AOS PROTOCOL AND PROCEDURE: AQUATIC MICROBIAL SAMPLING

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Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
A	01/21/2016	ECO-03455	Initial release, amalgamation of NEON.DOC.001200 and NEON.DOC.001201 and updated lake sampling depth guidelines.

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1 OVERVIEW

1.1 Background

This document describes the required protocols for conducting field sampling of microbes in aquatic habitats. Microbes mediate nutrient cycling in all habitats. Linking activity and community composition to chemistry measures will enable a mechanistic understanding of ecosystem function. Temperature, nutrient and carbon availability, physical dispersal in water flow, and competition control microbial community composition and activity so concurrent sampling ensures comparison between drivers and effects. Microbes also form biofilms in the benthos which are important to the productivity of the system. Collecting basic measures of biomass, enzymatic activity, and DNA will enable researchers and managers to assess changes in this key ecosystem group of organisms.

Aquatic microbes are different from those in terrestrial systems mainly due to dispersal across habitats. Stream flow transports bacteria downstream as well as into and between lakes. Additionally, large storm events can increase the similarity of microbial communities between sampling sites such as the inlet and outlet of a lake. The potentially large impact of stream flow on microbial communities has been incorporated into the NEON Aquatic Sample Strategy (RD[07]). Within the benthos, there is heterogeneity in community composition, particularly where substrata and flow rates differ. It is therefore important to ensure that microbes are collected using sterile technique, so that *in situ* diversity is preserved and cross contamination is minimized.

Concurrent sampling with environmental drivers as well as using sterile sampling techniques will enable comparison within a habitat and also across regions to determine patterns in biogeography as well as relationships with temperature changes or other habitat characteristics.

1.2 Scope

This document provides a change-controlled version of Observatory protocols and procedures. Documentation of content changes (i.e. changes in particular tasks or safety practices) will occur via this change-controlled document, not through field manuals or training materials.

1.2.1 NEON Science Requirements and Data Products

This protocol fulfills Observatory science requirements that reside in NEON's Dynamic Object-Oriented Requirements System (DOORS). Copies of approved science requirements have been exported from DOORS and are available in NEON's document repository, or upon request.

Execution of this protocol procures samples and/or generates raw data satisfying NEON Observatory scientific requirements. These data and samples are used to create NEON data products, and are documented in the NEON Scientific Data Products Catalog (RD[03]).

1.3 Acknowledgments

Collection techniques have been standardized to be consistent with the NEON water chemistry protocol and stream algal protocol. Collection techniques are based closely on the protocols of the USGS National Water Quality Assessment (Moulton et al. 2002), the EPA Rapid Bioassessment Program (Stevenson and Bahls 1999), Antarctic LTER programs (Lisle and Priscu 2004), Arctic Streams LTER program (Slavik et al. 2004), Methods in Stream Ecology (Lowe and LaLiberte 2006), and the University of Maryland Center for Environmental Studies Horn Point Laboratory, with the addition of the protocols of LaRouche et al. 2012. Lydia Zeglin (Kansas State University) and Lee Stanish (NEON FSU) also contributed to the methods provided in the following SOPs.

2 RELATED DOCUMENTS AND ACRONYMS

2.1 Applicable Documents

Applicable documents contain higher-level information that is implemented in the current document. Examples include designs, plans, or standards.

AD[01]	NEON.DOC.004300	EHS Safety Policy and Program Manual
AD[02]	NEON.DOC.004316	Operations Field Safety and Security Plan
AD[03]	NEON.DOC.000724	Domain Chemical Hygiene Plan and Biosafety Manual
AD[04]	NEON.DOC.050005	Field Operations Job Instruction Training Plan
AD[05]	NEON.DOC.014051	Field Audit Plan
AD[06]	NEON.DOC.000824	Data and Data Product Quality Assurance and Control Plan

2.2 Reference Documents

Reference documents contain information that supports or complements the current document. Examples include related protocols, datasheets, or general-information references.

RD[01]	NEON.DOC.000008	NEON Acronym List
RD[02]	NEON.DOC.000243	NEON Glossary of Terms
RD[03]	NEON.DOC.005003	NEON Scientific Data Products Catalog
RD[04]	NEON.DOC.001271	NEON Protocol and Procedure: Manual Data Transcription
RD[05]	NEON.DOC.003041	Datasheets for AOS Protocol and Procedure: Aquatic Microbial Sampling
RD[06]	NEON.DOC.001646	General AQU Field Metadata Sheet
RD[07]	NEON.DOC.001152	NEON Aquatic Sample Strategy Document
RD[08]	NEON.DOC.001154	AOS Protocol and Procedure: Aquatic Decontamination
RD[09]	NEON.DOC.003045	AOS Protocol and Procedure: Periphyton, Seston, and Phytoplankton Sampling
RD[10]	NEON.DOC.002905	AOS Protocol and Procedure: Water Chemistry Sampling in Surface Waters and Groundwater
RD[11]	NEON.DOC.001153	AOS Protocol and Procedure: Wadeable Stream Morphology Mapping

RD[12]	NEON.DOC.014048	TOS Protocol and Procedure: Soil Physical, Chemical, and Microbial Measurements
RD[13]	NEON.DOC.002494	Datasheets for AOS Sample Shipping Inventory
RD[14]	NEON.DOC.003046	AOS Protocol and Procedure: Aquatic Macroinvertebrate Sampling
RD[15]	NEON.DOC.003040	AOS Protocol and Procedure: Aquatic Plant, Bryophyte, Lichen, and Macroalgae Sampling
RD[16]	NEON.DOC.001197	AOS Protocol and Procedure: Bathymetry and Morphology of Lakes and Non-Wadeable Streams
RD[17]	NEON.DOC.002191	Datasheets for Secchi Depth and Depth Profile Sampling
RD[18]	NEON.DOC.002792	AOS Protocol and Procedure: Secchi Disk and Depth Profile Sampling in Lakes and Non-wadeable Streams

2.3 Acronyms

Acronym	Definition
^{o}C	Degrees Celsius
DI	De-ionized water
DNA	Deoxyribonucleic acid
HDPE	High-density polyethylene
L	Liter
LTER	Long Term Ecological Research
m	Meter
mL	Milliliter
µm	Micrometer
mm	Millimeter
PFD	Personal flotation device
qPCR	Quantitative polymerase chain reaction
qt	Quart
s	Second
USEPA	US Environmental Protection Agency
USGS	US Geological Survey

2.4 Definitions

Bryophyte: Aquatic moss, liverworts, or hornworts lacking true vascular tissues.

Cobble: Medium-sized rocks in the bottom, geologically defined as 64 to 256 mm diameter. Cobbles are larger than pebbles (4-64 mm), and smaller than boulders (>256 mm).

Epilimnion: Top layer of water of a stratified lake, denoted by highest temperatures and least dense water. Typically occurs in the summer.

Epilithon: Periphyton colonizing rock substrata.

Epipelon: Periphyton colonizing silt substrata.

Epiphyton: Periphyton colonizing surface of aquatic plants.

Epipsammon: Periphyton colonizing sand substrata.

Epixylon: Periphyton colonizing woody substrata.

Euphotic zone (or "Photic zone"): The upper layer of lake water where sunlight penetrates and photosynthesis can occur. Specifically, the depth to which 1% of surface light penetrates.

Hypolimnion: The dense bottom layer of a stratified lake that sits below the thermocline. This layer is cooler than the surface water and has less circulation.

Hydrograph: A diagram depicting the change in discharge (m^3) over a given time (s).

Integrated: A sample that is composed of multiple samples in the water column.

Littoral zone: Near-shore area of the lake/river, extends from the high-water mark to the shallow, submerged area of the lake/river; typically the area near shore where sunlight reaches the bottom.

Macrophyte: Aquatic plant with vascular tissues.

Metalimnion: The layer of water in a stratified lake that sits between the hypolimnion and the epilimnion. Often equated with the thermocline.

Pelagic: The part of the lake that is not near shore or close to the bottom.

Periphyton: Mixture of algae, cyanobacteria, microbes, and detritus that coats submerged surfaces in most bodies of water

Pool: An aquatic habitat unit in a river or stream created by local scour or impoundment and having a structural control. Pool water depth is above average, if all the running water in the stream was shut off, areas in the stream that would still hold water are pools. Pool water velocity is below average for the reach and because of that fine sediments deposit in pools . Pools are generally longer than they are wide (unless they are plunge pools), and are 1.5 x deeper at their maximum depth than they are at their crest.

Riffle: An aquatic habitat with swiftly flowing water but no surface water agitation, with relatively uniform flow.

Run: Shallow reaches flowing over rough bed material such as boulders and cobbles, creating ripples, waves, and eddies on the water surface.

S1 and S2: Locations of NEON aquatic sensors.

Sand: Small sediment particles, 0.25-4 mm diameter.

Secchi depth: Depth that visible light penetrates, usually approximately 10-15% of light transmission.

Seston: Organic and inorganic particles suspended in the water column.

Silt: Very small sediment particles, 3.9-62.5 µm diameter.

Step Pool: High-gradient streams (steep) where water cascades over a rock or woody snag, dropping into a pool. Due to the gradient and surrounding geology, this pattern continues down the stream step (drop)—pool—step—pool—etc.

Stratified: Layers within the system (e.g., warm and cold water layers indicate thermal stratification in a lake).

Thalweg: The portion of the stream channel through which the majority of the stream flow is transported. This is typically identified as the deepest portion of the flowing channel.

Thermocline: A distinct layer in a body of water where the change in temperature is more rapid than increasing depth - usually a change of more than 1 ^{o}C per meter. The denser and cooler layer below the thermocline is the hypolimnion, warmer upper layer is termed the epilimnion.

3 METHOD

The goal of the Aquatic Microbial Sampling Protocol is to determine structure and function of benthic and surface water microbial communities, and to provide data that can be linked to other AOS datasets.

Surface water microbes

Surface water microbes are collected 12 times per year in **wadeable streams** and 6 times per year in **lakes and non-wadeable streams**, at the same time and location as standard recurrent water chemistry samples (RD[10]). Surface water samples in lakes and non-wadeable streams are taken 6 times per year on the same day as standard recurrent water chemistry sampling. Sample timing is based on statistical analysis of environmental parameters. Details on sampling locations and timing are provided in the Surface Water Chemistry Sampling in Aquatic Habitats (RD [10]). Surface water samples are collected as 1) a preserved water sample for cell counts and 2) on filters for archive and analysis.

Benthic microbes

Benthic microbes are collected 3 times per year in **wadeable streams** at the same time and location as algal periphyton samples (RD[09], Appendix D). Benthic biofilms are collected in wadeable streams during periods of stable stream flow (e.g., when the stream is not flooding, defined as >3x median discharge for the preceding year) (Clausen and Biggs 1997, Stevenson and Bahls 1999) using a series of scrubbing procedures, depending on habitat and substratum type (Moulton et al. 2002). Additional details may be found in Periphyton and Seston Sampling in Aquatic Habitats (RD [09]). Microbial samples are collected both as whole sample (plant or sediment grab) and on filters for archive and analysis.

