Rhithron Associates, Inc.

Identification and enumeration of benthic diatoms using a voucher flora approach adapted for production taxonomy:

National Ecological Observatory Network (NEON)

DRAFT

Revision 3.0 2024

Corporate Approval

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Executive Summary

Rhithron began adapting a voucher flora approach for identification and enumeration of diatoms starting in 2020. Motivation to regularly incorporate a voucher flora into Rhithron projects for clients was inspired by a series of publications and workshops (Bishop *et al.* 2017; Lee *et al.* 2020; Potapova *et al.* 2022; Spaulding *et al.* 2021; Tyree *et al.* 2018, 2020; USEPA 2017). Detailed methods outlined how to use open-source software (GNU Image Manipulation Program, GIMP) (www.gimp.org) to organize digital images of diatom taxa into a voucher flora. Rhithron phycologists saw the enormous reference value of these vouchers for improving accuracy and precision of diatom identifications on a day-to-day basis. Rhithron subsequently adapted and modified the GIMP-based voucher flora approach to suit the unique demands of production taxonomy. Specifically, many of Rhithron's clients have multi-year contracts that include shipments of samples scattered throughout a contract time frame. Often clients provide samples from the same locations throughout these multi-year contracts. It was essential to establish a continuous client voucher that could be referenced and updated as samples were processed over the life of the contract. Rhithron phycologists found it effective and efficient to build a client specific voucher and update it regularly as samples were analyzed. Additionally, Rhithron phycologists found it very useful to have the taxonomic nomenclature, taxonomic concepts, references, and comments noted directly in the voucher. Finally, the voucher flora approach added an additional element of quality assurance that was reflected in results from routine internal diatom QC (e.g. re-analysis of same transects by QC phycologist). Rhithron's approach to constructing and managing an on-going voucher flora for clients is outlined in this document for consideration by the NEON working group.

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Introduction

The Rhithron laboratory processes and identifies diatoms and soft algae in periphyton and phytoplankton samples from clients throughout North America. Data generated from these samples must be consistently and reliably generated to support client goals, which are typically related to the assessment of water quality and habitat integrity in surface water systems. The laboratory 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 the required taxonomic resolution, must be faithfully followed. **This document outlines the procedures used in the analyses of diatom samples collected as part of the National Ecological Observatory Network (NEON) program**. These procedures are adapted from Rhithron's Periphyton SOP ver.21.2.b(Rhithron 2021). Rhithron's internal data quality objectives (see QAP Ver 21.1.a) are maintained by following these procedures.

All personnel working on the NEON project samples, in any capacity, must review the version-controlled Standard Operating Procedure (SOP) in its entirety prior to working on the project.

Additionally, if more than two months lapse without a review of the NEON SOP then each person shall review the SOP in its entirety.

Diatom Project Workflow

Samples received at Rhithron enter an organized workflow specifically for a diatom analysis project (Figure 1). **A project is defined as a shipment of client samples received by Rhithron (e.g., 15-25 samples) that will proceed through the workflow**. Clients typically have several Rhithron projects completed annually to accommodate their field sampling activities. The workflow diagram in Figure 1 illustrates the timing of key activities for each client's project. Each activity is presented in detail within this SOP.

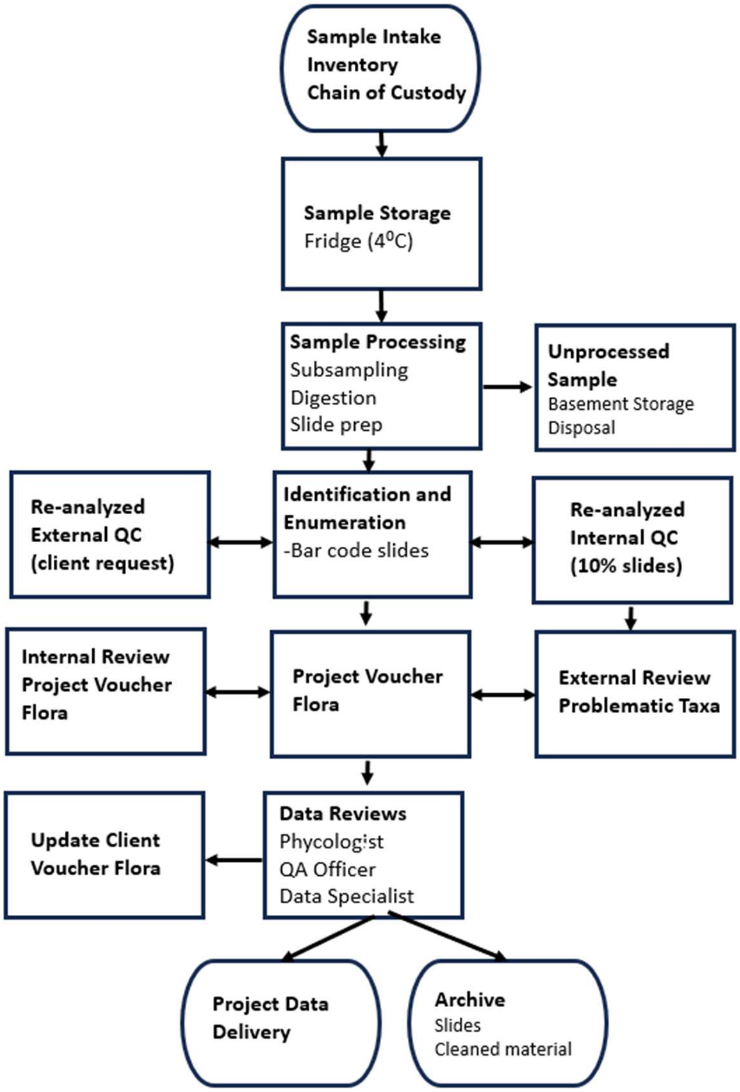
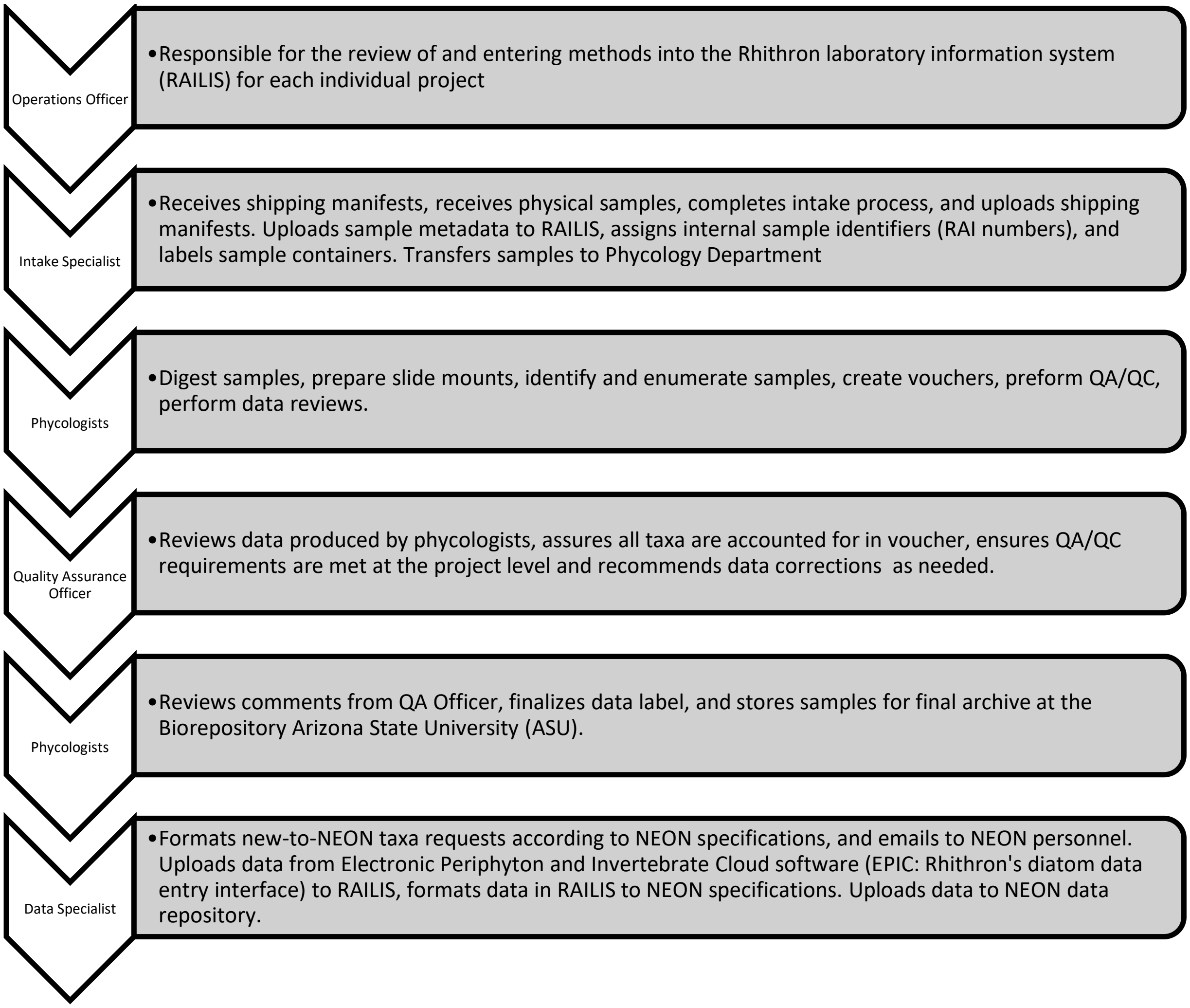


Figure 1. *Project workflow for diatom sample analysis.*

Laboratory Organization

The organizational chart in **Error! Reference source not found.** shows the Rhithron personnel responsible for the various tasks associated with the workflow and illustrates the pathways of communication that assure the quality of Rhithron's work.



