LIMNOpro
Aquatic Science

Standard Operating Procedure
Laboratory Methods: NEON-Zooplankton Version 1.1

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Contact

Primary
Daniel McEwen, Ph.D./CLP
Principal Scientist
Limnopro Aquatic Science, Inc
Email: dan@limnopro.com
Phone: 320-342-2210

Physical Address
700 15th Ave NE, Suite 300
Saint Stephen, Minnesota 56374

Shipping Address
5 11th Street SW
Saint Stephen, Minnesota 56375

STANDARD OPERATING PROCEDURE
LABORATORY METHODS: NEON-ZOOPLANKTON

Scope and Applicability

This standard operating procedure (SOP) is used to establish a uniform format for sample handling and analysis for samples from the National Ecological Observatory Network (NEON). This method is applicable to all samples received from NEON. All staff at Limnopro Aquatic Science, Inc., Saint Cloud, MN, performing zooplankton identification and measurements associated with NEON contracts must follow this SOP without deviation.

A. Sample Receiving

1. To receive samples, collect the delivered box as soon as possible, and store them at room temperature until sample check-in can be performed. Proceed to download Battelle-generated chain of custody (COC) and associated electronic files.

2. To collect the COC, within 14 days of shipment receipt, search email for the electronic chain of custody (COC) that will come from CLANotify@battelleecology.org email address. Each COC contains a sample receipt file (file name prefix “receipt_form”) that serves as the electronic version of the COC (e-COC). Check the electronic file into the laboratory information management system (LIMS). Proceed to unpack sample bottles and check for their condition.

3. To unpack samples and check for their condition, remove each individual sample bottle from the box and line them up on the processing table to ensure each is in good condition. Specifically, check each sample for container damage, preservative leakage, and for labels being present, complete and legible. Any leakage or damage, and any discrepancies between sample labeling and the e-COC document must be noted. For any samples received in compromised condition, take a digital photograph of the sample, record the nature of the compromise on the e-COC. If possible, rectify the compromised samples by adding preservative (70% ethanol), moving sample to new bottle, and/or rewriting labels. Create a notation on the e-COC for any rectifying activity on a sample bottle. Email Battelle to report any problems with the shipment and include digital photograph of compromised samples within 14 days. After unpacking samples and checking condition, immediately check in samples.

4. To check in samples, the label of each sample container must be checked against the e-COC and marked off as they are identified. For each bottle identifier that matches the sample code in the receipt_form, type in “Y” in the “sampleReceived” field. For any discrepancies between sample labels and e-COC, take a digital photograph that clearly shows the discrepancy. Email Battelle to report any

problems with the shipment and include a digital photograph of label mismatch within 14 days. Once all samples have been checked off against the e-COC as instructed by the COC email, upload the completed e-COC to the NEON data ingest portal, which is to be accessed at data.neonscience.org/web/external-lab-ingest. Place a round green sticker on the sample bottle indicating the sample bottle has been checked in and is ready for processing. Repack samples for storage.

5. To repack samples for storage, ensure caps are tightly sealed on storage bottles, that they contain ethanol by noting the mark on the label or checking for odor if not and store them at room temperature until sample processing occurs.

B. Sample Processing

1. To prepare a sample for processing, randomly select a bottle from a shipment in the order shipment was received.

2. To concentrate the sample, pour the entire volume of the bottle as sent through a 37-micron mesh net (i.e., “concentration net”) and collect the filtrate in a 1L glass wide-mouth jar t. Once the sample has filtered through the concentration net, pull the concentration net away from the 1L glass jar filtrate reservoir. Fill up the original empty sample bottle approximately one third of way with tap water, cap and invert several times. Uncap and pour the water with residual animals through the concentration net allowing the water to pass through and drain down the sink. Repeat this process of rinsing the original bottle out three times. If the sample contains a separate mesh net that was packed with the sample prior to shipping (i.e., “sampling net”), pull it out, and holding the 37-micron concentration net that contains all the animals over the sink, use a rinse bottle with a wide-bore tip filled with tap water to rinse any animals that might be adhered to the sampling net into the concentration net. Place the sampling net back into the original sample container.

