (or replicates for KC and NC).	
AVERAGE CONCENTRATION	AVG_CONC	Numeric	Calculated average of concentration values for a sample. Substitute 0.1 µg/L for any result recorded as <0.1 µg/L	
QA FLAG (if appropriate)	QA_FLAG	Text	Data qualifier codes associated with specific identifications of voucher samples. These codes	

			provide more information than those used when reporting receipt of samples. A technician may use alternative or additional qualifiers if definitions are provided as part of the submitted data package (e.g., as a separate worksheet page of the data submission file).	
			Flag	Definition
			ND	Concentration below detection. Unless the sample was diluted, the concentration will be 0.05 µg/L
			H	Sample did not meet the holding time and was not analyzed within 90 days.
			HI	Result indicated that a high concentration (i.e., outside calibration range)> 2.0 µg/L
			J	Concentration above detection but below reporting limit. Without dilution, these values are between 0.05 and 0.1 µg/L
			QCF	QC failure
			Q	Other quality concerns, not identified above
LABORATORY COMMENT	LAB_COMMENT	Text	Explanation for data flag(s) (if needed) or other comments.	

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

3.7.4 Dilutions (if needed)

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

1. 1:10 dilution
    a. Add 900 µL of distilled or deionized water to a clean 20 mL vial. (Note: Dilutions may also be made using the kit's diluent rather than distilled or deionized water.)
    b. Pipette 100 µL from the sample into the vial. (To provide more accurate dilutions and less chance of contaminating the diluent, the diluent should be added to the vial before the sample.)
    c. Mix by vortexing.
    d. Multiply final concentration and Abraxis' method detection limit of 0.05 µg/L by 10 to obtain the sample-specific detection limit of .5 µg/L.
2. 1:100 dilution
    a. Add 3.96 mL of distilled or deionized water to a clean, appropriately labeled glass vial. (Note: Dilutions may also be made using the kit's diluent rather than distilled or deionized water.)
    b. Vortex the sample to mix thoroughly, then pipette 40 µL from the sample and add to the water (or diluent) in the appropriate labeled vial. Vortex the sample again.

Multiply the final concentration and Abraxis' method detection limit of 0.05 µg/L by 100 to obtain the sample-specific detection limit of 50 µg/L.

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

3.8 Pertinent QA/QC Procedures

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

3.8.1 QC Samples

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

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

Each laboratory will run the QC samples following the same procedures used for the other samples. The External QC Coordinator will compare the results and assess patterns in the data (e.g., one laboratory being consistently higher or lower than all others). Based upon the evaluation, the External QC Coordinator may request additional information from one or more laboratories about any deviations from the method or unique laboratory practices that might account for differences between the laboratory and others. With this additional information, the External QC Coordinator will determine an appropriate course of action, which may include no action, flagging the data, or excluding some or all the laboratory's data.

3.8.2 Summary of QA/QC Requirements

Table 3.3 provides a summary of the quality control requirements described in **Sections** Error! Reference source not found. **and** Error! Reference source not found.**.** For cylindrospermopsin, the precision for a sample is reported in terms of the percent coefficient of variation (%CV) of its absorbance values. Relative Standard Deviation (RSD) is the same as the %CV. Because many of the plate reader software programs provides the CV in their outputs, the procedure presents the quality control requirement in terms of %CV instead of RSD. Accuracy is calculated by comparing the average concentration of the kit control with the required range (0.75 +/- 0.15).

Table 3.3: Cylindrospermopsin: quality control- sample analysis

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

Quality Control Activity	Description and Requirements	Corrective Action
Calibration	All of the following must be met: ○ Standard curve must have a correlation coefficient of ≥0.99; ○ Average absorbance value, $\bar{A}_0$, for S0 must be ≥0.80; and ○ Standards S0-S6 must have decreasing average absorbance values. That is, if $\bar{A}_i$ is the average of the absorbance values for S_i, then the absorbance average values must be: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5 > \bar{A}_6$	If any requirement fails: Results from the analytical run are not reported. All samples in the analytical run are reanalyzed until calibration provides acceptable results. At its discretion, the laboratory may consult with USEPA for guidance on persistent difficulties with calibration.
Kit Control	The average concentration value of the duplicates (or triplicate) must be within the range of 0.75 +/- 0.15 µg/L. That is, results must be between 0.60 and 0.90.	If either requirement fails: • Results from the analytical run are not reported • The laboratory evaluates its processes, and if appropriate, modifies its processes to correct possible contamination or other problems. • The laboratory reanalyzes all samples in the analytical run until the controls meet the requirements.
Negative Control	The values for the negative control replicates must meet the following requirements: ○ All concentration values must be < 0.1 µg/L (i.e., the reporting limit); and ○ One or more concentration results must be nondetectable (i.e., <0.05 µg/L)	
Sample Evaluations	All samples are run in duplicate. Each duplicate pair must have %CV≤15% between its absorbance values.	If %CV of the absorbance for the sample>15%, then: • Record the results for both duplicates using different start dates and/or start times to distinguish between the runs. • Report the data for both duplicate results using Quality Control Failure flag "QCF"; and • Re-analyze the sample in a new analytical run. No samples are to be run more than twice. If the second run passes, then the data analyst will exclude the data from the first run (which will have been flagged with "QCF"). If both runs fail, the data analyst will determine if either value should be used in the analysis (e.g., it might be acceptable to use data if the CV is just slightly over 15%).
Results Within Calibration Range	All samples are run in duplicate. If both of the values are less than the upper calibration range	If a result registers as "HIGH", then record the result with a data flag of

Quality Control Activity	Description and Requirements	Corrective Action
	(i.e., 2.0 µg/L for undiluted samples), then the requirement is met.	"HI." If one or both duplicates register as 'HIGH,' then the sample must be diluted and re-run. **No samples are to be run more than twice.** If samples are re-run, do not enter concentration information of the first run.
External Quality Control Sample	External QC Coordinator, supported by QC contractor, provides 1-2 sets of identical samples to all laboratories and compares results.	Based upon the evaluation, the External QC Coordinator may request additional information from one or more laboratories about any deviations from the method or unique laboratory practices that might account for differences between the laboratory and others. With this additional information, the External QC Coordinator will determine an appropriate course of action, including no action, flagging the data, or excluding some or all of the laboratory's data.

3.9 Sample and Record Retention

The laboratory shall retain:

1. The sample materials, including vials, for a minimum of 3 years from the date the EPA publishes the final report. During this time, the laboratory shall freeze the materials. The laboratory shall periodically check the sample materials for degradation.

2. Original records, including laboratory notebooks and the reference library, for a minimum of 10 years from the date that EPA publishes the final report.

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

4.0 ALGAL TOXIN IMMUNOASSAY PROCEDURE: MICROCYSTIN

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

4.1 Summary of Method

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

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

4.2 Health and Safety Warnings

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

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

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

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

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

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

4.3.1 Definitions

The following terms are used throughout the procedure:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Standard Deviation (S) shows variation from the average

4.4 General Requirements for Laboratories

4.4.1 Expertise

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

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

4.4.2 Quality assurance and quality control requirements

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

To demonstrate its ongoing commitment, the person in charge of quality issues for the organization shall sign the NLA 2022 QAPP Certification Page.

4.4.3 Personnel

Laboratory Technician: This procedure may be used by any laboratory technician who is familiar with the NLA 2022 QAPP, and this procedure in the NLA 2022 LOM. The laboratory technician also must be familiar with the use of a multichannel pipette and plate readers.

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

4.4.4 Equipment/Materials

The procedures require the following equipment and information:

- Eurofins Technologies (formerly Abraxis ADDA Test Kit, Product #520011

- Adhesive Sealing Film (Parafilm) for Micro Plates (such as Rainin, non-sterile, Cat. No. 96-SP-100): Used to cover plates during incubation.
- Data Template – See Error! Reference source not found.**2**
- Distilled or Deionized Water: For diluting samples when necessary.
- ELISA evaluation software
- Glass scintillation, LC, vials (two vials of 2 mL each)
- Glass vials with Teflon-lined caps of size:
    - 20 mL
    - 4 mL (for dilutions)
- Multichannel Pipette & Tips: A single-channel and an 8-channel pipette are used for this method.
- Norm-ject syringes (or equivalent)
- Paper Towels: For blotting the microtiter plates dry after washing.
- Permanent Marker (Sharpie Fine Point): For labeling samples, bottles, plates and covers.
- Plate Reader (e.g., Metertech Model M965 AccuReader; ChroMate®; or equivalent readers with software to read the microtiter plates and measure absorbances).
- Reagent Reservoirs (e.g., Costar Cat Number 4870): Plain plastic reservoir for reagents that accommodate the use of a multi-channel pipette.
- Test tubes: For dilutions, if needed.
- Timer: For measuring incubation times.
- Vortex Genie: For mixing dilutions.
- Whatman Glass fiber syringe filter (25mm, GF 0.45 µm filter)

4.5 Sample Receipt

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

Because EPA initiates tracking procedures designed to recover any missing shipment, the laboratory personnel responsible for tracking samples must start the following login steps within 24 clock hours of receiving a delivery (**Table 4.1** Microcystin: required data elements – login.

1. Report receipt of samples in the NARS IM sample tracking system (within 24 clock hours).
2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
    a. Verify that the sample IDs in the shipment match those recorded on the:
        i. Chain of custody forms when the batching laboratory sends the samples to the microcystins laboratory; or
        ii. Sample tracking form if the field crew sends the shipment directly to the state laboratory.
    b. Record the information in into NARS IM, including the Condition Code for each sample:

i. *OK*: Sample is in good condition
ii. *C*: Sample container was cracked
iii. *L*: Sample container is leaking
iv. *ML*: Sample label is missing
v. *NF*: Sample is not frozen

c. If any sample is damaged or missing, contact the EPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed.

3. Store samples in the freezer until sample preparation begins.
4. Maintain the chain of custody or sample tracking forms with the samples.

Table 4.1 Microcystin: required data elements – login

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

4.6 Procedure

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

4.6.1 Sample Preparation

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

5. All cycles: Keep the samples in dark or dimly lit areas (i.e., away from sunlight, but under incandescent lighting is acceptable).
6. First freeze-thaw cycle:
   a. Start with a frozen 500 ml sample.
   b. Thaw the sample to room temperature (approximately 25° C). Swirl the sample to check for ice crystals. At this temperature, no ice crystals should be present in the sample.

c. Shake well to homogenize the sample, then transfer 10 mL to an appropriately labeled clean 20 mL glass vial.

7. Second freeze-thaw cycle:
   a. Freeze the vial.
   b. Keep the large sample bottle (from the 500 mL initial sample) frozen for future use.
   c. Thaw the sample vial contents to room temperature.
8. Third freeze-thaw cycle:
   a. Freeze the vial.
   b. Thaw the vial contents to room temperature.
   c. Filter the vial contents through a new, syringe filter (0.45 µm) into a new, labeled 20 mL glass scintillation vial. Norm-ject syringes and Whatman Glass fiber syringe filters (25mm, GF 0.45 µm filter) or similar alternative are acceptable. One new syringe and filter should be used per sample.

4.6.2 Kit Preparation

The technician prepares the kits using the following instructions:

1. Check the expiration date on the kit box and verify that it has not expired. If the kit has expired, discard and select a kit that is still within its marked shelf life. (Instead of discarding the kit, consider keeping it for training activities.)
2. Verify that each kit contains all the required contents:

   - Microtiter plate
   - Standards (6) referenced in this procedure as follows with the associated concentration:
     - S0: 0 µg/L
     - S1: 0.15 µg/L
     - S2: 0.40 µg/L
     - S3: 1.0 µg/L
     - S4: 2.0 µg/L
     - S5: 5.0 µg/L
   - Kit Control (KC): 0.75 µg/L
   - Antibody solution
   - Anti-Sheep-HRP Conjugate
   - Wash Solution 5X Concentrate
   - Color Solution
   - Stop Solution
   - Diluent
   - Foil bag with 12 microtiter plate strips
3. If any bottles are missing or damaged, discard the kit. This step is important because Abraxis has calibrated the standards and reagents separately for each kit.
4. Adjust the microtiter plate, samples, standards, and the reagents to room temperature.

5. Remove 12 microtiter plate strips (each for 8 wells) from the foil bag for each kit. The plates contain 12 strips of 8 wells. If running less than a whole plate, remove unneeded strips from the strip holder and store in the foil bag, ziplocked closed, and place in the refrigerator.
6. Store the remaining strips in the refrigerator (4-8^{o} C).
7. Prepare a negative control (NC) using distilled water
8. The standards, controls, antibody solution, enzyme conjugate, color solution, and stop solutions are ready to use and do not require any further dilutions.
9. Dilute the wash solution with deionized water. (The wash solution is a 5X concentrated solution.) In a 1L container, dilute the 5X solution 1:5 (i.e., 100 mL of the 5X wash solution plus 400 mL of deionized water). Mix thoroughly. Set aside the diluted solution to wash the microtiter wells later.
10. Handle the stop solution containing diluted H_2SO_4 with care.

4.6.3 Insertion of Contents into Wells

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

11. While preparing the samples and kit, turn the plate reader on so it can warm up. The plate reader needs a minimum of 30 minutes to warm up.
12. Turn on the computer so that it can control and access the plate reader.
13. Print the template (**Error! Reference source not found.**) to use as reference when loading the standards, controls, and samples as described in the next step. Templates contain rows, labeled with a marking pen, of strips of 8 wells that snap into the blank frame. (If the laboratory wishes to use a different template, provide a copy to the EPA HQ Laboratory Review Coordinator for approval prior to first use.
14. Using the 100-µL pipette, add 50 µL, each, of the standards, controls, and samples to the appropriate wells in the plate. Place all six standards (0.00, 0.15, 0.40, 1.00, 2.0 and 5.0 µg/L), the kit control (0.75 µL), and negative control, in pairs, starting in the well in the upper left-hand corner of the kit as shown in **Error! Reference source not found.**. Verify that the software displays the same template or make any necessary corrections. Laboratories with access to an autopipetter may use said machinery after proper documentation of set up, training and calibration has been provided and approved by EPA HQ Laboratory Review Coordinator prior to first use.

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

Figure 4.1 Microcystin: sample template

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

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

30. After 20 minutes, remove the Parafilm and add 50 µL of stopping solution to the wells in the same sequence as for the color solution. This will turn the contents a bright yellow color. After adding the stopping solution, read the plate within 15 minutes.
31. Within 15 minutes of adding the stopping solution, use the microplate ELISA photometer (plate reader) to determine the absorbance at 450 nm. The software (i.e., commercial ELISA evaluation program) calculates the absorbance and concentration values of the samples from the calibration curve and the average values for each pair. Use a 4-parameter standard curve fit to determine the concentrations.
32. Dispose of solution in plates in a lab sink. Rinse plates and sink with water to dilute the weak acid present.
33. Perform QC evaluations of the data as follows:
    a. If the following failures occur, then the laboratory must reanalyze all samples in the analytical run:
        i. Standard curve with a correlation coefficient of less than 0.99 (i.e., R<0.99)
        ii. Standards S0-S5 must have decreasing absorbance values. First, calculate the average values for each standard. That is, if $\bar{A}_i$ is the absorbance average for S_i, then the absorbance averages must be:
        $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5$
        iii. The average absorbance of the standard S0 less than 0.8 (i.e., $\bar{A}_0 < 0.8$).
        iv. Two or more negative control samples with detectable concentrations of microcystins (i.e., values > 0.1 µg/L). If this occurs, then evaluate possible causes (e.g., cross-contamination between samples), and if appropriate, modify laboratory processes before the next analytical run.
        v. Results for control samples of outside the acceptable range of 0.75 +/- 0.185 ppb. That is, results must be between 0.565 and 0.935.
    b. If either, or both, of the following failures occur, then the sample must be reanalyzed (maximum of two analyses, consisting of the original analysis and, if necessary, one reanalysis):
        i. The concentration value registers as HIGH (exceeds the calibration range). Dilute the sample for the reanalysis per **Section Error! Reference source not found.**.
        ii. The %CV > 15% between the duplicate absorbance values for a sample.
34. Record the results, even if the data failed the quality control requirements in #23b, for each well in EPA's data template (see **Table 4.2Error! Reference source not found.** for required elements). The required entries are for the following columns:
    a. **TYPE** should be one of the following codes: S0-S5 for standards; KC, NC, or SC for controls; P (primary) or D (for duplicate) of unknown sample.
    b. **CONC** contains the numeric concentration value. Two special cases:
        i. Non-detected concentrations: If the sample is non-detected, then provide the sample-specific detection limit which is 0.1 µg/L if the sample is undiluted with a salinity <3.5 ppt. See **Section Error! Reference source not found.** for calculating the sample-specific detection limit for a diluted sample.

ii. If the result shows that it is HI',” this indicates that the sample value is outside of the calibration range and must be diluted and re-run using another analytical run. Leave the CONC column blank and record 'HI' in the DATA FLAG column.

c. **DATA FLAGS** have codes for the following special cases:

i. **ND** if the sample was non-detected;
ii. **J** if the value is detected but at a level below the reporting limit of 0.15 µg/L (for undiluted samples);
iii. **H** if sample did not meet the holding time and was not analyzed within 90 days.
iv. **HI** if the concentration value registers as HIGH (exceeds the calibration range).

d. **QUALITY FLAGS** have codes for the following special cases:

i. **QCF** if there is a QC failure per step 23 above. The QCF code must be used for all failures to facilitate data analysis.
ii. **Q** for any other quality issue (describe in **COMMENTS**)

e. **DILUTION FACTOR** is only required if the sample was diluted.

f. **DUP AVG** and **DUP CV** are required for duplicate samples and control samples (use all three values if the controls are used in triplicate).

g.

