



**Rhithron Associates, Inc.**  
**Standard Operating Procedures:**  
**National Environmental Observation Network**  
**Zooplankton Indicator**  
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**Corporate Approval**

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## **Introduction**

Rhithron processes and identifies zooplankton samples collected as part of the National Environmental Observation Network (NEON) program. The data generated from these samples need to be consistently and reliably generated to support the uses to which the data are put, typically, to assess water quality and habitat integrity in surface water systems. The methods and protocols applied to samples vary and depend on client-specifications and project goals. Thus, samples must be handled with the utmost attention and care and the client-specified protocol, including required taxonomic resolution, must be faithfully followed. This document outlines the procedures used in the analyses of zooplankton samples. Rhithron's internal data quality objectives (see the Quality Assurance Plan) are maintained by following these procedures

All staff working on the NEON project samples, in any capacity , must review the version controlled Standard Operating Procedure in its entirety prior to the staff member working on the project. Additionally, if greater than two months lapse without a review of the NEON SOP then each staff member shall review the SOP in its entirety.

## **Laboratory organization**

The organizational chart in Figure 1 shows the Rhithron personnel responsible for the various tasks associated with zooplankton sample analysis, and illustrates the pathways of communication that are used to assure the quality of Rhithron's work.

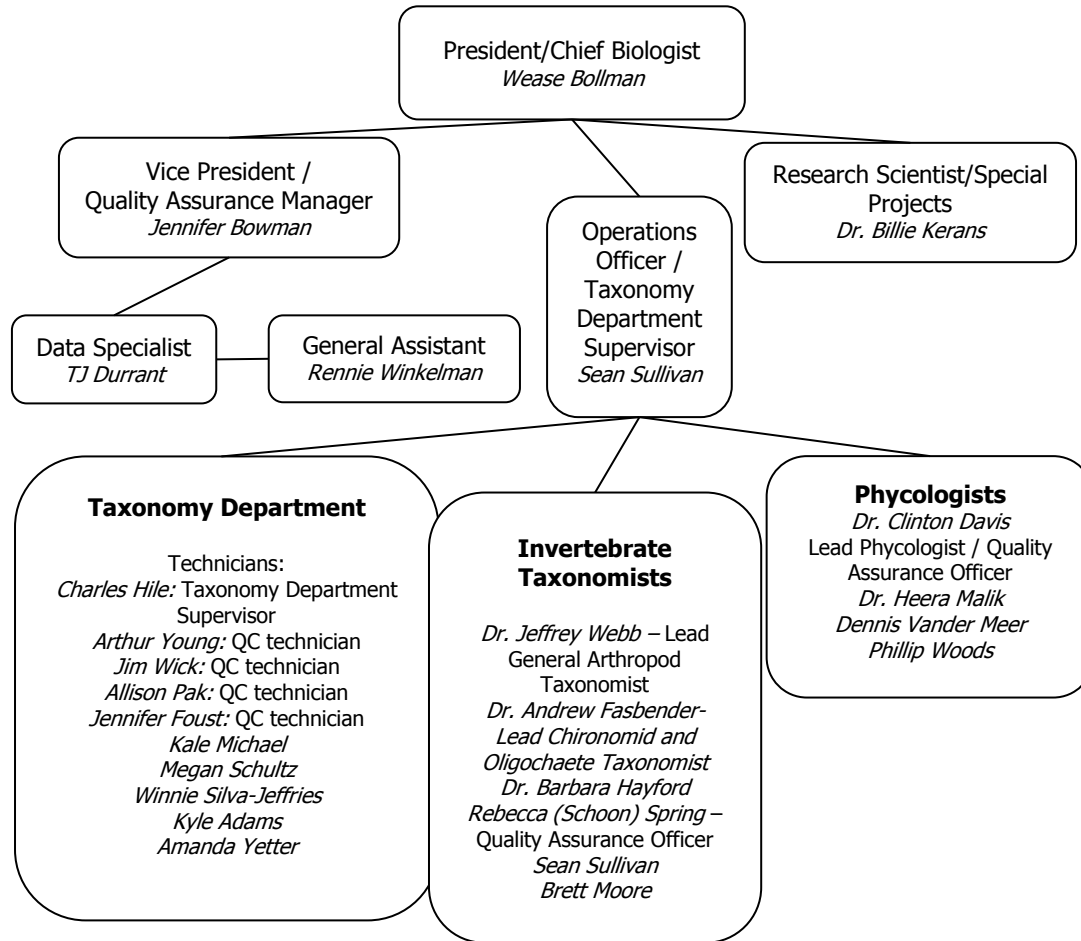


Figure 1. Rhithron Associates, Inc. organizational chart: November 2018

## Zooplankton samples

### ***Health and safety warnings***

In addition to the laboratory's usual requirements, the following health and safety procedures must be followed. 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 (benthic organisms or sediment) 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.