3. Using the same rinse bottle with tap water, concentrate animals to the apex of the concentration net. Place a funnel into the 50 ml Falcon tube. Flip over the concentration net mesh and rinse the sample through a funnel into the Falcon tube using fresh 70% ethanol. Fill the tube to between 25-50 ml of the ethanol, as much as is required, to rinse the animals off the concentration mesh. If animals are observed to be highly concentrated, fill the Falcon tube all the way to 50 ml with additional 70% ethanol. Add one drop of DAWN dish detergent to the Falcon tube to help with the clumping of organisms.

4. Label the Falcon tube with the mixedZooVialID AND mixedZooVialCode. These can be two separate labels.

a. mixedZooVialID: Using the LIMS, create, print off, and adhere a 1 x 1.5 inch label for a 50 ml Falcon tube. The mixedZooVialID will take the format of the NEON field sampleID (i.e., BARC.20250212.schindler.2)

b. mixedZooVialCode: Apply a unique barcode that Contractor will assign to the sample (not the barcode that came on the field sample)

  - Each barcode must be a unique value across all NEON samples handled by the contractor. Barcodes must be unique: 1 item = 1 barcode (no lots or batches)
  - Barcodes must have a human readable component
  - Barcodes printed or ordered by the Contractor must have a prefix “V” + 11 numbers. NEON will provide a PDF barcode template that can be used to print labels.
  - Example barcode for lab with the prefix ‘V”: V00000012345

V00000012345

  - Laboratory-generated labels should be oriented to fit on the side of vials, not overhang the vial bottom, not interfere with readability of the other label, and be readily readable by a barcode scanner.

Filtrate from the glass-mouth jar and the sampling net (if present), and the original sample jar can now be discarded as appropriate. Place a green sticker on the Falcon tube to indicate the sample is ready to be processed and store at room temperature until processing occurs. Proceed to process concentrated and prepared samples in Falcon tubes.

5. To process prepared samples in Falcon tubes, select one with a round green sticker and replace the green sticker with a yellow sticker to indicate the sample is currently undergoing processing. Next prepare a bench sheet for identification and counting by obtaining a bench sheet and recording the sample code, sample site/station, sample date, the date for start of identification of sample, the taxonomist name on the appropriate line, and estimated volume of the concentrated sample using the measurements on the Falcon tube. Falcon tube volume will be measured using the

volume markers on 50mL graduated centrifuge tube sets with gradations of 2.5 mL or less. Annual validation of graduated centrifuge tubes will completed as follows.

At the beginning of each award year, the Contractor will select 10 percent of the expected number of centrifuge tubes (e.g. Falcon tubes) to be used in the processing of NEON samples based on the estimated number of samples per year. If graduated centrifuge tubes will come from more than one vendor or shipment, tubes from all sources should be involved in the verification study for that year.

Using water, Contractor will fill the representative set of graduated centrifuge tubes to volumes that are similar to concentration volumes encountered with NEON samples (~25-50mL). The volume of water will be recorded for each tube based on the volumetric measurements on the graduated centrifuge tube. Water from each tube will then be transferred to a Class A graduated cylinder and the volumes recorded. Data will be organized in Excel as below and delivered to NEON via email or collaborative space (e.g. SharePoint) at the completion of the verification process.

Example of Graduated Centrifuge Tube Verification Data Table

Test #	Graduated Centrifuge Tube Measured Volume	Graduated Cylinder Measured Volume
2025-1	48	47
2025-2	32	32
2025-3	42	43

If variations in measurement exceed 10% difference, new materials should be obtained and tested. Provided Falcon tubes have met validation, proceed to subsample from the Falcon tube.