Table 4.2 Microcystin: required data elements – data submission

STAGE	FIELD	FORMAT	DESCRIPTION	
LOGIN	LAB ID	text	Name or abbreviation for QC laboratory	
	DATE RECEIVED	text	Date sample was received by lab	
	SITE ID	text	NLA site ID code as recorded on sample label or tracking form (blank if standard or control)	
	VISIT NUMBER	numeric	Sequential visits to site (1 or 2) (blank if standard or control)	
	SAMPLE ID	numeric	6-digit Sample ID number as recorded on sample jar or tracking form (blank if standard or control)	
	DATE COLLECTED	MMDDYY	Date sample was collected (blank if standard or control)	
	CONDITION CODE	text	Sample condition upon arrival at the laboratory (blank if standard or control)	
			Flag	**Definition**
			Blank or N	Not a sample (blank, standard, or control)
			OK	Sample is in good condition
			C	Sample container is cracked
			L	Sample or container is leaking
			ML	Sample label is missing
			NF	Sample is not frozen
			Q	Other quality concerns, not identified above
	CONDITION COMMENT	text	Comments about the condition of the sample.	

STAGE	FIELD	FORMAT	DESCRIPTION	
ANALYSIS	BATCH ID	Numeric	Batch identification code, assigned by lab	
	TECHNICIAN	text	Name or initials of technician performing the procedure	
	KIT EXPIRE DATE	MMDDYY	Expiration date on kit box	
	KIT ID	text	Kit identification code. If one does not exist, assign a unique code to each kit.	
	R2	numeric	R^2 from curve fit to the average absorbance values for the standards. Value is between 0 and 1.	
	TYPE	text	Type of solution being tested in the well	
			Code	**Definition**
			KC	Kit Control
			NC	Negative Control
			S0, S1, S2, S3, S4, S5	Standard
			QC	Quality control sample
			U	Sample of unknown concentration
	LOCATION	text	Location of well in the kit (e.g., B5 would be the fifth well from the left in the second row B)	
	PRIM_DUP	text	Primary or duplicate run of a sample of unknown concentration (see Figure 4.1)	
	CONC	numeric	Concentration or sample-specific detection limit of contents of well in µg/L. Sample-specific detection limit should be 0.1 µg/L for a sample with salinity <3.5 ppt which hasn't been diluted.	
	UNITS	text	The units of the concentration of the CONC column	
	MDL	numeric	Minimum detection limit in the same units as the CONC column	
	RL	numeric	Reporting Limit in same units as the CONC column	
	ABSORBANCE	numeric	Absorbance value	
	DILUTION FACTOR	numeric	10, 100, etc for number of times the sample was diluted. If not diluted, leave blank or record 1	
	CV_ABSORB	numeric	Calculated %CV of duplicate values of absorbance for a sample. Only calculated for TYPE=U, KC, or NC. Enter %CV. Value is between 0 and 100%.	
	AVG_ABSORB	numeric	Calculated average of absorbance values for a sample. Only provided for TYPE=U, KC, NC, or SC. Average value of the original sample and its duplicate (or replicates for KC and NC).	
	AVG_CONC	numeric	Calculated average of concentration values for a sample.	
	QA FLAG (if appropriate)	text	Data qualifier codes associated with specific identifications of voucher samples. These codes provide more information that those used when reporting receipt of samples. A technician may use alternative or additional qualifiers if definitions are provided as part of the submitted data package (e.g., as a separate worksheet page of the data submission file).	
			Flag	**Definition**
			ND	Concentration below detection. Unless the sample was diluted, the concentration will be 0.1 µg/L
			H	Sample did not meet the holding time and was not analyzed within 90 days.

STAGE	FIELD	FORMAT	DESCRIPTION	
			HI	Result indicated that a high concentration (i.e., outside calibration range)
			J	Concentration above detection but below reporting limit.
			QCF	QC failure
			Q	Other quality concerns, not identified above
	LAB COMMENTS	text	Explanation for data flag(s) (if needed) or other comments.	

4.6.4 Dilutions (if needed)

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

1:10 dilution

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

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

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

4.7 Quality Measures

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

4.7.1 Assistance Visits

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

4.7.2 QC Samples

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

4.7.3 Summary of QA/QC Requirements

Table 4.3Error! Reference source not found. provides a summary of the quality control requirements described in **Sections 4.5** and **4.6**.

Table 4.3 Microcystin: quality control – sample analysis

Quality Control Activity	Description and Requirements	Corrective Action
Kit – Shelf Life	Is within its expiration date listed on kit box.	If kit has expired, then discard or set aside for training activities.
Kit – Contents	All required contents must be present and in acceptable condition. This is important because Abraxis has calibrated the standards and reagents separately for each kit.	If any bottles are missing or damaged, discard the kit.
Calibration	All of the following must be met: ○ Standard curve must have a correlation coefficient of ≥0.99; ○ Average absorbance value, $\bar{A}_0$, for S0 must be ≥0.80; and Standards S0-S5 must have decreasing average absorbance values. That is, if $\bar{A}_i$ is the average of the absorbance values for S_i, then the absorbance average values must be: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3 > \bar{A}_4 > \bar{A}_5$	If any requirement fails: • Results from the analytical run are flagged QCF All samples in the analytical run are reanalyzed until calibration provides acceptable results.
Kit Control	The average concentration value of the duplicates (or triplicate) must be within the range of 0.75 +/- 0.185 µg/L. That is, results must be between 0.565 and 0.935.	If either requirement fails: • Results from the analytical run are not reported • The lab evaluates its processes, and if appropriate, modifies its processes to correct possible contamination or other problems.
Negative Control	The values for the negative control replicates must meet the following requirements: ○ All concentration values must be < 0.15 µg/L (i.e., the reporting limit); and	

Quality Control Activity	Description and Requirements	Corrective Action
	One or more concentration results must be nondetectable (i.e., <0.10 µg/L)	The lab reanalyzes all samples in the analytical run until the controls meet the requirements.
Sample Evaluations	All samples are run in duplicate. Each duplicate pair must have %CV≤15% between its absorbance values.	If %CV of the absorbances for the sample>15%, then: • Record the results for both duplicates. • Report the data for both duplicate results as Quality Control Failure "QCF"; and • Re-analyze the sample in a new analytical run. No samples are to be **run more than twice.** If the second run passes, then the data analyst will exclude the data from the first run. If both runs fail, the data analyst will determine if either value should be used in the analysis (e.g., it might be acceptable to use data if the CV is just slightly over 15%).
Results Within Calibration Range	All samples are run in duplicate. If both of the values are less than the upper calibration range (i.e., 5.0 µg/L for undiluted samples), then the requirement is met.	If a result registers as "HIGH", then record the result with a data flag of "HI." If one or both duplicates register as 'HIGH,' then the sample must be diluted and re-run. **No samples are to be run more than twice.** If samples are re-run, do not enter concentration information of the first run.
External Quality Control Sample	External QC Coordinator, supported by QC contractor, provides 1-2 sets of identical samples to all laboratories and compares results.	Based upon the evaluation, the External QC Coordinator may request additional information from one or more laboratories about any deviations from the Method or unique laboratory practices that might account for differences between the laboratory and others. With this additional information, the External QC Coordinator will determine an appropriate course of action, including no action, flagging the data, or excluding some or all of the laboratory's data.

4.8 Sample and Record Retention

The laboratory shall retain:

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

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

5.0 BENTHIC MACROINVERTEBRATE METHODS

This procedure is adapted from the *Wadeable Streams Assessment: Benthic Laboratory Methods* (USEPA. 2004),and is modified to facilitate processing and identification of benthic organisms collected in the littoral zone of lakes and reservoirs.

Benthic macroinvertebrate samples will be preserved in the field with ethyl alcohol (EtOH) and shipped from field crews to a contract batching laboratory. The contract batching laboratory will send the batched samples to the analysis laboratory. Preserved samples will arrive in the analysis laboratory and can be held for several months. If samples are not processed soon after receipt, then periodic evaluation of samples should occur to ensure that sufficient EtOH levels are maintained. Benthic invertebrate analysis laboratories will need to process samples in accordance with the time frame outlined in contractual agreements. Contractual agreements for delivery of data do not supersede indicator holding times.

5.1 Responsibility and Personnel 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 re-identify a randomly-selected 10% of the samples for QC, as noted below, to quantify enumeration and taxonomic precision, or consistency, as **percent difference in enumeration (PDE)** and **percent taxonomic disagreement (PTD)**, to help target corrective actions, and ultimately to help minimize problems during data analysis. Samples are sent to the laboratory from the field on a regular basis to avoid delays in processing and sample identification.

5.2 Precautions

5.2.1 Sorting and Subsampling Precautions

Because it can be difficult to detect the organisms in lake samples (due to inexperience, detritus, etc.), a person who has received instruction from senior biology staff familiar with processing benthic samples must have a QC check performed by qualified personnel (laboratory QC Officers) only. These QC checks will be performed in the pertinent QA and QC Procedures section. The laboratory QC Officers must perform these QC checks immediately following sorting of each grid.

Thoroughly clean all sorting equipment and make sure all equipment is free of organisms prior to sorting the next sample.

5.2.2 Taxonomy Precautions

The USEPA will supply a list of taxa that have been collected from previous iterations of the National Lakes Assessment (provided during laboratory initiation call). The laboratories will use this list as the primary source for taxonomic names to be used in the current NLA sample processing. During the processing of samples, if new taxa are encountered that are not part of the existing NLA taxa list then analysts must provide either a literature citation for this new taxa or its Integrated Taxonomic Information System (ITIS; Web at http://itis.gov) number, if available. New taxa will not be excepted unless either of these items are provided.

The analyst must prepare a list of primary and secondary technical literature used in completing the identifications and submit this list to the Project Quality Assurance Manager when samples are returned (see below).

5.3 Equipment/Materials

5.3.1 Sorting and Subsampling Equipment/Materials

- U.S. 35 sieve (500 µm)
- Round buckets
- Standardized gridded screen (370-µm)
- Mesh screen, 30 squares (6 cm^2 each) with white plastic holding tray[1]
- 6-cm scoop
- 6-cm^2 metal dividing frame ("cookie cutter")
- White plastic or enamel pan (6" x 9") for sorting
- Scissors
- Teaspoon
- India ink pens
- Dropper
- Fine-tipped forceps (watchmaker type, straight and curved)
- Specimen vials with caps or stoppers
- Sample labels for specimen vials
- 70-80% denatured ethanol (EtOH)
- Benthic Sample Log-In Form
- Benthic Macroinvertebrate Laboratory Bench Sheet (**APPENDIX B: SAMPLE LABORATORY FORMS**)
- Stereo zoom microscope (6-10X magnification)

5.3.2 Taxonomy Equipment/Materials

- Stereo dissecting microscope with fiberoptics light source (50-60X magnification)
- Compound microscope (10, 40, and 100X objectives, with phase-contrast capability)
- Petri dishes
- Microscope slides (1" x 3" flat, precleaned)
- Cover slips (appropriately sized)
- CMCP-10 (or other appropriate mounting medium)
- India ink pens
- Dropper
- Fine-tipped forceps (watchmaker type, straight and curved)

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

- Specimen vials with caps or stoppers
- Sample labels for specimen vials
- 70 - 80% denatured ethanol in plastic wash bottle
- Benthic Macroinvertebrate Taxonomic Bench Sheet
- Hand tally counter

5.4 Sample Receipt

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

1. Report receipt of samples to the NARS IM Team by completing and emailing the sample tracking spreadsheet with the sample login and sample condition information. (See **Section 1.2** of the manual for contact information).

2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
   a. Verify that the sample IDs in the shipment match those recorded on the sample tracking form.
   b. Record the information in **Table 5.1** for the NARS IM Team, including the Condition Code for each sample:
      i. *OK*: Sample is in good condition
      ii. *C*: Sample container was cracked
      iii. *L*: Sample container is leaking
      iv. *ML*: Sample label is missing
   c. If any sample is damaged or missing, contact the USEPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed. (See contact information in Chapter 2 of the Manual).

3. Store samples until sample preparation begins.

4. Maintain the sample tracking forms with the samples.

Table 5.1 Benthic macroinvertebrate login: required data elements

FIELD	FORMAT	DESCRIPTION
LAB	text	Name or abbreviation for laboratory
DATE RECEIVED	MMDDYY	Date sample was received by laboratory
SITE ID	text	NLA site id as used on sample label
VISIT NUMBER	numeric	Sequential visits to site (1 or 2)
SAMPLE ID	numeric	Sample id as used on field sheet (on sample label)
DATE COLLECTED	MMDDYY	Date sample was collected

FIELD	FORMAT	DESCRIPTION	
CONDITION CODE	text	Condition codes describing the condition of the sample upon arrival at the laboratory.	
		Flag	Definition
		OK	Sample is in good condition
		C	Sample container is cracked
		L	Sample or container is leaking
		ML	Sample label is missing
		Q	Other quality concerns, not identified above
CONDITION COMMENT	text	Comments about the condition of the sample.	

5.5 Procedure

5.5.1 General

1. Record receipt of samples in the laboratory on the Benthic Sample Log-In form (**APPENDIX B: SAMPLE LABORATORY FORMS**). Assign the appropriate chronological bench number to each sample. Store samples at room temperature until ready for processing.
2. Sample container(s) may arrive with very little alcohol to expedite shipping times and to account for hazardous material handling requirements. Inspect each jar THE SAME DAY THEY ARE RECEIVED and refill them with 70-80% EtOH if necessary. After refilling the sample containers, store them until sorting begins. Check samples periodically to ensure EtOH levels are sufficiently maintained.
3. Use a gridded screen to sort a randomized 500-organism subsample separately from the rest of the sample. Preserve the sorted organisms in one or more specimen vials with 70-80% EtOH.
4. For each sample, document the level of effort, or proportion of sample processed (e.g., number of grids processed), on the Benthic Macroinvertebrate Laboratory Bench Sheet (**APPENDIX B: SAMPLE LABORATORY FORMS**).
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.
   a. Station Name
   b. Station Location
   c. Station Number
   d. Date Sorted
   e. Sorter's Initials
   f. "1 of x" or "2 of x", etc. if the sample is sorted into >1 vial (where x is the total number of vials for the sorted sample)

5.5.2 Subsampling

1. Remove the lid from the sample container and remove the internal sample label (save the label—it will need to be returned to the sample container with the archived portion of the sample that does not get processed). Record the sample collection information on a Benthic

Macroinvertebrate Laboratory Bench Sheet. Header information required includes both project name and date the sample was collected. 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 any organisms found back 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 the tray to the level of the wall panels (it should be spread as evenly as possible), divide it among two or more trays.
4. NOTE: 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 laboratory, before subsampling has begun on that particular sample. Magdych 1981 describes an inexpensive, easily constructed elutriator. An example of an acceptable elutriation method is as follows:
    a. 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.
    b. 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.
    c. 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.
    d. Repeat this until the water runs clear.
    e. 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.
    f. 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.
    g. Repeat this process until all of the inorganic material has been elutriated and checked for heavier organisms, such as clams, mussels, or worms.
5. Spread the sample now in the circular sieve over the 30-grid Caton tray.
6. Place the gridded screen into the larger white tray. (Note: Some laboratories 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.
7. 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.
8. 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). A minimum of three grids (Canton tray or larger grid size), or 10% of the grids (if a grid of more than 30 squares [<6 cm^2 each] is used) are sorted from the sample to ensure that the subsample material is

representative of the overall sample. Remove all the material from the first grid. If two trays are being used to hold a large sample, remove the material from the same grid on the second pan. Remove the material 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:
    i. An organism belongs to the grid containing its head.
    ii. 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.
    iii. If the head of an organism lies on the line between two grids, all organisms on the top of a grid and those on the right side of a grid belong in that grid, and are picked with that grid.
c. Quarter the grid (if necessary, see **Section 5.5.3**, #2). 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.

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

5.5.3 Sorting

1. Randomly select at least 10% of the tray or three grids in the case of a Caton tray (assuming 30 grids).
2. If the number of organisms appears to exceed the target number (500 organisms) in the collective three grids, quarter each grid, and randomly select a quarter for initial sorting. Sort the quarter volume of the first grid. Sort the remaining two grids (quartered) in successive order (compositing of the first three grids is not done).
3. If the number of organisms is below the target number, then process another fraction of each grid 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 bias.
4. If the target is not reached when the three grids are fully processed (including organisms recovered during QC checks), randomly select subsequent grids and pick each 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, stop sorting for that sample on completion of the sorting of the corresponding fraction (i.e., the third grid quarter would not be processed).
5. If the target level of 500 organisms is not reach within 20 hours of sorting, stop sorting and preserve the remaining unsorted material in 70-80% denatured EtOH, and store for future sorting, if needed.