Operations Officer
- Responsible for the review of and entering methods into the Rhithron laboratory information system (RAILIS) for each individual project

Intake Specialist
- Receives shipping manifests, receives physical samples, completes intake process, and uploads shipping manifests. Uploads sample metadata to RAILIS, assigns internal sample identifiers (RAI numbers), and labels sample containers. Transfers samples to Phycology Department

Phycologists
- Digest samples, prepare slide mounts, identify and enumerate samples, create vouchers, preform QA/QC, perform data reviews.

Quality Assurance Officer
- Reviews data produced by phycologists, assures all taxa are accounted for in voucher, ensures QA/QC requirements are met at the project level and recommends data corrections as needed.

Phycologists
- Reviews comments from QA Officer, finalizes data label, and stores samples for final archive at the Biorepository Arizona State University (ASU).

Data Specialist
- Formats new-to-NEON taxa requests according to NEON specifications, and emails to NEON personnel. Uploads data from Electronic Periphyton and Invertebrate Cloud software (EPIC: Rhithron's diatom data entry interface) to RAILIS, formats data in RAILIS to NEON specifications. Uploads data to NEON data repository.

Figure 2: *Rhithron personnel flow chart for processing diatom projects.*

Health and Safety Warnings

Health and safety procedures must be followed to prevent laboratory injuries. All proper personal protection clothing and equipment (e.g., lab coat/apron, protective eyewear/goggles, nitrile/rubber gloves, fume hood) must be worn or applied. When working with potentially hazardous chemicals (e.g., glutaraldehyde) 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. All staff performing duties associated with chemicals in this SOP shall review the Rhithron Health and Safety Manual (Rhithron 2022)and review all Safety Data Sheets for any of the chemicals they will handle. Safety Data Sheets are maintained in the

Rhithron laboratory Safety Plan.

Project Set-up

Sample intake, inventory, and chain of custody

Rhithron is notified of sample shipment through the CLANotify system within the NEON framework, and receives the manifest and receipt forms at the time of shipping notification. Upon delivery the Intake Specialist takes custody of the delivered samples. All samples are shipped directly to the Rhithron laboratory, no off-site location is used.

The Intake Specialist assesses the condition of the samples and preservative needs, makes sure that all samples correspond with the NEON shipment manifest, and that all expected parts of the delivery have arrived safely. The Intake Specialist is also responsible for the completion of sample intake procedures, including generation of the project inventory report, chain of custody records, NEON receipt form and transfer of custody to the Phycology Department upon completion of the intake and inventory procedures.

Procedures

When a project arrives, all shipping containers are opened by the Intake Specialist and the manifest is located on the Rhithron server. The manifest is referred to during all of the following steps. Each sample jar is removed, and the level and integrity of preservative is checked, and recharged with glutaraldehyde (1% final concentration volume/volume) when required (e.g. decaying matter smell detected). The label of each sample container is checked against the manifest, and each sample is marked off on the manifest as it is identified. The NEON-provided barcode label is scanned. For samples with multiple bottles, all bottles are organized together and scanned. Any leakage or damage, and any discrepancies between sample labeling and the manifest document are noted. This information is reported to the Operations Officer immediately. The Operations Officer reports this information to NEON by replying (Reply All) to the shipping email notification to immediately notify pertinent parties to the problem and also by reporting this on the sample receipt forms. Discrepancies are rectified before manifests are signed, copied, and returned to the client.

Once all samples have been checked against the manifest, and all discrepancies have been rectified by the client, the NEON receipt form is completed by the Intake Specialist. Upon completion, the receipt form is reviewed by the Data Specialist and uploaded to the NEON data repository.

The Intake Specialist creates the Rhithron inventory file (RAI Inventory) from the client manifest, which is an Excel file saved to the Rhithron server in the NEON client folder. Entries in this file include the site name, client sample identifiers, replicate numbers, sample collection dates, the number of bottles in each sample (e.g., 1 of 2, 2 of 2, etc.), and any other distinguishing data. The RAI Inventory contains a number of worksheets: 1) the "*Client*" worksheet contains client Chain of Custody (COC) information, and any other information gathered by the Intake Specialist from bottle labels, 2) the "*Pre-check-in"* worksheet contains client information that is copied into the Rhithron Associates, Inc. Laboratory Information System (RAILIS) format for subsequent upload, and the creation of sample labels and RAI numbers (internal identifiers) assigned to each sample, 3) the "*Check-in*" worksheet contains client-corrected information consistent with the bottle labels, and 4) the "*Sample Upload*" sheet is used by the Intake Specialist to upload sample information to Rhithron's proprietary Electronic Periphyton and Invertebrate Cloud software (EPIC). The Intake Specialist files the physical copy of the client's COC document.

The Intake Specialist uploads the internal inventory into RAILIS and prints the sample labels output, which is generated by RAILIS. The sample label output consists of sample identifier labels for each of the sample containers, and labels for the processed diatom slides. Each label type includes the unique internal identifiers (RAI numbers) assigned to each sample. The Intake Specialist attaches the appropriate sample identifier labels to the sample bottles during check-in.

The Intake Specialist takes samples to the storage site. The location of samples within a project are recorded as coordinates within the storage site (e.g., Fridge 1: Shelf A). Location coordinates and preservative type are recorded in RAILIS. RAILIS generates a Project Inventory Report benchsheet and sample labels for use during processing; these are printed when the samples are about to be processed. The Intake Specialist notifies the Phycology Department of the status of the samples and their location in the storage site.

Sample storage and transfer to Phycology Department

Periphyton samples awaiting processing are stored under refrigeration in the processing laboratory storage site. RAILIS enables staff to track custody transfers, current sample location, and the progress of a project through laboratory analysis. Once sample intake procedures are complete, custody of the project and samples is transferred to the Phycology Department which is responsible for the security of samples stored while awaiting processing.

Project inventory reports are delivered to a current projects folder in the Phycology Department office area. All phycologists are notified of the project sample locations by the

Intake Specialist via email. Stored samples and sample fractions are checked monthly by phycologists to ensure sample integrity, and preservatives are added as needed.

Sample Processing and Diatom Slide Preparation

Goals of diatom sample processing includes digestion, which produces a "cleaned" subsample portion, free of organic matter, and containing a diatom assemblage representative of the sample. The subsample is then used to prepare a slide mount with an even distribution of this assemblage. Samples are processed in a standardized, repeatable manner. In general, the digestion of diatom sample material follows standard methods outlined in the Academy of Natural Sciences SOP for the National Assessment of Water Quality (NAWQA) program (Charles *et al.* 2002). Diatom processing procedures are applied to achieve the following outcomes:

- The client-specified protocol is followed precisely. Client specifications are reviewed by the Operations Officer, phycologists, and Intake Specialist before the processing of samples. If there is any question or uncertainty about any procedure or protocol detail, the Operations Officer is consulted for clarification before proceeding.
- The appropriate paperwork (i.e., sample labels, slide labels, Project Inventory Report benchsheet) is associated with the correct project samples.
- All data pertinent to the processing procedure, including initial sample volume, processed volume, and final digested volume are recorded and entered into the database appropriately.
- Cross-contamination among samples does not occur.

Materials and equipment

50 mL centrifuge tubes
RAILIS-generated sample labels
Liquinox® soap
Microwave digestion system (Milestone ETHOS EZ), 10 rotor digestion vials
70% nitric acid
Bottle-top dispenser (BRAND® Dispensette® S, Organic, Analog-adjustable, 2.5-25 mL volume range)
Distilled water (using Waterwise 9000 Countertop Distiller), in wash bottle
Graduated cylinder
Ultrasonic bath (Garosa 1.3L Model 113HTD)
Project Inventory Report benchsheet (pre-printed and specific to project)
Hot plate, temperature controlled
Glass microscope slides (2.5x7.5cm)
Glass cover slips (round 18mm diameter), No.1 thickness
Transfer pipettes, disposable (7mL capacity, 0.5mL graduation)
Razor blades, single edged
Naphrax™ mounting medium

Forceps
Drying pan
20 mL glass scintillation vials
90% acetone (PHARMCO, general use HPLC-UV grade), in 50 mL glass vial
Compound microscope (Leica DM1000), equipped with 10X and 63X objectives
PPE (gloves, goggles, lab coat/apron)
Fume hood
pH indicator strips

Subsampling, digestion, and slide prep

Step 1: Label 50 mL centrifuge tubes with the preprinted RAILIS-generated label for each diatom sample and place them in a centrifuge tube rack. Organize project samples into batches of 10, which is the capacity of the digester. For small projects, create batches that divide the samples as close to evenly as possible. For example, a project of 13 total samples will be a batch of 6 and a batch of 7.

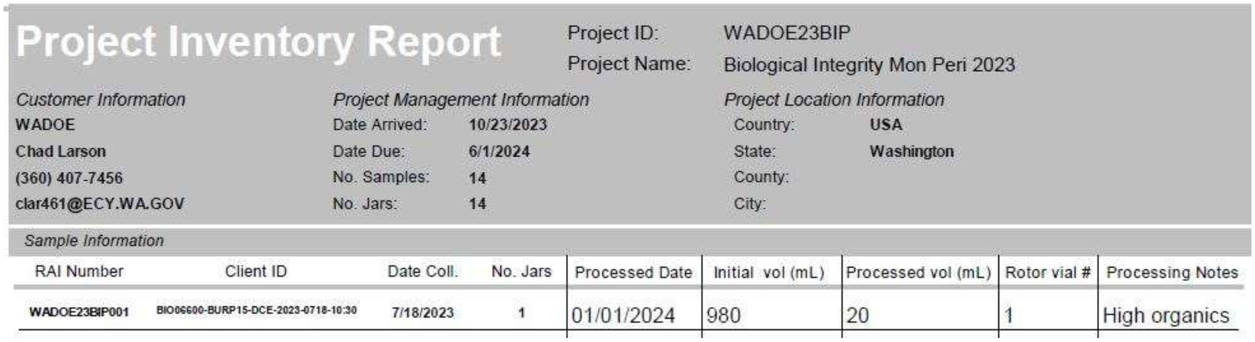
Step 2: Homogenize periphyton sample in its container(s) by thoroughly agitating by hand for one minute to dislodge diatom epiphytes from filamentous algae and to mix sample. Pour the entire periphyton sample into an appropriately sized graduated cylinder to obtain the initial total volume. Record the initial sample volume on the Project Inventory Report benchsheet in “Initial vol (mL)” column (**Error! Reference source not found.**).

