Standard Operating Procedures
Laboratory Analysis: NEON Benthic Macroinvertebrates

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Prepared by

EcoAnalysts, Inc.

1420 South Blaine Street, Suite 14
Moscow, Idaho 83843

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LIST OF EQUATIONS

This SOP addresses the laboratory operations and analyses for benthic macroinvertebrate samples. This plan describes data quality objectives, measurement and data acquisition, and information management for processing benthic macroinvertebrate samples.

QUALITY OBJECTIVES AND CRITERIA

Sorting Efficacy – Aliquot Method

At least 20% of each sample is re-sorted by a quality control technician, who did not originally sort the sample, to ensure at least 90% of the organisms have been removed. The QCs are performed by technicians who have shown to achieve 90% efficacy on a minimum of 90% of samples they process. QC technicians are trained in the QC process by the sorting lab manager. The QC technician QCs a minimum of 20% of the sorted material from a given sample to ensure at least 90% of the organisms have been removed. The estimated percent efficacy is calculated, using the following equation:

Equation 1. Sorting Efficacy

$$\boldsymbol{Sorting\ Efficacy}\ \% = \frac{\boldsymbol{Original\ count}}{(\boldsymbol{Original\ Count} + \left(\boldsymbol{QC\ count} * \left(\frac{\boldsymbol{QC\ total\ grids}}{\boldsymbol{QC'd\ grids}}\right)\right)} * \mathbf{100}$$

Where:
OriginalCount = the number of organisms picked prior to current QC
QCCount = the number of organisms found in the current Quality Control
sort QC'd grids = the number of grids sorted during the QC process
QC Total grids = the total number of grids in the QC Caton

Sorting efficacy is measured as the estimated percent of the total organisms found during the original sorting process. If the estimated percent sorting efficacy is 90% or greater, the sample passes the quality control check. If the estimate is less than 90%, the full sample pickate is re-sorted. When this happens, the sample undergoes the quality control process again until it passes the 90% efficacy requirement, but the original count is now a summation of organisms picked during sorting, QC, and resort to ensure proper efficacy calculations.

If a technician consistently sorts below the 90% efficacy requirement, they will be removed from the project until accuracy improves.

Taxonomic Precision and Accuracy

Taxonomic precision is quantified by comparing whole-sample identifications completed by a second taxonomist who did not perform the primary identification. Accuracy of taxonomy is qualitatively evaluated through specification of target hierarchical levels (e.g., family, genus, or species) and the specification of appropriate technical taxonomic literature or other references (e.g., identification keys, voucher specimens). To calculate taxonomic precision for benthic macroinvertebrate samples, 10% of the samples are randomly selected for re-identification. Comparison of the results of whole sample re-identifications provides a Percent Taxonomic Disagreement (PTD) calculated as:

Equation 2. Percent Taxonomic Disagreement (PTD)

$$\boldsymbol{PTD} = \left[\mathbf{1} - \left(\frac{\boldsymbol{a}}{\boldsymbol{N}}\right)\right] * \mathbf{100}$$

where
a = the number of taxonomic agreements
N = the total number of individuals in the larger of the two counts.

The lower the PTD, the more similar taxonomic results are and the overall taxonomic precision is better. A

Measurement Quality Objective (MQO) of ≤15% is will be followed for taxonomic differences. Individual samples exceeding 15% are examined for taxonomic areas of substantial disagreement, the reasons for disagreement investigated, corrective measures taken where needed, and data is corrected. Sample enumeration is another component of taxonomic precision. Final specimen counts for samples are dependent on the taxonomist, not the rough counts obtained during the sorting activity. Comparison of counts is quantified by calculation of percent difference in enumeration (PDE), calculated as:

Equation 3. Percent Difference in Enumeration

$$PDE = \frac{|n_1 - n_2|}{n_1 + 2n_2} * 100$$

Where:
n_1= the number of individuals counted by the original taxonomist
n_2= the number of individuals counted by the QC taxonomist

An MQO of ≤5% will be followed. Individual samples exceeding 5% are examined to determine reasons for the exceedance. Original and QC taxonomists will discuss how to resolve the difference in enumeration, and data may be edited accordingly.

MQO Evaluation

PTD and PDE will be calculated by the Lead Freshwater Taxonomists prior to the data for each sample group being validated and provided to the PM to input into the NEON data deliverables format.. For samples exceeding these MQOs, corrective actions can include defining the taxa for which re-identification may be necessary (potentially even by a third party), for which samples (even outside of the 10% lot of QC samples) it is necessary, and where there may be issues of nomenclatural or enumeration problems. Samples will be marked as QC'd and denotated in the data delivery.

Samples will be identified using the most appropriate technical literature that is accepted by the taxonomic discipline and reflects the accepted nomenclature, which includes the NEON standard taxonomic effort document. Where necessary, the Integrated Taxonomic Information System (ITIS, http://www.itis.usda.gov/) will be used to verify nomenclatural validity and spelling. New taxa will be added to the established reference collection.