6. Remove the macroinvertebrates from the detritus with forceps. Sort all samples under a minimum of 6x (maximum of 10x) dissecting microscope. Perform QC checks using the same power microscope. Place picked organisms in an internally-labeled vial (or larger container, if necessary) containing 70-80% denatured EtOH.
7. Keep a rough count of the number of organisms removed and enter the number of organisms found in each grid under the appropriate 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.
8. 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, Culicidae, Cladocera, or Copepoda)
    - Incidentally-collected terrestrial taxa.
9. Also, do not count fragments such as legs, antennae, gills, or wings.
10. 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).
11. Once it is picked by the initial sorter, an experienced, certified, laboratory QC Officer must check each sample for missed organisms before another sample is processed. The laboratory QC Officer will count any missed organisms found and place them into the sample vial, or other suitable sample vial. The laboratory QC Officer will note the number of organisms missed on the Benthic Macroinvertebrate Laboratory Bench Sheet and add that number to the final count of the sample.
12. 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 just enough water to cover the sample. Randomly choose slices and count organisms that are wholly contained within the slices. If an organism is lying between two slices, use the criteria in **Section 5.5.2** #8 (B) to determine which slice it belongs in. Choose slices until you reach the target number (500 +20%). As with picking grids and quarters, you must pick an entire pie slice, even if the sample goes over 500 organisms as long as it remains under 600 total organisms.
13. Once the QC check of the material in the pan has been completed, remove the material from the pan and place it in a separate container with preservative (70-80% EtOH). Label the container "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.
14. After the laboratory QC Officer completes the QC check, 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 0.5" long and found in less than one eighth of the tray holding the entire sample. Place any organisms found into a vial labeled "L/R" for "Large/Rare."

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

15. Return all material not subsampled (remaining on the grid) to the original container with the preservative. This container will include the original sample labels. Prepare two additional labels "Unsorted Sample Remains" and place one inside the container and attach the other to the outside of the container. Replace the lid and tighten securely. Archive the container until all appropriate QC checks are completed (subsampling and taxonomy). The decision to discard any sample portion should be done only following joint approval of the laboratory QC Officer and the Project Manager.
16. Record the sorting date each sample was completed near the top right corner of the bench sheet.

5.5.4 Taxonomy Procedures

1. The taxonomic target for benthic invertebrates is identified in **Section 5.5.1 #3**
2. Upon receipt of a set of sample vials from the project cooperator or the contractor batch laboratory, remove the sample tracking form from the shipping container, and sign and date it in the "received by" space to verify that the samples were received. 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 sample tracking form with the samples; it will be needed to return the samples.
3. Empty one sample vial at a time into a small Petri dish. Add 80% denatured EtOH to keep the organisms covered. Remove the internal sample label and complete the top portion of a Benthic Macroinvertebrate Taxonomic Bench Sheet, using the information from the label or that provided by the project facilitator.
4. View the sample under the stereo dissecting microscope and remove similar organisms to other dishes (keep these covered with 80% EtOH). Identify organisms to the correct taxonomic level for the project (usually genus, **Table 5.2**). However, according to the laboratory manager's discretion, a taxonomist can identify any organism finer than the target level if he or she is 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. Use the following steps to compare the final taxa list for each site to that of the provided USEPA NLA taxa list.

    a. Merge the USEPA provided NLA taxa list with the laboratory electronic bench sheet data by merging the TARGET_NAME from the NLA taxa list to the TAXON_NAME from the individual sample data.
    b. Any taxa in the individual sample data that do not match a name from the NLA taxa list should be checked for the following potential issues. If after this is completed and it is determined that the non-matched taxa is unique then this taxa name can be included, but only after either a literature citation or an ITIS number are provided.
        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. Identifying to a lower level than 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 with 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)

5. 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. Label the slides with the same sample number or log-in number as the alcohol specimens.
6. 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.
7. 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; the analysts should match the detached head and thorax parts to ensure that double counting of individuals does not occur.
   b. oligochaetes, heads with a sufficient number of segments;
   c. the mollusk shell (bivalve or gastropod) is occupied by a specimen;
   d. the specimen is the sole representative of a taxon in the sample.
8. If early instar or juvenile specimens can be identified, they are counted as separate taxon.
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 the totals for each developmental stage and the total number of each taxon in the cells at the bottom of the sheet. Cross-check to be sure the totals were summed correctly. 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 identified). The taxonomist must choose an appropriate specimen(s) to represent each taxon in the master taxa list. The specimen(s) must be removed from the sample and placed in 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 must maintain a master list of taxa recorded. The contract lead will coordinate any necessary inter-laboratory 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 EtOH, and cap tightly.
13. Re-package the samples and slide-mounted specimens carefully, and sign and date the sample tracking form in the next "relinquished by" space. The samples must be shipped, properly packed in a box, by overnight carrier to the Project Facilitator, and receipt must be confirmed by the person doing the shipping. Each taxonomist must retain a full set of bench sheet copies and ship the original bench sheets to the contract lead. Ship samples and bench sheets separately.

5.5.4.1 Taxonomic Level of Effort

This is the Standard Taxonomic Effort list for benthic macroinvertebrates (**Table 5.2**). 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. For 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 doddsl*). If the minimum taxonomic level cannot be achieved due to immature, damaged, or pupal specimens this should be noted in the data file with a "flag" variable (e.g., IM = y, DD = y, PP = y).

Table 5.2 Required level of taxonomic identification for benthic macroinvertebrates.

Phylum	Class			Required Taxonomic Identification	Notes
ANNELIDA					
	Branchiobdellida			Family	
	Hirudinea			Genus	
	Oligochaeta			Genus	
	Polychaeta			Family	
ARTHROPODA					
	Arachnoidea				
		Acari		Genus	
	Insecta				
		Coleoptera		Genus	
		Diptera	**Except in the following cases:**	Genus	
			Chironomidae	Genus	this may not be possible for some groups, which should be identified to at least tribe or subfamily
			Dolichopodidae	Family	
			Phoridae	Family	
			Scathophagidae	Family	
			Syrphidae	Family	
		Ephemeroptera		Genus	
		Hemiptera		Genus	
		Lepidoptera		Genus	
		Megaloptera		Genus	
		Odonata		Genus	
		Plecoptera		Genus	
		Trichoptera		Genus	
	Malacostraca			Genus	
		Amphipoda		Genus	
		Decapoda		Genus	
		Isopoda		Genus	

Phylum	Class			Required Taxonomic Identification	Notes
		Mysidacea		Genus	
COELENTERATA					
MOLLUSCA					
	Bivalvia			Genus	
	Gastropoda		**Except in the following case:**	Genus	
			Hydrobiidae	Family	
NEMERTEA				Genus	

5.6 Pertinent QA/QC Procedures

5.6.1 Sorting and Subsampling QC

1. A QC Analyst 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 acceptable criteria for percent sorting efficiency (PSE), which is 90%. This will not only apply to inexperienced sorters, but also to those initially deemed as "experienced." Qualification will only occur when sorters achieve PSE ≥ 90% for five samples consecutively.
2. The laboratory QC Officer will calculate PSE for each sample as follows:

   Equation 5.1 Percent sorting efficiency (PSE).

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

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

3. 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 laboratory QC Officer. In the event that an individual fails to achieve ≥ 90% sorting efficiency, he or she will be required to sort an additional five samples and continue to have their sorting efficiency monitored. However, if he or she shows marked improvement in sorting efficiency prior to completion of the next five samples, achieving ≥ 90% sorting efficiency, the laboratory QA Officer may, at his/her discretion, consider this individual to be "experienced". Do not calculate PSE for samples processed by more than one individual.
4. After individuals qualify, 10% (1 out of 10, randomly selected) of their samples will be checked.
5. If an "experienced" individual fails to maintain a ≥ 90% PSE as determined by QC checks, the laboratory QC Officer will perform QC checks 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.

5.6.2 Taxonomic QC

5.6.2.1 Internal Taxonomic QC

As directed by the Indicator QC Coordinator, an in-house QC Analyst will conduct an internal 10% re-identification of all samples identified by that laboratory to ensure that each meets the acceptable criteria for percent identification efficiency which is 90%.

If the individual fails to maintain a ≥ 90% identification as determined by QC checks, previous samples will be re-counted and identified.

5.6.2.2 External Taxonomic QC

1. Upon receipt of the data, the Indicator QC Coordinator for macroinvertebrates will randomly select 10% of the samples. The Indicator QC Coordinator will then have the original laboratory send those samples to a QC taxonomist (another experienced taxonomist who did not participate in the original identifications). The original laboratory will complete a sample tracking form and send with the samples.
2. The QC taxonomist will perform whole-sample re-identifications, taking care to ensure inclusion of all slide-mounted specimens and completing another copy of the Benthic Macroinvertebrate Taxonomic Bench Sheet for each sample. Label each bench sheet with the term "QC Re-ID." As each bench sheet is completed, fax it to the Project Facilitator.
3. The Indicator QC Coordinator will compare the taxonomic results (counts AND identifications) generated by the primary and QC taxonomists for each sample and calculate percent difference in enumeration (PDE) and percent taxonomic disagreement (PTD) as measures of taxonomic precision (Stribling et al. 2003) as follows:

   Equation 5.2 Percent difference in enumeration (PDE).

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

   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.

   Equation 5.3 Percent taxonomic disagreement (PTD).

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

   where $comp_{pos}$ is the number of agreements (positive comparisons) and N is the total number of specimens in the larger of the two counts.
4. The recommendation for PDE is 5% or less.
5. A PTD of 15% or less is recommended for taxonomic difference (overall mean $<$ 15% is acceptable). Individual samples exceeding 15% are examined for taxonomic areas of substantial disagreement, and the reasons for disagreement investigated. A reconciliation call between the primary and secondary taxonomist will facilitate this discussion. Results greater than this value is investigated and logged for indication of error patterns or trends.
6. Corrective actions include determining problem areas (taxa) and consistent disagreements and addressing problems through taxonomist interactions. These actions help to rectify disagreements resulting from identification to a specific taxonomic level.

5.6.2.3 Taxonomic QC Review & Reconciliation

The Indicator QC Coordinator prepares a report or technical memorandum to quantify aspects of taxonomic precision, assess data acceptability, highlight taxonomic problem areas, and provide recommendations for improving precision. This report is submitted to the HQ Project Management Team, with copies sent to the primary and QC taxonomists. Another copy is maintained in the project file. Significant differences may result in the re-identification of samples by the primary taxonomist and a second QC check by the secondary taxonomist.

All samples are stored at the laboratory until the Project Lead notifies the laboratory regarding disposition.

Table 5.3 Laboratory quality control: benthic indicator.

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
SAMPLE PROCESSING (PICK AND SORT)			
Sample residuals examined by different analyst within laboratory	10% of all samples completed per analyst	Efficiency of picking ≥ 90%	If < 90%, examine all residuals of samples by that analyst and retrain analyst
IDENTIFICATION			
Sorted samples re-identified by different analyst within laboratory	10% of all samples	Accuracy of contractor laboratory picking and identification ≥ 90%	If picking accuracy < 90%, all samples in batch will be reanalyzed by contractor
Independent identification by outside taxonomist	All uncertain taxa	Uncertain identifications to be confirmed by expert in particular taxa	Record both tentative and independent IDs
Use standard taxonomic references	For all identifications	All keys and references used must be on bibliography prepared by another laboratory	If other references desired, obtain permission to use from Project Facilitator
Prepare reference collection	Each new taxon per laboratory	Complete reference collection to be maintained by each individual laboratory	Benthic Laboratory Manager periodically reviews data and reference collection to ensure reference collection is complete and identifications are accurate
External QC	10% of all samples completed per laboratory	PDE ≤ 5% PTD ≤ 15 %	If PDE > 5%, implement recommended corrective actions. If PTD > 15%, implement recommended corrective actions.
DATA VALIDATION			
Taxonomic "reasonable-ness" checks	All data sheets	Genera known to occur in given lakes or geographic area	Second or third identification by expert in that taxon

6.0 FECAL INDICATOR: ENTEROCOCCI

6.1 Scope and Application

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

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

6.1.1 Summary of Method

Each sub-sample has previously been filtered aseptically and folded inward in half three times to form an umbrella or in half and rolled up and then inserted into sterile sample extraction tubes containing sterile glass beads or Roche MagNA Lyser Green BeadsTM (actually siliconized white ceramic beads in a green capped tube). Extraction tubes containing filter concentrates (retentates) have been stored on dry ice until transport to the analytical laboratory by air courier. Filter concentrates will be shipped by air courier on dry ice from the field to the analytical team at EPA New England Regional Laboratory. Filter concentrates received by NERL staff will be subjected to DNA extraction procedures and subsequently analyzed by Draft EPA Method 1606 or 1607 for Total Enterococcus along with modifications to the QA/QC procedures described below. The laboratory methods are summarized in **Table 6.4** of **Section 6.15**.

6.1.2 Definitions of Method

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

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

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

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

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

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

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

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

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

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

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

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

6.2 Interferences

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

6.3 Health & Safety Warnings

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

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

6.4 Personnel Qualifications

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

6.5 Equipment and Supplies

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

6.6 Reagents & Standards

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

6.7 Preparations Prior to DNA Extraction & Analysis

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

3. 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.
4. **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).
5. 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.*

6. 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.
7. *Enterococcus faecalis* DNA for Standards.
8. Frozen Reference Stock (20-µL) at 2.89 x 10^6 GEQs per µL.
9. Dilute 10-µL of the Frozen Reference stock 363-fold to a final volume of 3,630 µL AE buffer. Aliquot 20-µL volumes into many 200-µL microfuge tubes and store frozen at -20 ºC. The net concentration of *Enterococcus* GEQs is 8,000 / µL. Each week perform a series of 10-fold and 4-fold dilutions from one thawed tube of the 8,000 GEQ/µL standard solution to create 800

GEQ/µL, 80 GQ/µL and 20 GEQ/µL standard solutions. The analyst performs *Enterococcus* qPCR upon duplicate 5-µL volumes of each of the four standards yielding a Standard Curve of Log GEQs ENT versus Ct value from which the assays "efficiency" is subsequently calculated in the Relative Quantitation EXCEL Spreadsheet.

a. Make *Enterococcus faecalis* calibrator filter samples:
    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.

10. 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.
11. 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 ≥ 15-min. exposure to high intensity germicidal (254 nm) ultraviolet light.

6.8 Procedures for Processing & qPCR Analysis of Sample Concentrates.

6.8.1 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 (≤ 50-mL) are acceptable if suspended particulates hinder the filtering of the standard 50-mL volume but equivalent volumes for each filter replicate were requested. Filtration of lower sample volumes necessitated modifications to Method 1606 which are directed by the Analysis Decision Tree (ADT; **Section 6.15.1**).

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

12. 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.
13. 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.

14. 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.
15. Spin the supernatant tubes for 5-min at 14,000 rpm at Room Temperature. Recover >350-µL supernatant and transfer to new 1.7-mL tube. When all samples in a batch have been extracted transfer dilute 20-µL of DNA extract (2nd supernatant) five-fold (5X) in 80-µL AE buffer (sans SS-DNA) and store at 4˚C for qPCR assays. (If supernatant, 5X and even 25X sample dilutions possess dark pigment and exhibit severe qPCR inhibition in Sketa assays, consider extracting replicate filters of samples using the Modified MagNA Pure LC DNA Isolation Protocol (see **Section 6.15.2**).

6.8.2 Sample Analysis by *Enterococcus* qPCR

6.8.2.1 Preparation of qPCR assay mix

16. 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.
17. 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.
18. 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.
19. Using a micropipettor, prepare assay mix of the *Enterococcus*, and Salmon DNA reactions in separate, sterile, labeled 1.7 mL microcentrifuge tubes as described in **Table 6.1**.
20. 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.
21. 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.
22. Set 32 Smart tubes in Cepheid Racks in PCR cabinet along with micro-pippetors and expose to germicidal UV lamp for 15-min.
23. 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.
24. 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.

25. 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 6.15.2**).

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

DNA equivalent to the amount in the Salmon-spiked Lysis Binding Buffer added to each sample filter lysate during the MagNA Pure LC DNA extraction process.

The "Unknown" and "Control" sample extracts whether processed using the SAE buffer or MagNA Pure LC Kit III reagents are analyzed according to the Cepheid SmartCycler *Enterococcus* and Sketa qPCR protocols described in Appendix A of the Draft EPA Method 1606 with C*t* determination made by the software using Manual Determination (equivalent of Fit Points Method of Roche LightCycler) with the fluorescence threshold set at 8.0 units which enables uniform analysis and comparability of all samples' qPCR results.

6.8.3 Sample analysis sequence for SmartCycler

Example: For analyses on a single 16-position SmartCycler, calibrator samples and water samples are analyzed in separate runs and a maximum of 6 water samples (or 2 replicates of 3 samples) are analyzed per run, as described in **Table 6.2** and **Table 6.3** of **Section 6.15**.

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

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

6.9 Storage & Timing of Processing/Analysis of Filter Concentrates

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

6.10 Chain of Custody

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

Field Sampling forms and NRSA 2018-2019 Sample Tracking EXCEL Spreadsheet shall be consulted to determine if a sample has been properly preserved during collection and transport prior to analysis and that it has passed all criteria permitting its analysis. The qPCR results of samples exceeding established criteria or whose associated field/lab blanks had positive *Enterococcus* qPCR detections of DNA shall be flagged.

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

The Data Quality Objectives and the Laboratory QC Procedures are listed and summarized in **Table 6.5** and **Table 6.6** of **Section 6.15**.

The number of field blanks (dilution buffer only) shipped by field crews performing the resampling of 91 re-visited rivers and streams represents a frequency of 5-10% of the total number of samples extracted and analyzed by qPCR. All field blanks (negative controls) will be extracted and analyzed by qPCR for the detection of *Enterococcus*. The blanks will be analyzed in these cases to insure that positive detections in field samples are not due to contamination by sampling crews.