Step 3: Pour a subsample of 25 mL (minimum 5 mL) into the corresponding labeled centrifuge tube. Note and enter the volume on the Project Inventory Report benchsheet in “Processed vol (mL)” column (**Error! Reference source not found.**).

Steph 4: Between samples, ensure that there is no cross-contamination by thoroughly rinsing the graduated cylinder. Completely fill and rinse the graduated cylinder at least 3 times with distilled water.

Step 5: Record processing date (month/day/year) on the Project Inventory Sheet in “Processed Date” column (**Error! Reference source not found.**). Record any notable features of the unprocessed sample material (e.g. high detritus, sediments, green algae clump) in the “Processing Notes” column (**Error! Reference source not found.**).

Step 6: Repeat steps 2 through Step 5 until all samples have been subsampled for diatom processing.



Project Inventory Report

Project ID: WADOE23BIP
Project Name: Biological Integrity Mon Peri 2023

Customer Information
WADOE
Chad Larson
(360) 407-7456
clar461@ECY.WA.GOV

Project Management Information
Date Arrived: 10/23/2023
Date Due: 6/1/2024
No. Samples: 14
No. Jars: 14

Project Location Information
Country: USA
State: Washington
County:
City:

Sample Information

RAI Number	Client ID	Date Coll.	No. Jars	Processed Date	Initial vol (mL)	Processed vol (mL)	Rotor vial #	Processing Notes
WADOE23BIP001	BIO06600-BURP15-DCE-2023-0718-10:30	7/18/2023	1	01/01/2024	980	20	1	High organics

Figure 3 *.Project Inventory Report benchsheet example, illustrating key sample information (i.e., initial volume, processed volume, processing notes) to be recorded at the bench during the subsampling procedure.*

Step 7: Top off the centrifuge tubes to 50mL with distilled water. Spin the centrifuge tubes in the centrifuge for 15 minutes at 2500 RPM. After centrifuging, carefully remove the supernatant from each tube, using the vacuum spigot, and being sure to **stop vacuuming at 7.5mL in the tube to avoid removing the settled material**.

Step 8: Transfer the remaining material, including the settled material, from the centrifuge tube to a rotor digestion vial from the microwave digester. The rotor digestion vials are already labeled from 1 to 10 (Figure 4). Using distilled water, rinse the centrifuge tube with 7-10mL, adding this rinsate to the digestion vial. Try to obtain a total volume of approximately 15 mL of sample material: this will affect the final acid-to-sample-volume ratio: see Step 9. Be careful not to overdo the rinsing. Insert each rotor digestion vial into the rotor and record the position of each sample on the Project Inventory Report benchsheet in the “Rotor vial #” column (**Error! Reference source not found.**).

Step 9: For this step, wear protective equipment, including acid-resistant gloves, a lab jacket or apron, and eye protection. This step must be performed inside the fume hood, with the sash down. Confirm the bottle-top dispenser is set to 15mL and carefully add 15mL of 70% nitric acid to each of the rotor digestion vials using the bottle top dispenser. The final acid-to-sample-volume ratio should be as close to 1 (equal parts acid and sample) as possible.

Step 10: Secure the rotor digester vials into the digester blocks using a torque wrench set to 22.5 Nm.

Step 11: Place blocks in microwave digester verifying rotor vial, block, and turret position (Figure 4). Carefully secure temperature probe in block #1 (refer to Milestone ETHOS EZ manual for proper probe installation and care). Temperature probe should fit in smoothly (i.e. does not require force), if not, inspect probe for damage and note alignment of digestion components.

Figure 4. Digester components labeled 1-10 to track sample locations during digestion process. Red circles indicate example of rotor digester vial #7 location in digester block #7 and placed in position #7 within the digester turret.

Step 12: Run the program for the digester. The temperature will increase from room temperature to 140°C for the first 20 minutes. Over the next 40 minutes the temperature will gradually increase to 170°C. The final phase will be a 10-minute cool down period. Total run time will take 70 minutes.

Step 13: Remove the blocks individually from the digester, place them in the fume hood, and allow them to cool for 45 to 60 minutes.

Step 14: Remove the rotor vials from the blocks and transfer the digested material to the labeled centrifuge tubes using distilled water to rinse the rotor vials.

Step 15: Carefully dilute the nitric acid by topping off the centrifuge tubes with distilled water using a wash bottle. Avoid splashing and overspray. Centrifuge the samples for 15 minutes at 2500 RPM. Carefully siphon the liquid down to 7.5 mL using the vacuum spigot. Repeat dilution-centrifuge-siphon steps at least 6 times, then check with pH indicator strips to ensure the acid is sufficiently neutralized to greater than 5 pH.

Step 16: Record the final volume after digestion on the Project Inventory Report benchsheet in the "Processing Notes" column (**Error! Reference source not found.**). Typically, digested diatom samples should be 7.5 mL at this step.

Step 17: Steps 17 through 27 will result in the assembly of 2 diatom slide mounts for each sample, a lower-density slide mount and a higher-density slide mount. This helps to ensure that the phycologist has a slide that can be easily analyzed. Cover slips are cleaned in 90% acetone and wiped dry with laboratory wipes prior to use. Discard any cover slips that have debris that cannot be removed. Level the hot plate. **Label a slide with a RAILIS-generated sample label AND write the rotor digester vial number above the sample RAI number**. Place two (2) cleaned round 18mm cover slips for each sample on the labeled slide (Figure 5). Record the rotor digester vial number on the Project Inventory Report benchsheet and ***ensure that the rotor digester vial number matches the RAI sample number.***

Step 18: Using a clean transfer pipette for each sample, mix the solid and liquid portions remaining in the centrifuge tubes and transfer this material to a 20mL vial labeled with the RAILIS-generated sample label. Gently sonicate clean diatom material to break up clumps by smoothly dipping vial in/out of sonication bath 10 times. Sonicator water temperature should be room temperature so temperature control should be off.

Step 19: In steps 19 and 20, the low dilution (higher-density) and high dilution (lower-density) subsamples are prepared from the material in the 20 mL vial. Visually evaluate the biomass of diatom material in the vial by holding it up to a light source, and swirling the vial to suspend the solid materials. Diatom frustules will appear as “white sparkles” while presence of sand particles will add a brown/darkening of the sample. Based on observations and experience, determine whether the diatom biomass is relatively high: water may be added to high biomass samples. If water is added, the total sample volume should not be allowed to exceed 20 mL. Update the final digested volume recorded in the Project Inventory Report benchsheet in the “Processing Notes” column (Figure 3) to reflect the addition of water. Needing to concentrate samples in cases of very low biomass is extremely rare, however centrifuging-siphoning to a 1 mL can be achieved.

Step 20: Choose a low (e.g., 50) and a high (e.g., 100) dilution factor, based on observation and experience. Prepare the low dilution cover slip first, followed by the high dilution cover slip. Agitate vial thoroughly before subsampling. Mix dilutions in a clean graduated glass beaker by using a clean transfer pipette to add 1 mL of cleaned material and bring up to the final volume with distilled water. For example, 1 mL sample + 49 mL water will result in a dilution factor of 50. **Confirm the location of the labeled slide that matches the diluted sample** and transfer approximately 1 mL of a diluted mixture onto a clean cover slip. **Low dilution goes on the left-side cover clip** and **high dilutions go on the right-side cover slip** (Figure 5). Rinse the graduated beaker thoroughly with distilled water between samples. Repeat dilution steps for high dilution cover slip, transferring the second diluted mixture to the right-side cover slip. In the Processing Data tab in EPIC (Figure 7), record the dilution/concentration factor (DCF) for the low dilution cover slip in the Slide Dilution Concentration Factor field, and record the DCF for the high dilution cover slip in the Notes field.

Step 21: Left-over cleaned material in the 20 mL vial is stored at room temperature in a project-labeled box. Lids are left loose to allow water to evaporate leaving dried diatom frustules. Cleaned material in barcoded vials are checked after 10 days to determine if additional time is needed for desiccation. The lids of dried materials are secured at this time.

Figure 5. *Set-up for drying a low dilution and a high dilution cover slip for each sample. In this example,* 2 samples are shown (058, 059) for this client's project (ECOA22NL05).

Step 22: Allow the cover slips to dry overnight at room temperature (~70°F). Examine the cover slips the following morning to see if all liquid has apparently evaporated. Temperature and humidity will vary by season/day, and this can impact drying times.

Step 23: Scan dried cover slips under the microscope to evaluate valve density and dispersion, and the quality of the dried subsample. At 10X magnification, assess for the presence of sediment, clumping of frustules, residue, or other problems. Using the 63X objective, evaluate valve density. Ideally, each field of view (FOV) at 63X should have approximately 20 diatom valves visible. Additional processing steps, including additional sample rinses to remove residues or dilutions for high valve density or excessive sediment, may be needed.

Step 24: With a hot plate set to the lowest setting (i.e. warm to the touch), gently heat the dried cover slips for 1 hour to remove any remaining moisture, which may not be visible to the naked eye. Using the Project Inventory Report benchsheet, label slides on the left side with the corresponding RAILIS-generated sample label and **write the dilution factor above RAI numbers**. ***Carefully refer to the benchsheet and to the previously-labeled slide to ensure that the RAI numbers on all of the slides corresponding to the sample are in agreement.*** Place labeled slides on a hot plate set to 250°C inside the fume hood, arranging along the edge in the following order: project sample 001 low dilution, project sample 001 high dilution, project sample 002 low dilution, project sample 002 high dilution, etc. (Figure 6). Using a transfer pipette, place a small pea-size drop of Naphrax on each heated slide. Naphrax should be easily pipetted, having a viscosity similar to glycerin. If the Naphrax is too viscous, it is likely that the toluene has evaporated, and new Naphrax is needed.