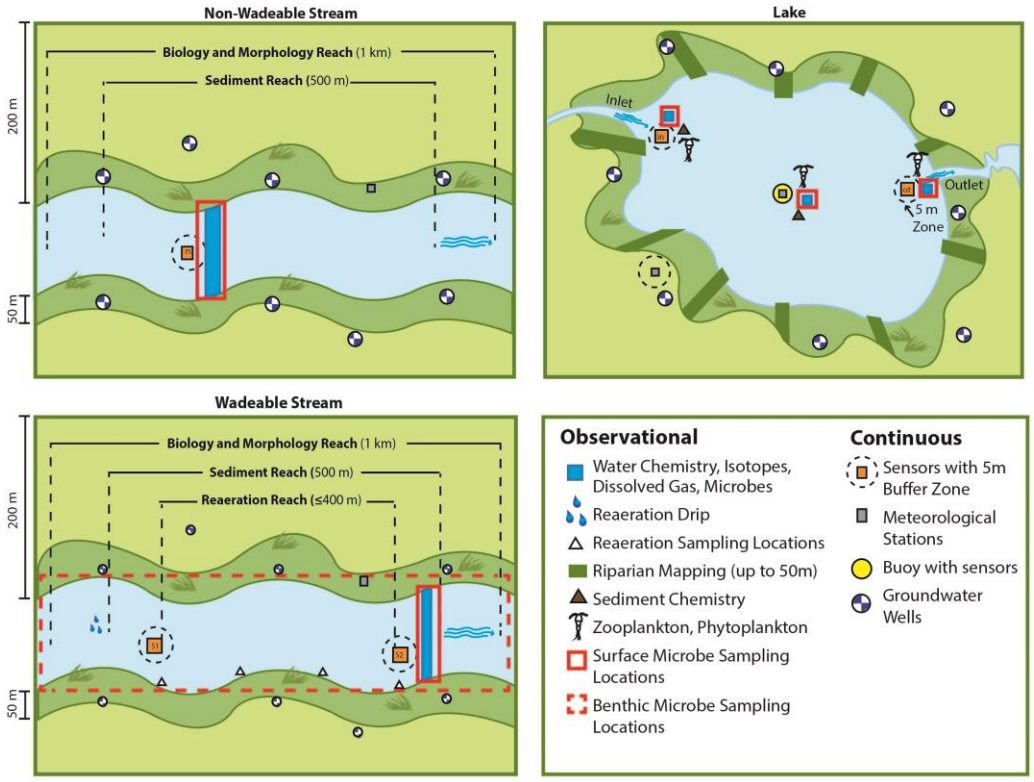

Figure 1. Generic site layouts with microbe sampling locations in red.

Standard Operating Procedures (SOPs), in Section 7 of this document, provide detailed step-by-step directions, contingency plans, sampling tips, and best practices for implementing this sampling procedure. To properly collect and process samples, field technicians **must** follow the protocol and associated SOPs. Use NEON's problem reporting system to resolve any field issues associated with implementing this protocol.

The value of NEON data hinges on consistent implementation of this protocol across all NEON domains, for the life of the project. It is therefore essential that field personnel carry out this protocol as outlined in this document. In the event that local conditions create uncertainty about carrying out these steps, it is critical that technicians document the problem and enter it in NEON's problem tracking system.

The procedures described in this protocol will be audited according to the Field Audit Plan (AD[05]). Additional quality assurance will be performed on data collected via these procedures according to the NEON Data and Data Product Quality Assurance and Control Plan (AD[06]).

4 SAMPLING SCHEDULE

4.1 Sampling Frequency and Timing

Surface water microbes

Surface water microbes will be collected 12 times per year in **wadeable streams** and 6 times per year in **lakes and non-wadeable streams**. Sampling will occur at the same time, location and sampling depth as standard recurrent water chemistry samples (RD[10]). Microbial samples may be collected either just before or just after chemistry sample collection as long as the water column remains undisturbed.

Benthic microbes

Benthic microbial sampling in **wadeable streams** occurs three times per year at each site, roughly spring, summer, and autumn. Benthic microbial samples are collected at the same time and location as periphyton samples (RD[09]) and may be collected before, after, or during periphyton sampling as long as they are collected from undisturbed substrate. Sampling must be scheduled within the first 21 days of the 1 month window specified in Appendix D with a minimum of two weeks between sampling dates. Accommodations for local weather conditions (e.g., late ice-off) may be made that cause the sample date to fall outside of the pre-determined window. Use NEON's problem reporting system to report sampling efforts that take place outside of the defined sampling window.

4.2 Criteria for Determining Onset and Cessation of Sampling

Surface water microbes

At **lake and non-wadeable stream sites**, sampling will occur with monthly or bi-monthly recurrent water chemistry sampling, including dates that require sampling under the ice at northern sites. At northern lakes, sample microbes with every other chemistry bout as the bi-monthly schedule changes due to ice-on and ice-off.

Benthic microbes

For **wadeable stream** benthic samples, a range of dates for each site were determined *a priori*, based on historical data including ice on/ice off, streamflow, the accumulation of degree days, weather, and riparian phenology (Appendix D). Benthic microbes will be collected together during periods of stable stream flow (Stevenson and Bahls 1999).

4.3 Timing for Laboratory Processing and Analysis

Filtered samples and grab samples (sediment and plant material) must be flash-frozen in the field and may be held at the domain lab at -80 ^{o}C for up to 30 days before shipping. Cell count samples must be

preserved in formalin in the field and may be held at the domain lab at 4 °C for up to 14 days before shipping. For additional storage and shipping timelines see SOP F.

4.4 Sample Timing Contingencies

All samples from one sampling bout must be collected within one day (i.e., all samples per site as detailed in this protocol). A minimum of 2 weeks between sample periods shall be observed.

Table 1. Contingent decisions

Delay/ Situation	Action	Outcome for Data Products
Hours	If weather conditions deteriorate and conditions become unsafe (e.g. approaching thunderstorm, rapid increase of water level in the **wadeable stream**), or the **lake/non-wadeable stream** becomes too windy (>32 km hr^{-1}) and has unsafe wave heights (>1 m) so that the boat cannot be held stationary over a sampling point while at anchor, return to shore and wait in a safe location for 30 minutes. If conditions improve, resume sampling, if not, return to the Domain Support Facility and sample at another time.	None as long as samples are collected within the pre-determined sampling window. If waiting for favorable conditions causes sampling to occur outside of the sampling window, data must be flagged.
	If circumstances occur that delay sampling (e.g., lightning), but sampling can be continued the same day while still meeting the streamflow or weather requirements, continue to collect samples after the delay. If conditions do not improve, discard any previously collected samples at the site or at the Domain Support Facility and start over on the next appropriate sampling day.	None as long as samples are collected within the pre-determined sampling window. If waiting for favorable conditions causes sampling to occur outside of the sampling window, data must be flagged.
14 Days	If flooding occurs on or prior to the targeted sampling date in a **wadeable stream** (>3x median discharge for the preceding year; Clausen and Biggs 1997) or unsafe wading conditions occur (Lane and Fay 1997), wait a minimum of 14 days after the water level drops below 3x median discharge and is safely wadeable and biofilm can recolonize.	None as long as samples are collected within the pre-determined sampling window. If waiting for flooding to diminish causes sampling to occur outside of the sampling window, data must be flagged.
	Preserved cell count samples may be held for a maximum of 14 days at 4 °C in the domain lab if circumstances do not allow shipping to the external lab.	Sample degradation is possible during the 14 day window, however data shipped within this window do not need to be flagged.

6 months	Frozen samples (filters, sediment, and plant material) may be held for up to 6 months at -80 ^{o}C in the domain lab if circumstances do not allow shipping to the external lab.	Holding samples >30 days affects external lab schedules, staffing, and budgets and delays data release on the NEON portal. However, sample integrity is not affected and samples do not need to be flagged if held for ≤6 months.

4.5 Sampling Specific Concerns

1. Care must be taken to avoid contaminating samples with re-suspended bed sediment.
   a. In **wadeable streams**, minimize contamination by entering the stream downstream of the sampling location.
      i. The sampling location should be located in a flowing section of the stream that is deep enough to sample without disturbing the stream sediments.
      ii. If sediments are disrupted, wait until the area has visually cleared before sampling.
   b. In **lakes and non-wadeable streams**, contamination may be minimized by anchoring the boat upwind (or upstream) of the sampling site, and using an anchor line 2 times as long as the depth of the lake or stream.
2. Equipment must be sterilized in the lab, and any equipment used for multiple samples in the field must be field-sterilized during sampling. Gloves are required to maintain the cleanliness of the sampling equipment and to decrease contamination of microbial samples by human or cross-site microbes while in the field.
3. Take care to keep track of the volume of water used to scrub the sample and the volume of water used for filtering, these data are very important for conversion to higher data products.
4. Failure to completely mix sample before filtering can result in skewed results. All subsamples are meant to be representative of one-another, so careful mixing is a necessity.
5. Should it begin to rain during **surface microbe** sampling, collect surface water samples in 4-L containers and return the containers on ice to the lab or base-camp where samples can be subsampled or filtered within 3-4 hours of sample collection.
   a. Water jugs must be shaken before sub-sampling or filtration to re-suspend particulates and homogenize water. If at any point you believe contamination has occurred during subsampling, discard samples and resample.
6. For **benthic microbe** sampling in **wadeable streams**, sampling too soon after a disturbance event (e.g., flooding, drying, or wildlife crossing a stream) can dramatically decrease biomass and diversity, be sure to wait at least 14 days for recolonization to occur.

5 SAFETY

This document identifies procedure-specific safety hazards and associated safety requirements. It does not describe general safety practices or site-specific safety practices.

Personnel working at a NEON site must be compliant with safe field work practices as outlined in the Operations Field Safety and Security Plan (AD[02]) and EHS Safety Policy and Program Manual (AD[01]). Additional safety issues associated with this field procedure are outlined below. The Field Operations Manager and the Lead Field Technician have primary authority to stop work activities based on unsafe field conditions; however, all employees have the responsibility and right to stop their work in unsafe conditions.

Technicians must wear protective nitrile gloves when handling microbial samples to prevent cross-contamination and to protect the technician from chemicals used in preservation. Safety Data Sheets (SDS) shall be made available for all chemicals used in this work (glutaraldehyde). Whenever chemicals are used, follow requirements of the site-specific Chemical Hygiene and Biosafety Plan (AD[03]) for laboratory safety and NEON EHS Safety Policy and Program Manual (AD[01]), Section HC-03, Hazard Communication.