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

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

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

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

6.12 Method Performance

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

6.13 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 2018-2019 database stored on a computer server in Corvallis, Oregon.

6.14 Waste Management & Pollution Prevention

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

All ethanol used shall be consumed by ignition or evaporation. Volumes of ethanol remaining at the end of the project can be stored for later use in a flammable cabinet or disposed of through appropriate hazardous waste disposal vendors. Reagent ethanol shall be contained in screw cap tubes along with the filter forceps to sterilize the latter and to prevent ethanol spillage during transport between sampling sites.

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

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

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

Reagent	Volume/Sample (multiply by # samples to be analyzed per day)
Sterile H_2O	1.5 µL
Bovine Serum Albumen (20 mg/mL)	2.5 µL
TaqMan® master mix	12.5 µL
Primer/probe working stock solution	3.5 µL*

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

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

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

** Diluted equivalently to the water samples*

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

Sample Description*	Quantity Samples	PCR Assay Master Mix	Quantity PCR Reactions
Water samples, (5-fold dilution)	26	*Enterococcus*	26
Method blank or Sample PCR Reaction Duplicate, (1- or 5-fold dilution)	1	*Enterococcus*	1
Non-diluted SAE Buffer	1	*Enterococcus*	1
Water samples, (1- or 5-fold dilution)	26	Salmon DNA	26
Method blank or Sample PCR Reaction Duplicate, (1- & 5-fold dilution)	1	Salmon DNA	1

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

Table 6.4 Enterococci: laboratory methods

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

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

Table 6.5 Enterococci: parameter measurement data quality objectives

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

C = critical, N = non-critical quality assurance classification.
**AQM = Absolute Quantitation Method; RQM = Relative Quantitation Method;*
SPC = Sample Processing Control (Salmon DNA / Sketa); CCEs = Calibrator Cell Equivalents

Table 6.6 Enterococci: laboratory QC procedures – enterococci DNA sequences

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

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

6.15.1 *Enterococcus* qPCR Analysis Decision Tree (ADT)

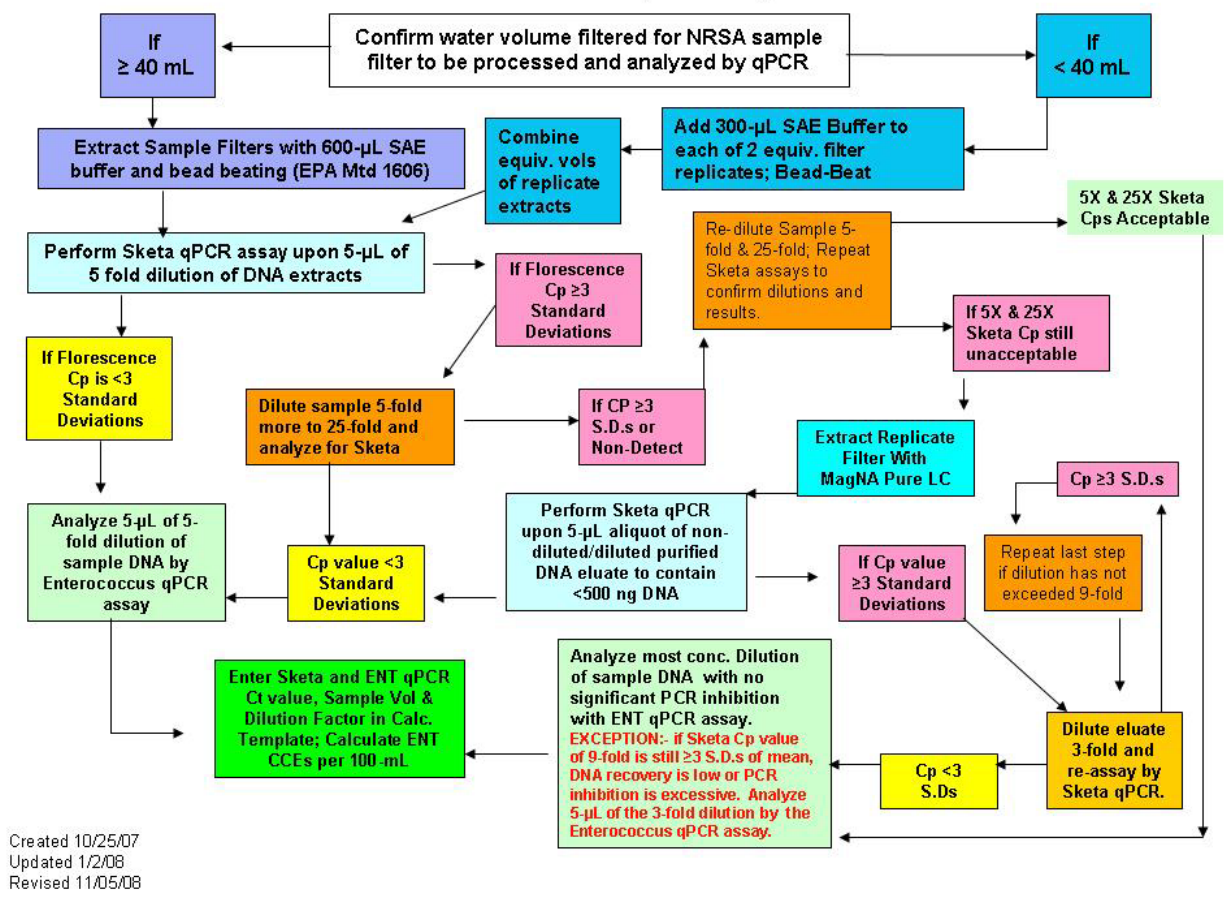

Figure 6.1 Enterococci: qPCR analysis decision tree (ADT)

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

26. Pre-warm the MagNA Pure LC DNA Isolation Kit III Lysis Buffer to 65 ºC in waterbath. Quickly pipette 260-µL of warm Lysis Buffer (un-amended) into each "Green Bead" tube with filter (preserved after filtration temporarily on ice or during long-term storage in freezer). Shake tube 5 to 10 seconds to mix buffer with beads and filter. Let stand at RT until batch of 16 samples (including positive control LFB or LFM and negative control LB samples) have all had Lysis Buffer and had their caps sealed tight. Leave water bath on to use during 30-minute Proteinase K treatment period.
27. 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.
28. 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.
29. 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.
30. 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.
31. 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.
32. 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.
33. 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.
34. 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.
35. 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.
36. 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.

37. 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.
38. 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.
39. 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.
40. 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.
41. 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.

7.0 PHYTOPLANKTON METHODS

This method is adapted from protocols used for the U.S. Geological Survey National Water Quality Assessment program (Charles et al. 2003) to identify and enumerate taxa in phytoplankton samples. The method involves microscopic examination of preserved phytoplankton samples from integrated samples collected from the euphotic zone of the water column.

Phytoplankton samples will be preserved in the field with Lugol's solution and shipped from field crews to a contract batching laboratory. The contract batching laboratory will send the batched samples to the analysis laboratory. Preserved samples will arrive in the analysis laboratory and can be held for several months. Phytoplankton analysis laboratories will need to process samples in accordance with the time frame outlined in contractual agreements. Contractual agreements for delivery of data do not supersede indicator holding times.

7.1 Responsibility and Personnel Qualifications

This procedure may be used by any person who has received training in processing and/or identification of phytoplankton samples. It is important that all taxonomists maintain contact with other taxonomists through professional societies and other interactions and keep abreast with the pertinent literature, because taxonomic groupings and nomenclatural basis for species identifications are updated frequently. A second taxonomist will re-identify a randomly-selected 10% of the samples for QC, as noted below, to quantify taxonomic precision, or consistency, as **percent difference (PD)**, to help target corrective actions, and ultimately to help minimize problems during data analysis. Samples are sent to the laboratory from the field on a regular basis to avoid delays in processing and sample identification.

7.2 Precautions

Wear appropriate clothing for safety precautions, such as nitrile gloves, rubber apron, long pants, etc.

7.3 Equipment/Materials

- Compound microscope (with 10, 40, 100X objectives with 10 - 15X ocular, and epifluorescence capability)
- Utermöhl sedimentation chamber
- Pasteur pipette
- Volumetric cylinder
- Bench sheet
- Phytoplankton Sample Log-In Form
- Phytoplankton Laboratory Sheet
- Labels

7.4 Sample Receipt

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

1. Report receipt of samples to the NARS IM Team by completing and emailing the sample tracking spreadsheet with the sample login and sample condition information. (See **Section 1.2** of the manual for contact information).

2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
   a. Verify that the sample IDs in the shipment match those recorded on the sample tracking form.
   b. Record the information in **Table 7.1** for the NARS IM Team, including the Condition Code for each sample:
      i. *OK*: Sample is in good condition
      ii. *C*: Sample container was cracked
      iii. *L*: Sample container is leaking
      iv. *ML*: Sample label is missing
   c. If any sample is damaged or missing, contact the USEPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed. (See contact information in Chapter 2 of the Manual).

3. Store samples until sample preparation begins.

4. Maintain the sample tracking forms with the samples.

Table 7.1 Phytoplankton login: required data elements.

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

7.5 Procedure

7.5.1 Prepare Utermöhl Sedimentation Chamber

1. Use a light amount of vacuum grease to attach a cover glass to the bottom of an Utermöhl sedimentation chamber. It is critical that the cover glass be clean and grease free.
   - For tubular varieties of settling chambers, seal a cover glass to the threaded end of the tube and screw the tube into the base assembly.
   - For a plate chamber type of settling chamber, attach the cover glass on the bottom of the base, lock it into place with the metal ring and seal the cylinder on top of the base using a light amount of vacuum grease.
2. Homogenize the concentrated samples by repeatedly inverting the sample bottle. Place a 10-mL aliquot of the sample into the assembled settling chamber. Let the sample settle for at least 8 hours.
3. For the plate chamber type of Utermöhl chamber, drain the volumetric cylinder by sliding over the drainage hole. Slide the cover plate over the chamber without allowing air bubbles to form. Analysis should proceed within a few hours of removing the cylinder.

7.5.2 Choose Count Method

7.5.2.1 Determine random fields

1. Using a high oil microscope objective (10-15X objective, 100-1500X total system magnification), identify and enumerate algae in selected, random fields. Enumerate between 8 and 100 fields from each Utermöhl chamber. If necessary, use a second chamber.
2. Choose a random starting place in the upper left-hand quadrant of the counting chamber and approximate the number of fields that must be analyzed (400 natural units [**Section 7.5.3]** need to be counted with a minimum of 8 and maximum of 100 random fields).
3. Develop a pattern that allows for equal probability of landing in any area of the cell or chamber with the exception of the edges and the center. A maximum pattern with 100 fields is made by having an 8x8 grid, and then subtracting 3 or 4 fields in either direction of the center.

7.5.2.2 Determine transects

1. Using a high oil microscope objective (10-15x objective, 100-1500x total system magnification) with a calibrated stage, identify and enumerate algae along transects, either horizontally or vertically across the Utermöhl plate chamber.
2. Without looking into the microscope, choose a location near the left edge in the upper third of the chamber (if vertical transects are analyzed, choose a location near the top edge in the left third of the chamber). Make a transect by moving only the horizontal stage control (or vertical control for vertical transects) a measured distance.
3. Develop a pattern for the transects that will avoid the center and edges of the chamber. A second Utermöhl chamber can be used, if necessary (400 natural units need to be counted with a minimum of one complete transect).

7.5.3 Identify and Enumerate 400 Natural Algal Units

1. Species-level resolution is the taxonomic requirement for phytoplankton which likely means using a magnification of 1000X or higher.
2. Using the pattern developed above, move the microscope stage to a new position in the pattern. Make all movements of the microscope stage without looking through the objectives.

3. Identify and enumerate all algal forms in the field of view: enumerate algal forms using natural counting units. Natural counting units are defined as one for each colony, filament, diatom cell (regardless if colonial or filamentous) or unicell. With the exception of diatoms, identify algal forms to species. Develop a method of selecting taxa that are only partially in view. For example, only count taxa that are partially in the field of view if they are on the left side. If they are on the right do not count.
4. Count only "living" diatoms at the time of collection. If there is any protoplast material in the frustule, the diatom is considered to have been living when collected.
5. Differentiate diatoms to the lowest practical taxonomic level. This will usually be genus, but use of categories such as naviculoid, cymbelloid, centric, nitzschoid is appropriate.
6. Count the number of algal cells comprising each multicellular counting unit.
7. Tabulate the data on a bench sheet **APPENDIX B: SAMPLE LABORATORY FORMS**, mechanical or electronic tabulator.
8. Repeat steps 1 - 4 until 400 natural algal units have been enumerated. Again, count only "living" diatoms as part of the required 400 natural algal units.
9. Add and record the tallies of each taxon on the bench sheet. Record the number of cells for multicellular counting units in parentheses beside the tally of natural counting units.
10. Record the number of fields or the total transect length for the area that was observed.

7.5.4 Identify and Enumerate Larger, Rarer Taxa

There is an additional procedure that can be used for samples with low concentrations (less than five natural counting units) of large cells or colonies (maximum dimension greater than 100 µm).

1. Using a low-power objective (10-15X), scan 20 fields or 4 transects. Count the larger, rarer taxa (as defined above).
2. Enumerate as natural units and estimate the number of cells in each. Record the counts of each of the taxa on the bench sheets, noting the scan area (i.e., total area for the 20 microscope fields or 4 transects). Multiply the number of larger, rarer taxa by the ratio of the total area scanned in the regular count to the area scanned in this count.
3. Record that number as the total count for that taxon.

7.5.5 Measure Cell Biovolumes

1. For each group of samples, measure the dimensions of the taxa that contribute most to sample biovolume. Cell biovolumes of all identified taxa will be quantified on a per milliliter basis. Use formulae for solid geometric shapes that most closely match the cell shape (Hillebrand et al., 1999) to estimate biovolume. Base biovolume calculations on measurements of 10 organisms per taxon for each sample where possible.
2. Biovolumes for each abundant taxon (i.e., occurring in more than 5% in any one sample) should be based on measurements of 10 cells or more
3. Biovolumes for each common taxon (i.e., occurring 2 – 5% in any one sample) should be based on measurements of one or more cells.
4. Biovolumes for each rare taxon (i.e., occurring in 0.1 – 2% in any one sample) should be based on measurements from literature descriptions of taxa, previous measurements of the taxon, or measurements of one or more cells.
5. For taxa with substantial size variation (e.g., diatoms), designate size classes based on sample quality to determine average cell size (biovolume). For each taxon, measure 10 cells from each size class (assuming that sufficient numbers are available). Use mean biovolumes within each size class

to calculate the total biovolume contributed by the taxon to its representative sample (Burkholder and Wetzel, 1989).

7.6 Calculation and Reporting

1. The calculation of phytoplankton abundance depends on the apparatus used during analysis. Biovolume values are determined by multiplying the abundance (cells/mL) by the average biovolume of each cell (μm^3). Phytoplankton abundance (cells/mL) is calculated as follows:

Equation 7.1 Phytoplankton abundance.

$$cells/mL = \left(\frac{count \times chamber \times 1000mL}{numfields \times field \times mlsettled} \right) / 1000$$

where *count* = number of cells counted, *chamber* = chamber area (in mm^2), *numfields* = number of microscope fields, *field* = microscope field area (in mm^2), and ml settled = number of ml settled in Utermöhl chamber.

2. Prepare a spreadsheet file containing the count data, using the columns (fields) as shown in the **(APPENDIX B: SAMPLE LABORATORY FORMS: Phytoplankton Measurement Data Sheet**. Submit the file electronically to the USEPA**.**

7.7 Pertinent QA/QC Procedures

Table 7.2 provides a summary of quality control procedures for the phytoplankton indicator.

7.7.1 Internal Taxonomic QC

An in-house QC Analyst will randomly select 5 of the samples counted and identified by individual taxonomists to ensure that each meets the acceptable criteria for percent identification efficiency which is 90%. If the individual fails to maintain a ≥ 90% identification as determined by QC checks, previous samples will be re-counted and identified.

7.7.2 External Taxonomic QC

EPA may implement an external taxonomic QC review process for phytoplankton. If EPA implements an external QC process, upon receipt of the data after initial identification, the Indicator QC Coordinator for phytoplankton randomly selects 10% of the samples for external QC analysis. The Indicator QC Coordinator will direct the original laboratory to send those samples to a QC taxonomist, a second experienced taxonomist who did not participate in the original identifications. The original laboratory will complete a sample tracking form and send it with the samples.

7.7.2.1 Plankton Re-identification

Duplicate processing (duplicate the processing steps presented in **Section 7.5.1 – 7.5.5**).

The remaining concentrated sample will be sent to the QC taxonomist.

1. Using the same volume as the original Utermöhl chamber, prepare a duplicate Utermöhl chamber cell and enumerate 400 natural algal units. Complete another copy of the Taxonomic Bench Sheet for each sample. Label each bench sheet with the term “QC Dup-ID.” As each bench

sheet is completed, the laboratory sends it (through email or fax) to the Indicator QC Coordinator.

2. The Indicator QC Coordinator compares the taxonomic results generated by the primary and QC taxonomists for each sample and calculate percent difference using:

Equation 7.2 Percent difference.

$$PctDiff = 100 - \sum \min(a, b)$$

where *a* and *b* are the relative proportions recorded for a given taxon by the primary taxonomist (a) and the QC taxonomist (b).