Step 25: Carefully invert the cover slip using forceps so that the dried diatom material is placed on the heated slide, on top of the drop of Naphrax.

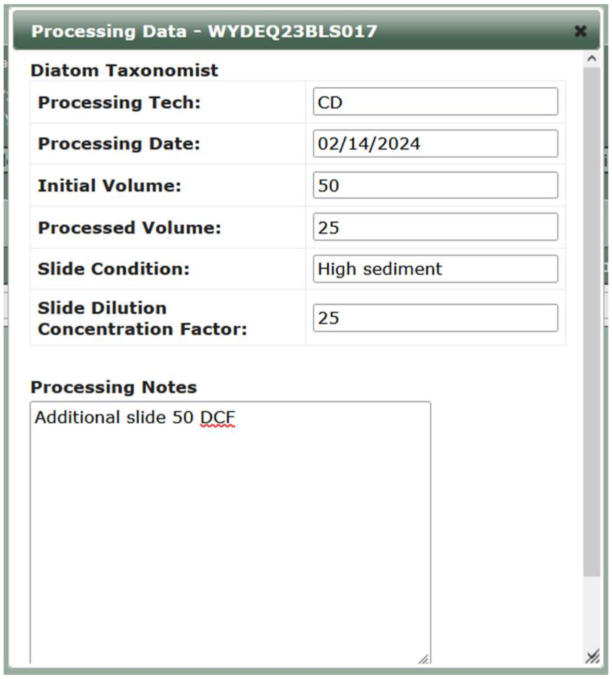
***Figure 6**. Arrangement of slides on hot plate in preparation for Naphrax addition and cover slip gluing. In this example, 2 project samples are shown (058, 059) for this client's project (ECOA22NL05), each having slides with a low dilution (50DCF) and high dilution (100 DCF).*

Step 26: Allow the slides and cover slips to sit on the hot plate briefly (typically 3-5 minutes) to allow the toluene to boil out of the Naphrax. Bubble formation should cease when all toluene has been removed. Remove the slides from the heat and gently press the cover slips with forceps to remove any bubbles in the Naphrax.

Step 27: Allow the slides to cool for a few minutes and carefully scrape excess Naphrax from around the cover slips using a razor. The Naphrax should be brittle and easily removed. If otherwise (e.g., sticky, soft), return the slide to the hot plate for additional heating to remove toluene.

Step 28: Scrub all labware using Liquinox © solution and rinse with tap water followed by triple rinse with distilled water.

Step 29: Enter metadata from sample processing into the Processing Data tab (Figure 7) in EPIC, the diatom data entry interface. Enter the initials of the processor, the processing date, initial volume, processed volume, slide condition, DCF value of "best" slide and general processing notes (e.g., additional slide made 50 DCF).



Diatom Taxonomist

Processing Tech:	CD
Processing Date:	02/14/2024
Initial Volume:	50
Processed Volume:	25
Slide Condition:	High sediment
Slide Dilution Concentration Factor:	25

Processing Notes

Additional slide 50 DCF

Figure 7. *Example of sample processing metadata, entered into the Processing Data tab in Rhithron diatom data entry interface EPIC.*

Sample processing QA/QC plan

Sample volumes and acid volumes are carefully monitored by the processing staff in order to maintain equivalent processing procedures for all samples. Great care is taken to ensure that contamination does not occur during processing by thoroughly cleaning all equipment, and using the microwave digester, which isolates each sample during the digestion procedure.

Identification and Enumeration

All analysis of diatom samples is performed by Rhithron staff phycologists. Phycologists have 5 to 20 years of experience in the species identification of diatoms, and each holds Society for Freshwater Science (SFS) Level 1 diatom genus or level 2 diatom species taxonomic certifications. The following methods are used to ensure reproducible diatom identification and enumeration data from processed sample material. Phycologists analyzing the diatom slides should note this very important project data element:

- New clients having their first project voucher flora completed will have that project voucher saved as the on-going client voucher that will be referenced, and added to, in subsequent projects. The first project voucher is a time-consuming process as all taxa encountered are effectively “new” and documented following the standardized approaches outlined in this Identification and Enumeration section.

- **Identified taxa are assigned species names at the bench by referencing images in a client voucher flora**. **If a specimen does not match any taxon in the client voucher, the phycologist captures an image and assigns a name, as described in Step 5 below.** Once the current project is completed, all new images and taxa are compiled into the project voucher flora and reviewed by at least two Rhithron phycologists. Phycologists discuss disagreements and review literature as a group. A consensus is generally reached, with an occasional “majority rule” approach, and on rare instances a “tied” result requiring consultation with an external phycologist for input. At this point, initial bench names are subject to change. **New taxa in the project voucher flora are added to the on-going client voucher for reference when identifying diatoms in future client projects.**

Materials and equipment

Compound Microscope (Leica DM2500), equipped with differential interference contrast (DIC)
Diamond scribe, mounted on objective turret
100X oil objective (1.4NA)
10X, 20X,40X objectives
Oil condenser
Digital camera (Leica DMC2900)
Networked computer located at the microscope for access to EPIC data entry software
GNU Image Manipulation Program (GIMP)
Measurement calibration slide (1mm ruler, 0.01mm resolution)

Slide analysis

Step 1: For a sample, select one of the prepared slides (high density or low density) for analysis. Use a Leica DM 2500 compound microscope equipped with DIC, an oil condenser, and 100X objective (1.40NA, 1000x magnification) for identification and enumeration.

Step 2: Scan the slide using coarse (100X) magnification to confirm adequate density and valve dispersion. Also note areas of optical distortions that should be avoided, such as areas near the cover slip edges. Additionally, the second slide can also be scanned as an alternative with a higher/lower DCF value. Confirm that the correct DCF value is entered in EPIC for the chosen slide (Figure 7). Sometimes neither slide is deemed adequate, and the phycologist can choose to re-process the sample, obtaining a different DCF, in order to obtain a more readable diatom slide.

Step 3: Using the diamond scribe, scribe a vertical transect line in an area free of optical distortions and avoids the cover slip edges. A horizontal “start” line is scribed to mark the beginning of the vertical transect and the count proceeds along the right side of the transect line, as viewed through the microscope (Figure 8). Another horizontal line is scribed at the “end” of the transect when counting along the transect ceases. Record transect location, start, and end locations using the X/Y coordinates on the stage Vernier scale (e.g., transect 1 start 38/109, transect 1 end 38/93). Several transects may be required to attain the target count of 600 valves; each transect should be scribed as described above. Record these data in the Sample Comments tab, in the Comments field within EPIC (Figure 9). Record the total length and number of transects when the sample identification and enumeration are completed (Figure 9).

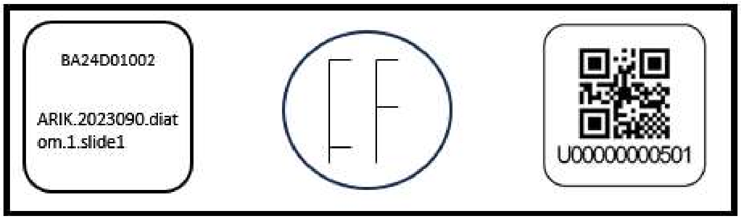
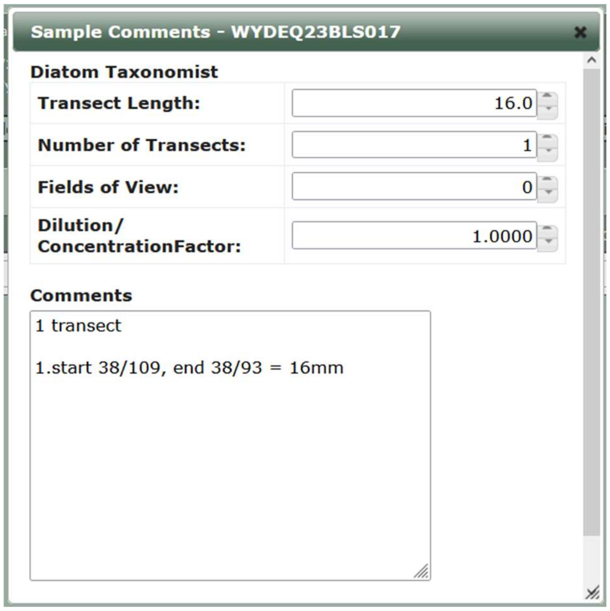


Figure 8. *Example of scribed lines on cover slip. Vertical scribed lines are viewed top-to-bottom on the right-hand side of the transect line. Starting and ending points are indicated by horizontal scribed lines. RAI slide label on the left side of coverslip and NEON unique barcode to the right of the coverslip.*



Sample Comments - WYDEQ23BLS017

Diatom Taxonomist

Transect Length:	16.0
Number of Transects:	1
Fields of View:	0
Dilution/ ConcentrationFactor:	1.0000

Comments

1 transect

1.start 38/109, end 38/93 = 16mm

Figure 9. *Example of transect metadata entered into Sample Comments tab in Rhithron diatom data entry interface EPIC. Fields of view are not recorded using the transect method. Dilution/Concentration Factor is not recorded here.*

Step 4: A diatom valve or frustule (2 valves) is counted if greater than half of the specimen is within the FOV (Figure 10); this includes specimens lying at the edge of the FOV as well as broken valves. A fragment is given a count of 1 if over half of the valve is visible within the FOV, including the central area. For raphid species, both central raphe endings should be visible.