See Section 10 in the NEON Operations Field Safety and Security Plan (AD[02]) for aquatic-specific field safety requirements. In addition, the following safety requirements must be followed:

1. Activities in **wadeable streams** should only be performed when flow conditions are safe. Do not attempt to wade a stream where velocity x depth is ≥ 10 ft^2/s (0.93 m^2/s; Lane and Fay 1997).
2. In **lakes and non-wadeable streams**, site-specific hazards may be encountered that cause technicians to conduct sampling from the boat, without dismounting from the vessel. In addition, technicians are required to use extra caution in waters where alligators are present and to make sure a safe distance from hazards is maintained.
3. When working around ice, refer to (AD[02], Section 10.3 Winter Water Safety. Do not attempt to walk on frozen lake is depth of ice is less than 6" (+15cm) or operate UTV or snowmobile on frozen lake if depth of ice is less than 8" (20cm). Use caution and good judgment to carefully evaluate site conditions including ice strength. Local guidelines from natural resource officials, property owners or hosts, and domain managers should be consulted regarding work on ice, prior to deploying employees and equipment. Do not continue if the risk is too great.
4. All personnel must be wearing a personal flotation device (PFD) prior to entering a boat.
5. All employees shall have access to a form of communication with other team members such as a two-way radio.
6. Technicians should be aware of any site-specific hazards and to the waters of that particular location (i.e. current status, tidal charts, water release from dams, etc.).

6 PERSONNEL AND EQUIPMENT

6.1 Equipment

The following equipment is needed to implement the procedures in this document. Equipment lists are organized by task. They do not include standard field and laboratory supplies such as charging stations, first aid kits, drying ovens, ultra-low refrigerators, etc.

Table 2. Equipment list – General equipment

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
RD[11] or RD[12]	R	Site-specific Stream Morphology or Bathymetry Map	Determining sampling locations	1	N
	R	Handheld GPS unit (with batteries, ±4 m accuracy) or Humminbird™	Navigating to sampling locations	1	N
	R	Clipboard	Recording data	1	N
	R	Cryogenic gloves	Handling dry ice	1 pair	N
	R	Cooler (9-28 qt)	Field sample storage; use size appropriate for number of samples being collected	2	N
	R	Waders (hip or chest) or knee boots	Boating or wading	1 pair per person	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Consumable items					
RD[06]	R	Aquatic Field Metadata Sheet	Recording metadata	1	N
RD[05]	R	Field datasheets (all-weather paper)	Recording data	1	N
	R	Pre-printed adhesive labels (all-weather, 1”x2”)	Labeling samples	20	N
	R	Pencils	Recording data	2	N
	R	Permanent markers	Labeling samples	2	N
	R	Ice or chemical ice packs	Keeping cell count samples cool	2	N
	R	Dry ice	Flash-freezing samples in the field	0.5-1 kg	Y
	R	Nitrile gloves, powderless	Sterile collection methods	10	N
	R	Resealable bags, gallon	Containing sterilized equipment	20	N

R/S=Required/Suggested

Table 3. Equipment list – Lab sterilization

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
	R	Wash basin	Container for sterilization solutions	1	N
MX100373	R	Peristaltic pump head	Filtering DI water	1	N
MX100383	R	18V drill pump	Filtering DI water	1	N
MX100364	R	Peristaltic pump tubing	Filtering DI water with peristaltic pump	1	N
MX100662	R	Flexible tubing, 3/8” inner diameter	Filtering water with peristaltic pump	2	N
MX100376	R	Tubing connector	Attaching C-flex to peristaltic tubing	1	N
	R	1 L HDPE bottle or 4 L HDPE jug with lid, sterilized	Container for filtered DI	1	N
Consumable items					
	R	Clean bench paper or paper towels	Storing/drying equipment	1 roll	N
	R	ETOH, 95%	Sterilizing equipment	1 L	Y
	R	Phosphate-free detergent (Alconox or Liquinox)	Sterilizing equipment	<1 gallon	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
MX107386	R	0.22 µm Sterivex SVGP® L10RC capsule filter with luer lock outlet	Filtering DI	2	N
	R	Deionized water	Rinsing equipment, preparing filtered DI	4 gallons	N
	R	Gallon zip top bags	Storing sterilized equipment	5	N

R/S=Required/Suggested

Table 4. Equipment list – Surface water microbe sampling equipment

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
MX100393	R	Kemmerer sampler with rope and messenger	**Lakes and non-wadeable streams**, collecting water	1	N
	S	Ice auger	**Lake** sampling under ice	1	N
	R	Sterile 4 L HDPE jug	Collecting or integrating samples	2-6	N
MX100662	R	Flexible tubing, 3/8” inner diameter	Filtering water with peristaltic pump	2	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
MX100376	R	Tubing connector	Attaching C-flex to peristaltic tubing	1	N
MX106253	R	Filter adapters for tubing (cut-off 3 mL syringe)	Connecting peristaltic tubing and C-flex tubing	2	N
MX100373	R	Peristaltic pump head	Filtering water	1	N
MX100383	R	18V drill pump	Filtering water	1	N
MX100364	R	Peristaltic pump tubing	Filtering water with peristaltic pump	1	N
	R	500 mL plastic graduated cylinder	Measuring filtered water volume	1	N
	R	10 mL adjustable pipette	Pipetting cell count samples into vials	1	N
	S	140 mL syringes	Alternative to peristaltic pump	2	N
Consumable items					
	R	Small zip tie or small hose clamp	Attaching flexible tubing to hose connector	1	N
	R	10 mL pipette tips	Pipetting cell count samples into vials	1 per station	N
MX106249	R	20 mL glass scintillation vials (cap not included)	Cell count sample container	1 per	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
				station	
MX107204	R	20 mL scintillation vial caps, polyethylene with cone-shaped liner	Cell count samples container lid	1 per station	N
MX107386	R	0.22 µm Sterivex SVGP® L10RC capsule filter with luer lock outlet	Microbe sample filters	2 per station	N
	R	Luer lock male closures	Capping the Sterivex® filters	4 per station	N
	R	Luer lock female closures	Capping the Sterivex® filters	4 per station	N
	S	Filtered DI (0.2 µm filter)	Field-sterilization and rinsing for multiple stations	1-4 L	N
	S	Alcohol pads, 70% isopropyl, individually-wrapped	Field-sterilization for multiple stations	20	N

R/S=Required/Suggested

Table 5. Equipment list – Benthic microbe sampling equipment (**wadeable streams** only)

Item No.	R/S	Description	Purpose	Conditions Used	Quantity	Special Handling
Durable items						
	R	Template (35 mm plastic slide cassette)	Sampling area for epilithon and epixylon scrubbing	Cobbles and wood	1	N
	R	Larval insect tray, plastic	Scrubbing container in which sample is collected	Cobbles and wood	1	N
	R	Wash bottle, unitary, 125 mL	Rinsing substrate and larval tray into syringe	Cobbles and wood	1	N
	R	140 mL HDPE syringe	Filtering samples	All	2	N
	R	Spatula (metal, flat)	Epipsammon and epipelon collection	Sand and silt	1	N
	R	Sterilized scissors	Epiphyton collection	Plant surfaces	1	N
	S	ETOH rinse bottle	Field sterilization	All	1	N
Consumable items						
	R	Filtered DI (0.2 µm filter)	Field sterilization and rinsing	All	1-4 L	N
	R	Alcohol pads, 70% isopropyl, individually-	Field sterilization	All	20	N

Item No.	R/S	Description	Purpose	Conditions Used	Quantity	Special Handling
		wrapped				
	S	ETOH, 95%	Field sterilization, refill ETOH wash bottle above	All	500 mL	Y
	R	Nylon toothbrushes for each habitat type (new toothbrush for each sample)	Epilithon and epixylon scrubbing	Cobbles and wood	≤8	N
MX106246	R	15 mL plastic centrifuge tubes with lids	Epipsammon and epipelon sample container	Sand and silt	≤8	N
	R	Whirl-paks®, 24 oz.	Epiphyton sample container	Plant surfaces	≤8	N
MX107386	R	0.22 µm Sterivex SVGP® L10RC capsule filter with luer lock outlet	Microbe sample filters	Cobbles and wood	≤16	N
	R	Luer lock male closures	Capping the Sterivex® filters	Cobbles and wood	≤16	N
	R	Luer lock female closures	Capping the Sterivex® filters	Cobbles and wood	≤16	N

R/S=Required/Suggested

Table 6. Equipment list – Sample processing and preservation

Item No.	R/S	Description	Purpose	Conditions Used	Quantity	Special Handling
Durable items						
MX106253	R	3 mL HDPE syringe, luer lock end	Filtering formaldehyde	Surface water, cobbles, and wood	1	N
	R	Freezer (-80 °C)	Sample storage	Sterivex® filters, whole sample grabs	1	N
Consumable items						
MX106257	R	10% formaldehyde, buffered (sodium borate or phosphate)	Preserving samples	Surface water, cobbles, and wood	1 L	Y
MX106239	R	0.2 µm Acrodisc® filter	Filtering formaldehyde	Surface water, cobbles, and wood	2	N

R/S=Required/Suggested

Table 7. Equipment list – Shipping materials

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
	R	Dry ice shipping container	Shipping filters	1	N
	R	Cooler, 9 qt.	Shipping cell count samples	1	N
Consumable items					
	R	Vermiculite, Grade 2	Absorbing liquid leaks and cushioning shipment	As needed	N
	R	Trash bag to line cooler (~13 gallon size)	Protecting against leaks	1	N
	R	Resealable plastic bags (gallon and quart size)	Protecting against leaks	6	N
	R	Dry Ice	Shipping filters	As needed	Y
	R	Ice or chemical ice packs	Shipping cell counts	As needed	N
RD[13]	R	Shipping inventory	Provides sample information to external lab	1	N

R/S=Required/Suggest

6.2 Training Requirements

Additionally, technicians must complete protocol-specific training for safety and implementation of this protocol as required in Field Operations Job Instruction Training Plan (AD[04]).

All personnel required to operate a boat shall be trained through an approved program. All other personnel using a boat shall be aware of boating safety procedures.

Personnel will be trained in field protocols associated with this document, and trained in safe working practices for aquatic field work. Technicians must also be trained in safe handling of formalin (AD[03]).

6.3 Specialized Skills

Where applicable, personnel will be licensed to operate a boat and able to safely handle a motor and drive a boat safely.

6.4 Estimated Time

The time required to implement a protocol will vary depending on a number of factors, such as skill level, system diversity, environmental conditions, and distance between sample plots. The timeframe provided below is an estimate based on completion of a task by a skilled two-person team (i.e., not the time it takes at the beginning of the field season). Use this estimate as framework for assessing progress. If a task is taking significantly longer than the estimated time, a problem ticket should be submitted.