## ***Project set-up***

### **Goals**

The goals of Zooplankton project set-up procedures are to prepare samples for processing by the Taxonomy Department and while maintaining the integrity of the samples and to generate the required paperwork and computer files. The General Assistant (Figure 1) receives the samples when they arrive and is responsible for making sure that required procedures are followed.

### **Sample intake, inventory and chain of custody**

Samples that Rhithron receives for processing are collected by clients, and delivered by commercial or postal carriers. Samples generally arrive at the laboratory's front door and the General Assistant signs delivery documents.

#### ***Scope***

Using the following procedures, the General Assistant assesses the condition of the samples and preservative needs, makes sure that all samples correspond with a chain of custody or inventory (COC) provided by the client, and that all expected parts of the delivery have arrived safely. The procedures in this section pertain to zooplankton sample deliveries, with alcohol (ethanol solution) preservation.

#### ***Personnel***

The General Assistant is responsible for the completion of sample intake procedures, including generation of the project inventory report (which serves as the sample sign-out document for the Taxonomy Department) upon completion of the intake and inventory procedures.

#### ***Procedures***

When packages arrive, all shipping containers should be opened by the General Assistant and the COC should be located. The COC should be referred to during all following steps. Each sample jar should be removed and the level and integrity of preservative must be checked, recharging ethanol solution when required. 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 samples with multiple jars, all jars need to be organized together. Any leakage or damage, and any discrepancies between sample labeling and the COC document must be noted. This information must be reported to the Quality Assurance Manager (Figure 1) immediately who reports to NEON by telephone or email immediately. Any discrepancies must be rectified before custody documents are signed, copied, and returned to the client.

Once all samples have been checked off against the COC, and all discrepancies have been rectified by the client, the client-provided COC must be signed and copied. The original COC is returned to the client via fax, email or USPS by the Quality Assurance Manager.

The General Assistant creates the Rhithron Associates Incorporated (RAI) Inventory file (an Excel file saved to the client folder). Entries in this file include the site name, client sample identifiers, replicate numbers, sample collection dates, the number of jars in each sample (e.g., 1 of 2, 2 of 2, etc.), and any other distinguishing data. The RAI Inventory file contains a number of worksheets: 1) "*Client*" Client COC information, or information gathered by the General Assistant from jar labels, 2) "*Pre-check-in*" Client information that is copied into

Rhithron-Laboratory Information Management System (LIMS) format for subsequent upload and to create sample jar labels and RAI numbers (internal identifiers) that are assigned to each sample, 3) "*Check-in*" Client information with an additional column with correct client info and consistent with the jar labels, it is a record of the jar/COC checking activity and also for recording discrepancies (communicates to Quality Assurance Manager the discreps he/she has to rectify with NEON) and also the Data Technician (Figure 1) adds discrepancies to the macroinvertebrate project LIMS (RAILIS) for documentation and 4) "*Sample Upload*" This sheet is used by the Data Technician. The General Assistant also places the physical copy of the COC into the General Assistant's filing cabinet

The General Assistant uploads the internal inventory into the Rhithron Associates, Inc. Laboratory Information System (RAILIS) and makes the sample jar labels (RAILIS output) which consist of the unique laboratory sample identification RAI numbers and labels for the all the sample jars. The General Assistant attaches these labels to the sample jars during check-in.

The General Assistant takes samples to the storage site. He/she records the location of the samples in the storage site, the type of preservative, whether the samples were decanted by the client in the project table in RAILIS. The General Assistant then notifies the Data Technician (and copies the Operations Officer (Figure 1)) that the sample data are ready for upload.

The Data Technician then gets custody of the samples and data. He/she uploads the internal inventory created by the General Manager into RAILIS and sample metadata into Rhithron's proprietary Electronic Periphyton and Invertebrate Cloud software (EPIC). RAILIS also generates the subsampling benchesheets, Project Inventory Report and vial labels for use during sorting that are printed by Operations Officer when the samples are about to be processed.