Step 5: Identify diatoms to the lowest possible taxonomic level, generally to species, by

referencing the client voucher flora. As identifications proceed, enter taxon names, counts, and comments into the Taxon, Count, and Comments fields, respectively, in the Taxonomic Data Entry interface, which is part of the EPIC software. The Taxonomic Data Entry interface is error-protected for taxonomic nomenclature. For specific EPIC data entry procedures, see the EPIC Instruction Manual for Rhithron Phycologists(Rhithron 2014)**.** Taxa that are not in the on-going client voucher flora are documented with images (see recommendations in section **Digital images and voucher floras**), identified following standard taxonomic references, and assigned current nomenclature (e.g., Spaulding et al 2021, Cantonati et al. 2017). Rhithron's taxonomic resource library includes an extensive collection of books and documents in hard copy/electronic form on the Rhithron network server. Taxa that are not found in this resource library are identified to genus and assigned provisional names (e.g., *Navicula* sp1) in the Comments field for the taxon entry. Occasionally, genus-level identification is uncertain (e.g., when the specimen is obscured); in these cases the specimen is designated as "undetermined", and names such as "Undetermined Centric" and "Undetermined Pennate" are used. These names should be used sparingly, and, ideally, only provisionally. Phycologists should use a combination of sample images, additional scanning of slides, and experience with taxa and existing vouchers to help achieve target-level identifications for these troublesome specimens.

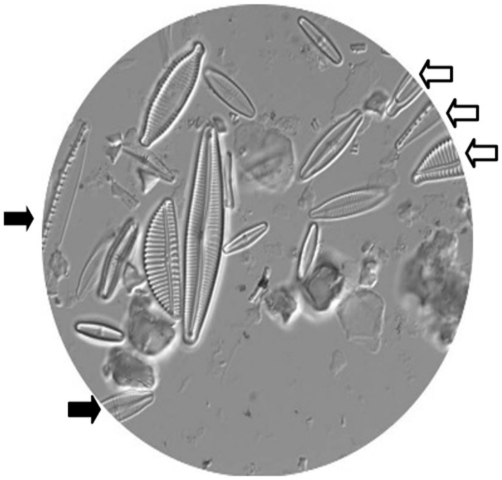


Figure 10. *Example of a field of view with arrows marking diatoms partially in view. Specimens marked with a solid black arrow would be included in the count, whereas those with a white arrow would be excluded.*

NEON WORKING GROUP NEEDS TO MAKE A DECISION ON HOW TO HANDLE NON-TARGET TAXA ENUMERATION (we suggest either going with step 6a OR step 6b)

Step 6a: (**NO AGGREGATION**) Diatom valves that are oriented in girdle (side) view, are damaged, or are obscured because of tilting or debris may not be able to be identified to target-level. These must be documented consistently during the analysis. Phycologists can make a temporary genus-level entry (e.g., *Nitzschia*) with a descriptive note (e.g., girdle, 20x3um cf *N. fonticola*) in the Comments field (See Table 1) in EPIC. Ideally, the identity of such specimens can be resolved as more specimens are confidently identified during the analysis. Situations do arise where assigning these girdle/obscured/damaged valves to a species cannot be made with confidence. A common example would be *Achnanthidium* girdle views where multiple species from the genus can have very similar girdles. For such a case, enter "*Achnanthidium*" and make a note "girdle" in the Comments field (Table 1). All girdle views that look similar will be counted in this entry throughout the entire analysis of the slide (e.g., 300 girdle valves counted). *Rhoicosphenia*, *Gomphonema,* and *Eunotia* girdle views, *Planothidium* and *Psammothidium* rapheless valves, and *Cocconeis* raphe valves are all possible situations that may require this approach. **Decisions on how to analyze non-target taxon entries (e.g., aggregations) will be left to the end user of the dataset**.

Table 1: *No aggregation of non-target taxa (i.e., girdle views not added proportionally to species entries)*

Taxon	Count	Comments
Achnanthidium minutissimum	90	
Achnanthidium rivulare	10	
Achnanthidium	300	Girdle

Step 6b: (**AGGREGATION**) Diatom valves that are oriented in girdle (side) view, are damaged, or are obscured because of tilting or debris may not be able to be identified to target-level. These must be documented consistently during the analysis. Phycologists can make a temporary genus-level entry (e.g., *Nitzschia*) with a descriptive note (e.g., girdle, 20x3um cf *N. fonticola*) in the Comments field for the taxon in EPIC. Ideally, the identity can be resolved as more species are confidently identified during the analysis. Situations do arise where assigning these girdle/obscured/damaged valves to a species cannot be made with confidence. A common example would be *Achnanthidium* girdle views where multiple species from the genus can have very similar girdles. For such a case, enter a <u>temporary entry</u> of "*Achnanthidium*" and make a note "girdle" in the Comments field for that taxon. All girdle views that look similar will be counted in this entry throughout the entire analysis of the slide (e.g., 300 counted). In this example, two *Achnanthidium* species are identified in the sample (e.g., *A.minutissimum* and *A.rivulare*): the *Achnanthidium* in girdle view could be either of these species. Upon completion of sample identification to the specified target count, genus-level girdle view counts are assigned to the 2 species identified, in proportion to the relative abundance for each confirmed species. For example, if *A.minutissimum* and *A. rivulare* had counts of 90 and 10 respectively, and if there were 50 genus-level counts of *Achnanthidium* "girdle", 45 of these 50 counts are assigned to *A. minutissimum*, and the remaining 5 are assigned to *A. rivulare*. A. minutissimum now has a

count of 135, and A. rivulare has a count of 15, and the genus-level *Achnanthidium* “girdle” entry is deleted (Table 2). *Rhoicosphenia*, *Gomphonema* and *Eunotia* girdle views, *Planothidium* and *Psammothidium* rapheless valves, and *Cocconeis* raphe valves are all possible situations that may require this outlined approach. High numbers of girdle/obscured counts that are processed this way should be noted in the taxon entry Comment field (e.g., added 45 *Achnanthidium* girdles, Table 2).

Table 2: *Aggregation of non-target taxa (i.e., girdle views added proportionally to species entries)*

Taxon	Count	Comments
Achnanthidium minutissimum	135	Added 45 Achnanthidium girdles
Achnanthidium rivulare	45	Added 5 Achnanthidium girdles

Step 7: Count each specimen along the scribed transect(s) until the target count is achieved for each sample. Enter taxon names, counts, and other information as you identify and count specimens. Continue identifications and counts until any one of the following criteria is met: 1) the target count is reached OR 2) a maximum of **5 transects is analyzed, OR 3) 4 hours, excluding time spent imaging and/or learning new taxa, is spent in the analysis.**

Step 8: When counts and identifications are complete, attach the barcode sticker to the analyzed slide, on the right side of the coverslip (opposite Rhithron sample label) (Figure 8). Barcode numbers for each sample are recorded in the shipping manifest by the Intake Specialist when slides are sent to the Biorepository at Arizona State University. Additional slides that were not analyzed will be archived at the NEON biorepository at ASU.

Digital images and voucher floras

Images should be taken during analysis to document new taxa and improve identification accuracy and precision. In general, the following recommendations should be considered:

- Images are needed for **all new taxa that are not documented in the client diatom voucher.** If a diatom voucher flora does not yet exist for a client, the first project voucher created is copied and saved as the client voucher that will be referenced and added to for future projects. A subfolder for the new client voucher flora should be created in the appropriate client folder on the server. Use a descriptive name for the subfolder, such as NEON23_Diatom Voucher Flora.
- Record the cover slip XY coordinates for each new taxon in the Reference Collection tab (see Figure 10). For more details, see the section: Metadata for image reference collections, below.
- In addition to new taxa, images should be taken for 1) difficult taxonomic groups (e.g., *Fragilaria, Gomphonema, Nitzschia, Sellaphora*), 2) any taxa that require further scrutiny and “keying out” in references, or 3) specimens that closely fit existing species concepts in the client voucher but have attributes that “expand” the concept.
- Images should be of sufficient quality to illustrate the important morphological characteristics that define the species. Ask yourself, “Could I really ID this specimen to species with this image?”. If in doubt, share the image with another phycologist to get some feedback. Bad images are not worth reviewing or adding to a voucher.
- Capture images using the grey scale setting in the camera software.
- Always include a scale bar within the image. Place the scale bar in the lower right corner of the image. The scale bar should be 10 microns long and vertically oriented.
- Name the image file in the following format Taxon_Sample ID_Magnification_YearMonthDay. For example: Gomphonema parvulum_NEON24001_1000x_20240721.
- Export images into the project subfolder (e.g., NEON24_Raw Diatom Images) on the Rhithron server.

Step 1: Review raw diatom images for the project in the project subfolder on the Rhithron server. Decide which images/taxa should be processed and included in the project voucher flora. Priority is given to taxa that are 1) not currently documented in the on-going client voucher flora or 2) abundant taxa in extremely difficult taxonomic groups. Inclusion in the project voucher flora is not limited to these taxa; phycologists can include as many taxa as they like in the project voucher. Remember to occasionally check the taxonomic concepts in the on-going client voucher flora to make sure the species concept has not drifted during current analysis.

Step 2: Process selected images and arrange them into the project voucher flora using the GIMP software. Details for how to process images and create a voucher are outlined in materials provided by DONA Diatom Web Academy workshop, May 2020 (https://diatoms.org/news/online-voucher-flora-workshop)(Lee *et al.* 2020). Arrange taxa images by genus or morphology (depending on number of images) into separate flora plates (e.g., NEON24_Achnanthidium_plate1.psd, NEON24_Surirelloids_plate1.psd). Differences in camera age and quality on Rhithron's scopes requires that GIMP-processed images (Scaled Image in X and Y resolution in GIMP) should have a standardized resolution of 72 dpi.