Field sampling requires two technicians for three hours per site in addition to other chemistry and biological sampling occurring concurrently, plus travel to and from the site. There is no lab processing associated with this protocol.

7 STANDARD OPERATING PROCEDURES

SOP A Preparing for Sampling

1. Make sure all supplies are packed and peristaltic pump batteries are charged and the pump is in good working condition.
2. Create a filter adapter for the peristaltic pump.
   a. Remove the plunger and cut off the top of a 3 mL syringe (Figure 2).
   b. Connect 3/8" inner diameter C-flex tubing to the top of 3 mL syringe.

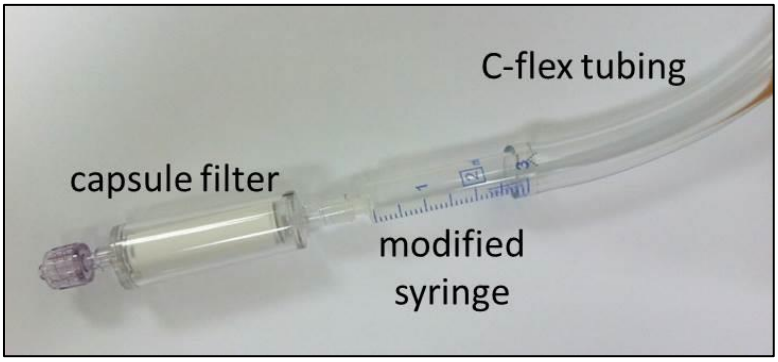

Figure 2. Filter adapter for peristaltic pump

3. Prepare filtered DI for rinsing.
   a. Filter DI using the peristaltic pump setup (with sterilized tubing) and a 0.2 µm Sterivex® filter.
   b. Store in sterilized 1 L HDPE bottle or 4 L jug for up to 2 weeks.
4. Sterilize any equipment that is reused between sampling bouts.
   a. Equipment to be sterilized includes:
      i. **Surface microbes**: 4 L jug, 140 mL syringe, graduated cylinder (if using prior to filtering), filter adapter (Figure 2), pump tubing, Kemmerer (**lakes and non-wadeable streams** only).
         1) Having an additional piece of peristaltic pump tubing, hose connector, and filter adapter that can be dedicated to microbe sampling (i.e., not used for water chemistry) may be easier for logistics.
      ii. **Benthic microbes (wadeable streams)**: 125 mL wash bottle, brushes, slide template, larval tray, spatula, scissors.
   b. Prepare workspace:
      i. Keep a labeled wash bottle of 70% ETOH on hand for rinsing.
      ii. Rinse the lab bench with 70% ETOH and allow to dry.
      iii. Spread bench paper on ETOH sterilized benchtop so you have a place to set things to dry.
   c. Wash in detergent solution.
      i. Prepare a 0.2% (v.v) solution of detergent and DI water in a wash basin.
      ii. Wearing nitrile gloves, submerge equipment thoroughly in detergent solution. Use bottle brush and/or hands to clean equipment.

d. Rinse with 0.2 µm filtered DI water.
   i. Remove equipment from detergent solution and rinse thoroughly in DI. Place on clean bench top paper.
e. Rinse with 70% ETOH.
   i. Allow to thoroughly dry (minimum drying time 5-10 minutes).
f. When dry, cap bottles and place all other equipment in clean gallon Ziploc bags to store or transport to the field.

5. Attach pre-printed labels (Figure 3, RD[05]) to bottles and capsule filters (Figure 4), use permanent marker to fill out labels as much as possible before going into the field. Leave filter volume blank until filter has been completed.
   a. Surface water samples
      i. **Wadeable streams** surface water: 1 sample
      ii. **Lakes** surface water: 3 samples
      iii. **Non-wadeable streams** surface water: 1 sample
   b. Benthic samples
      i. **Wadeable streams** benthic, dominant habitat: 5 samples
      ii. **Wadeable streams** benthic, secondary habitat: 3 samples

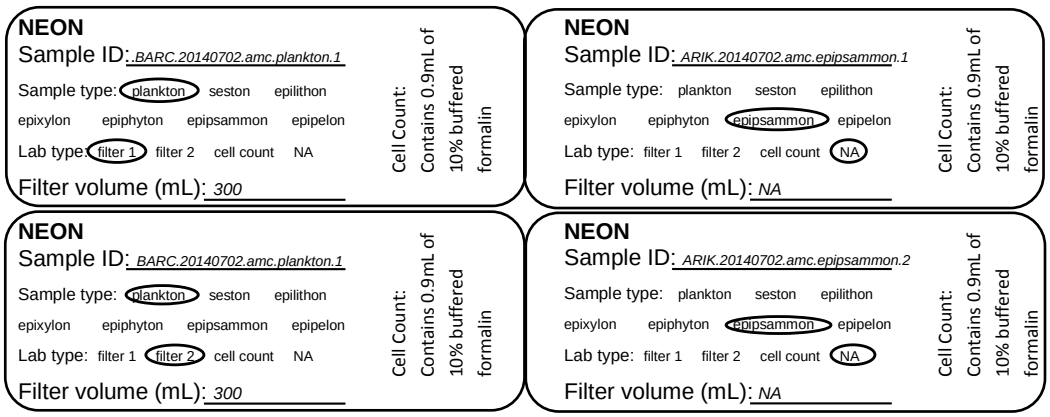

Figure 3. Example NEON microbes label. Adhere right side of label to filter or vial first, label will overlap on filters.

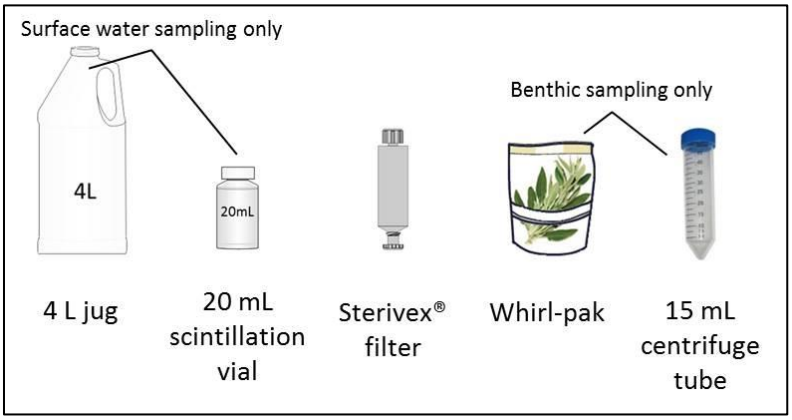

Figure 4. Sample containers.

6. Use the Acrodisc® 0.2 µm filter attached to a sterile 3 mL syringe, add filtered buffered formalin preservative to 20 mL vials for cell counts (0.9 mL per 18 mL sample) making sure to wear gloves.
7. Prepare coolers, frozen ice packs or water ice (cell counts), and dry ice (filters and grab samples).
8. Fill out the General Aquatic Field Metadata Sheet (RD[08]) upon every field sampling visit, and Secchi and Depth Profile Datasheet (RD[17]) in **lakes and non-wadeable streams**.
9. **Always wear nitrile gloves.** Field sampling methods should be as sterile as possible. Have a clean/sterile place to set equipment on the boat or stream bank. Plastic containers or organizers work well.

SOP B Field Sampling: Surface Water Microbes

B.1 Wadeable Stream Collection

1. Samples will be collected at one location near the **wadeable stream** S2 sensor set (ss).
   a. Collect surface water samples prior to or upstream of any benthic sampling.
2. Collect and integrate whole water samples in containers.
3. Sample in the same location where water chemistry samples are collected (ss). Confirm that no one is wading upstream during sampling.
4. **Wearing clean nitrile gloves**, rinse both 4 L HDPE bottles 3 times with stream water, shake vigorously. Discard rinse water into stream. Do not set bottle cap down as this increases the chance of contamination.
5. Standing in the thalweg, hold 4 L bottles several centimeters below the water surface with the opening pointed upstream. Tilt bottle slightly underwater to allow stream water to fill bottle. Fill one to two 4 L jugs to ensure sufficient water collection, any extra may be discarded.
6. Return to the stream bank and proceed to SOP B.3 for Surface Water Processing.

B.2 Lakes and Non-wadeable Stream Collection

SELECTING SAMPLING LOCATIONS

1. **Lakes** will be sampled at three locations in conjunction with water chemistry samples: the central/deepest part of the lake ('c0', 'c1', 'c2', or 'c3'), the inlet ('in'), and the outlet ('ot'). N**on-wadeable streams** will be sampled at the sensor (rs) in conjunction with water chemistry sampling.
2. Refer to Section B.2 in Water Chemistry Sampling in Surface Waters and Groundwaters (RD[10]).
3. Determine the stratification conditions at the buoy from the Secchi Disk and Depth Profile Sampling in Lakes and Non-Wadeable Streams (RD[18]), Section 7, SOP C.
4. Sample in the same locations and depths where water chemistry samples are collected RD[10]).
   a. Center (buoy)
      i. Unstratified ('c0'): 0.5 m (±5 cm)
      ii. Stratified (two samples: 'c1' and 'c2'): 0.5 m (±5 cm) **AND** the midsection of the hypolimnion depth.
   b. Inlet ('in'): 0.5 m (±5 cm)
   c. Outlet ('ot'): 0.5 m (±5 cm)
5. If sampling under ice, core through ice and sample as above as long as there is at least 0.5 m of water depth below the ice.
   a. If the water depth is <0.5 m, move to a location within 10 m of the original sampling location and note the new GPS location.
   b. If sampling at the inlet or outlet, do not move further than 20 m from the shoreline or below a depth of 1.5 m.

SAMPLE COLLECTION

1. Navigate to the sampling location. Gently lower anchors at the bow and allow boat to float back with wind or current to sampling location. Drop a second anchor at the stern to hold boat in place.
   a. Allow ~5 minutes for sediments to settle after lowering the anchor; you can use this time to prepare the sampling equipment.
   b. Use an bow anchor line 2 times the depth to prevent contamination from sediments.
   c. The boat must be anchored at the bow and stern in order to collect representative water column samples and prevent the boat from spinning.
2. Always sample near the bow of the boat to minimize the effects of the motor on the water column. When anchored, the bow of the boat tends to orient itself with the bow into the wind or current.
3. **Wearing gloves**, prepare the Kemmerer sampler (Figure 5) for sampling. Pull the stoppers from the collection cylinder by holding the top and bottom stopper and giving a short, hard pull to the bottom stopper.
   a. A short, hard pull is important to keep the stoppers open. If the stoppers don't stay open, pull harder.
4. Tie the free end of the Kemmerer line to a cleat on the boat to prevent losing the sampler.
5. Carefully lower the Kemmerer sampler over the side of the boat into the water. Hold the messenger in one hand. Ensure it is secured properly to the line.
6. Rinse the Kemmerer by dunking it in the water body to be sampled 3 times.
7. Continue to lower the Kemmerer sampler until it reaches the desired depth by using the depth markings on the line attached to the sampler.
8. When you reached the desired depth, gently move the sampler up and down to ensure water from the correct depth is in the sampler. Drop the messenger to release the clamps and seal the sampler.
9. Pull the sampler back into the boat and dispense sample water into the collection bottles through the spout (Figure 5a).