## **Sample storage and transfer to the Taxonomy Department**

Zooplankton samples and projects awaiting processing are stored in the storage building behind the Rhithron laboratory. The storage building is locked and alarmed for unauthorized entry, and is equipped with a fire suppression system and fire alarms.

### **Procedures**

The General Assistant or designee transfers samples to the secure storage area. Each project is assigned to an individual storage shelf or area designated only for that project and the location is noted in RAILIS. The samples and project remain in the custody of the Taxonomy Department until samples have been processed, at which time custody of the samples and project is transferred to the Technical Department. Stored samples and sample fractions are checked monthly for sample integrity, and preservative is added as needed. Sample and project custody are tracked and recorded using the custom LIMS project mapping function.

## ***Sample processing***

### **Goal**

The goal of zooplankton sample processing procedures is to produce a random sub-sampling of a raw sample as delivered to Rhithron by NEON and identifications to the requisite taxonomic resolution. Sub-samples and identification must be produced in a standardized, repeatable manner, and subsampling and identification are quality-assured by the application of QC procedures to at least 10% of subsamples. Subsampling and identification procedures must be applied so as to achieve the following outcomes:

- The target count of organisms is achieved within the specified tolerance limits (300 individuals).
- The appropriate paperwork is associated with the correct sample.
- All data pertinent to the sub-sampling procedure, including fraction of sample used to obtain the target number of organisms, volume of sample, volume analyzed, condition of the sample, any problems associated with sorting, and quality assurance procedure outcomes and statistics, etc. are recorded in EPIC.
- Cross-contamination between samples does not occur.

## **Considerations**

### ***The Protocol and Procedures***

Rhithron's standard operating procedure for subsampling of zooplankton samples is described in the following sections. These procedures are applicable to ethanol-preserved samples which are to be subsampled to a minimum 300 count. A complete project procedures page accompanies each project and every analyst assigned to a project must review the project-specific protocols before beginning to process any samples. If there are any questions or uncertainty about any procedure or protocol detail, the Operations Officer should be consulted for clarification before proceeding.

### ***The sample inventory and sign-out sheet***

Each project has an associated internal sample inventory, which provides spaces for sample sign-out, located in the sample staging area. The General Assistant prints the inventory sheet during the process of sample intake, and delivers it to the technical laboratory with the benchsheets and labels for the project. The inventory sheet serves as an internal chain-of-custody document for the Taxonomy Department and includes the protocol for that project.

### ***Sample metadata***

Taxonomists record sample volumes, subsample volumes, and dilution concentration factors by entering the information into an electronic data entry interface (EPIC). Each sample is identified uniquely within the interface, in order to prevent data association with the wrong sample. Metadata for each sample is recorded by the General Assistant immediately after all steps of sample check-in. All interface fields pertaining to sample preparation and sorting, and QC procedures must be filled out completely.

## **Scope and personnel qualifications**

Rhithron Taxonomists have processed hundreds of zooplankton samples from across North America and have the experience and taxonomic certainty to complete zooplankton analysis. Taxonomic concepts and keys are standardized to harmonize data. Additional training, if necessary, is completed in-house among taxonomists. Outside macrozooplankton systematists are consulted when needed.

## **Materials and equipment**

Ethanol solution (40% Ethanol), in wash bottle  
Nitrile examination gloves  
Stereoscopic microscope (Leica S8APO)  
Compound Microscope (LeicaDM1000 with 10x, 20x, 40x, 63x, and 100x (oil) objectives)

Fiberoptic or LED illuminator  
Digital Camera calibrated to each microscope  
3x lighted magnifier  
Specimen handling tools (forceps, needles, pipette)  
Petri dishes/ watch glasses  
Disposable pipettes  
Graduated cylinders (50mL, 100mL, 200mL, 500mL, 1000mL)  
Hensen-Stempel Pipette  
Biological stains (Chlorozal black, Methyl Blue)  
45 micron sieve (US Std. #325)

## **Methods**

### ***Sample preparation***

Before beginning the preparation procedure, the Taxonomist should be sure that all equipment is thoroughly cleaned and free of organisms. He/she should carefully examine sieves, graduated cylinders, watch glasses, pipettes, and all other equipment using a 3x lighted magnifier before proceeding.

Samples are emptied into an appropriately sized graduated cylinder and the original volume of the sample is recorded in the electronic data entry software (EPIC). Sample contents are returned to the sample container after volumetric measurements are recorded.