Step 3: The images in a voucher flora must have the current taxon name to the top right of the species images (Figure 11). Record taxonomic concept references, name changes in client voucher flora, and additional notes below the image (Figure 11). Specifically, the taxonomic concept should be referenced with details (e.g., plate, figures) from publications (see example Figure 12C,E), or the plate name and OTU from publicly available voucher flora (see example Figure 12A,D).

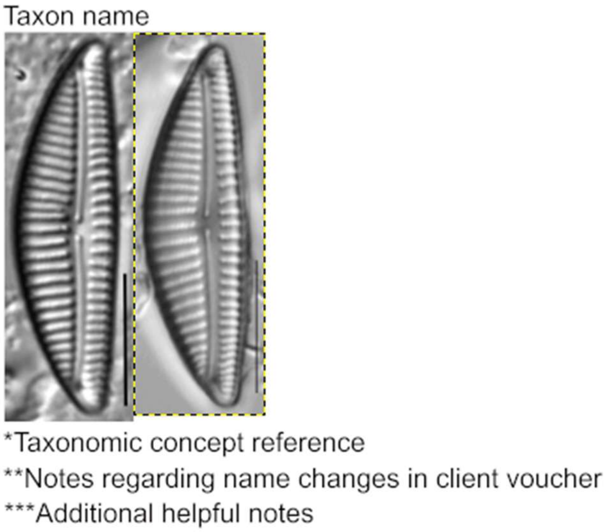


Figure 11. *Labeling and notes template for a taxon in a voucher flora plate.*

Step 4: Email all phycologists when the project voucher flora is complete, and request reviews by at least two.

Step 5: Review comments and requests for additional raw images to aid in a further review should be sent via email. A meeting to discuss opinions is held to evaluate all the review comments.

Step 6: Once a consensus is reached for a new taxon it can be added to the on-going client voucher flora. The voucher flora is consulted and cited (e.g., NEON_Achnanthidium_plate1.psd) for future identifications. Consultation with external phycologists may be required if a consensus cannot be reached on the naming of a new taxon.

Step 7: Create a "species complex" if necessary. A complex or "slash group" may be warranted if phycologists cannot agree on how to separate morphotypes consistently (e.g., *Achnanthidium minutissimum* complex, Figure 12A).

Step 8: Export the finished project voucher flora in two formats: psd and jpg. A GIMP specific format ".psd" will allow users to copy/paste images into the on-going client voucher flora. The shareable static version ".jpg" will allow voucher flora plates to be delivered easily to clients upon completion of the project.

Updates to client voucher flora

Updates to species concepts in the client voucher flora are often needed, given the ever-evolving field of diatom taxonomy and interpretation of species concepts. It is essential that the timing/date of concept changes be documented in the on-going client vouchers. Several situations that lead to revision of concepts in client voucher floras include:

- Name change in the literature (i.e., a name change based on the same type specimen, see S. Spaulding DONA post on 23 May 2023)(Spaulding 2023a; b). Cited literature can be a recent publication or new literature discovered by Rhithron phycologists. A descriptive note should be made in the client voucher beneath the taxon images (e.g., 2010-2017 reported as *Diatoma mesodon*, updated 27 February 2018 to *Odontidium mesodon,* Figure 12B).
- A newly described taxon is published which clearly resembles an existing taxon in the client voucher flora. A descriptive note should be made in the client voucher beneath the images (e.g., 2014-2020 reported as *Gomphosphenia* sp 1, updated 12 January 2021 to *Gomphosphenia praegnans*, Figure 12E).
- Two species (or more) in the client voucher are deemed "problematic" for consistent separation and need to be combined. Typically, the problematic species do not have solid distinguishing features visible with DIC optics that are resolvable among the phycologists. The issue often becomes apparent during review of internal QCs and subsequent years of reviewing and using the client voucher. A descriptive note should be made in the client voucher beneath the combined images (e.g., 2008-2010 reported separately as *G. parvulum* and *G. exillissimum*, combined 6 May 2011 into *Gomphonema parvulum* complex, Figure 12D).
- A single species needs to be split into 2 species (or more) in the client voucher due to the realization that solid distinguishing features are apparent and agreed upon by the phycologists. Often this realization comes about due to a recently published or discovered reference that offers clarity and guidance. A descriptive note should be made in the client voucher beneath the images of the taxa that were split out (e.g., 2009-2017 reported as *Achnanthidium minutissimum*, updated 3 March 2018 to *Achnanthidium affine*, Figure 12C).

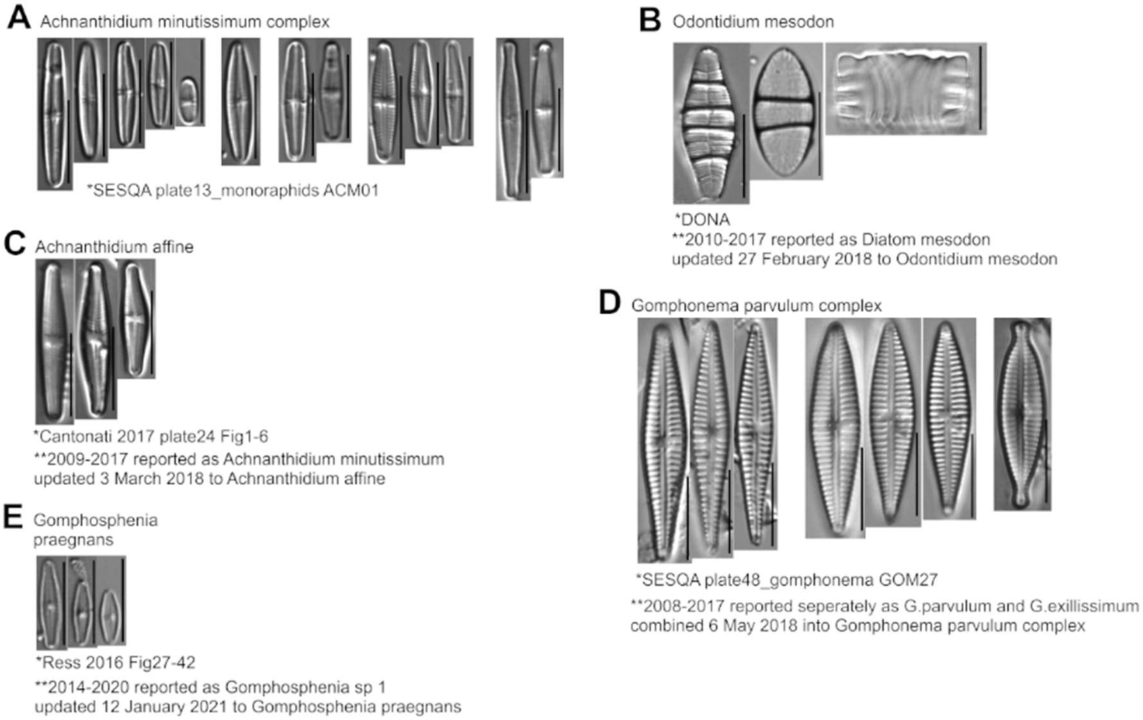


Figure 12. *Example of an on-going client voucher with taxonomic concept references and the types of descriptive notes for recording name/nomenclature changes.*

Metadata for image reference collections

Additional metadata is documented for all new taxa identified in samples. Metadata for a new taxon includes image location, image magnification, XY coordinates of a representative specimen, and taxonomic references.

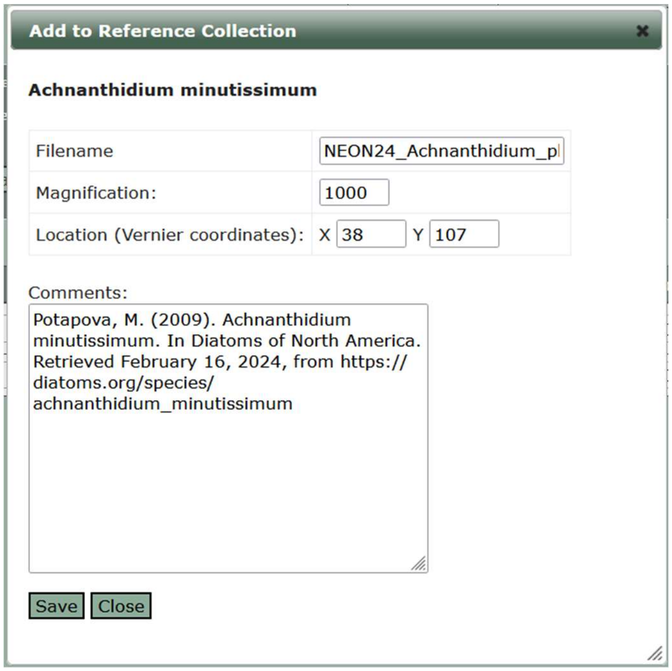
Step 1: Add metadata for a new taxon entry by clicking on the plus symbol under the Reference column in EPIC. All metadata recorded in steps 2-5 need to be entered into this Reference Collection tab (Figure 13).

Step 2: Record the magnification used to take the diatom image (typically 1000X).

Step 3: In the Location (Vernier coordinates) field, record the Vernier (XY) coordinates to pinpoint the specimen location on the slide. **Record XY coordinates immediately when encountering a new taxon for the client voucher** as it is time-consuming to “back track” to the specimen location.

Step 4: Record the taxonomic reference used for identification in the Comment field (Figure 13). Include the full citation information available for the reference (e.g., Potapova, M. (2009). Achnanthidium minutissimum. In Diatoms of North America. Retrieved February 19, 2024, from https://diatoms.org/species/achnanthidium_minutissimum). Commonly referenced USGS voucher floras listed on DONA’s voucher flora archive webpage (https://diatoms.org/practitioners/voucher-flora-archive) (Spaulding *et al.* 2021) can be cited with abbreviations (SESQA, NESQA, PNSQA), however include plate number (e.g., gomphonema1_MERGEreconcile_PNSQA) and the OTU code (e.g., PNSQA GOM52).

