LIMNOpro
Aquatic Science

Standard Operating Procedure
Laboratory Methods: NEON-Zooplankton Version 1.0

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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.

Sample Receiving

1. To receive samples, upon arrival place box in refrigerator for storage at 4 °C until processed as check-in. Each box will be assigned a unique identifier that serves as a "batch". The Batch ID will be used in a laboratory information management system (LIMS). For samples with multiple jars, all jars need to be organized together. Each sample will be identified to a unique Batch ID number. No later than 16 business hours after receiving samples, unpack sample bottles and check for their condition.

2. To unpack samples and check for their condition, pull the box from the refrigerator, and locate the chain of custody (COC) form, and set it aside. Next, 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 and legible. Any leakage or damage, and any discrepancies between sample labeling and the 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 COC, and the sample identification number. If possible, rectify the compromised samples by adding preservative (40% ethanol), moving sample to new bottle, and/or rewriting labels. Create a notation on the COC for any rectifying activity on a sample bottle. Email the client to report any problems with the shipment and include digital photograph of compromised samples within 16 business hours. After unpacking samples and checking condition, immediately check in samples.

3. To check in samples, the label of each sample container must be checked against the COC and marked off as they are identified. The NEON supplied barcode is scanned and metadata stored in the scanner until download. For each bottle identifier that matches COC, place a small checkmark using pencil on both the sample label and line on the COC. For any discrepancies between sample labels and COC take a digital photograph that clearly shows the discrepancy. Email the client to report any problems with the shipment and include digital photograph of label mismatch within 16 business hours. If all sample labels match COC, indicate such on the COC. Once all samples have been checked off against the COC, the client-provided COC must be signed and copied. The original COC is returned to the client

via fax, email or USPS. 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.

4. To repack samples for storage, ensure caps are tightly sealed on storage bottles and place them in refrigerator at 4 °C, preserved in 40% ethanol until sample processing occurs.

Sample Processing

1. To prepare a sample for processing, randomly select a sample from storage. Print off the bench sheet associated with the sample from the LIMS. Confirm the barcode sample ID on the bench sheet with the barcode sample ID on the bottle. At the top of the sheet, record the original sample volume, start date/time and taxonomist name on the appropriate line. Replace the green round sticker on the bottle with a yellow sticker to indicate the sample is currently being processed. Proceed to subsampling.

2. To subsample for processing that will lead to a minimum of 300 animals per sample, rotate the original sealed bottle gently 10 times to mix the contents. Immediately, insert a 1 mL Hensen-Stempel pipette into the sample and further homogenize, mixing it in a random fashion (not swirling). The sub-sample is captured during the mixing process to avoid bias due to sinking of heavier planktonic organisms. Add subsampled contents to a Syracuse glass. Dissemble the Hensen-Stempel pipette and rinse using running tap water to clean any residual organisms. Estimate the count for total organisms.

    a. If the estimated count is less than 400, sample an additional 1 mL using the same process until a minimum count of 300 would be estimated to be achieved. Based on that estimate Proceed to identification and measurement.

    b. If the estimated count is greater than 400 do an appropriate dilution using 40% ethanol that will generate between 300-400 animals per mL. For example, if original count of organisms is estimated at 600 individuals use 40% ethanol to rinse animals from the Syracuse dish back into the original sample container. Mix original sample and pour 20 mL into a graduated 50 mL falcon tube. Add 20 mL of 40% ethanol to the tube to bring to a volume of 40 mL. Mix well and then use a Hensen_Stempel pipette to extract 1 mL and proceed as described above. Record details of the dilution on the bench sheet (e.g., 1:1). Proceed to identification and measurement.

3. To identify and measure animals, scan the sample in the Syracuse dish for morphospecies that require identification. For each unique morphospecies, mount five (5) on a temporary slide for examination with compound microscope for

identification. Record the taxonomic reference used to key the organism. Pick an additional 15 individuals for each taxonomic unit and transfer those to a clean Syracuse glass with distilled water and a drop of DAWN dish detergent. Use the microscope camera to take a photograph of all 15 individuals. Use the built-in software to measure the length of each individual. 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 0.1mm. Body fragments should not be counted unless the head is attached. If damaged, the record may be recorded as "Sample condition" = "Damaged, affecting measurement". Proceed to create a voucher if a new species is encountered.