### ***Subsampling***

Samples contained in the original sample container are homogenized by gentle agitation of the contents (e.g. aspiration with pipette). Care should be taken to not swirl the sample contents as zooplankton can adhere to the walls of the container. Once the sample is homogenized a 1mL aliquot of the sample is retrieved from the centrifuge tube using a Hensen-Stemple pipette (APHA 2005), and deposited into a watch glass/Syracuse dish. The density of specimens is evaluated. A target of 300 (EcoAnalysts 2017) organisms is desired. Dilutions or concentrations may be necessary to achieve target densities. Any dilution or concentrations performed on the sample contents are recorded in the electronic data entry software (EPIC). Concentrations are achieved through settling the sample overnight, dilutions are achieved through the addition of 40% ethanol solution.

### ***Identification and Enumeration***

Contents of the Syracuse dish are identified and enumerated to the requisite taxonomic targets (Lowest Practical Taxonomic Level). Taxonomic target levels are: Genus and species for Cladocera, Cyclopoida, Calanoida, and Anostraca, Rotifera, Hydracarina and order level for Harpacticoida. Body length (width for Rotifera) measurements for at least the first 15 individuals of each taxon are taken and recorded in the data entry software. Slide mounting may be necessary to identify specimens to the required taxonomic levels. Biological stains such as Chlorazol Black or Methyl Blue may be used to stain Macrozooplankton and assist in identification. The entire 1mL aliquot is identified, additional aliquots are fully identified until a minimum of 300 individuals are counted. The number of 1mL aliquots is recorded in Rhithron's data entry software as the volume analyzed.

Taxa occurring within these groups are not to be counted

- Terrestrial Invertebrates
- Vertebrates



- Insects
- Molluscs
- Exuviae
- Headless organisms

Identified aliquots are preserved in 40% ethanol in a separate labeled 20mL container for taxonomic quality control.

A reference collection is maintained on all taxa encountered in samples, both physical specimens are kept as voucher specimens and a photomicrograph with scale bar is also maintained.

## Quality Assurance/Quality Control

### ***Quality Assurance***

Quality assurance is described in the Rhithron QAP (ver 18.1.a), and is maintained through the adherence to the processes and procedures described in this SOP to ensure sample integrity throughout sample processing

### ***Quality Control***

Quality control measures are described the Rhithron QAP (ver 18.1.a), and is maintained through routine checks of taxonomic precision, accuracy, and data accuracy.

#### ***QA/QC plan***

Accomplishment of the performance objectives is evaluated by the following QA/QC plan that examines the adherence to Rhithron-specific and client-specific protocols.

Accuracy of taxonomy is evaluated by adherence to target taxonomic resolution requirements, and by the use of appropriate technical taxonomic literature or other references (e.g., identification keys, voucher specimens). Bias is minimized by the use of taxonomic literature and resources that are accepted by the industry and reflects the most current accepted nomenclature. A bibliography of Rhithron's taxonomic library is maintained in a literature database. Consultation with experts and systematists occurs frequently. High quality optical equipment is used and regularly maintained. Geographic distributions of identified animals are checked and experts consulted when uncertainties arise, to assure credible identifications. Taxonomic discrepancies are examined and discussed by the original taxonomist and the QC taxonomist. Discussions may include the Taxonomy Department Supervisor, Project Manager, Quality Assurance Officer as well as other staff taxonomists. Discrepancies and disagreements that cannot be resolved internally are submitted via vouchered specimens or digital photographs to experts or systematists for resolution. Taxa lists may be changed when disagreements are resolved.

Taxonomic precision is assessed by the re-identification of a randomly-selected 10% of samples in a blind procedure. The results of the QC process are evaluated by the calculation of the PDE,PTC and the PTD. The percent taxonomic disagreement (PTD) is calculated by the following equation:

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

where  $comp_{pos}$  is the number of agreements and  $N$  is the total number of organisms in the larger of the 2 counts. The lower the PTD, the more similar are taxonomic results and the overall taxonomic precision is better. Rhithron's quality objective for PTD is 15% or less. The percent difference in enumeration (PDE) is calculated by the following equation:

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

where *n1* is the number organisms counted by the original taxonomist, and *n2* is the number of organisms counted by the QC taxonomist. The lower the PDE, the more precise the enumeration. Rhithron's quality objective for PDE is 5% or less.