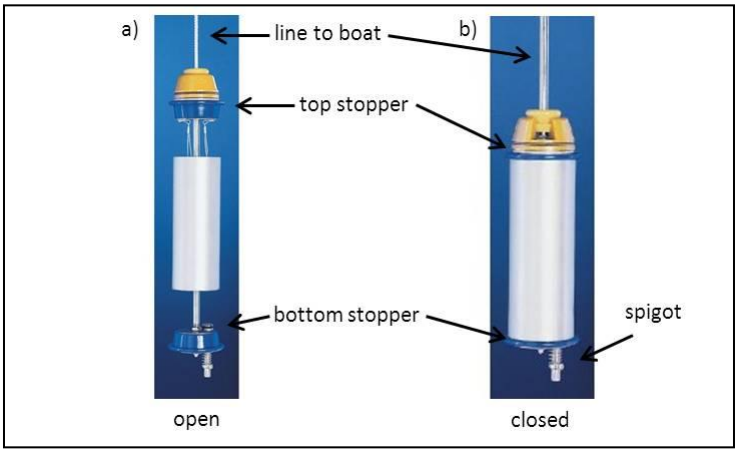

Figure 5. Vertical Kemmerer water sampler in the a) open or cocked position and b) closed (after dropping the messenger) position.

10. Rinse sample bottles (4 L jug) 3 times with source water at each location.
    a. Hold the bottle cap in a gloved hand, setting the cap down increases the risk of contamination.
    b. Discard rinsewater away from the location where you are sampling.
11. Collect samples to be integrated in 4 L jug.
12. Repeat for each sample, rinsing the Kemmerer with source water at each new location.
13. Proceed to Section B.3 for Surface Water Processing.

B.3 Surface Water Processing

Processing may be conducted at the domain support facility in case of inclement weather within 4 hours of collection provided 4 L jugs are appropriately labeled. Field processing and preservation is preferred. Two Sterivex® filters and 1 cell count vial are produced per surface water sample (Figure 6).

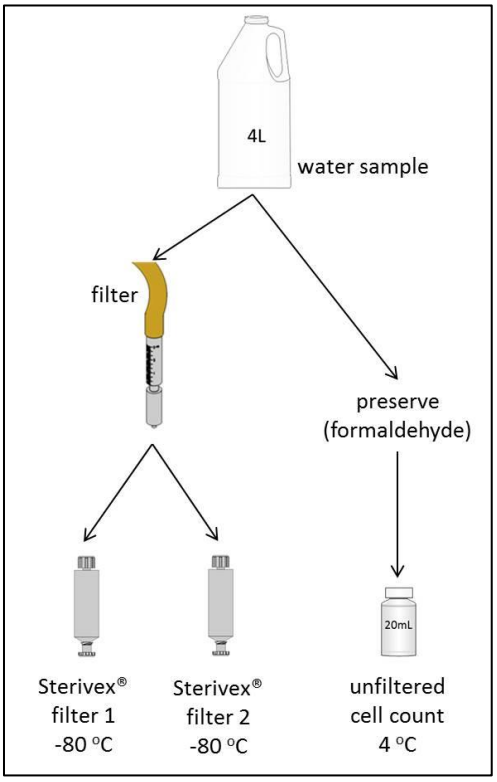

Figure 6. Schematic for surface water sample partitioning

CELL COUNTS

1. Shake 4 L jug to mix sample.
2. Use adjustable pipette with new sterile tips (if tips are not sterile, transport clean tips from the lab to the field in clean Ziploc bags) to transfer 18 mL of sample from 4 L jug to 20 mL glass scintillation

vial containing 0.9 mL buffered formalin preservative. Samples must be immediately preserved with formalin.

3. Recap scintillation vial and invert 5 times to mix.
4. Place cell count vial in cooler with ice packs, do not freeze.
5. Fill out field data sheet in pencil (RD[05]).

FILTERS

1. **WEAR GLOVES**, the same gloves can be worn while collecting the sample.
2. Set-up filter apparatus on stream bank, boat, or other relatively level surface:
   a. Set peristaltic pump speed below manufacturer specifications (45 psi for Millipore Sterivex SVGP® filter). If pump speed is set too high, the filter can rupture.
   b. Check that the 3 mL syringe adapter is in place to connect pump tubing to the capsule filter (Figure 2).
   c. Attach 3/8" inner diameter C-flex tubing to the peristaltic pump. Use a zip tie or small hose clamp to firmly attach the tubing to the hose connector.
3. Label capsule filter with an adhesive label marked in permanent marker (Figure 3).
4. Rinse tubing by pumping ~100 mL of sample water completely through the tube.
5. After tubing is flushed with sample water, attach Sterivex® filter to luer lock end of 3 mL syringe/filter adapter.
6. Begin pumping water through the filter using peristaltic pump. Make sure the tube is filled with water to reduce air and reduce the potential to blow a hole in the filter.
   a. A clean, sterilized 140 mL syringe maybe be also used, connected directly to the capsule filter, if peristaltic pump is unavailable.
7. Filter >500 mL (≥1 L if possible, 2 L maximum) of surface water through the filter, discarding the filtrate in a 500 mL graduated cylinder to keep track of the filter volume. Stop filtering when filter clogs with sample or filtering becomes very slow.
   a. You may either pump filtered water into the graduated cylinder to measure the volume filtered (recommended, Figure 7), or pump water out of the graduated cylinder into the filter (this requires sterilization of the graduated cylinder).
   b. Filter <500 mL if filter becomes clogged and record filter volume.

Figure 7. A field technician demonstrates pumping sample water out of the 4 gallon HDPE jug, through the peristaltic pump, the trough the Sterivex® filter, and into a graduated cylinder to measure the volume filtered.

8. Filter two capsule filters per site for each parent sample (Figure 6). Filters should remain in original packaging until filtration.
9. When finished, remove filter from set up. Using the 140 mL syringe, draw air into the syringe and push air gently through the filter with 140 mL syringe until dry. If measuring water volume after it has been filtered, include this water in the sample volume as it has passed through the filter.
10. Cap ends of filter with luer lock male and female end caps (Figure 8).

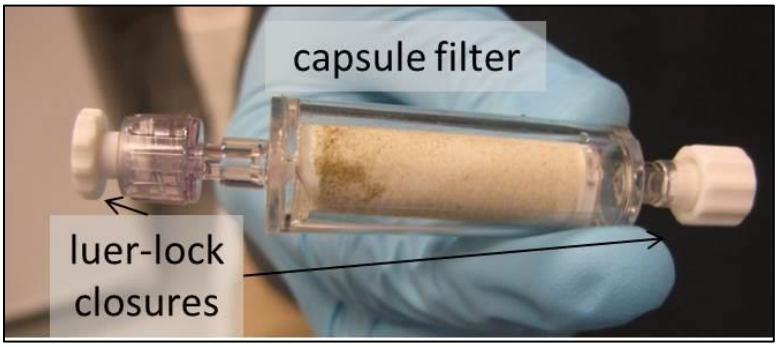

Figure 8. Capsule (Sterivex®) filter and luer lock closures

11. Record filter volume on field data sheet and adhesive label (Figure 9, RD[05]) for each sample.

NEON Microbe Collection

Site (4-letter code): *POSE*
Date (YYYYMMDD): *20140702*
Local time (HH:MM): *14:30*
Recorded by: *jstewart@neoninc.org*
Collected by: *sparker@Field-ops.org*
Sampling protocol: *NEON.DOC.003044* **Rev:**

Location ID	Habitat	Sample type	Lab type	Replicate	Sample ID	Substratum size class [stream benthic]	Sample volume (mL) [stream benthic]	Depth 1 (m) [lakes, rivers]	Depth 2 (m) [lakes, rivers]	Filter volume (mL)
ss	pool	seston	filter 1	1	POSE.20140702.amc.seston.1					500
ss	pool	seston	filter 2	1	POSE.20140702.amc.seston.1					500
ss	pool	seston	cell count	1	POSE.20140702.amc.seston.1					NA
us of sensors	riffle	epilithon	filter 1	1	POSE.20140702.amc.epilithon.1	cobble	125			50
us of sensors	riffle	epilithon	filter 2	1	POSE.20140702.amc.epilithon.1	cobble	125			50
us of sensors	pool	epiphyton	NA	2	POSE.20140702.amc.epiphyton.2	sand	NA			NA
ds of sensors	pool	epiphyton	NA	3	POSE.20140702.amc.epiphyton.3	sand	NA			NA

Figure 9. Example of field data sheet (RD[05]).

12. Place samples in a zippered plastic bag and flash-freeze on dry ice in the field. Ensure that samples remain frozen until returning to the domain lab.
13. Proceed to Sample Preservation (SOP C.6).

SOP C Field Sampling: Benthic Microbes (wadeable streams only)

Benthic biofilm microbial samples are collected in wadeable streams at the same time and location as algal periphyton samples (see RD[09]), but collected independently, i.e., from different cobbles within the same habitats. Benthic microbes do not need to be collected on the same day as surface water microbes.