SAMPLE RECEIVING

Immediately upon receipt of benthic macroinvertebrate samples, all containers are inspected for damage or leakage. Sample labels are checked against chain of custody forms and/or packing slips and any discrepancies are noted. Compromised sample condition will be reported to those on the shipment email. Completed receipt records are reported to the client within one business day of sample receipt. Chain of custody logs are reported, throughout the project, according to timelines and methods requested by the client. Samples are logged into the EcoAnalysts, Inc. custom LIMS database and assigned a unique sample tracking number.

ANALYTICAL METHODS

Equipment List

- **250µm stainless steel mesh sieves**
- **Plastic volumetric measuring container**
- **Large volume Tupperware**
- **250µm mesh bottom caton**
- **95% ethanol**
- **Flammable safety-labeled wash bottles**
- **5mL glass shell vials with plug closures**
- **20mL plastic and glass scintillation vial with poly seal screw caps**
- **4 oz PET plastic jars**
- **32 oz PET plastic jars**
- **Tally counters**

- **Glass Petri dishes**
- **Watch glasses**
- **Forceps**
- **Slides and cover slips**
- **Slide trays**
- **Warming trays**
- **CMCP 10**
- **CMC 1019**
- **Slide boxes**
- **Goose neck LED light**
- **Leica S6 or S7 dissecting microscopes**
- **Leica DEM dissecting microscopes**
- **Leica DM 1000 or Zeiss Stemi 2000 dissecting microscope**
- **Leica DM 750 compound microscope with 40-100-400x objectives**
- **Fume hoods**
- **Taxonomic references**

Sorting Benthic Macroinvertebrate Samples

A sample is checked out by a sorting technician via the LIMS. A sorting bench sheet is printed that contains the EcoAnalysts sample identification information and sorting protocols assigned to it. The sorter records the primary matrix type and approximates the volume of detritus prior to sieving. The standard descriptors for the types of sample matrix are: Inorganic, Coarse Organic, Fine Organic, Vegetation, and Filamentous Algae.

The sample is prepped for subsampling by rinsing the matrix with tap water into a 250um mesh sieve. If the sample matrix is made up of a significant percentage of inorganic material, the organic material will be elutriated from the inorganic material prior to sorting.

For elutriation, the whole sample is washed into a shallow pan of water. At this time any large pieces of organic material can be rinsed and inspected thoroughly by the original technician and a secondary technician for attached and burrowing aquatic invertebrates. If large organic matter (e.g. sticks, large leaf material) is deemed removable from the sample, it is retained separately as sample residues. The sample is agitated with water to separate any organic matter from inorganic sediments. After agitating the sample in water, the lighter organic material is poured back into the sieve. The inorganic portion of the sample remaining in the pan is repeatedly washed and decanted into the sieve until no more organic matter remains in the pan with the inorganic material.

The remaining inorganic sediments are inspected under a magnifying lamp (3X) to look for any invertebrates too heavy to have been elutriated (e.g. mollusks, snails, stone-cased Trichoptera, etc.). If there are significant numbers of heavy invertebrates in the inorganic material – too many to easily remove under the magnifying lamp – the inorganic and organic matrix is recombined into the sieve and entire sample matrix will be prepared for subsample. If there are not significant numbers of heavy invertebrates in the inorganic material, they are removed under the magnifying lamp and placed with the organic matrix. A second technician inspects the inorganic material for organisms until it is determined there are no more invertebrates in the inorganic fraction of the sample. Unless otherwise requested, the inorganic elutriate is discarded.

The organic material and other contents of the sieve are then evenly distributed into the bottom of a Caton -style tray. These are trays of various sizes consisting of uniform grids, each grid being 2 inches per side and the bottom is constructed of 250-micron mesh.A grid (or a standardized portion of a grid) is randomly selected, and its contents transferred to a Petri dish. If an organism falls across a grid line, they are only selected for the grid in which their head resides. The material in the Petri dish is sorted under a dissecting microscope (minimum magnification = 10X). The benthic macroinvertebrates are counted as they are placed into vials containing 70% ethanol. The material is subsampled until a minimum of 300 organisms have been enumerated, or the total abundance of material has been processed. The number of grids, or portions of grids, processed to reach the minimum target count are counted.

Sorters are trained to pick and count only benthic macroinvertebrates, with heads, that were alive during sampling and contain the attributes required for taxonomic identification. Organisms picked may include sub-aquatic organisms or other specified organisms according to the specific study design. Specimens rejected according to EcoAnalysts' standard includes: terrestrials, vertebrates, copepoda, zooplankton, exuviae, and any organism without a head. Photo reference guides may be utilized to assist in identifying rejects while sorting. However, more than 300 organisms are enumerated during sorting to account for taxonomists rejecting specimens. Organisms are enumerated, with a tally counter, into three specific groups: generals, chironomids, and oligochaetes. If there is unsorted material remaining after the target count is reached, a large/rare scan will be conducted. Organisms deemed relatively large or rare to the sample (in comparison with the target taxa enumerated in the final count) are found by a naked eye scan in the unsorted sample remnants and are not counted but picked and placed in a separate vial.