## Electronic data

### *Data entry*


Taxonomic data are entered by taxonomists, using a proprietary data-entry software application (EPIC v.1.7). The EPIC software uses drop-down taxa lists and incorporates several required fields for each taxonomic data entry. Required fields include correctly-spelled taxonomic name, count, uniqueness code, life stage, qualifier, and comments. Direct data entry by taxonomists minimizes errors due to misspellings, data loss or corruption at transfer, and maximizes completeness and thoroughness of the data.

The procedures for data entry are as follows:

1. Access log-in screen using Firefox at [www.rhithron.managementsolutionsofva.com](http://www.rhithron.managementsolutionsofva.com).
2. Enter your user name and password.
3. Select the appropriate taxonomist type from the drop down menu.
4. Select the "Sample Entry" Tab from the upper right hand corner buttons.
5. Select the appropriate Project ID from the drop down menu.
6. Select the appropriate Sample ID from the drop down menu.
7. Move your cursor to the highlighted row and Taxon column.
8. Begin typing your taxon's name, a list of approved taxa will appear, select your taxon from the dropdown menu, then press Tab.
9. Enter the count of organisms belonging to this taxon, then press Tab.
10. Select the "Uniqueness" from the drop down menu of the taxon, then press Tab.
11. Select the life stage from the drop down menu of the taxon, then press Tab.
12. Select the "Qualifier" from the drop down menu, if one is needed, then press Tab.
13. Select the "Portion" from the drop down menu of the sample the organisms came from, then press Tab.
14. Type any comments regarding the identification, then press Tab.
15. Press Enter and/or Click the "Save" button and the line of data is saved.
16. Repeat this process until all data have been entered for the sample.
17. To end the sample Click the "Sample Comments" tab and fill in the required data and comments about the sample as a whole. Click "Save Changes". Check the box for sample portion completion under your name and taxonomist type. The sample is now complete and you can move to the next assigned sample.


### *Reference collection specimens*

Data for an individual taxon must be entered prior to entering reference collection data.


1. After data for the taxon has been entered select the  icon in the left most column of the appropriate taxon's row.
2. A pop-up window will appear.
3. Select the number of organisms you are depositing into the reference collection from the drop down menu.
4. Enter the Vial and or Slide number for the appropriate taxon.
5. Optional\*\*\* include any narrative comments regarding the specimen(s).
6. Press "Save".

### ***Editing Lines of Data***


Data are saved line by line and you can only edit data that you have created.

After having saved a line of data, to reactivate the line of data select the  icon in the furthest right column of the line you wish to edit. This will reactivate the line of data and allow you to edit any of the column data. Select Save to save the changes.


### ***Deleting data***

To delete a line of data, select the  icon; a pop-up box will appear asking you if you wish to delete the line of data. DOUBLE CHECK THE DATA, and press "OK".

### ***Morphometric data***

Measurements are made via a calibrated ocular micrometer installed in each microscope, or with calibrated digital imaging software. To add measurement data to a given taxon, select the  icon; a pop-up box (below) will appear and you will be required to enter the quantity, width (if necessary to nearest mm) and length (nearest mm). Multiple lines of data can be entered for binned organisms to the nearest mm. A check box can be used to indicate that the specimens were damaged and an estimated length was recorded. Red lettering will indicate that the number of measurements does not match the number of specimens of a given sample. This is a QA measure to ensure that all measurements meet the minimum criterion.

Morphometric data are transferred to our Laboratory Information Management System and extracted for data deliverables including the mean mad and min lengths of zooplankton and mean widths for Rotifera.

**Morphometry** 

The number of measurements doesn't match the number of specimens.

**Microcyclops rubellus (Unknown) Total Count: 4**

Quantity	Width	Length	Estimated
3 ▾	0	1	<input type="checkbox"/>
1 ▾			<input type="checkbox"/>

**Add Row**

**Save Changes** **Close**

**Morphometry** ✕

**Microcyclops rubellus (Unknown) Total Count: 4**

Quantity	Width	Length	Estimated
3 ▾	0	1	<input type="checkbox"/>
1 ▾	0	2	<input type="checkbox"/>

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***References***

APHA (2005) *Standard Methods for the Examination of Water and Wastewater*. 21st ed.  
EcoAnalysts (2017) Standard Operating Procedures for Laboratory Analysis: Zooplankton Indicator. November 27, 2017