It is recommended, but not required, that three technicians work in the field together on the benthic microbes and periphyton protocols:

- 1 person collecting periphyton samples
- 1 person wearing sterile gloves collecting benthic microbe samples
- 1 person wearing sterile gloves to assist the microbe sampler with items on the bank

C.1 All Benthic Substrata

1. Sample the sample habitat/substratum type as for the periphyton protocol (RD[09]).
   a. Determine the dominant habitat and second-most dominant habitat based on the Stream Morphology Map (RD[10]) for the site. Habitat suggestions for NEON sites are provided in Appendix E.
   b. The habitat type chosen should be present during all sampling bouts.
   c. All 5 (or 3) replicate samples must be taken from the same habitat type on each sampling bout, unless a major event (i.e., a flood or dewatering of the stream) causes significant changes to the stream channel and the habitat type is no longer present.
   d. If there is only one clear habitat at the site, sample two different types of substrata using the sampling methods below (e.g., in a slow-moving run, collect 5 epilithon samples and 3 epipsammon samples).
2. Target substratum types in order of sampling preference (see Definitions, 2.4). If the preferred substratum is present in high enough density, that takes precedence over a less preferred, more dominant substratum type (e.g., if silt is the dominant substratum type but cobbles are present in high enough density to sample consistently, cobble sampling takes priority; Porter et al. 1993).
   a. Cobble (epilithon) → follow sampling procedure SOP C.3
   b. Woody snag (epixylon) → follow sampling procedure SOP C.3
   c. Plant surface (epiphyton) → follow sampling procedure SOP C.4
   d. Sand (epipsammon) → follow sampling procedure SOP C.5
   e. Silt (epipelon) → follow sampling procedure SOP C.5
3. Choose sampling locations with shallow, flowing water that appear to be historically wetted (i.e., usually underwater). Avoid areas that have been recently dried. Signs of recent drying include: extremely shallow areas, rocks that have nothing growing on them, and rocks that are not slippery to the touch.
4. Choose sampling locations that are representative of the periphyton/biofilm cover of the reach (i.e., not extremely dense or extremely sparse cover relative to nearby substrata).
5. Do not collect samples within a 5 m radius of the aquatic instrumentation.
6. Do not sample substrata where you or other field technicians have walked or locations that appear recently disturbed (e.g., overturned rocks, footprints, dislodged plants, other evidence of wildlife, cattle, humans, etc.).
7. Avoid substrata that are close to the stream/river bank or lake shore and may be exposed to frequent drying.
8. Choose sampling locations that are exposed to ambient light (e.g., not under a log or cut bank).
9. Unless sampling epiphytes, avoid substrata that are heavily colonized with aquatic plants, bryophytes, invertebrates, or have leaf litter clinging to the surface. You may brush some invertebrates off the surface, but the presence of aquatic plants and bryophytes may skew the results.
10. Avoid substrata that have noticeably tumbled or been recently disturbed.

11. Do not collect all replicate samples from the same location.
    a. In **wadeable streams**, collect samples from several different runs/riffles/pools, or if that is not possible, from different portions of the same run/riffle/pool.
12. Start sampling at the bottom of the reach, working upstream so as not to stir up sediments in the water column which can decrease visibility.

C.2 Contingent decisions

Table 8. Contingent decisions for sampling in a wadeable stream.

Situation	Action	Outcome for Data Products	Considerations
Wadeable stream site with <200 m aboveground stream length due to stream size	Habitat available may be insufficient to accommodate all 8 replicate samples without causing harm to the stream. Reduce sampling by collecting samples only in the dominant habitat type (5 samples total).	Lower resolution for diversity metrics.	If the decision is made to decrease the number of samples collected for this protocol, it must also be reflected in the other wadeable stream biology protocols (RD[09], RD[14], RD[15]).
Wadeable stream site with seasonal drying	If the stream experiences seasonal drying such that the chosen habitat types have disappeared, select the next dominant habitat type in C.1, or use the sub-dominant habitat if still present.	Less standardization of the dataset.	Habitat types sampled should be present throughout the year. The decision to switch habitat types should only occur during periods of extreme drying such that typical habitat types are no longer present at the site.

C.3 Epilithon (Rock Scrubs) and Epixylon (Wood Scrubs) Microbes

1. Using 1”x2” adhesive labels (RD[05]), label two capsule filters for each parent sample (Figure 11).
    a. **Wadeable streams**, dominant habitat: 10 filters/labels
    b. **Wadeable streams**, secondary habitat: 6 filters/labels
2. **Wear gloves.** Replace gloves between habitat types.
    a. Field-sterilize gloves with alcohol wipes between samples in the same habitat type.
3. Select five cobbles and/or pieces of woody debris from the dominant habitat or three from the secondary habitat. Select cobbles and/or pieces of woody debris that meet both the requirements in SOP C.1 AND the following:

a. Larger than the scrubbing template (i.e., > 2 inches in diameter).
b. **These are NOT composite samples.** Only one cobble/piece of woody debris is required per sample.

4. Note the dominant substratum size class at the sampling location on the field data sheet (Figure 9, RD[05]).
5. Place cobble/woody debris top-side up in the white larval tray. Take care to keep the cobble top-side up. This is the surface that has been exposed to the sunlight, and will be the portion of the cobble that you sample.
6. Be sure to keep cobbles/woody debris moist with native water until scrubbing.
7. Proceed to a location on the stream bank or boat to process the sample. This location should be out of direct sunlight if possible.
8. Field sterilize and prepare equipment at stream bank.
   a. A clean, sterile tray or tote is recommended for setting equipment on the stream bank (Figure 10).
   b. Wearing gloves, rinse the 140 mL syringe 3 times in 0.2 µm filtered DI and discard rinse water (water filtered at domain support facility).
   c. Wipe surfaces of all reusable equipment (e.g., larval tray, scrubbing template) with new alcohol wipes. After cleaning, rinse with filtered DI.

Figure 10. Template placement for epilithic microbe sampling protocol. Note sterile organizer used to hold equipment on the stream bank.

9. Fill the sterilized 125 mL wash bottle with 0.2 µm filtered DI to the "fill line" (as marked on bottle).
   a. Make sure that bottom of the meniscus lines up with the "fill line".

b. **Since this is a volume-based sampling technique, it is important to keep the sample volume consistent and record the volume of water used.**

10. Holding cobble/woody debris underwater, briefly rinse any leaves and/or large invertebrates from surface using stream water (you can use your hand to gently sweep insects off the cobble/woody debris surface, but take care not to scrub hard and dislodge biofilm).
    a. Recheck cobble, if there are more than 10 invertebrates attached to the substratum within your template, discard and choose a new cobble/piece of woody debris.
    b. If there is growth of aquatic plants or bryophytes that falls within your template, discard and choose a new cobble.
11. Place cobble/woody debris right-side up (the side exposed to the sun) in white tray and pour any excess water out of the tray.
12. Place white slide template on top of cobble/woody debris (surface that was exposed to light at the stream bottom; Figure 10). Check cobble/woody debris again for colonization of invertebrates, bryophytes, or plants.
13. Holding the template firmly in place on the cobble/woody debris, begin scrubbing inside the template (scrub gently if woody debris).
    a. Both cobbles and woody debris are scrubbed using a **toothbrush** for this protocol.
    b. Use a new toothbrush for each sample, discard after use.
    c. Be sure to hold the template in place, as slipping would change the area you are sampling (Figure 10).
    d. Scrubbing should be similar to brushing your teeth.
14. Periodically rinse the inside area of the template using the 0.2 µm filtered D1 in the 125 mL wash bottle **while holding the template in place**. Allow water to run into the white tray – **DO NOT DISCARD** rinse-water.
15. Continue scrubbing until the inside of the template is clean.
16. Remove template. There should be a clean rectangle left from your scrubbing. This is harder to see on woody substrates.
17. Using the wash bottle, rinse the template (front and back), the cobble/woody debris, scrub brush, and gloved fingers into the tray. If you need more water for rinsing, refill the 125 mL wash bottle with 0.2 µm filtered DI water and keep track of the volume used.
18. If there is any remaining water in the 125 mL wash bottle, empty this into the tray. All rinse-water should now be in the white tray and should total 125 mL (or more if additional water was needed for rinse). **DO NOT DISCARD.**
19. Discard cobble/woody debris in the stream where it will not be sampled again during this bout, or on the stream bank. There is no composite sampling for this protocol.
20. Carefully swirl contents of tray (scrubbed material + rinse-water) to re-suspend the sample.
21. Carefully pour sample water into 140 mL syringe with a luer lock end cap attached. There may be some sand left in the bottom of the tray, this is ok. If you feel that the sample wasn't properly mixed, you may pour the sample back in the tray and swirl again.

22. Attach Sterivex® filter to end of 140 mL syringe. Filter ~50 mL of sample, or until filter becomes clogged.
    a. You do not need to filter all of the material in the syringe.
    b. Keep track of the volume filtered.
23. After filtering the sample, remove the filter from the syringe, pull in air, replace the filter and push air through the filter to dry prior to freezing. This may require more than 1 pass of air from the syringe.
24. Cap both ends of the capsule filter tightly with luer lock caps, place in a Whirl-pak® bag and flash-freeze on dry ice in the field.
25. Repeat Steps 22-24 for second Sterivex® filter (Figure 11).

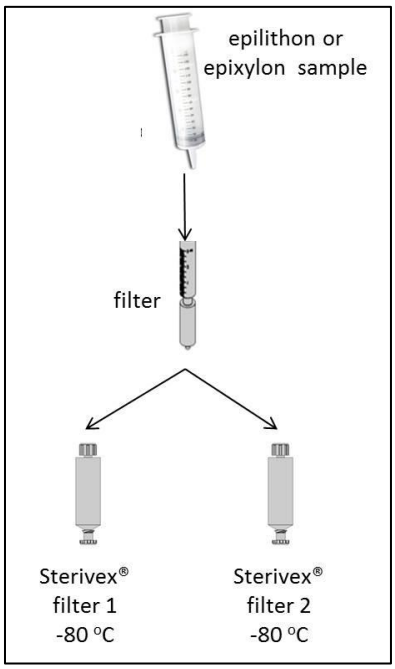

Figure 11. Schematic for benthic biofilm sampling via scrub slurry (SOP C).

26. Fill out field data sheet in pencil (Figure 9, RD[05]).
27. Rinse tray, template, and syringe with filtered DI and field sterilize with alcohol wipes and/or ETOH before starting next sample.
28. Process remaining cobbles/woody debris in the reach one at a time.
29. Discard sampled cobble/woody debris in stream or on the bank before you leave the site.
30. Recheck labels and place all sample filters on dry ice. Use resealable bags to contain all samples from the same site.

C.4 Epiphytic (Aquatic Plant Surfaces) Microbes

1. Using 1”x2” adhesive labels (RD[05]), label one Whirl-pak® per sample (Figure 3).
   a. **Wadeable streams**, dominant habitat: 5 Whirl-paks®
   b. **Wadeable streams**, secondary habitat: 3 Whirl-paks®
2. **Wear gloves.** Replace gloves between habitat types.
   a. Field-sterilize gloves with alcohol wipes between samples in the same habitat type.
3. Select five plants if the dominant habitat and three if the secondary habitat. Select plants for sampling that are well-colonized with epiphytes (Figure 12) and that meet both the requirements in SOP C.1 AND the following:
   a. Sample from only 1 plant species to standardize sampling.
   b. The plant species should be common (i.e., accounts for >50% of the aquatic plants) in the reach throughout the year.
   c. Plants should not covered by sediments.
4. Using sterile scissors, cut approximately 5 cm length of heavily colonized plant (include leaves and stem if present).
   a. Sample only portions of plant that are underwater.
   b. Reuse scissors from sample to sample. Field-sterilize with alcohol pads and rinse with filtered DI between samples.
   c. Be gentle with the plants as epiphyton/sample material may be easily dislodged.

Figure 12. Example of epiphytes growing on reeds in a Colorado stream.