6. To subsample, target a minimum of 300 animals per sample. Rotate the concentrated sample in the 50-ml Falcon tube gently 10 times to mix the contents. Immediately, insert a pipette with a disposable 1 mL wide-bore pipette tip into the sample and further homogenize, mixing it in a random fashion by pumping the pipette up and down several times. Pipettes will be calibrated annually for NIST traceability using a third party vendor. A 1 mL subsample is captured during this mixing process to avoid bias due to sinking of heavier planktonic organisms. Add subsampled contents to a gridded Sedgewick Rafter Cell. Allow animals to sink and settle and stop swirling for 5 – 10 minutes. Identify animals to the following target resolution or lowest practical level:

Taxonomic group	Target
Cladocera	Genus
Cyclopoida	Order
Calanoida	Order
Anostraca	Genus
Rotifera	Genus
Acari	Subclass
Chaoborus	Genus
Harpacticoida	Order
Ostracoda	Class

7. Identify and count all zooplankton under a compound microscope using 40X magnification by moving up and down columns of 9 grid cells (3 x 3) per field. Count and identify animals that overlap on the right and bottom grid lines but not on the left or top as viewing the slide. Monitor liquid levels on the slide under the microscope as the ethanol will evaporate quickly. Add distilled water as necessary to keep organisms from drying out on the slide.

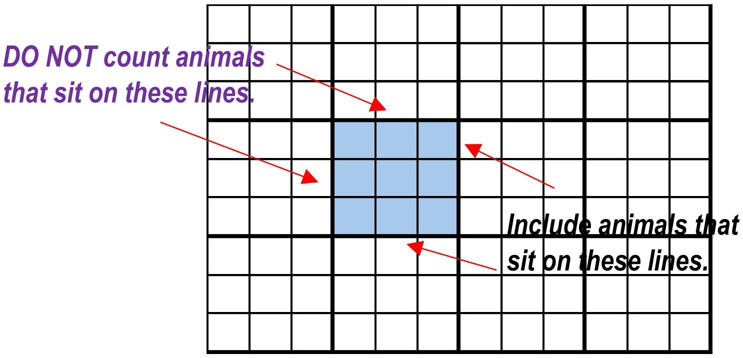


Illustration 1. Count all zooplankton within the nine-grided blue area of a Sedgewick Rafter slide and then move up to the next 9 grid blue area and so on until all slide has been counted. A nine-grided field is an ideal field under a compound scope at 40X magnification. Each individual grid is 1 mm2.

If higher magnification is required to identify smaller rotifers or where animals are highly concentrated, move through individual grids one at a time through the 3 x 3 grid.

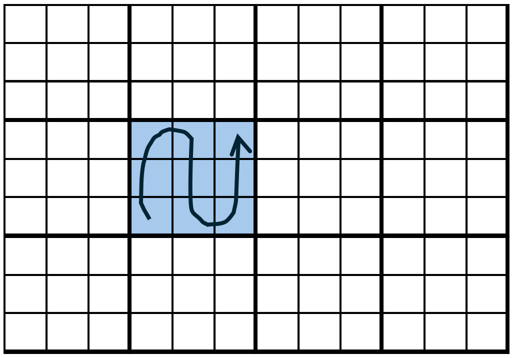


Illustration 2. If animals are small or densely packed on the slide, it may be necessary to use higher magnification and scan each of the nine-grids individually as shown.

8. Once the entire Sedgewick Rafter slide has been processed, determine if a minimum of 300 animals has been counted. If not, add another 1 mL of the concentrated sample and repeat the process until 300 animals have been counted. Record the final volume counted to the top of the bench sheet in the appropriate space. Note that the goal is to process between 1.0 – 3.0 ml of the concentrate. If after processing the first slide, there are fewer than 100 animals, the subsample is too dilute. If this is the case, repeat above “Sample Processing: Step 2” but decrease the volume used in the concentration step in the Falcon tube to a value < 25 ml that will yield 300 plus organisms in 1 ml subsample. Once enough animals have been identified and counted, proceed to measure.

9. To measure animals, scan through the sample from the beginning of the Sedgewick Rafter Slide. Use the microscope camera to photograph 15 individuals selected as first encountered for each taxonomic unit. Use the software to measure the length and width of each rotifer genera or length only for all other taxonomic units. All measurements are linear and made as follows:

    - Copepod measurements are total length including the caudal rami, excluding antennae and terminal setae of caudal rami.
    - Cladoceran measurements are total length, including helmets and excluding tail spines/mucros.
    - Leptodora lengths are taken from the distal end of the ocular lobe to the posterior end of the abdomen, excluding the furcae. If specimens are bent, usually two measurements are taken and summed.
    - Chaoborus length measurements are from the anterior point of the head capsule to the posterior end of the abdomen. If specimens are bent, usually two measurements are taken and summed.