Laser-printed labels containing the internal sample ID, NEON sample ID, vial count, and contents: generals, chironomids, oligochaetes, or large/rare. information is placed in the vial(s). The total number of organisms removed (not including large and rare organisms), the number of grids sorted out of the total, the time spent sorting, and the final volume of the remaining sample volume are all recorded on the sorting bench sheet, as well as comments significant to the preparation, sorting, and/or condition of the sample.

Taxonomic Identification of Benthic Macroinvertebrates

Taxonomists are assigned a sample aggregate group for identification in their area of expertise, which is typically a shipping batch from a domain. The taxonomist then selects a sample for identification entries via the LIMS and empties it organisms into a petri dish or watch glass. Oligochaetes are permanently mounted on slides for genus/species identification. Under a dissecting and/or compound microscope, the invertebrates are identified to the lowest practical level, generally genus/species. Taxon names will be harmonized with historic data. The taxonomist enters each taxon directly into the project database using a unique taxonomic code (this is done while at the microscope). The number of individuals of each taxon is counted and entered into the database. The taxonomist measures size class to the nearest whole mm. Size class is measured from the tip of the head to the end of the abdomen. Size class may be extrapolated if the individual is damaged, or may be recorded as "Sample condition" = "Damaged, affecting measurement".

T A reference collection containing organisms new to the NEON data set will be prepared, where at least one specimen (preferably 3-5 specimens) of each taxon encountered is placed into a 1-dram vial containing 95% ethanol and is properly labeled with identity and sample number. Chironomidae reference specimens are permanently slide mounted in CMC 1019 and labeled with the sample number and taxonomic determination. Additional reference collection handling information is provided in the Sample Archiving and Return Section, below.

10% of the samples are randomly selected for re-identification by a QC taxonomist. All specimens in those samples that were not set aside for the reference collection are re-identified. The final data is adjusted according to the recommendations of both taxonomists. Taxonomic references used for the taxonomic analysis of samples will be provided.

DATA MANAGEMENT

Data is directly entered into the LIMS database. Throughout the project and sample analysis, data entry is double checked for accuracy, and validated by the laboratory managers. The appropriate data are combined for each sample to obtain the sorting statistics and comprehensive taxa lists and counts.

Quality assurance data sheet checks are part of the sample validation process, and include scanning for apparent entry errors, measurement errors, omissions, and anomalies. Suspect data are flagged and/or excluded from use. Data may be presented in table, graph, and chart format. Unusual data are rechecked to verify their accuracy.

Data is formatted on client data ingest sheets and returned by uploading directly to the client project data portal or returning to a filesharing folder.

SAMPLE ARCHIVING AND RETURN

Excess sample debris from sorting will be stored for 6 months after data delivery and will then be safely disposed. Identified organisms will be stored in permanent mounting media on slides or in 95% ethanol in 20mL glass scintillation vials with poly seal screw cap or 4oz plastic jars at room temperature. Barcode labels will be created for each vial and slide produced during taxonomy. The unique identifier will indicate what type of organism group is being stored: general vial, chironomid vial, oligochaete vial, large/rare vial, chironomid, or oligochaete slide. This information will be added to the data delivery. Organisms will also be stored for 6 months prior to shipping to long term archiving. EcoAnalysts will provide shipping manifests per shipment.

Reference collections will be stored until the client provides a location for long term storage. Depending on size or taxonomic grouping reference collection organisms may be stored on slides, 2mL glass shell vial, 20mL glass scintillation, or 4oz plastic jar. Organisms will be labeled with taxonomic ID, internal sample ID, and NEON sample ID. Barcode and unique identifier will be added to slides and containers for archiving and added to the data delivery. Sample material will be stored in 95% ethanol at room temperature for six months prior to data delivery for that sample lot. After the six months hold EcoAnalysts will request action for these materials, if no direction is given, use of non-climate control storage may be utilized.

Table of Revisions

Revision Number	Revision Date	Revisor	Reason for Revision
1	20191127	M. Payne	Including comprehensive language for the NEON project
2	20201111	M. Payne	Revised language regarding QC, stating PTD and PDE will happen prior to data workup
6*	20240512	M. Payne	*Table of revisions to match title.

SOP Revision Approvals

Macroinvertebrates Identification Revision 4			
Title	**Name**	**Signature**	**Date**

Taxonomist Experience and Protocol Review Tracking

Macroinvertebrates Identification Revision 4				
Name	**Title**	**Year of Experience**	**Date of review**	**Signature**

Date document was printed: _____

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