3. Values will be a combination of subsampling error and taxonomic error; the MQO is that the two counts will have a percent difference of ≤ 50.
4. If it appears that high percent difference for soft-bodied phytoplankton are due to subsampling inconsistency, then determine and implement appropriate corrective actions working with the Indicator QC Coordinator. In addition, disagreements resulting from identification to a specific taxonomic level, creating the possibility to double-count "unique" or "distinct" taxa shall be rectified through corrective actions working with the Indicator QC Coordinator.

7.7.3 Taxonomic QC Review & Reconciliation

The Indicator QC Coordinator prepares a report or technical memorandum to quantify aspects of taxonomic precision, assess data acceptability, highlight taxonomic problem areas, and provide recommendations for improving precision. This report is submitted to the HQ Project Management Team, with copies sent to the primary and QC taxonomists. Another copy is maintained in the project file. Significant differences may result in the re-identification of samples by the primary taxonomist and a second QC check by the secondary taxonomist.

All samples are stored at the laboratory until the Project Lead notifies the laboratory regarding disposition.

Table 7.2 Laboratory quality control: phytoplankton indicator.

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
IDENTIFICATION			
Independent identification by outside taxonomist	All uncertain taxa	Uncertain identifications to be confirmed by expert in particular taxon	Record both tentative and independent IDs
Use standard taxonomic references	For all identifications	All keys and references used must be on bibliography prepared by another laboratory	If other references desired, obtain permission to use from Project Facilitator
Prepare reference collection	Each new taxon per laboratory	Complete reference collection to be maintained by each individual laboratory	Laboratory Manager periodically reviews data and reference collection to ensure reference collection is complete and identifications are accurate

External QC (if implemented)	10% of all samples completed per laboratory	Efficiency (PD) ≤ 50%	If PD > 50%, implement recommended corrective actions
DATA VALIDATION			
Taxonomic "reasonable-ness" checks	All data sheets	Genera known to occur in given lakes or geographic area	Second or third identification by expert in that taxon

8.0 PESTICIDE SCREEN: ATRAZINE

This method describes the application of enzyme linked immunosorbent assay (ELISA) to the determination of atrazine and related atrazine occurrence and concentration in surface water samples. The Eurofins Technologies (formerly Abraxis) magnetic particle atrazine kit is used for this analysis.

You will filter the lake water sample, add the filtered water to a disposable test tube with an enzyme conjugate, and then add paramagnetic particles with atrazine-specific antibodies. After allowing for a 15-minute reaction between the sample and reagents, you apply a magnetic field to the test tube that retains the paramagnetic particles (with atrazine and labeled atrazine bound to the antibodies on the particles in proportion to their original concentration) and allow the unbound reagents to be decanted. After decanting, wash the particles with the washing solution. You will detect the presence of atrazine and related atrazines by adding the color solution. After an incubation period, the reaction is stopped and stabilized by the addition of a dilute acid (Stopping Solution). Because the labeled atrazine (conjugate) was in competition with any unlabeled atrazine in the sample for the antibody sites, the color developed is inversely proportional to the concentration of atrazine in the sample. The detection limit for this method is 0.03 µg/L and the reporting limit is 0.05 µg/L.

The field crews will ship chilled atrazine pesticide screen samples to the contract batching laboratory as part of the batch frozen shipment. The chilled samples will be placed in an insulated tube in a separate component of the dry ice liner to prevent freezing during shipment. The contract batching laboratory will store samples in the refrigerator and send the batched samples to the analysis laboratory in coolers on wet ice. Samples will arrive in the analysis laboratory chilled, and they can be held in a refrigerator or cold room for several weeks. Atrazine pesticide screen analysis laboratories will need to process samples within the 90-day holding time and in accordance with the time frame outlined in contractual agreements.

The methods listed below follow the methods used by Minnesota Pollution Control Agency (MPCA) based on the ELISA kit instructions.

8.1 Responsibility and Personnel Qualifications

All laboratory personnel are trained in advance in the use of equipment and procedures used during the implementation of this SOP. All personnel are responsible for complying with all of the QA/QC requirements that pertain to this indicator.

8.2 Precautions

The stopping solution contains diluted sulfuric acid (H_2SO_4). Avoid contact of the stopping solution with skin and mucous membranes. If this reagent comes in contact with the skin, wash with water. Consult state, local, and federal regulations for proper disposal of all reagents.

8.2.1 Storage and Stability

Store all reagents at 2-8°C. Do not freeze reagents. Before use, allow the solutions to reach room temperature (20-25°C). Reagents may be used until the expiration date on the box. The test tubes and the washing solution require no special storage condition and may be stored separately from the reagents.

8.3 Equipment

- Abraxis Atrazine Kit (each kit contains Atrazine Antibody Coupled Paramagnetic Particles, Atrazine Enzyme Conjugate, Atrazine Standards, Control, Diluent/ Zero Standard, Color Solution, Stopping Solution, Washing Solution, and test tubes)
- Precision pipets capable of delivering 250 and 500 µL and a 1.0 mL repeating pipet
- Vortex mixer
- Magnetic separation system
- Photometer capable of readings at 450 nm

8.4 Sample Receipt

Because USEPA initiates tracking procedures designed to recover any missing shipment, the laboratory personnel responsible for tracking samples must start the following login steps upon receiving a delivery.

1. Report receipt of samples to the NARS IM Team by completing and emailing the sample tracking spreadsheet with the sample login and sample condition information. (See **Section 1.2** of the manual for contact information).

2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
   a. Verify that the sample IDs in the shipment match those recorded on the sample tracking form
   b. Record the information in **Table 8.1** for the NARS IM Team, including the Condition Code for each sample:
      i. *OK*: Sample is in good condition
      ii. *C*: Sample container was cracked
      iii. *L*: Sample container is leaking
      iv. *ML*: Sample label is missing
      v. *W:* Sample is warm (>4°), record the temperature in the comment field, and perform the assay
   c. If any sample is damaged or missing, contact the USEPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed. (See contact information in Chapter 2 of the Manual).

3. Store samples in the refrigerator until sample preparation begins.

4. Maintain the sample tracking forms with the samples.

Table 8.1 Atrazine login: required data elements.

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

8.5 Procedure

8.5.1 Test preparation

1. Filter all lake water samples with a 0.2 µm filter (e.g., Anotop or Arcodisc) to remove particles.
2. If the atrazine concentration of a sample exceeds 5 ppb, you will need to repeat the test with a diluted sample. A ten-fold or greater dilution of the sample is recommended with an appropriate amount of Diluent/ Zero Standard or Sample Diluent (e.g., make a ten-fold dilution by adding 100 µL of the sample to 900 µL if Diluent/ Zero Standard). Mix the dilution thoroughly before assaying. Perform the assay according to the Assay Procedure and calculate the final results by multiplying the value obtained by the dilution factor.
3. Bring reagents to room temperature and thoroughly mix the antibody coupled paramagnetic particles before use.

8.5.2 Procedural notes and precautions

- A consistent technique is important for optimal performance. For the greatest precision, treat each tube in an identical manner.
- Add reagents directly to the bottom of the tube while avoiding contact between the reagents already added to the tube and the pipet tip. This will help assure consistent quantities of reagent in the test mixture.

- Avoid cross contamination and carryover of reagents by using clean pipets for each sample addition and by avoiding contact between reagent droplets on the tubes and the pipet tips.
- Avoid foam formation during vortexing.
- Mix the antibody coupled paramagnetic particles just prior to pipeting.

8.5.3 Assay procedure

1. Label test tubes for standards, controls, and samples (**Table 8.2**).
2. Add 200 or 250 µL of the appropriate standard, control, or sample to the test tube.
3. Add 250 µL of Atrazine Enzyme Conjugate to each tube.
4. Mix the Atrazine Antibody Coupled Paramagnetic Particles thoroughly and add 500 µL to each tube.
5. Vortex for 1 to 2 seconds minimizing foaming.
6. Incubate for 15 minutes at room temperature.
7. Separate in the Magnetic Separation System for two minutes.
8. Decant and gently blot all tubes briefly in a consistent manner.
9. Add 1mL of washing solution to each tube and allow them to remain in the magnetic separation unit for two minutes.
10. Decant and gently blot all tubes briefly in a consistent manner.
11. Repeat steps 9 and 10 one additional time.
12. Remove the rack from the separator and add 500 µL of Color Solution to each tube.
13. Vortex for 1 to 2 seconds minimizing foaming.
14. Incubate for 20 minutes at room temperature.
15. Add 500 µL of Stopping Solution to each tube.
16. Add 1 mL Washing Solution to a clean test tube. Use as a blank in Step 17.
17. Within 15 minutes after the addition of the stopping solution, read the absorbance at 450 nm with a photometer.

8.5.4 Results

1. Calculate the mean absorbance value for each of the standards.
2. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for the standard by the mean absorbance value for the Diluent/ Zero Standard.
3. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical logit (Y) axis versus the corresponding atrazine concentration on the horizontal logarithmic (X) axis.
4. $\%B/B_0$ for controls and samples will then yield levels in ppb of atrazine by interpolation of the standard curve.

Some instrument manufacturers make photometers that allow for automatic calculation of calibration curves. Refer to instrument operating manuals for detailed instructions.

Table 8.2 Test tube labeling for atrazine assay.

Tube Number	Contents of Tube
1,2	Diluent/ Zero Standard, 0 ppb
3,4	Standard 1, 0.1 ppb
5,6	Standard 2, 1.0 ppb
7,8	Standard 3 5.0 ppb
9	Control
10	Sample 1
11	Sample 2
12	Sample 3

8.5.5 Data Entry

Required data elements that laboratories must provide to the USEPA, are identified in the USEPA's data template, available separately from the USEPA. If the laboratory applies its own QC codes, the data transmittal must define the codes.

8.6 Pertinent QA/QC Procedures

8.6.1 Internal QC

1. A control solution at approximately 3 ppb of atrazine is provided in the atrazine kit. Include a control in every run and treat it in the same manner as an unknown sample.
2. Prepare and incubate one duplicate sample for every 10 samples analyzed.
3. Table 8.3 provides a summary of the quality control requirements.

Table 8.3 Atrazine: quality control requirements.

Quality Control Activity	Description and Requirements	Corrective Action
Kit – Shelf Life	Is within its expiration date listed on kit box.	If kit has expired, then discard or set aside for training activities.
Kit – Contents	All required contents must be present and in acceptable condition. This is important because Abraxis has calibrated the standards and reagents separately for each kit.	If any bottles are missing or damaged, discard the kit.
Calibration	All of the following must be met: ○ Standard curve must have a correlation coefficient of ≥0.99; ○ Average absorbance value, $\bar{A}_0$, for S0 must be >0.80; and ○ Standards S0-S3 must have decreasing average absorbance values. That is, if $\bar{A}_i$ is the average of the absorbance values for S_i, then the absorbance average values must be: $\bar{A}_0 > \bar{A}_1 > \bar{A}_2 > \bar{A}_3$	If any requirement fails: • Results from the analytical run are not reported. • All samples in the analytical run are reanalyzed until calibration provides acceptable results. At its discretion, the lab may consult with EPA for guidance on persistent difficulties with calibration.

Quality Control Activity	Description and Requirements	Corrective Action
Kit Control	The average concentration value of the duplicates (or triplicate) must be within the range of 3 ppb +/- 10%.	If the requirement fails: • Results from the analytical run are not reported • The lab evaluates its processes, and if appropriate, modifies its processes to correct possible contamination or other problems. • The lab reanalyzes all samples in the analytical run until the controls meet the requirements.
Negative Control	The values for the negative control replicates must meet the following requirements: ○ All concentration values must be < 0.05 µg/L (i.e., the reporting limit)	
Sample Evaluations	Samples are run in duplicate: requires 1 in 10 duplication of samples; Each duplicate pair must have %CV≤10% between its absorbance values.	If the requirement fails when applying the: *All* samples in the kit, not just the sample that failed, must be run a second time. No samples are to be run more than twice.
Results Within Calibration Range	If the result is less than the upper calibration range (i.e., 5.0 µg/L for undiluted samples), then the requirement is met.	If a result registers as "HIGH", then record the result with a data flag of "HI." If the result registers as 'HIGH,' then the sample must be diluted and re-run. If the sample is evaluated using a duplicate pair, if one or both results register as 'HIGH', then the sample must be diluted and re-run. No samples are to be run more than twice. The lab reports both the original and diluted sample results.
External Quality Control Sample	External QC Coordinator, supported by QC contractor, provides 1-2 sets of identical samples to all laboratories and compares results.	Based upon the evaluation, the External QC Coordinator may request additional information from one or more laboratories about any deviations from the method or unique laboratory practices that might account for differences between the laboratory and others. With this additional information, the External QC Coordinator will determine an appropriate course of action, including no action, flagging the data, or excluding some or all of the laboratory's data.

8.6.2 External QC

1. For the national lab, analyze 10 provided spiked samples (blind sample) provided by the USEPA HQ Laboratory Review Coordinator. For the state labs, analyze 5 provided spiked samples (blind sample) provided by the USEPA HQ Laboratory Review Coordinator. After processing the samples, the laboratory will send the results to the USEPA HQ Laboratory Review Coordinator. The results will be compared to the known concentrations and a determination made.

9.0 FISH TISSUE FILLET (Whole Fish Composite Sample)

Laboratory methods incorporated into EPA Office of Science and Technology QA documents.

10.0 WATER CHEMISTRY and CHLOROPHYLL *A*

This chapter describes the analysis requirements for water quality samples. The purpose is to determine concentrations of water quality parameters and chlorophyll *a* in water quality samples collected in the NLA 2022 and related studies.

10.1 Analytical Parameters

A total of 20 parameters are determined from each bulk water chemistry sample collected (**Table 10.1**). In addition, chlorophyll *a* is determined from a separate, discrete sample following the same performance-based methods approach as proposed for water chemistry analytes.

Table 10.1 Water chemistry parameters measured for the National Lakes Assessment 2022.

Analyte	Units	Comments
Conductivity	µS/cm at 25˚C	
pH	Standard (Std) Units	
Turbidity	NTU	
Acid Neutralizing Capacity (ANC)	µeq/L (20 µeq/L=1 mg as $CaCO_3$)	
Dissolved Organic Carbon (DOC)	mg /L	
Ammonia-N (NH_3-N)	mg /L	The method measures ammonia and ammonium; the relative proportion between these two analytes depends on pH. Typically, NLA (and other NARS) samples consist of mostly ammonium
Nitrate-Nitrite (NO_3-NO_2)	mg/L	Note different preservation methods and holding times depending on whether the lab is using ion chromotography (IC) or flow injection analysis (FIA)
Total Nitrogen (TN)	mg/L	
Total Phosphorus (TP)	µg /L	
Total Dissolved Nitrogen (TDN)	mg/L	Supplemental analysis in NLA 2022
Total Dissolved Phosphorous (TDP)	µg /L	Supplemental analysis in NLA 2022
Sulfate (SO_4)	mg /L	
Chloride (Cl)	mg /L	
Nitrate (NO_3)	mg /L	May be obtained as part of nitrate-nitrite determination (use FIA to obtain nitrate-nitrite and nitrite separately, then calculate difference for nitrate), or as a direct measurement (e.g., ion chromatography)
Calcium (Ca)	mg /L	
Magnesium (Mg)	mg /L	
Sodium (Na)	mg /L	
Potassium (K)	mg /L	
Silica (SiO_2)	mg /L	
True Color	PCU	Performance objectives based on use of visual estimation method
Chlorophyll *a*	µg/L (in extract)	

10.2 Sample Receipt

Because USEPA initiates tracking procedures designed to recover any missing shipment, the laboratory personnel responsible for tracking samples must start the following login steps upon receiving a delivery.

1. Report receipt of samples to the NARS IM Team by completing and emailing the sample tracking spreadsheet with the sample login and sample condition information. (See **Section 1.2** of the manual for contact information).

2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
   a. Verify that the sample IDs in the shipment match those recorded on the sample tracking form
   b. Record the information in **Table 10.2** for the NARS IM Team, including the Condition Code for each sample:
      i. *OK*: Sample is in good condition
      ii. *C*: Sample container was cracked
      iii. *L*: Sample container is leaking
      iv. *ML*: Sample label is missing
      v. *W*: Sample is warm (>7°), record the temperature in the comment field, and perform the assay
   c. If any sample is damaged or missing, contact the USEPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed. (See contact information in Chapter 2 of the Manual).

3. Store samples in the refrigerator until sample preparation begins.

4. Maintain the sample tracking forms with the samples.

Table 10.2 Water Chemistry login: required data elements.

FIELD	FORMAT	DESCRIPTION	
LABORATORY ID	text	Name or abbreviation for laboratory	
DATE RECEIVED	MMDDYY	Date sample was received by laboratory	
SITE ID	text	NLA site id as used on sample label	
VISIT NUMBER	numeric	Sequential visits to site (1 or 2)	
SAMPLE ID	numeric	Sample id as used on field sheet (on sample label)	
DATE COLLECTED	MMDDYY	Date sample was collected	
CONDITION CODE	text	Condition codes describing the condition of the sample upon arrival at the laboratory.	
		Flag	Definition
		OK	Sample is in good condition
		C	Sample container is cracked
		L	Sample or container is leaking
		ML	Sample label is missing
		W	Sample is warm (>7°)
		Q	Other quality concerns, not identified above
CONDITION COMMENT	text	Comments about the condition of the sample. If the condition code='W' then provide the temperature	

10.3 Sample Processing and Preservation

Due to the short holding time of these samples, samples will be shipped overnight by the field crews and must be preserved by close of business (COB) the day after sample collection. If expected samples do not arrive or arrive after the acceptable time frame (24 hours after the samples were collected), laboratories must flag those samples on the sample check-in spreadsheet provided by the NARS IM Team and notify the NARS IM Coordinator (see **Section 1.2**).