4. To create a voucher for a new taxonomic unit not already in the reference collection, 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 Dropbox 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 Dropbox. Proceed to store sample for enumeration.

5. To store sample for enumeration, rinse the contents of Syracuse dish into a 10 ml glass vial and increase volume to 6 mL total using 40% ethanol. Proceed to identify and enumerate organisms.

6. To identify and enumerate animals determine the taxonomic affiliation to lowest practical level without dissection and count them with the following targets:

Taxonomic group	**Target**
Cladocera	Genus, species
Cyclopoida	Genus, species
Calanoida	Genus, species
Anostraca	Genus, species
Rotifera	Genus, species
Hydracarina	Genus, species
Diptera	Family
Harpacticoida	Order

a. To identify and enumerate macrozooplankton, gently agitate the 10 mL glass vial containing the subsample. Use a wide-tipped 2 mL pipette to extract a 2 mL sample and add to Ward plankton counting wheel, and add one drop of dish detergent, cover, and let settle for a minimum of 15 minutes. Count and identify all macrozooplankton (do not count rotifers, which will be counted in a separate step). Remove individuals that cannot be identified to lower practical level by viewing in the counting wheel and mount on a slide for identification. Rinse the animal(s) back into the subsampled vial. Repeat this process three times until all individuals in the sample are counted. Rinse and store counted samples to 10 ml glass vials as described in #4. Proceed to identify and enumerate microzooplankton (i.e., rotifers).

b. To identify and enumerate microzooplankton (rotifers) begin by concentrating individuals to 3 ml sample. Mix the 10 mL glass vial by inversion a minimum of 10 times and pouring the sample through a 35 micron net. Rinse animals using 40% ethanol to 3 ml into a 5 ml glass vial. Using a wide-bore pipette transfer a 1 ml aliquot to a gridded Sedgewick Rafter slide. All the sample to settle for a minimum of 15 minutes. Identify and enumerate all rotifers. Repeat the process two more times until the entire subsample has been processed. Proceed to store sample for storage or archive.

7. To store sample or archive, rinse the subsampled, picked, and identified individuals back into the 10 mL glass vial, seal the cap with parafilm, add a waterproof label that includes the barcode and use lab tape to take the entire vial within their original sample container. Record the stop date/time on the bench sheet. Replace the green circle stick with a red circle sticker. Place the sample back in the refrigerator for QA. Proceed to enter bench sheet data to LIMS. Proceed to Quality Assurance/Quality Control.

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. After original samples have been processed, a random number generator will select a minimum of 10% of samples from each batch processed. The 10 mL subsamples contained in the glass vials selected by random number generator will be processed for both the Percent Taxonomic Difference (PTD) and Percent Difference in Enumeration (PDE) (Stribling et al. 2008). After QC is performed, values will be entered on NEON standard datasheets.

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 required datasheet and an indication that QC was performed for a given sample will be recorded.

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 required datasheet and an indication that QC was performed for a given sample will be recorded.

Proceed to sample archiving.

Zooplankton Sample Archive Preparation

After sorting and identifying specimens as described above, all processed will be stored refrigerated at 4 °C preserved in 40% ethanol for up to six months following data return to allow NEON to perform a post-analytical review of the data. After the six-month period, Limnopro will ship the archive samples to the NEON Biorepository, located at Arizona State University in Tempe. Every sample/subsample sent to the Biorepository for archiving will get a unique identifier and QR Barcode that is specified on the labels and entered/submitted in the respective Biorepository shipment datasheets. Container size and labeling requirements will be matched to NEON instructions.

Zooplankton Data Return

Data from these samples will be uploaded to the NEON portal prior to shipping to the Biorepository 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
Attachment2i_zooplankton_perVial	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