- Rotifer lengths exclude anterior and posterior spines (posterior spine is included in single-spine Keratella, e.g.- Keratella cochlearis). Widths taken are maximum widths of the body.

Prior to each series of measurements, calibration of the software will be checked using a 0.01 mm micrometer. Measurements are binned to the nearest 0.01mm for rotifers and 0.1mm for all other taxonomic units. Body fragments should not be counted unless the head is attached. If damaged, the record may be recorded as "Sample condition" = "Damaged, affecting measurement". Record measurements to the bench sheet.

10. If any new or difficult to identify genera are encountered not currently part of the NEON taxonomic database create a slide mount and digital image of the individual(s). Record any individuals removed from the final processed sample for the reference collection or slide mounts. Save image files labeled with the original NEON sample ID and taxon abbreviation or scientific name to the Biorepository's GLOBUS folder for inclusion into the Symbiota portal. File names should not have spaces. Create a supplemental datasheet using client provided format detailing image file names, scientific names, identified by, etc. to be included in the GLOBUS space.

11. Once processing of sample is complete, return the identified portion of the sample to the appropriate concentrated 50mL Falcon tube. At that point, the yellow round sticker on the Falcon tube will be replaced with a red round sticker, indicating the sample has been processed and complete. Wrap to seal the cap on the Falcon tube using vinyl tape and place in box to ready for archive and shipping back. Store the sample at room temperature until return to the client. Proceed to enter bench sheet data to LIMS.

C. Quality Control

Quality Control (QC) requires the recount and reidentification of 10% of all samples in their entirety from a taxonomist different than the original taxonomist. A random number generator will select a minimum of 10% of samples. Samples selected for QC will be done at the same date and sequentially in time by two different taxonomists. Once QC samples have been distributed to the Sedgewick Rafter slide, taxonomist 1 will proceed to count and identify all zooplankton as described above and report data to a bench sheet. Following this, taxonomist 2 will do identifications and counts on the same slide and record data to their own bench sheet. If after the first distribution of animals is counted on the slide does not reach 300 organisms at a minimum, the slide will be rinsed and an additional distribution from the samples will be made to the slide and the process will continue until a minimum of 300 animals are counted.

Once a minimum of 300 animals are counted for the original count, the following will be used in order to determine both the Percent Taxonomic Difference (PTD) and Percent Difference in Enumeration (PDE) (Stribling et al. 2008) will be estimated as described below.

1. Percent Taxonomic Differences (PTD): Percent taxonomic difference must be <=15% between the sample identified by Taxonomist 1 and the re-identified (quality checked) sample identified by Taxonomist 2 (Stribling et al. 2008).

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

   where a is the number of matches between taxonomist 1 and taxonomist 2 for a single sample and N is the total number of individuals identified in the larger of the 2 counts for the sample.

   If PTD is >15%, the taxonomist 2 will update taxonomic identifications and reconcile any other data that have been affected. The QC results will be presented as numerical values in the perSample datasheet and an indication that QC was performed for a given sample will be recorded in the perTaxon datasheet.

2. Percent Different in Enumeration (PDE): Percent difference in enumeration must be <5% between the sample enumerated by Taxonomist 1 and the re-identified (quality-checked) sample enumerated by Taxonomist 2.

   $$PDE = \left(\frac{\left|n_1 - n2\right|}{n_1 + n_2}\right)100$$

   where n_1 is the number of individuals counted by taxonomist 1 and n_2 is the number of individuals counted by taxonomist 2.

   If PDE is >5% taxonomist 2 will update counts per taxon and reconcile any other data that have been affected. The QC results will be presented as numerical values in the perSample datasheet and an indication that QC was performed for a given sample will be recorded in the perTaxon datasheet.

   After QC is performed, values will be entered on NEON standard datasheets. Proceed to sample archiving.

D. Zooplankton Sample Archive Preparation

All completed processed samples will be stored at room temperature preserved in 70% ethanol for up to one year following data return to allow NEON to perform a post-analytical review of the data. Limnopro will ship the archive samples to the NEON Biorepository, located at Arizona State University in Tempe once they receive notification from NEON that all returned data has been confirmed and the samples may be shipped to archive. Samples will be shipped via ground to the NEON Biorepository following hazardous material requirements as appropriate.