Upon receipt of samples, inspect each sample and review the tracking form that was included with the samples. Samples damaged during the shipping process are flagged by the laboratory on the sample check-in spreadsheet upon receipt and inspection. Store samples at 4°C in darkness until aliquots are ready to be prepared. If possible, prepare aliquots the same day as samples are received, but no later than 48 hours after receipt. Laboratories should be familiar with and ensure that samples meet all defined target holding times. Any sample that does not meet holding time requirements is flagged and evaluated to determine if the exceedance impacts either sample integrity or any potential end uses of the data (USEPA 2002). Results from samples that exceeded target holding times are not rejected outright.

10.3.1 Water Chemistry Samples

Figure 10.1 illustrates sample preparation processing for the water chemistry indicators, including filtering and acidifying, for the various analytes.

1. Use 0.4µm pore size polycarbonate filters for all filtration.
2. Rinse vacuum filter funnel units thoroughly with reverse-osmosis (RO) or deionized (DI) water (ASTM Type II reagent water) five times before each use and in between samples. After placing a filter in the funnel unit, run approximately 100 mL of RO or DI water through the filter, with vacuum pressure, to rinse the filter. Discard the rinse water.
3. Place the appropriate sample bottle under the funnel unit and filter sample directly into the bottle. If a new filter is needed, remove the sample bottle, and rinse the new filter with 100 mL of RO or DI water before continuing.
4. After all filtered and unfiltered aliquots are collected, add ultra-pure acid (HNO_3 or H_2SO_4, depending on the analyte, see **Table 10.3**) to the sample in the aliquot container. Cap tightly and invert the bottle several times to mix.
5. Store all aliquots except the cation aliquot (filtered, acidified with HNO_3) in a refrigerator at 4°C.

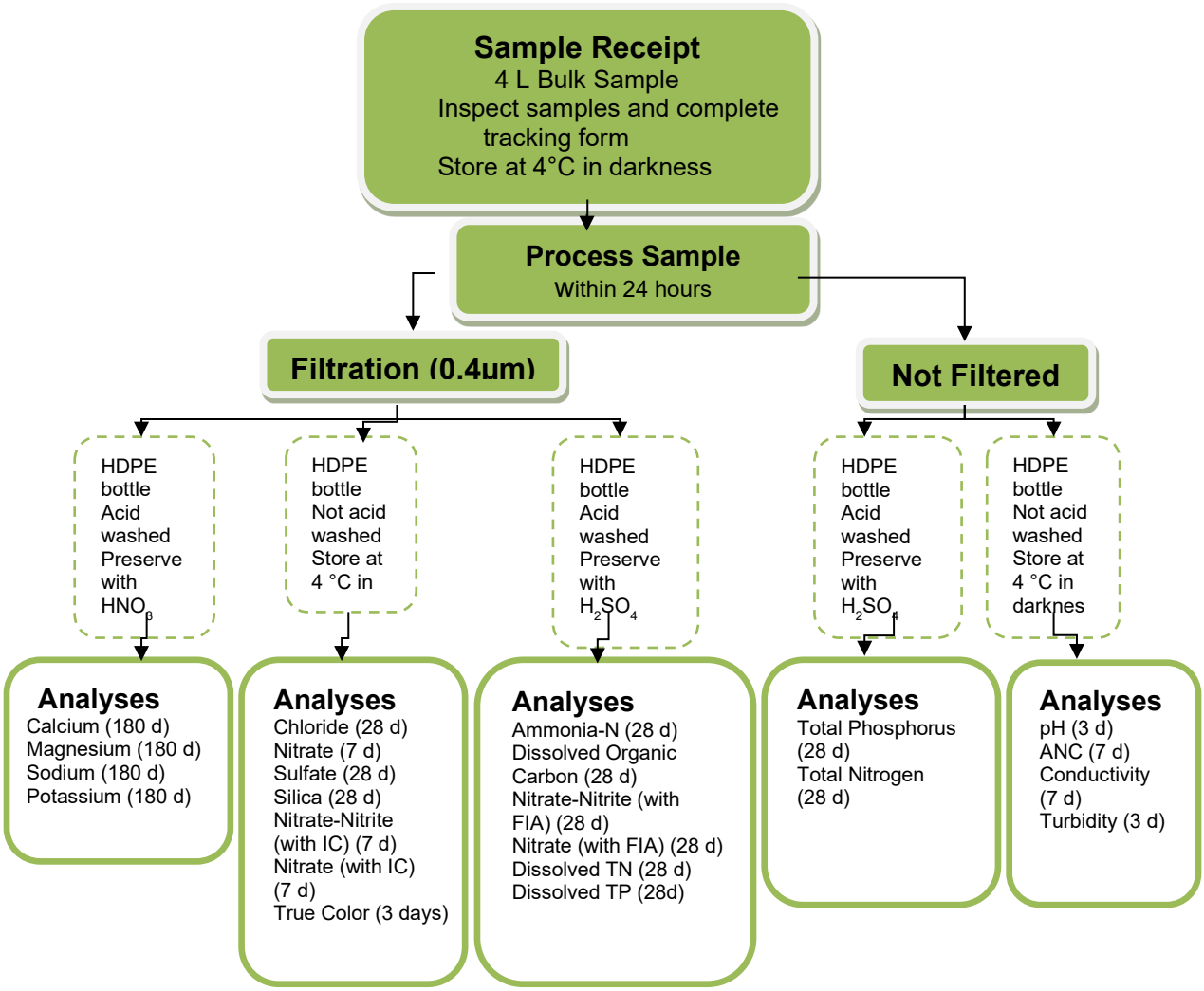

Figure 10.1 Water chemistry sample processing procedures.

Table 10.3 Acid preservatives added for various analytes.

Preservatives	
H_2SO_4	**HNO_3**
DOC	Ca
NH_3-N	Mg
TN	Na
TP	K
TDN	
TDP	
NO_2-NO_3	

10.3.2 Chlorophyll *a* Samples

Chlorophyll *a* samples are filtered in the field, placed in a labeled centrifuge tube in a dark cooler, and stored on ice until arrival at the laboratory. Store the filter in the centrifuge tube in the freezer at –20 ± 2°C for no more than thirty days before analysis.

10.4 Performance-based Methods

As an alternative to specifying laboratory methods for sample analysis, a performance-based approach that defines a set of laboratory method performance requirements for data quality is utilized for this survey. Method performance requirements for this project identify lower reporting limit (LRL), precision, and bias objectives for each parameter (**Table 10.5**). The LRL is the lowest value that needs to be quantified (as opposed to just detected), and is equal to or above the value of the lowest non-zero calibration standard. It is set to double the long-term method detection limit (LT-MDL), following guidance presented in Oblinger, Childress et al. (1999).

Precision and bias objectives are expressed in both absolute and relative terms following Hunt and Wilson (1986). The transition value is the value at which performance objectives for precision and bias switch from absolute (≤ transition value) to relative (> transition value). For pH, the objectives are established for samples with lower H^+ (or OH^-) concentrations (pH between 5.75 and 8.25) and higher H^+ (or OH^-) concentrations (pH < 5.75 or > 8.25).

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

Analytical methods used for past surveys (USEPA ORD-Corvallis) are summarized in **Table 10.4**. Participating laboratories may use alternative analytical methods for each target analyte if they can satisfactorily demonstrate the alternative method can achieve the performance requirements as listed in **Table 10.5**. Information is provided by the laboratory to the NLA Quality Team. The team reviews the information to determine whether the laboratories meet the necessary requirements. The information from this process is maintained in the NLA 2022 QA files by the USEPA HQ Laboratory Review Coordinator.

Table 10.4 Summary of analytical methods used by NLA 2022 (PESD Laboratory, USEPA ORD-Corvallis).

Analyte	Summary of Method[e]	References[f]	WRS SOP[g]
pH (laboratory)	Automated, using ManSci PC-Titrate w/ Titra-Sip autotitrator and Ross combination pH electrode. Initial pH determination for ANC titration	EPA 150.1 (modified), APHA 4500H	WRS 16A.2 (April 2021)
Specific conductance @ 25°C	Electrolytic, Man-Tech TitraSip automated analysis OR manual analysis, electrolytic	EPA 120.1, APHA 2510	WRS 16A.2 (April 2021) WRS 11A.5 (April 2021)
Acid neutralizing capacity (ANC)	Automated acidimetric titration to pH≤3.5, with modified Gran plot analysis	EPA 310.1, APHA 2320	WRS 16A.2 (April 2021)
Turbidity	Nephelometric; Man-Tech TitraSip automated analysis, OR Manual analysis using Hach turbidimeter (high turbidity samples)	EPA 180, APHA 2130	WRS 16A.2 (April 2021) WRS 13A.4 (April 2021)
True color (Hach Kit)	Visual comparison to calibrated glass color disk.	EPA 110.2 (modified), APHA 2120	WRS 15A.4 (April 2021)
Dissolved Organic Carbon (DOC)	UV promoted persulfate oxidation to CO_2 with infrared detection	APHA 5310-C, EPA 415.3	WRS 21A.5 (May 2021)
Nitrate+Nitrite, as N (fresh waters)	Ion Chromatography OR FIA automated colorimetric (cadmium reduction)	EPA 300.1; SW-846 9056A; APHA 4110B EPA 353.2 APHA 4500-NO_3-N-E Lachat 10-107-04-1-C	WRS 36A.2 (April 2021 WRS 40A.7 (February 2021)
Nitrate	Measured as part of Nitrate-Nitrite when using IC or calculated after measuring Nitrate+Nitrite using FIA.	See above	See above
Ammonia, as N (fresh waters)	FIA automated colorimetric (salicylate, dichloroisocyanurate)	Lachat 10-107-06-3-D	WRS 30A.5 (April 2021)
Silica, dissolved (SiO_2) (fresh waters)	FIA automated colorimetric (molybdate, stannous chloride)	EPA366.0,APHA 4500-SiO2 F, Lachat 10-114-27-1-B	WRS 32A.6 (February 2021)
Total nitrogen (TN)	Persulfate Digestion; FIA Automated Colorimetric Analysis (Cadmium Reduction, sulfanilamide)	EPA353.2 (modified) APHA 4500-N-C (modified) ASTM WK31786 Lachat 10-107-04-1-C (modified)	WRS 34A.6 (February 2021)
Total phosphorus (TP)	Persulfate Digestion; Automated Colorimetric Analysis (molybdate, ascorbic acid)	EPA 365.1 (modified), APHA 4500-P-E USGS I-4650-03 Lachat 115-01-1-B (modified)	WRS 34A.6 (February 2021)
Dissolved nitrogen	Persulfate Digestion; FIA Automated Colorimetric Analysis (Cadmium Reduction, sulfanilamide)	EPA353.2 (modified) APHA 4500-N-C (modified) ASTM WK31786	WRS 34A.6 (February 2021)

[e] FIA=Flow injection analysis.

[f] APHA= American Public Health Association, Standard Methods for the Examination of Water and Wastewater, . EPA=Methods for Chemical Analysis of Water and Wastes.

[g] WRS= Willamette Research Station. References are to laboratory SOP. Available upon request. (contact the Project Lead). The WRS laboratory is now known as the Pacific Ecological Surveys Division but SOP names have not yet been changed.)

Analyte	Summary of Method[e]	References[f]	WRS SOP[g]
		Lachat 10-107-04-1-C (modified)	
Dissolved phosphorus	Persulfate Digestion; Automated Colorimetric Analysis (molybdate, ascorbic acid)	EPA 365.1 (modified), APHA 4500-P-E USGS I-4650-03 Lachat 115-01-1-B (modified)	WRS 34A.6 (February 2021)
Major anions, dissolved chloride, nitrate, nitrite, sulfate	Ion Chromatography	EPA 300.1; SW-846 9056A; APHA 4110B	WRS 40A.7 (February 2021)
Major cations, dissolved calcium, sodium, potassium, magnesium,	Inductively-coupled Plasma Atomic Emission Spectroscopy (ICP-AES)	EPA 200.7; SW6010D	WRS 52A.14 (January 2017)
Chlorophyll *a* (Chl-a)	Extraction 90% acetone analysis by fluorometry	EPA 445.0 , EPA 446.0	WRS 71A.5 (February 2021)

10.5 Pertinent QA/QC Procedures

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

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

The analytical laboratory demonstrated in previous studies that it can meet the required LRL (USEPA 2004). QA/QC procedures outlined in this manual and the NLA 2022 QAPP will be followed to ensure these LRLs are met for the NLA 2022.

10.5.1 Laboratory Performance Requirements

Table 10.5 summarizes the pertinent laboratory performance requirements for the water chemistry and chlorophyll *a* indicators.

10.5.2 Laboratory Quality Control Samples

Table 10.6 summarizes the pertinent laboratory quality control samples for the water chemistry and chlorophyll *a* indicators.

Table 10.5 Laboratory method performance requirements for water chemistry and chlorophyll *a* sample analysis.

Analyte	Units	Potential Range of Samples[h]	Lower Reporting Limit[i]	Transition Value[j]	Precision Objective[k]	Bias Objective[l]
Conductivity	µS/cm at 25˚C	2-34,000	2.0	20	± 1 or ±10%	± 1 or 5%
pH (laboratory)	Std Units	3.5 to 10	N/A	5.75, 8.25	≤5.75 or ≥8.25: ±0.07 5.75- 8.25: ±0.15	≤5.75 or ≥8.25: ±0.05 5.75- 8.25: ±0.10
Turbidity	NTU	0 to 1,000	2.0	10	± 1 or ±10%	± 1 or ±10%
Dissolved Organic Carbon (DOC)	mg /L	0.2 to 160	0.20	≤ 1 > 1	± 0.10 or ±10%	± 0.10 or ±10%
Ammonia as N(NH_3-N)	mg /L	0 to 3	0.02	0.02	± 0.002 or ±10%	± 0.002 or ±10%
Nitrate-Nitrate (NO_3-NO_2)	mg /L	0 to 360 (as nitrate)	0.02	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Total Nitrogen (TN)	mg/L	0.01 to 36	0.02	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Total Phosphorus (TP)	µg /L	0 to 11,000	4	20	± 2 or ±10%	± 2 or ±10%
Dissolved Nitrogen	mg/L	NA	0.02	0.10	± 0.01 or ±10%	± 0.01 or ±10%

[h] Estimated from samples analyzed at the PESD-Corvallis laboratory between for NLA 2017.

[i] The lower reporting limit is the lowest value that needs to be quantified (as opposed to just detected) and is equal to or above the value of the lowest nonzero calibration standard. It is set to 2 times the long-term detection limit, following USGS Open File Report 99-193 *New Reporting Procedures Based on Long-Term Method Detection Levels and Some Considerations for Interpretations of Water-Quality Data Provided by the U.S. Geological Survey National Water Quality Laboratory* (USGS 1999).

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

[k] For 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.

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

Analyte	Units	Potential Range of Samples[h]	Lower Reporting Limit[i]	Transition Value[j]	Precision Objective[k]	Bias Objective[l]
Dissolved Phosphorus	µg /L	NA	4	20	± 2 or ±10%	± 2 or ±10%
Sulfate (SO_4)	mg /L	0 to 7,000	0.50	2.5	± 0.25 or ±10%	± 0.25 or ±10%
Chloride (Cl)	mg /L	0 to 12,000	0.20	1	± 0.10 or ±10%	± 0.10 or ±10%
Nitrate (NO_3)	mg /L	0 to 30	0.02	0.1	± 0.01 or ±10%	± 0.01 ±10%
Calcium (Ca)	mg /L	0.04 to 550	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Magnesium (Mg)	mg /L	0.0.3 to 1000	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Sodium (Na)	mg /L	0.08 to 8,000	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Potassium (K)	mg /L	0.01 to 440	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
Silica (SiO_2)	mg /L	0.02 to 70	0.10	0.5	± 0.05 or ±10%	± 0.05 or ±10%
True Color	PCU	0 to 250	5	50	±5 or ±10%	±5 or ±10%
Chlorophyll *a*	µg/L (in extract)	3.5 to 4,000	0.5	15	± 1.5 or ±10%	± 1.5 or ±10%

Table 10.6 Laboratory quality control samples: water chemistry indicator.