Step 5: In the Filename field, record the location of the reference image. Images in voucher floras will have the name of the specific project plate where the image/taxon is located (e.g., NEON24_Achnanthidium_plate1.jpg).



Add to Reference Collection

Achnanthidium minutissimum

Filename	NEON24_Achnanthidium_pl
Magnification:	1000
Location (Vernier coordinates):	X 38 Y 107

Comments:

Potapova, M. (2009). Achnanthidium minutissimum. In Diatoms of North America. Retrieved February 16, 2024, from https://diatoms.org/species/achnanthidium_minutissimum

Figure 13. *Example of metadata entered for a new taxon.*

Quality Assurance and Quality Control

QA/QC summary

To ensure project integrity, these important rules are followed:

- Phycologists work as a team and continually communicate to improve data quality for the entire project.
- Phycologists adhere to the client-specified protocol (e.g., target valve count, taxonomic resolution, new taxa/image documentation).
- Project images and voucher flora are reviewed by all Rhithron phycologists, to assure that identifications are accurate and consistent.

- All client-requested deliverables are provided, including citations for reference materials and project/client voucher floras.
- All calculations are double-checked for accuracy.
- A summary of QA/QC procedures and results are documented and delivered to the client along with a summary of sample processing procedures.
- Re-identification and enumeration of the same transect(s) is conducted for at least 10% of samples to determine comparability of sample analysis, such as the Percent Taxonomic Disagreement parameter (PTD), (Stribling *et al.* 2003, 2008)). Rhithron follows the NRSA 2023-2024 QAPP minimum data quality objective (MDQO), which is PTD ≤25% (USEPA 2022).

Internal re-identification of identified transect(s)

Ten percent of samples from a client's project are identified by a second Rhithron phycologist along the same scribed transect line(s) that were used in the original identification. QC samples are randomly selected, and the re-identification proceeds as a "blind" examination, that is, the QC phycologist does not have access to the original identification data. The initial identifications are compared with the QC identifications, and the Percent Taxonomic Disagreement (Stribling *et al.* 2003) parameter is calculated (Figure 14).

Percent Taxonomic Disagreement is calculated as:

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

Where $comp_{pos}$ is the number of agreements and N is the total number of taxa identified.

Additionally, the relative percent difference in transect lengths (RPDTL, Figure 14) is calculated to determine if the same transects were read. No MDQO exists for the relative percent difference in transect length parameter. Taxonomic and transect length discrepancies are examined and discussed between the original phycologist and the QC phycologist.

Relative Percent Difference of Transect Length (RPDTL) is calculated as:

$$RPDTL = \left(\frac{(TL_1 - TL_2)}{\frac{(TL_1 + TL_2)}{2}} \right) X\ 100$$

Where TL_1 and TL_2 are the Transect Lengths in mm reported from the primary and secondary taxonomists respectively.

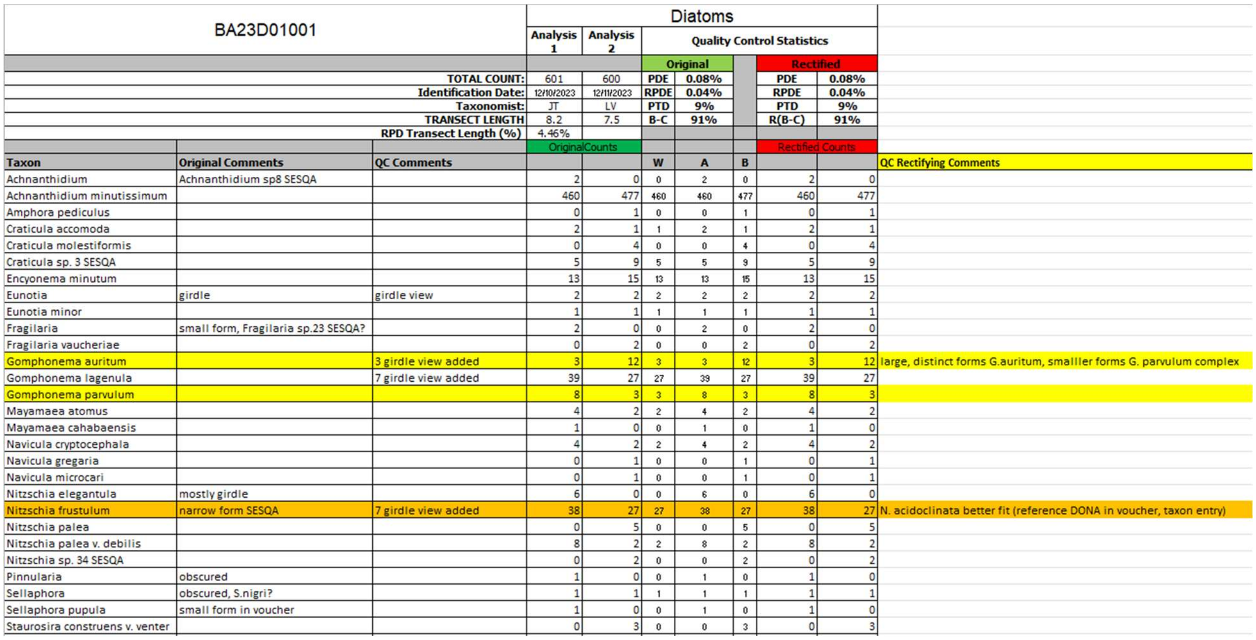
$$PDE = \left(\frac{Count_1 - Count_2}{Count_1 + Count_2} \right) X\ 100$$

Where Count 1 and Count 2 are the Sum total of all diatoms enumerated by each phycologist.

$$PTC_{abs} = ABS\left(\left(\frac{Target_{count1}}{Total_{count1}}\right) - \left(\frac{Target_{Coun}}{Total_{count2}}\right)\right)$$

Where the Target counts are the number of individuals identified to the required taxonomic target (usually species) for each respective taxonomist, and Total Counts are the total number of individuals identified in the sample from each respective taxonomist.

Difficult taxa are further discussed with the entire phycology team to come to a consensus on the handling of the taxa, and to provide a basis for taxonomic precision. Data changes or nomenclatural edits are applied to the entire project dataset, not just the sample that underwent QC. Changes to taxonomic concepts in the client voucher flora may also be applied warranted.



BA23D01001			Diatoms							
			Analysis 1	Analysis 2	Quality Control Statistics					
					Original			Rectified		
TOTAL COUNT:			601	600	PDE	0.08%		PDE	0.08%	
Identification Date:			12/10/2023	12/11/2023	RPDE	0.04%		RPDE	0.04%	
Taxonomist:			JT	LV	PTD	9%		PTD	9%	
TRANSECT LENGTH			8.2	7.5	B-C	91%		R(B-C)	91%	
RPD Transect Length (%)			4.46%							
			OriginalCounts					Rectified Counts		
Taxon	Original Comments	QC Comments			W	A	B			QC Rectifying Comments
Achnanthidium	Achnanthidium sp8 SESQA		2	0	0	2	0	2	0	
Achnanthidium minutissimum			460	477	460	460	477	460	477	
Amphora pediculus			0	1	0	0	1	0	1	
Craticula accomoda			2	1	1	2	1	2	1	
Craticula molestiformis			0	4	0	0	4	0	4	
Craticula sp. 3 SESQA			5	9	5	5	9	5	9	
Encyonema minutum			13	15	13	13	15	13	15	
Eunotia	girdle	girdle view	2	2	2	2	2	2	2	
Eunotia minor			1	1	1	1	1	1	1	
Fragilaria	small form, Fragilaria sp.23 SESQA?		2	0	0	2	0	2	0	
Fragilaria vaucheriae			0	2	0	0	2	0	2	
Gomphonema auritum		3 girdle view added	3	12	3	3	12	3	12	large, distinct forms G.auritum, smalller forms G. parvulum complex
Gomphonema lagenula		7 girdle view added	39	27	27	39	27	39	27	
Gomphonema parvulum			8	3	3	8	3	8	3	
Mayamaea atomus			4	2	2	4	2	4	2	
Mayamaea cahabaensis			1	0	0	1	0	1	0	
Navicula cryptocephala			4	2	2	4	2	4	2	
Navicula gregaria			0	1	0	0	1	0	1	
Navicula microcari			0	1	0	0	1	0	1	
Nitzschia elegantula	mostly girdle		6	0	0	6	0	6	0	
Nitzschia frustulum	narrow form SESQA	7 girdle view added	38	27	27	38	27	38	27	N. acidoclinata better fit (reference DONA in voucher, taxon entry)
Nitzschia palea			0	5	0	0	5	0	5	
Nitzschia palea v. debilis			8	2	2	8	2	8	2	
Nitzschia sp. 34 SESQA			0	2	0	0	2	0	2	
Pinnularia	obscured		1	0	0	1	0	1	0	
Sellaphora	obscured, S.nigri?		1	1	1	1	1	1	1	
Sellaphora pupula	small form in voucher		1	0	0	1	0	1	0	
Staurosira construens v. venter			0	3	0	0	3	0	3	

Figure 14. *Internal diatom QC analysis example for re-identification of same transect(s). Calculations are made in Excel within locked cells in order to avoid miscalculation of QC metrics. Important fields to note in the spreadsheet include the Analysis 1 and Analysis 2 fields, and the Quality Control Statistics fields. Analysis 1 includes the counts made in the original identification, and Analysis 2 includes the counts made by the QC phycologist. Where "0" occurs in one of these fields, a discrepancy in the identification of one or more specimens is indicated. In the Quality Control Statistics fields, calculations for the Percent Difference in Enumeration (PDE), and the Percent Taxonomic Disagreement (PTD), among other parameters, are given. Values for these parameters are given for a preliminary comparison of the two analyses in the Original (green filled) fields. During the QC process, taxonomic discrepancies are discussed. Discrepancies may be attributable to changes in nomenclature, further identification of difficult specimens, or duplication of entries, etc. If consensus is reached, the data are rectified to reflect the agreed-upon changes, which are elaborated in the Taxon and Comments fields. A*

comparison of the rectified data is then conducted, and quality control statistics are computed and reported in the Rectified (red filled) fields.