5. Place plant material in a Whirl-pak®. Do not add water to sample.
6. Label outside of Whirl-pak®.
7. Close Whirl-pak® bag and flash-freeze on dry ice. Place all Whirl-paks® from the same site in one plastic bag so they are organized and not damaged by dry ice.

a. Close the Whirl-pak® by holding the wire tabs at either side of the bag (Figure 13), then whirl the bag at least 3 complete revolutions to form leak-proof seal. Rather than whirling, you may also fold the top over as tightly as possible at least 3 times. Bend the wire ends over onto the bag to complete.

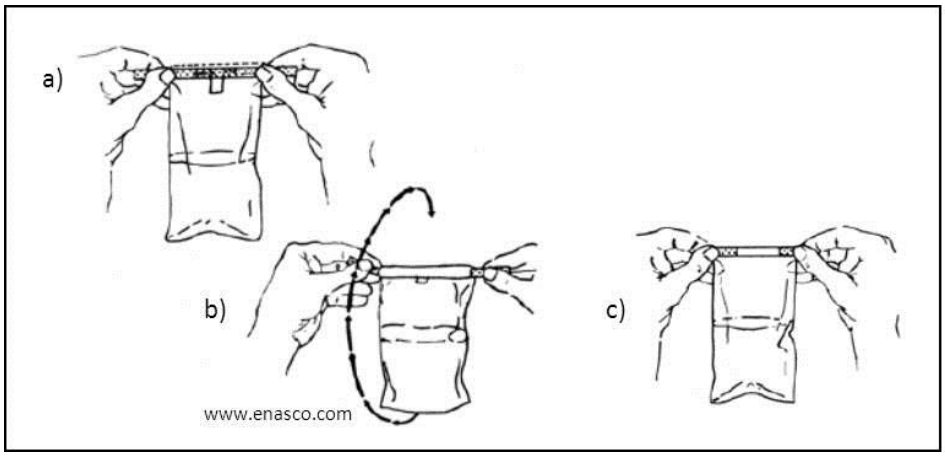

Figure 13. How to close a Whirl-pak® bag: a) hold the wire tabs; b) whirl the bag 3 complete revolutions (or fold the top over); and c) fold the wire ends over to close

8. Fill out field data sheet in pencil (RD[05]).
9. Repeat above steps until 5 samples (if this is the dominant habitat) or 3 (if this is the secondary habitat) have been collected.
10. No further filtering or processing occurs at the domain lab. Freeze samples at -80 ^{o}C and send to external lab for extraction (similar to TOS soil microbe samples, RD[12]).

C.5 Epipsammon (Sand) and Epipelon (Silt) Microbes

1. Using 1”x2” adhesive labels (RD[05]), label one sterile 15 mL plastic tube per sample (Figure 4).
   NOTE: Tubes may NOT be reused from the last sampling trip.
   a. **Wadeable streams**, dominant habitat: 5 tubes/labels
   b. **Wadeable streams**, secondary habitat: 3 tubes/labels
2. **Wear gloves.** Replace gloves between habitat types.
   a. Field-sterilize gloves with alcohol wipes between samples in the same habitat type.
3. Select five locations if this is the dominant habitat and three if this is the secondary habitat. Select locations to sample that meet the requirements in SOP C.1.
4. Note the dominant substratum size class (i.e., silt or sand) at the sampling location on the field data sheet (Figure 9, RD[05]).
5. Fill the 125 mL wash bottle with 0.2 µm filtered DI if needed for rinsing.
6. Uncap sterile centrifuge tube and carefully press the tube into the upper 3 cm of substrate (like a cookie cutter). Take care not to disturb the substratum before placing tube on bottom. If substratum is disturbed, find a new sampling location.

7. Slide spatula under tube to enclose the sample. Holding the tube tightly to the spatula, lift out of water. Gently rinse excess silt not enclosed by tube from spatula. It is okay if minimal sample is lost.
8. Hold spatula + sample + tube over white sampling tray and invert tube.
9. Carefully slide spatula from top of tube and cap tightly. Take care not to spill sample. You may use your gloved fingers to help get the sample into the bottle from the spatula.
    a. No filtering occurs with these samples, these are whole samples that will be extracted at the external lab.
    b. These are not composite samples.
10. Fill out field data sheet in pencil (Figure 9, RD[05]).
11. Field-sterilize and rinse spatula with filtered DI before starting next sample.
12. Repeat steps 1-11 until samples have been collected from all selected locations.
13. Recheck labels and place all sample filters on dry ice. Use resealable bags to contain all samples from the same habitat.
14. No further filtering or processing occurs at the domain lab. Freeze samples at -80 ^{o}C and send to external lab for extraction (similar to TOS soil microbe samples, RD[12]).

C.6 Sample Preservation

CELL COUNT SAMPLES

1. If filtered formalin was not already added to the 20 mL scintillation vials in the lab (SOP A), cell counts must be preserved immediately in the field. Add 0.9 mL of 0.2 µm (Acrodisc® filter) filtered buffered formalin to cell counts samples using a 3 mL syringe or pipet for every 18 mL of sample.
    a. Maximum time to preserve cell counts = 4 hours.
2. Recap bottle, and invert to mix ~5 times.
3. Put samples in cooler with ice packs. Do not freeze.
4. Chill sample at 4°C upon return to lab. Samples should only be held for 7 days before shipping, 14 days at a maximum.

STERIVEX® FILTERS

1. Flash-freeze capped Sterivex® filters on dry ice in the field immediately after collection.
2. Freeze filters at -80 °C upon return to lab. Filters may be held for up to 30 days before shipping.

WHOLE SEDIMENT (EPIPSAMMON, EPIPELON) OR PLANT (EPIPHYTON) SAMPLES

1. Flash-freeze whole samples on dry ice in the field immediately after collection.
2. Freeze whole samples at -80 ^{o}C upon return to the lab. Samples may be held for up to 30 days before shipping.

C.7 Ending the Sampling Day

1. Sterilize and dry equipment and place in new, clean zip-top bags for the next sampling bout.
   a. Having a microbe-dedicated set of peristaltic pump tubing, connector, and c-flex tubing may be logistically helpful, or you can sterilize and reuse the tubing used for water chemistry.
2. Refreshing the sampling kit
   a. Replace Whirl-pak® bags and filters, nitrile gloves, luer locks, resealable bags, toothbrushes, etc. Refer to equipment list in Section 6.1.
   b. Discard and replace any broken templates and used toothbrushes.
   c. Restock the cell count preservation vials.
3. Equipment maintenance, cleaning and storage
   a. Charge drill pump batteries.
   b. Wash all equipment that has come in contact with site water and does not need to be sterilized according to the NEON Aquatic Decontamination Protocol (RD[08]).
   c. Dry all items completely before storing.

SOP D Laboratory Sampling and Analysis

Surface water microbes

If inclement weather prevents field filtering and preservation, surface water samples may be collected in bulk and processed back at the Domain Support Facility following the field standard operating procedure outlined in SOP C.6. Samples must be processed within 4 hours of collection.

All microbe samples

Samples should be stored as outlined in preservation, SOP C.6.

SOP E Data Entry and Verification

As a best practice, field data collected on paper datasheets should be digitally transcribed within 7 days of collection or the end of a sampling bout (where applicable). However, given logistical constraints, the maximum timeline for entering data is within 14 days of collection or the end of a sampling bout (where applicable). See RD[04] for complete instructions regarding manual data transcription.

Enter all data from the field datasheets into the AOS database, PDA, or Web UI if available.

SOP F Sample Shipment

Information included in this SOP conveys science-based packaging, shipping, and handling requirements, not lab-specific or logistical demands. For that information, reference the CLA shipping document on NEON's CLA intranet site.

Shipments are to include a hardcopy of the "per sample" tab of the shipping inventory (RD[13]) as well as an electronic shipping inventory that is emailed to the receiving laboratory and to the contact in NEON Collections and Laboratory Analysis at the time of shipment. The shipment tracking number (shipment ID) must be included in the electronic version of the shipping inventory as well as the email, but is not necessary on the hard copy.

F.1 Cell Count Shipping

1. Cell count samples must not freeze. Take care to avoid shipping at times when the samples may be subject to sitting for long periods in a frozen warehouse (e.g., note lab weekend and holiday receiving schedule).
2. Place scintillation vials into one or several gallon-size resealable zip-top bags, grouped by site.
3. Line a cardboard box or 9 qt cooler with a plastic bag to prevent leaks.
4. Place all vials right-side up inside the liner bag. Add packing material (Vermiculite or other) to take up excess space in container and cushion samples.

a. If using 9 qt coolers, include return shipping label for external lab to send cooler back.

5. Include shipping inventory/manifest in additional zip-top bag.
6. Ship overnight on ice or ice packs. Formalin in these concentrations is not considered hazardous.

F.2 Filters, Plant, and Sediment Sample Shipping

1. All filters and whole samples must remain frozen at -80 ^{o}C. Do not ship on Fridays and ensure that the receiving lab will be open when the shipment arrives (e.g., take note of holiday schedules).
2. Place filters and samples in resealable zip-top bags grouped by site/date.
3. Place inside small dry-ice shipper.
4. Keeps filters/bags from directly touching dry ice using cardboard or additional bags.
5. Include shipping inventory/manifest in additional zip-top bag.
6. Follow instructions for shipping overnight on dry ice in AD[03].

F.3 Handling Hazardous Material

Formalin in the concentration and volume shipped by NEON for this protocol is not considered hazardous.

F.4 Supplies/Containers

See sections F.1, F.2, and Table 7 for specific shipping materials.

F.5 Timelines and Conditions

1. **Cell counts**: Formalin-preserved samples may be stored at 4 ^{o}C for up to 7 days. Do not freeze.

a. Cell count samples may be held for up to 14 days if necessary with a data flag. Holding times longer than 14 days requires disposal of sample.

2. **Sterivex® filters**: Filters may be stored at -80 °C for up to 30 days.
   a. Frozen samples may be held for up to 6 months if the lab is not able to receive samples (Table 1).
3. **Whole samples (plants, sediment):** Samples may be stored at -80 °C for up to 30 days.
   a. Frozen samples may be held for up to 6 months if the lab is not able to receive samples (Table 1).

F.6 Grouping/Splitting Samples

Group samples by site per bout.

F.7 Return of Materials or Containers

Include return shipping label with WBS code if any shipping materials need to be returned to the domain support facility (e.g., cooler).

F.8 Shipping Inventory

Shipments are to have a hardcopy of the shipping inventory (RD[13]) sent in each box as well as an electronic shipping inventory that is emailed to the receiving laboratory and to the contact in NEON Collections and Laboratory Analysis at the time of shipment. Also include the shipment tracking number in the email.

F.9 Laboratory Contact Information and Shipping/Receipt Days

See the CLA shipping document on NEON's CLA intranet site.