QC Sample Type and Description	Analytes	Description	Frequency	Acceptance Criteria	Corrective Action
Laboratory/ Reagent Blank	All		Once per analytical batch prior to sample analysis	Control limits ≤ LRL	Prepare and analyze new blank. Determine and correct problem (e.g., reagent contamination, instrument calibration, or contamination introduced during filtration) before proceeding with any sample analyses. Reestablish statistical control by analyzing three blank samples.
Filtration Blank	All dissolved analytes	ASTM Type II reagent water processed through filtration unit	Prepare once per week and archive Prepare filter blank for each box of 100 filters, and examine the results before any other filters are used from that box.	No analytes >LRL	Measure archived samples if review of other laboratory blank information suggest source of contamination is sample processing.
Method Detection Limit Check Standard (MDL-C)	All analyses except true color and turbidity	Prepared so concentration is four to six times the LT-MDL objective	Once per day	Target LT-MDL value (which is calculated as a 99% confidence interval)	Confirm achieved LRL by repeated analysis of LT-MDL QCCS. Evaluate affected samples for possible re-analysis.
Initial and Continuing Calibration Verification			Analyze ICV after calibration. Analyze CCV after every 10 samples and at the end of analytical batch.	±10% or method criteria	Perform corrective action and repeat all associated samples since last successful CCV. Alternatively, recalibrate and re-analyze all samples since last successful CCV.
Analytical Duplicate Sample	All analyses		One per 10 samples	Within precision objective	If results are below LRL: Prepare and analyze duplicate from different sample (volume permitting). Review precision of batch. Check preparation of duplicate sample.
Standard Reference Material (SRM)	When available for a particular analyte		One analysis in a minimum of five separate batches	Manufacturers certified range	Analyze standard in next batch to confirm suspected imprecision or bias. Evaluate calibration standards for contamination and

QC Sample Type and Description	Analytes	Description	Frequency	Acceptance Criteria	Corrective Action
					preparation error. Correct before any further analyses of routine samples are conducted. Reestablish control by three successive reference standard measurements that are acceptable. Qualify all sample batches analyzed since the last acceptable reference standard measurement for possible reanalysis.
Matrix Spike Samples	Only prepared when samples with potential for matrix interferences are encountered		One per batch	Control limits for recovery cannot exceed 100±20%	Select two additional samples and prepare fortified subsamples. Reanalyze all suspected samples in batch by the method of standard additions. Prepare three subsamples (unfortified, fortified with solution approximately equal to the endogenous concentration, and fortified with solution approximately twice the endogenous concentration).

10.5.3 Data Reporting, Review, and Management

Checks made of the data in the process of review and verification are summarized in **Table 10.7**. Data reporting units and significant figures are given in **Table 10.8.** The NLA 2022 Project QA Officer is ultimately responsible for ensuring the validity of the data, although performance of the specific checks may be delegated to other staff members.

Table 10.7 Data validation quality control for water chemistry indicator.

Activity or Procedure	Requirements and Corrective Action
Range checks, summary statistics, and/or exploratory data analysis (e.g., box and whisker plots)	Correct reporting errors or qualify as suspect or invalid.
Review holding times	Qualify value for additional review
Ion balance: Calculate percent ion balance difference (%IBD) using data from cations, anions, pH, and ANC. See **Equation 10.1.**	• If total ionic strength ≤100 µeq/L – %IBD ≤ ±25%. • If total ionic strength > 100 µeq/L – %IBD ≤±10%. • Determine which analytes, if any, are the largest contributors to the ion imbalance. Review suspect analytes for analytical error and reanalyze. – Flag = unacceptable %IBD • If analytical error is not indicated, qualify sample to attribute imbalance to unmeasured ions. Reanalysis is not required. – Flag = %IBD outside acceptance criteria due to unmeasured ions
Conductivity check: Compare measured conductivity of each sample to a calculated conductivity based on the equivalent conductance of major ions in solution (Hillman et al., 1987)	• If measured conductivity ≤ 25 µS/cm, – ([measured - calculated] ÷ measured) ≤ ±25%. • If measured conductivity > 25 µS/cm, – ([measured - calculated] ÷ measured) ≤ ±15%. • Determine which analytes, if any, are the largest contributors to the difference between calculated and measured conductivity. • Review suspect analytes for analytical error and reanalyze. • If analytical error is not indicated, qualify sample to attribute conductivity difference to unmeasured ions. Reanalysis is not required.
Review data from QA samples (laboratory PE samples, and inter-laboratory comparison samples)	Indicator QC Coordinator determines impact and possible limitations on overall usability of data based on the specific issue.

Table 10.8 Data reporting criteria: water chemistry indicator.

Measurement	Units	No. Significant Figures	Minimum No. Decimal Places
DO	mg/L	2	1
Temperature	°C	2	1
pH	pH units	3	2
Carbon, dissolved organic	mg/L	3	1
ANC	µeq/L	3	1
Conductivity	µS/cm at 25 °C	3	1
Calcium, magnesium, sodium, potassium, chloride, nitrate, and sulfate	µeq/L	3	1
Silica	mg/L	3	2
Total phosphorus	µg/L	3	0
Total nitrogen	mg/L	3	2
Total dissolved phosphorus	µg/L	3	0
Total dissolved nitrogen	mg/L	3	2
Nitrate-Nitrite	mg/L	3	2
Ammonia-N	mg/L	3	2
Turbidity	NTU	3	0
True color	PCU	2	0
Chlorophyll *a*	ug/l	3	2

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

Equation 10.1 Percent ion difference (%IBD)

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

where ANC is the acid neutralization capacity; cations are the concentrations of calcium, magnesium, sodium, potassium, and ammonium (converted from mg/L to µeq/L); anions are the concentrations of chloride, nitrate, and sulfate (converted from mg/L to µeq/L), and H^{+} is the hydrogen ion concentration calculated from the antilog of the sample pH. Factors to convert major ions from mg/L to µeq/L are presented in **Table 10.9**.

For the conductivity check, equivalent conductivities for major ions are presented in **Table 10.10**.

Table 10.9 Constants for converting major ion concentration from mg/L to µeq/L

Analyte	Conversion from mg/L to µeq/L[m]
Calcium	49.9
Magnesium	82.3
Potassium	25.6
Sodium	43.5
Ammonia-N	55.4
Chloride	28.2
Nitrate	16.1
Sulfate	20.8

Table 10.10 Factors to calculate equivalent conductivities of major ions.[n]

Ion	Equivalent Conductance per mg/L (µS/cm at 25 °C)	Ion	Equivalent Conductance per mg/L (µS/cm at 25 °C)
Calcium	2.60	Nitrate	1.15
Magnesium	3.82	Sulfate	1.54
Potassium	1.84	Hydrogen	3.5×10^{5} [o]
Sodium	2.13	Hydroxide	1.92×10^{5}
Ammonia-N	4.13	Bicarbonate	0.715
Chloride	2.14	Carbonate	2.82

10.5.4 Data Entry

Required data elements that laboratories must provide to the USEPA, are identified in the USEPA's data template, available separately from the USEPA. If the laboratory applies its own QC codes, the data transmittal must define the codes.

[m] Measured values are multiplied by the conversion factor.

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

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

11.0 ZOOPLANKTON METHODS

This method is used to identify and enumerate species of lake zooplankton collected with vertical plankton net tows using the NLA 2022 method. Macrozooplankton are counted from a sample using a course (150 µm) mesh nets. Microzooplankton, especially rotifers, nauplii, copepodites <0.6 mm long, and cladocerans <0.2 mm long, are counted from a sample collected using a fine mesh net (50 µm). Both the course and fine mesh nets include a reducing collar with diameter of 20 cm (30 cm to 20 cm cowling).

Zooplankton samples will be preserved in the field with EtOH and shipped from field crews to a contract batching laboratory. The contract batching laboratory will send the batched samples to the analysis laboratory. Preserved samples can be held for several months, but zooplankton analysis laboratories will need to process samples in accordance with the time frame outlined in contractual agreements. Contractual agreements for delivery of data do not supersede indicator holding times.

11.1 Responsibility and Personnel Qualifications

This procedure may be used by any person who has received training in processing and/or identification of zooplankton samples. It is also important that the taxonomist maintains contact with other taxonomists through professional societies and other interactions, and keep abreast of the pertinent literature, because taxonomic groupings and nomenclatural basis for taxonomy and nomenclature are updated frequently. A second taxonomist will re-identify a randomly-selected 10% of the samples for QC, as noted below, to quantify taxonomic precision, or consistency, as **percent taxonomic disagreement (PTD)**, help target corrective actions, and ultimately help minimize problems during data analysis. Samples are sent from the field to the laboratory on a regular basis during the project to avoid delays in processing and specimen identification.

11.2 Precautions

Wear appropriate clothing for safety precautions, such as nitrile gloves, rubber apron, long pants, etc. Follow all laboratory safety and waste disposal guidelines regarding the disposal of formalin (37% formaldehyde) solutions.

11.3 Equipment/Materials

- Dissection microscope (magnifications: 10X-50X)
- Compound microscope (magnifications: 40X-400X with phase-contrast capability)
- Hensen-Stempel pipettes (1, 2, and 5 mL)
- Graduated cylinders (100-, 250-, and 500mL)
- Folsom plankton Splitter
- Ward counting wheel or other suitable counting chamber
- Utermöhl counting chamber or Sedgwick-Rafter counting cell (1 mL vol) with cover slips
- Ring nets with 50, 500 and 1000 µm Nitex mesh
- Mechanical or electronic tally counters
- Microscope slides, 1 x 3 inch
- Cover slips
- Tubes for concentrating plankton samples (see below)
- Small sieves with 45 and 140-µm mesh
- 50-µm Nitex mesh Heavy duty rubber bulb Microprobe

- 150-µm Nitex mesh Heavy duty rubber bulb Microprobe
- Micro-forceps
- 100- to 500-mL glass jars with split fractions written on labels
- Zooplankton Sample Log-In Form
- Zooplankton Laboratory Sheet
- Labels

Construct the first plankton concentrating tube by covering one end of a wide glass tube (such as a chromatography tube) with 50-µm mesh. Secure the mesh with O-rings and attach a heavy-duty bulb to the other end to provide suction. Construct the second plankton concentrating tube by covering one end of a wide glass tube (such as a chromatography tube) with 150-µm mesh. Secure the mesh with O-rings and attach a heavy-duty bulb to the other end to provide suction.

The following reagents are needed:

- Formalin (37% formaldehyde solution)
- 95% EtOH
- 5% Sodium hypochlorite solution (unscented bleach)
- Rose Bengal stain dissolved in EtOH
- Dilute solution of laboratory detergent

11.4 Sample Receipt

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

1. Report receipt of samples to the NARS IM Team by completing and emailing the sample tracking spreadsheet with the sample login and sample condition information. (See **Section 1.2** of the manual for contact information).
2. Inspect each sample THE SAME DAY THEY ARE RECEIVED:
   a. Verify that the sample IDs in the shipment match those recorded on the sample tracking form.
   b. Record the information in **Table 11.1** for the NARS IM Team, including the Condition Code for each sample:
      i. *OK*: Sample is in good condition
      ii. *C*: Sample container was cracked
      iii. *L*: Sample container is leaking
      iv. *ML*: Sample label is missing
   c. If any sample is damaged or missing, contact the USEPA HQ Laboratory Review Coordinator to discuss whether the sample can be analyzed.
3. Store samples until sample preparation begins.
4. Maintain the sample tracking forms with the samples.

Table 11.1 Zooplankton login: required data elements.

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

11.5 Procedure

11.5.1 Zooplankton Stratified Splitting

1. Record all zooplankton samples received at the laboratory in a logbook or sample log form (See **APPENDIX B: SAMPLE LABORATORY FORMS: Zooplankton Sample Log In Form**). Add approximately 1 to 3 mL of Rose Bengal stain solution to each sample bottle to aid in finding the smaller organisms. Process samples one at a time. Shake jar to mix water sample. Under the hood, rinse the first sample jar taken with the 50-µm mesh net through a 45-µm mesh sieve with deionized (DI) water to remove the EtOH; the second sample bottle, taken from the 150-µm mesh net, is rinsed through a 145-µm mesh sieve with DI water to remove the EtOH. The two mesh size samples are treated as individual samples for processing and identification and are to be recorded on the laboratory bench sheet with the sample number and corresponding mesh size.
2. Be sure to rinse the corresponding sample bottles thoroughly with reverse osmosis (RO)/DI/distilled water into the 45-µm mesh and 145-µm mesh sieve to remove any residual organisms adhering to walls of the bottle. Rinse all containers from which zooplankton are transferred thoroughly, including the Folsom splitter, glass jars, and counting chambers. Wash the sample into a glass jar. Add a small amount of dilute laboratory soap to each sample, at this time, to prevent organisms from sticking to the sides of the containers and from floating at the surface of the sample.
3. Stir the sample gently to break up algal clumps and then pour the entire sample into the Folsom plankton splitter. Stir the sample again to distribute animals uniformly and split the sample by immediately rotating the splitter before the organisms can settle. Rinse the inside of the splitter well to remove organisms that may stick to the sides. Rinse one sub-sample from the splitter receiving trays and

save it in a labeled jar indicating the fraction of total original volume of sample bottle (1/2).

4. Place the second sub-sample from the split in the Folsom plankton splitter and divide again. Save one sub-sample in a labeled jar indicating the fraction of the total original volume it contains (1/4).
5. Repeat Steps 3 and 4 as many times as necessary until the last 2 sub-samples contain at least total of 400, and a maximum of 480 (400+20%), macrozooplankton each (not including rotifers and nauplii). These 2 sub-samples represent equal fractions of the original sample. Save one sub-sample in a jar labeled "A", and save the other sub-sample in a jar labeled "B". This process may vary depending on the density of organisms in the sample. If the minimum count is reached in the "A" subsample, then there is no need to identify individuals from subsample "B". Write the final split factor used, on the identification and enumeration bench sheets (see **APPENDIX B: SAMPLE LABORATORY FORMS: Zooplankton Enumeration Data Sheet in**).

11.5.2 Taxonomy Procedures

11.5.2.1 Taxonomic Level of Effort

The USEPA will supply a list of taxa that have been collected from previous iterations of the NLA (provided during laboratory initiation call). This list should be used as a guide for the appropriate taxa names to be used while processing samples. However, this list will clearly not include all potential taxa that may be encountered, but should assist in ensuring consistency between surveys. When possible the following resources should be used to identify zooplankton to species: Edmondson (1959), Pennak (1978), Smith and Fernando (1978), Stemberger (1979), the online Free-living and Parasitic Copepods (Including Branchiurans) of the Laurentian Great Lakes: Keys and Details on Individual Species and the online Image-Based Key to the Zooplankton of the Northeast, USA, produced by the University of New Hampshire Center for Freshwater Biology (cfb.unh.edu). Other resources and keys can be utilized, but should be provided to USEPA before processing of samples begin.

11.5.2.2 Macrozooplankton Identification and Enumeration (Excluding Rotifers and Nauplii)

Macrozooplankton are counted and identified from samples collected with the coarse mesh (150 µm) plankton net.

1. Species-level resolution will be the taxonomic requirement for macrozooplankton.
2. The taxonomist must examine and enumerate as many sub-samples needed to reach the target count of 400 to 480 organisms and record the information on the appropriate form (see **APPENDIX B: SAMPLE LABORATORY FORMS: Zooplankton Enumeration Data Sheet**).
3. Concentrate the sub-sample by using the small sieve or the condensing tube and place in a circular (or other suitable) counting chamber.
4. Identify all macrozooplankton under a dissecting microscope and enumerate using a mechanical or electronic tally counter.
5. Count the first two sub-samples which likely contain 400 organisms (**Section 11.5.1**, step 5) first, and count additional subsamples to reach enumeration target, if need. Examine and enumerate all macrozooplankton. If the minimum of 400 organisms in the first of the two original subsamples, then stop. There will be no need to examine the second of the first two subsamples. During identification and enumeration, make measurements on selected individuals. For dominate taxa, measure a minimum of 20 individuals. For subdominant taxa (taxa encountered less than 40 times during enumeration), measure 10 individuals. For rare taxa (taxa encountered less than 20 times during enumeration), measure 5 individuals. If rare taxa are in a position that makes it difficult to measure (e.g. odd angle), then remove these individuals after identification and enumeration and measure them separately. Additionally, while enumerating and identifying samples, especially note invasive species such as *Bythotrephes* and *Cercopagis.*

11.5.2.2.1 General Analysis and Guidelines

1. Mount organisms requiring higher magnification for identification on slides and examine at 100 - 1000x magnification under a compound microscope.
2. While counting macrozooplankton, make sure that all organisms are settled to the bottom. It is possible to sink floating macrozooplankton by gently pressing them down using the microprobe or by adding a drop of dilute laboratory detergent.
3. If a sample cannot be completely counted and archived within 2 days, keep the sample in the refrigerator and add a few drops of formalin to the jars to prevent organisms from clumping. Sample analysis should not extend beyond four days.
4. Place voucher specimens in a labeled vial and preserve with 95% EtOH. The label in the vial should include genus/species name, date preserved, analyst initials, station number, and sample number. A second taxonomist should confirm the voucher specimens.

11.5.2.2.2 Large Taxa Scan

Observe non-counted sample portion for the following: *Leptodora, Chaoborus, Craspedacusta sowerbii, Mysidae, Ostracoda*, and *Hydracarina*. Spend minimal effort here, < ~1-2 minutes. If detected, enter "yes" in the LARGE_RARE column on spreadsheet, and put the number counted in the L/R_AUND column.

11.5.2.3 Microzooplankton *(*Rotifers, Nauplii, and Crustaceans*)*

Microzooplankton are counted and identified from samples collected with the fine mesh (50 µm) plankton net.