E. Zooplankton Data Return

Data will be uploaded to the NEON portal based on the following schedule

Data Ingest Template	Type of Data Return	Upload Frequency
General Data Return		
Receipt form – provided as an email attachment with each automated shipping notification, used to report condition of each sample/shipment	'receipt' load group on NEON Data Portal	Upload after the receipt of each sample shipment
Zooplankton Taxonomy Date Return		
Attachment2g_zooplankton_perSample	Zooplankton_Lab_Data' load group on NEON Data Portal	Each completed analytical batch
Attachment2h_zooplankton_perTaxon	Zooplankton_Lab_Data' load group on NEON Data Portal	Each completed analytical batch
Taxonomic Table Update Request		
Attachment2k_taxon_request_template	Email to NEON technical representative	Up to 1 request per month
Biorepository Shipments		
Attachment3a_template_receipt_form_for_Biorepository shipments	shipment' load group on NEON Data Portal	Per Shipment to Biorepository

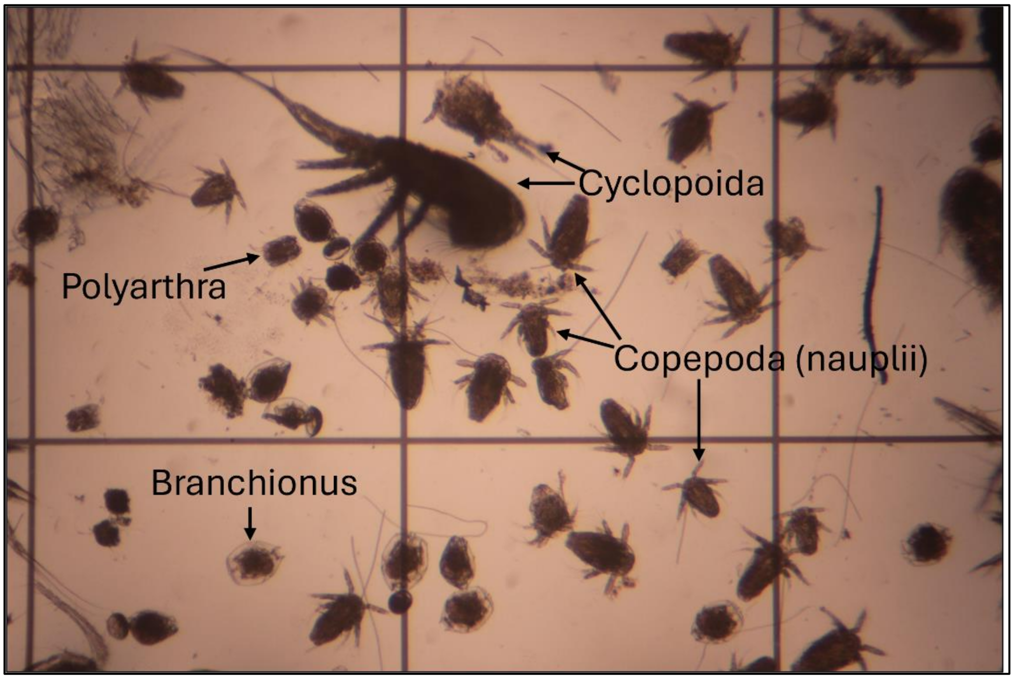
F. Auditing, Meeting, and Reporting:

Quarterly teleconferences for the duration of the contract with Battelle as well as a debrief teleconference at the conclusion of the contract. Additional covered auditing activities for the award may include filling out end of the year audit form (i.e., zooplanktonTaxID_Evaluation criteria and Supporting Documentation) and CAR forms that match in effort to prior years' provision of the same.

Processing Equipment

Processing supplies including 50 ml Falcon tubes, original sample bottles, 1 L glass jar with attached funnel and 37 micron net, pipette with wide bore 1 mL tips and Sedgewick Rafter slide.

Example Slide



Example camera view at 40X of diversity of zooplankton on Sedgewick Rafter slide with grids measuring 1 mm^2.