8 REFERENCES

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APPENDIX A DATASHEETS

The following datasheets are associated with this protocol:

Table 9. Datasheets associated with this protocol

NEON Doc. #	Title
NEON.DOC.003041	Datasheets for AOS Protocol and Procedure: Aquatic Microbial Sampling
NEON.DOC.001646	General AQU Field Metadata Sheet
NEON.DOC.002494	Datasheets for AOS Sample Shipping Inventory
NEON.DOC.002191	Datasheets for Secchi Depth and Depth Profile Sampling

These datasheets can be found in Agile or the NEON Document Warehouse.

APPENDIX B QUICK REFERENCES

B.1 Microbe Sampling Preparation

Step 1 – Check the microbe field sampling kit to make sure all supplies are packed.

Step 2 – Makes sure equipment has been sterilized.

Step 3 – Prepare adhesive labels.

Step 4 – Ensure the General AQU Field Metadata Sheet (RD[06]) is completed per field site visit and Secchi and Depth Profile datasheet in lakes and non-wadeable streams (RD[17]).

Step 5 – Determine habitat sampling locations and sampler type.

1. In **wadeable streams**:
   a. Sample **surface water** near the S2 sensor, same location as water chemistry.
   b. Determine **benthic** habitat from the Stream Morphology Map (RD[11]) and periphyton sampling locations.
   c. Determine sampler type based on the habitats present and the order of preference for sampling substratum.
2. In **lakes and non-wadeable streams**:
   a. Sample **surface water** at the sample locations as water chemistry.
   b. Determine the depths of the thermocline and the euphotic zone.
   c. Determine sampling depths (same as water chemistry). If the site is stratified, collect 1 sample from the epilimnion and 1 sample from the hypolimnion at the buoy location.

B.2 Steps for Surface Water Microbe Sampling

Step 1 – Collect samples:

1. Wear gloves
2. In **wadeable steams**, collect in the thalweg using 4 L jugs.
3. In **lakes and non-wadeable streams**, collect samples using the Kemmerer and integrate the samples in a 4 L jug.

Step 2 – Process the samples

1. Wear gloves
2. Cell counts:
   a. Collect in 20 mL glass scintillation vial.
   b. Add 0.9 mL of 0.2 µm (Acrodisc® filter) filtered buffered formalin.
   c. Chill sample at 4°C upon return to lab.
   d. Ship on ice overnight, but do not freeze.
3. Sterivex® filters (2 replicates):

a. Filter >500 mL of stream water through each capsule filter (2 filters total) until filter clogs.
b. Flash-freeze on dry ice in the field.
c. Freeze at -80 °C upon returning to the lab.
d. Ship on dry ice overnight.

B.3 Steps for Benthic Biofilm Sampling in Wadeable Streams

Step 1 – Determine habitat and sampling method.

Step 2 – Collect samples:

1. **Wear gloves**.
2. Epilithic (rock scrubs) and epixylic (wood scrubs) microbes:
    a. Scrub the cobbles or wood into a larval tray and add to 140mL syringe
    b. Sterivex® filters:
        i. Filter ~50 mL through each capsule filter (2 filters total).
        ii. Flash-freeze on dry ice in the field.
        iii. Freeze at -80 °C upon returning to the lab.
        iv. Ship on dry ice overnight.
3. Epipsammic (sand) and epipelic (silt) microbes:
    a. Collect samples in a centrifuge tube.
    b. Flash-freeze on dry ice in the field.
    c. Freeze samples at -80 ^{o}C upon returning to the lab.
    d. Ship on dry ice overnight.
4. Epiphytic (aquatic plant surfaces) microbes:
    a. Cut approximately 5 cm length of heavily colonized plant.
    b. Place in a Whirl-pak®
    c. Flash-freeze on dry-ice in the field.
    d. Freeze samples at -80 ^{o}C upon returning to the lab.
    e. Ship on dry ice overnight.

APPENDIX C REMINDERS

Before heading into the field: Make sure you…

- ☑ Collect and prepare all equipment including labels.
- ☑ Pre-print adhesive labels.
- ☑ Sterilize equipment.

Sample collection: Be sure to…

Surface microbes

- ☑ Field-sterilize equipment between samples. Sterilize all equipment that is reused between sampling bouts.
- ☑ Wear clean nitrile gloves.
- ☑ Filtering a second 4 L bottle may be required for very clear water to collect all samples.
- ☑ Preserve cell count samples with formalin in the field, flash freeze Sterivex® filters in the field.

Benthic microbes in wadeable streams

- ☑ Field-sterilize equipment between samples. Sterilize all equipment that is reused between sampling bouts.
- ☑ Determine the dominant habitat and second-most dominant habitat based on the Stream Morphology Map (RD[11]). Sample in same locations as periphyton.
- ☑ Choose the appropriate sampler.
- ☑ Start sampling at the bottom of the reach, working upstream so as not to decrease visibility and disrupt benthic biofilm communities.
- ☑ Spread replicate samples out along the reach.
- ☑ For epilithon/epixylon samples, keep the sample volume consistent and record the volume of water used. Do not discard rinse water from the larval tray as this is your sample.
- ☑ Do not sample anywhere you or other field technicians have walked, or locations that appear recently disturbed.

Sample processing: Be sure to…

- ☑ Filtering can be conducted in the domain lab in cases of inclement weather within 4 hours of collection provided 4L jugs are appropriately labeled.
- ☑ Keep track of the volume of sample filtered.
- ☑ DO NOT FREEZE cell count samples.
- ☑ Flash-freeze filters and grab samples on dry ice in the field.

APPENDIX D ESTIMATED DATES FOR ONSET AND CESSATION OF SAMPLING

Preliminary date ranges for benthic biological sampling bouts in **wadeable streams**. Surface water samples follow monthly water chemistry sampling dates. Also see the Site Specific Sampling Strategy Document on NEON's FOPS intranet site.

Domain	Site	Bout 1	Bout 2	Bout 3
D01	Hop Brook	11Apr-9May	9Jul-6Aug	3Oct-31Oct
D02	Mill Run*	19Mar-16Apr	5Jul-2Aug	18Oct-15Nov
D02	Posey Creek	19Mar-16Apr	5Jul-2Aug	18Oct-15Nov
D04	Rio Guilarte	26Jan-23Feb	21Jun-19Jul	9Nov-7Dec
D04	Rio Cupeyes	24Jan-21Feb	21Jun-19Jul	10Nov-8Dec
D06	Kings Creek	23Mar-20Apr	3Jul-31Jul	3Oct-31Oct
D07	Leconte Creek	15Mar-12Apr	30Jun-28Jul	12Oct-9Nov
D07	Walker Branch	9Mar-6Apr	1Jul-29Jul	19Oct-16Nov
D08	Mayfield Creek	5Mar-2Apr	29Jun-27Jul	31Oct-28Nov
D10	Arikaree River	21Mar-18Apr	4Jul-1Aug	20Sep-18Oct
D11	Pringle Creek	17Feb-17Mar	29Jun-27Jul	23Oct-20Nov
D12	Blacktail Deer Creek	1May-29May	13Jul-10Aug	30Aug-27Sep
D13	Como Creek	20May-17Jun	14Jul-11Aug	30Aug-27Sep
D13	West St. Louis Creek	2May-30May	5Jul-2Aug	3Sep-1Oct
D14	Sycamore Creek	17Feb-17Mar	29Jun-27Jul	21Oct-18Nov
D15	Red Butte Creek	29Mar-26Apr	6Jul-3Aug	29Sep-27Oct
D16	McRae Creek	10Apr-8May	11Jul-8Aug	23Sep-21Oct
D16	Martha Creek	6Apr-4May	5Jul-2Aug	22Sep-20Oct
D17	Convict Creek*	31Mar-29Apr	8Jul-5Aug	15Sep-13Oct
D17	Providence Creek*	19Mar-16Apr	1Jul-29Jul	25Sep-23Oct
D18	Oksrukuyik Creek	21May-18Jun	29Jun-27Jul	7Aug-4Sep
D19	Caribou Creek	2May-30May	26Jun-24Jul	18Aug-15Sep

*soft sites as of November 2015

APPENDIX E SITE-SPECIFIC INFORMATION: HABITAT AND SUBSTRATA RECOMMENDATIONS

See the Site Specific Sampling Strategy Document on NEON's FOPS intranet site.

Domain	Site	Benthic habitat 1 (5 reps)	Benthic habitat 2 (3 reps)	Surface water
D01	Hop Brook	Riffles (epilithon)	Pools (epipsammon)	ss (S2)
D02	Mill Run*			ss (S2)
D02	Posey Creek	Riffles (epilithon)	Pools (epipelon)	ss (S2)
D03	Ichawaynochaway Creek			rs
D03	Lake Barco			c0, in, ot
D03	Lake Suggs			c0, in, ot
D04	Rio Guilarte	Riffles (epilithon)	Pools (epipelon)	ss (S2)
D04	Rio Cupeyes	Riffles (epilithon)	Runs (epilithon)	ss (S2)
D05	Crampton Lake			c1, c2, in, ot
D05	Pickerel Creek*			ss (S2)
D06	Kings Creek	Riffle/run	Pools	ss (S2)
D06	McDowell Creek			rs
D07	Leconte Creek	Riffle (epilithon)	Pools (epipsammon)	ss (S2)
D07	Walker Branch	Riffle (epilithon)	Runs (epixylon)	ss (S2)
D08	Mayfield Creek	Runs/riffles (epixylon)	Runs (epipsammon)	ss (S2)
D08	Black Warrior River			rs
D08	Tombigbee River			rs
D09	Prairie Lake			c0, in, ot
D09	Prairie Pothole			c0, in, ot
D10	Arikaree River	Runs (epiphyton)	Pools/Runs (epipsammon)	ss (S2)
D11	Pringle Creek			ss (S2)
D11	South Pond at Klemme			c0, in, ot
D12	Blacktail Deer Creek	Riffles (epilithon)	Riffles/Runs (epixylon)	ss (S2)
D13	Como Creek	Riffles (epilithon)		ss (S2)
D13	West St. Louis Creek	Riffles (epilithon)	Pools (epipsammon)	ss (S2)
D14	Sycamore Creek	Riffles (epilithon)	Runs/Pools (epipsammon)	ss (S2)
D15	Red Butte Creek	Riffles (epilithon)	Runs (epilithon)	ss (S2)
D16	McRae Creek	Riffles (epilithon)	Step pools (epixylon)	ss (S2)
D16	Martha Creek	Riffles (epilithon)	Pools (epixylon)	ss (S2)
D17	Convict Creek*			ss (S2)
D17	Providence Creek*			ss (S2)
D18	Oksrukuyik Creek	Riffles (epilithon)	Pools (epipelon)	ss (S2)
D18	Toolik Lake			c1, c2, in, ot
D19	Caribou Creek	Riffles (epilithon)	Pools (epipsammon)	ss (S2)

*soft sites as of November 2015