1. Species-level resolution is the taxonomic requirement for rotifers, copepods <0.6 mm long, and *cladocerans* <0.2 mm long. Nauplii will be identified to the lowest possible taxonomic unit.
2. Selection of the split level from which a sub-sample for rotifer enumeration is based on estimates made during macrozooplankton enumeration (rotifers and small crustaceans are visible in the dissecting microscope).
3. Take two separate 1-mL sub-samples from the appropriate split. Count and identify microzooplankton from these two sub-samples (see **Section 11.5.2.4**). In cases where abundances are particularly low, use more than one 1-mL sub-sample for each count (see step 6).
4. Mix the sample thoroughly, and withdraw a 1-mL sub-sample with a Hensen-Stempel pipette (or other pre-calibrated large-bore pipette).
5. The 1-mL sub-sample should contain 400 rotifers, crustacean, and nauplii.
6. If the sub-sample contains less than 400 organisms, take a different sub-sample from a jar with a larger fraction of the original sample volume. If the sub-sample contains more than 480 organisms, use another sub-sample from a jar with a smaller fraction.
7. It is also permissible to use a second 1-mL aliquot if the original aliquot has less than 400 organisms. Count this second aliquot in the same manner as the first and combine the results to make a final count.
8. In cases of extremely low microzooplankton densities, concentrate the sample prior to taking sub-samples with the pipette. The maximum number of 1-mL aliquots counted at the lowest possible split level is 3 per count (i.e., a total of 6 mL), even if the sum does not reach 400 organisms.

11.5.2.3.1 Preparation and Microzooplankton Enumeration

1. Place the sub-sample in an Utermöhl counting chamber or Sedgwick-Rafter cell and cover with a glass cover slip.
2. Identify and enumerate all rotifers, microzooplankton, nauplii, and *Dreissena* veligers and post-veligers under a compound microscope at 100x magnification. Record results on the appropriate form. Make measurements on selected individuals at this time, and follow dominate, subdominant, and rare (**Section 11.5.2.2**). See measurement parameters for macro- and microzooplankton in **Sections 11.5.2.4.1** and **11.5.2.4.2** respectively.

3. After the counts are completed, measure the volume of the split used, including the volume of the aliquots, and record this information.

11.5.2.4 Measurement of Macrozooplankton and Microzooplankton

11.5.2.4.1 Crustaceans

To determine size distribution, measure zooplankton by use of a calibrated eyepiece micrometer during the identification and enumeration process.

Measure the first 20 encounters per species per sample as follows:

Cladocera: Length from the top of the head to the base of the caudal spine or to the end of the carapace.

Copepoda: Length from tip of the head to the insertion of spines into the caudal ramus.

Mysis: Carapace length, or the length from the tip of the head to the cleft in the telson.

Bythotrephes: Body length, excluding the caudal process.

Cercopagis: Body length, from the top of the eye to the end of the caudal claws.

NOTE: *If the organisms are curved or bent, make several straight line measurements and sum to obtain total length.*

11.5.2.4.2 Rotifers

Measure at least 20 encounters per species as follows:

1. Loricate forms: body length from corona to the opposite end at the base of spine (if present).
2. Non-loricate forms: body length from corona to the opposite end, excluding spines, paddles, "toes" or other extensions.

11.6 Calculating and Reporting

Report zooplankton densities as number of organisms per cubic meter, which is calculated in the following equations.

11.6.1 Volume of water filtered

Equation 11.1 Volume of water filtered.

$$V = L \times A$$

where:

V = Volume of water filtered (m^3)
L = Length of vertical tow*
A = Area of the mouth of the net (m^2) = 0.0314 m^2 for 0.2-m diameter net

*Field crews are to collect a cumulative tow length of 5 m for each net. Therefore, the tow length should be 5 m unless otherwise noted by the field crew in the comments.

11.6.2 Macrozooplankton Densities

Equation 11.2 Microcrustacean densities.

$$D = \frac{N \times S}{V}$$

where:

D = Density of organisms in number per cubic meter
N = Number of organisms
S = Spilt factor
V = Volume of water filtered (from above calculation)

11.6.3 Microzooplankton Densities

Equation 11.3 Microzooplankton densities.

$$D = \frac{\left(N \times V_s \times S\right)}{N_a \times V}$$

where:

D = Density of organisms in number per cubic meter
N = Number of organisms
N_a = Number of 1mL aliquots examined
V_s = Volume of sub-samples from which aliquots were taken
S = Spilt factor
V = Volume of water filtered (from above calculation)

11.6.4 Zooplankton Biomass Estimates

Biomass estimates will be based on established length/width relationships (Dumont et al. 1975; McCauley, 1984; Lawrence et al. 1987). The lengths or the lengths and widths of each species encountered will be measured and will be equal to 20 for common species and lesser for more rare taxa. For cladocerans, the length will be measured from the tip of the head to the end of the body (shell spines excluded). For copepods, the length will be determined from the tip of the head to the insertion of the caudal ramus. The length of rotifers will be measured from the tip of the head to the end of the body (spines, toes, etc. excluded). In accordance with McCauley (1984), biomass will be computed for the appropriate number of individuals for each sample location and the arithmetic mean biomass will be multiplied times the species abundance to produce a species biomass for each sample. More detailed discussion of the methodology is given in Havens et al (2011), Beaver et al. (2010), and Havens & Beaver (2010).

11.6.5 Results of Laboratory Processing, Sample Archiving

Prepare a completed data sheet with list of taxa and number of individuals of each taxon for each sample. In addition, you should organize and archive the full complement of specimens (in containers of preservative and/or on permanent slide mounts), the "counted" sample (in jars, vials, or slide mounts), the concentrated split sample, and the unused sample split/fraction. All sample components should be clearly-labeled to associate multiple vials and slides as a single sample. Labels should be as Sample ID "A," jar/vial 1 of x, and Sample ID "A," slide 1 of x; and Sample ID "A," unused sample fraction (1/2 original volume).

Required data elements that laboratories must provide to the USEPA, are identified in the USEPA's data template, available separately from the USEPA. If the laboratory applies its own QC codes, the data transmittal must define the codes.

11.7 Pertinent QA/QC Procedures

Table 11.2 provides a summary of quality assurance/quality control procedures for the zooplankton indicator.

11.7.1 Taxonomic QC

11.7.1.1 Internal Taxonomic QC

As directed by the Indicator QC Coordinator, an in-house QC Analyst will randomly select 5 of the samples counted and identified by individual taxonomists to ensure that each meets the acceptable criteria for percent identification efficiency which is 90%.

If the individual fails to maintain a ≥ 90% identification as determined by QC checks, previous samples will be re-counted and identified. EPA may also calculate or work with the lab to calculate the proportional analysis found in the External Taxonomic QC section below using internal QC information.

11.7.1.2 External Taxonomic QC

1. EPA may implement an external taxonomic QC review process for zooplankton. If EPA implements an external QC process, upon receipt of the data after initial identification, approximately 10% of the samples (for each laboratory) are randomly-selected for evaluation of taxonomic precision by the Indicator QC Coordinator. Following primary identification and enumeration, the remaining sample from each of the randomly selected samples are sent by the original laboratory to a QC taxonomist for complete sample processing and re-enumeration/identification. The laboratory will send the remaining field collected sample with a sample tracking form. Differences between the two samples are an indication of taxonomic precision.
2. Because the laboratory and QC taxonomist will be looking at different subsamples from the original field collected sample, the QC process will utilize the relative abundance of each taxon identified by both the laboratory and QC for each sample to determine the precision of taxonomic identifications. To determine the precision of taxonomic identifications the Indicator QC Coordinator will utilize a Bray-Curtis Dissimilarity index to compare taxonomic results from two independent taxonomists, using the formula:

   Equation 11.4 Bray-Curtis Dissimilarity *(BC_d)*.

$$BC_d = \frac{\sum|x_i - x_j|}{\sum(x_i + x_j)}$$

   where x_i *and* x_j are the specific counts from two different taxonomists for each taxon identified in each subsample.
3. A BC_d of 0.25 or less is recommended for taxonomic difference (overall mean ≤ 0.25is acceptable). Individual samples exceeding 0.25 are examined for taxonomic areas of substantial disagreement, and the reasons for disagreement investigated. A reconciliation call between the primary and secondary taxonomist will facilitate this discussion. Results greater than this value are investigated and logged for indication of error patterns or trends.
4. Corrective actions include determining problem areas (taxa) and consistent disagreements and addressing problems through taxonomist interactions. These actions help to rectify disagreements resulting from identification to a specific taxonomic level.

11.7.1.3 Taxonomic QC Review & Reconciliation

The Indicator QC Coordinator prepares a report or technical memorandum to quantify aspects of taxonomic precision, assess data acceptability, highlight taxonomic problem areas, and provide recommendations for improving precision. This report is submitted to the HQ Project Management Team, with copies sent to the

primary and QC taxonomists. Another copy is maintained in the project file. Significant differences may result in the re-identification of samples by the primary taxonomist and a second QC check by the secondary taxonomist. Each laboratory prepares reference/voucher samples. These samples will be identified and digitally referenced (a photograph with taxonomic information superimposed on the photograph and in the file name) and will be included in an electronic file folder on the NARS SharePoint Site.

All samples are stored at the laboratory until the Project Lead notifies the laboratory regarding disposition.

Table 11.2 Laboratory quality control: zooplankton indicator.

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
IDENTIFICATION			
Independent identification by outside taxonomist	All uncertain taxa	Uncertain identifications to be confirmed by expert in particular taxa	Record both tentative and independent IDs
Use standard taxonomic references	For all identifications	All keys and references used must be on bibliography prepared by another laboratory	If other references desired, obtain permission to use from Project Facilitator
Prepare reference collection	Each new taxon per laboratory	Complete reference collection to be maintained by each individual laboratory	Laboratory Manager periodically reviews data and reference collection to ensure reference collection is complete and identifications are accurate
External QC	10% of all samples completed per laboratory	Efficiency (BC_d) ≤ 0.25	If BC_d > 0.25, implement recommended corrective actions.
DATA VALIDATION			
Taxonomic "reasonable-ness" checks	All data sheets	Genera known to occur in given lake or geographic area	Second or third identification by expert in that taxon

12.0 RESEARCH INDICATOR: Environmental DNA (eDNA)

Information on this indicator is contained in other research documents.

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APPENDIX A: LABORATORY REMOTE EVALUATION AND VERIFICATION FORMS

Document Request Form – **Chemistry** Laboratories

The USEPA and its state and tribal partners will conduct a survey of the nation's lakes, ponds, and reservoirs. This National Lakes Assessment is designed to provide statistically valid regional and national estimates of the condition of lakes. Consistent sampling and analytical procedures ensure that the results can be compared across the country.

As part of the National Lakes Assessment (NLA) 2022, the Quality Assurance Team has been requested to conduct a technical assessment to verify quality control practices in your laboratory and its ability to perform **chemistry** analyses under this project. Our review will be assessing your laboratory's ability to receive, store, prepare, analyze, and report sample data generated under the USEPA's NLA.

The first step of this assessment process will involve the review of your laboratory's certification and/or documentation. Subsequent actions may include (if needed): analysis of Proficiency Testing samples and/or a site visit. All laboratories will need to complete the following form:

- ☐ A *signature* on the attached Laboratory Signature Form indicates that your laboratory will follow the quality assurance protocols required for chemistry laboratories conducting analyses for the NLA 2022.

In order for us to determine your ability to participate as a laboratory in the NLA, we are requesting that you submit the following documents (if available) for review:

- ☐ Documentation of a successful *quality assurance audit* from a prior survey that occurred within the last 5 years (if you need assistance with this please contact the individual indicated below)
- ☐ A copy of your Laboratory's *accreditations and certifications* if applicable (i.e. NELAC, ISO, state certifications, etc…)

If your laboratory can provide either documentation of a prior audit or accreditation, no other documentation is needed. If ***neither*** of the above is complete, please provide the following information.

- ☐ A copy of your Laboratory's *Quality Manual*
- ☐ *Standard Operating Procedures* (SOPs) for your laboratory for each analysis to be performed (if not covered in NLA 2022 Laboratory Manual)
- ☐ Other documentation supporting your laboratory's ability to meet the required level of data quality (if available)

This documentation may be submitted electronically via e-mail to forde.kendra@epa.gov. Questions concerning this request can be submitted to guenzel.lareina@epa.gov (202-566-0455) or forde.kendra@epa.gov (202-564-0417).

Laboratory Signature Form – Chemistry Laboratories

I _____certify that the _____laboratory, located in _____, will abide by the following standards in performing **chemistry** data analysis and reporting for the National Lakes Assessment (NLA).

1.) Utilize procedures identified in the NLA 2022 Laboratory Operations Manual (or equivalent). If using equivalent procedures, please provide procedures manual.
2.) Read and abide by the NLA 2022 Quality Assurance Project Plan (QAPP) and related Standard Operating Procedures (SOPs).
3.) Have an organized IT system in place for recording sample tracking and analysis data.
4.) Provide data using the template provided in the Laboratory Operations Manual.
5.) Provide data results in a timely manner. This will vary with the type of analysis and the number of samples to be processed. Sample data must be received no later than March 1, 2023 or as otherwise negotiated with the USEPA.
6.) Participate in a laboratory technical assessment or audit if requested by an USEPA NLA staff (this may be a conference call or on-site audit).

Signature _____ Date _____

Document Request Form - **Biology** Laboratories

The USEPA and its state and tribal partners will conduct a survey of the nation's lakes, ponds, and reservoirs. This National Lakes Assessment is designed to provide statistically valid regional and national estimates of the condition of lakes. Consistent sampling and analytical procedures ensure that the results can be compared across the country.

As part of the National Lakes Assessment (NLA) 2022, the Quality Assurance Team has been requested to conduct a technical assessment to verify quality control practices in your laboratory and its ability to perform **biology** analyses under this project. Our review will be assessing your laboratory's ability to receive, store, prepare, analyze, and report sample data generated under the USEPA's NLA 2022.

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

- ☐ A *signature* on the attached Laboratory Signature Form indicates that your laboratory will follow the quality assurance protocols required for chemistry laboratories conducting analyses for the NLA 2022.

In order for us to determine your ability to participate as a laboratory in the NLA, we are requesting that you submit the following documents (if available) for review:

- ☐ Documentation of a successful *quality assurance audit* from a prior survey that occurred within the last 5 years (if you need assistance with this please contact the individual listed below)
- ☐ A copy of your Laboratory's *accreditations and certifications* if applicable (i.e. NELAC, ISO, state certifications, etc…)

If your laboratory can provide either documentation of a prior audit or accreditation, no other documentation is needed. If ***neither*** of the above is complete, please provide the following information:

- ☐ Documentation of NABS *certification* for the *taxonomists* performing analyses (if available)
- ☐ A copy of your Laboratory's *Quality Manual*
- ☐ *Standard Operating Procedures* (SOPs) for your laboratory for each analysis to be performed (if not covered in NLA 2022 Laboratory Manual)
- ☐ Other documentation supporting your laboratory's ability to meet the required level of data quality (if available)

This documentation may be submitted electronically via e-mail to forde.kendra@epa.gov. Questions concerning this request can be submitted to guenzel.lareina@epa.gov (202-566-0455) or forde.kendra@epa.gov (202-564-0417)

Laboratory Signature Form – Biology Laboratories

I _____ certify that the _____ laboratory, located in _____, will abide by the following standards in performing **biology** data analysis and reporting for the National Lakes Assessment (NLA).

1.) Utilize procedures identified in the NLA 2022 Laboratory Operations Manual (or equivalent). If using equivalent procedures, please provide procedures manual.
2.) Read and abide by the NLA 2022 Quality Assurance Project Plan (QAPP) and related Standard Operating Procedures (SOPs).
3.) Have an organized IT system in place for recording sample tracking and analysis data.
4.) Use taxonomic standards outlined in the NLA 2022 Laboratory Manual.
5.) Participate in taxonomic reconciliation exercises during the field and data analysis season, which include conference calls and other laboratory reviews.
6.) Provide data using the template provided in the Laboratory Operations Manual.
7.) Provide data results in a timely manner. This will vary with the type of analysis and the number of samples to be processed. Sample data must be received no later than March 1, 2023 or as otherwise negotiated with the USEPA.
8.) Participate in a laboratory technical assessment or audit if requested by USEPA NLA staff (this may be a conference call or on-site audit).

Signature _____ Date _____

APPENDIX B: SAMPLE LABORATORY FORMS

Benthic Macroinvertebrate Laboratory Bench Sheet

Project Name/Number _____ Sample ID_____

Waterbody Name _____ Site ID _____

Sorter (initially spread sample)_____ 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				

Phytoplankton Measurement Data Sheet

Site ID _____ Sample #_____ Lake_____

Laboratory #_____ Date Collected_____

Analyzed by_____

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Zooplankton Sample Log In Form

Date Received	Sample Type	Sample Number	Site_ID	Lake Name	Tow Depth (m)	Laboratory Tracking #	Notes

Zooplankton Enumeration Data Sheet

Sample # _____ Site_ID _____ Laboratory # _____

Date Collected _____ Depth of tow (m)_____ Analyzed by _____

Working Volume (mL) _____ Milliliters in subsample (rotifers) _____ Split _____

↓Taxa / Count →	A	B	C	D
Total Mature Copepoda				
Total Immature Copepoda				
Total Cladocera				
Total Rotifera				
Total Other Organisms				

Note: For Rotifers only A and B counts are made.

Zooplankton Measurement Data Sheet

Sample #_____ Site_ID_____ Laboratory #_____

Date Collected_____ Depth of tow (m)_____ Analyzed by_____

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

APPENDIX C: STATE SAMPLE TRACKING SPREADSHEET

Provided on the NARS SharePoint site or from the Laboratory Review Coordinator.

APPENDIX D: REPORTING TEMPLATES

Templates will be provided on the NARS SharePoint Site.