Table 3: *Quality control parameters, associated measurement quality objectives, and potential corrective actions.*

Quality Control Parameter	Measurement Quality Objective	Corrective Action
Percent Taxonomic Disagreement (PTD)	25%	Review of taxa of concern, make necessary data changes throughout sample set. Additional samples may need secondary identification. Voucher adjustments
Percent Difference in Enumeration (PDE)	1%	Discuss slides, optics and counting requirements review SOP- to assure that counting technique is faithfully followed.
Relative Percent Difference in Transect Length (RPDTL)	10%	Discuss slides, optics, and counting, External QC may result in higher RPDTL due to FOV widths among scopes
Percent Taxonomic Completeness Absolute Difference (PTCabs)	10%	Review of voucher concepts, discuss obscured views, or girdle views

External re-identification of identified transect(s) by external phycologist

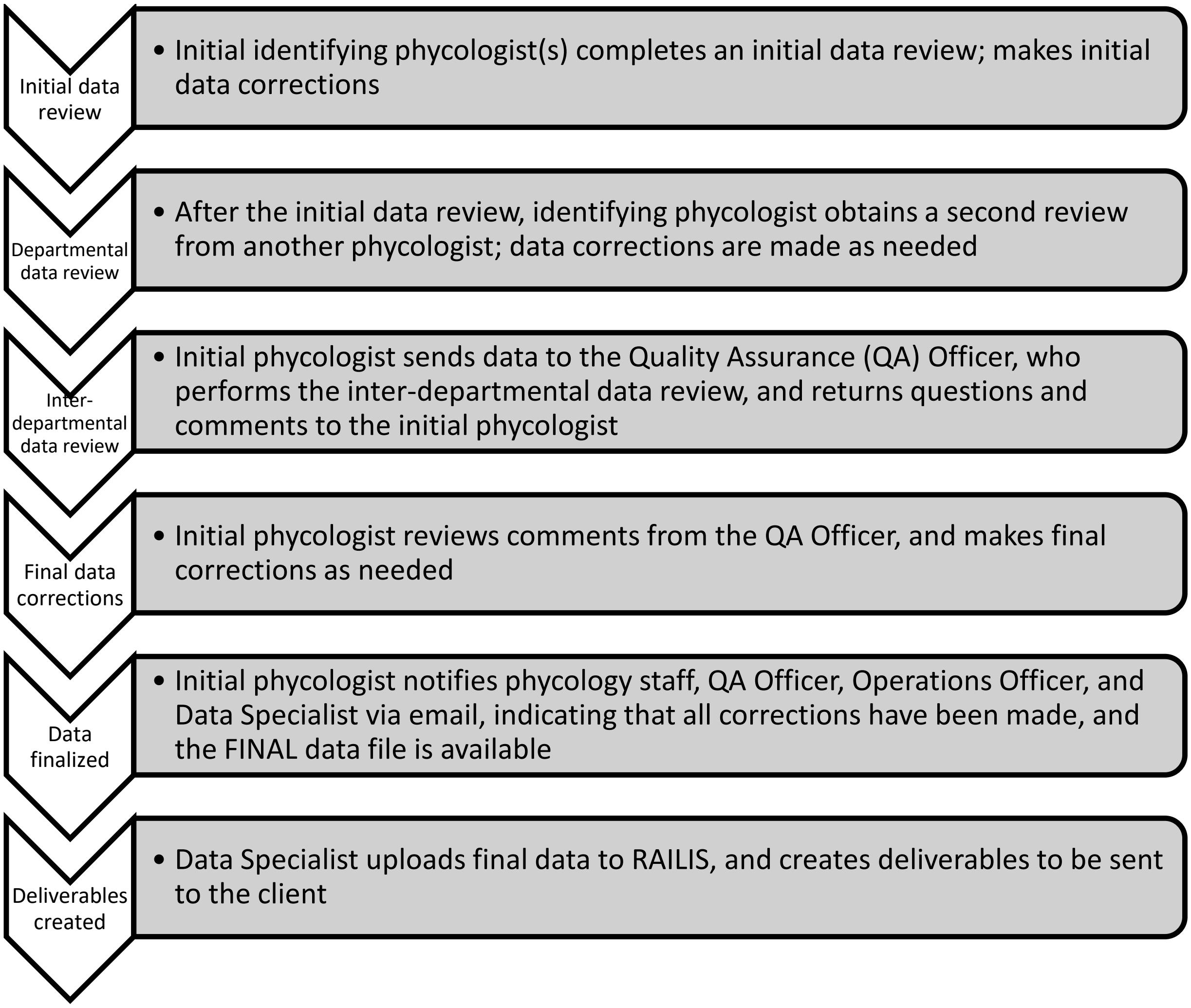
NEON WORKING GROUP NEEDS TO MAKE A DECISION ON WHETHER TO INCLUDE RE-ANALYSIS OF XX% SLIDES IN PROJECTS BY AN EXTERNAL/NON-RHITHRON PHYCOLOGIST. ADDITIONALLY, OR ALTERNATIVELY, AN EXTERNAL REVIEW OF PROJECT/CLIENT VOUCHERS COULD BE DONE PERIODICALLY.

Rhithron will send analyzed diatom slides to another phycologist for external QC upon client request. Pertinent information will be shared with the external phycologist so that, ideally, the re-analysis can be conducted exactly like Rhithron's internal QC procedures (e.g., same transects etc.). The project and on-going client voucher flora will be provided to the external QC taxonomist to review and use during re-analysis of the QC slides. Experience has shown that common issues that arise with external QC comparisons are 1) original transects are not located/followed and 2) microscopes have different field of view dimensions (note: all Rhithron phycologist scopes have 250-micron diameter FOVs at 1000X). These two issues complicate diatom QC comparisons and are documented when comparing the taxa lists and calculated similarity metrics. Rhithron phycologists review and discuss the results as a group. A summary presentation is drafted during the discussion that highlights QC metric scores, images of difficult/problematic taxa and potential actions to rectify the project data. Subsequently, the external QC summary is shared with the external phycologist. The summary is discussed to guide any changes that need to be made to project data and vouchers.

Data Review

Diatom data for each project undergoes a multistep review procedure, which results in thorough reviews and data corrections (Figure 15). Initial and second reviews are completed, and the data is sent on to Rhithron's Quality Assurance (QA) Officer. In addition to data review, the QA Officer highlights any new taxa in the project that were not previously on the client's taxa list or included in the voucher. The initial phycologist makes corrections and edits the data after each review. Common edits to diatom datasets include:

- Genus-level taxon entries that need a description in Comment field regarding why target-level was not reported. Description examples might include girdle views only, obscured valves, damaged, and provisional names (e.g., *Nitzschia* sp1).
- Nomenclature needs to be brought up to date.
- Metadata is missing or does not agree with the Project Inventory Report benchsheet. Project metadata, especially those data transcribed from benchseets to data fields, are evaluated for accuracy, precision and completeness during the initial data review by the original phycologist, secondary phycologists and the QC officer.
- Duplicate entries.
- Total count less than target without comment "maximum effort 5 transects/4h".
- Transect length discrepancy in Sample Comments.
- Review bench sheet entries that match data entry fields.



- Initial identifying phycologist(s) completes an initial data review; makes initial data corrections
- After the initial data review, identifying phycologist obtains a second review from another phycologist; data corrections are made as needed
- Initial phycologist sends data to the Quality Assurance (QA) Officer, who performs the inter-departmental data review, and returns questions and comments to the initial phycologist
- Initial phycologist reviews comments from the QA Officer, and makes final corrections as needed
- Initial phycologist notifies phycology staff, QA Officer, Operations Officer, and Data Specialist via email, indicating that all corrections have been made, and the FINAL data file is available
- Data Specialist uploads final data to RAILIS, and creates deliverables to be sent to the client

Figure 15. *Data review flow chart.*

Instrument and equipment inspection, maintenance and calibration

All microscopes and equipment needed to process diatom samples are inspected regularly according to manufacture specifications. Rhithron contracts with Pacific Microsystems LLC, Seattle, Washington to perform annual maintenance on all microscopes. Digital image calibrations are routinely checked against stage micrometers to assess for potential drift in measurement accuracy.

Data Delivery and Sample Material Shipment

Finalized data are transferred from EPIC to RAILIS by the Data Specialist via an electronic upload. All data, including taxonomic data and metadata are formatted to the client’s specifications and uploaded to the NEON data repository. Labeled and barcoded material are retrieved from the phycologists, sample material barcodes are scanned and additional metadata from RAILIS are formatted to the necessary shipping manifest specifications. Sample shipping manifests are uploaded to NEON and sample materials are securely packaged and sent to the biorepository (ASU). Sample fractions will be in three fractions 1) air-dried 20mL glass scintillation vial of digested diatom material,2) slide that was used for the identification and enumeration, and 3) the duplicate slide that is not associated with any taxa count data. Scintillation vials will be packaged in flats of 100 (10x10) with cardboard dividers. Slides will be packaged in 100 count slide boxes (50 samples per box), additional padding will be added to slide boxes to assure minimal movement of slides during transport.

Accrual Year Data Review

After all samples from NEON's designated accrual year have been successfully processed a cumulative data review will commence. While similar to the project data reviews, special attention is paid to the voucher flora and consistency over the accrual year's samples. During this data review taxa are discussed from throughout the accrual year, and any previous years, to assure precision among sample sets. Any necessary data changes to sample taxa count information or vouchers are communicated to NEON staff. The image voucher is finalized and an associated taxa translation table (alias tracking) spreadsheet containing synoptic notes is created to accompany the version-controlled Voucher Flora. The new finalized voucher flora in both PDF and .psd formats are delivered to NEON biologists for archive.

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