AOS PROTOCOL AND PROCEDURE: MICROBES IN WADEABLE STREAMS

PREPARED BY	ORGANIZATION	DATE
Heather Adams	AQU	06/07/2013
Stephanie Parker	AQU	01/29/2015
Keli Goodman	AQU	06/07/2013

APPROVALS	ORGANIZATION	APPROVAL DATE
Dave Tazik	SCI	06/01/2015
Mike Stewart	PSE	05/07/2015

RELEASED BY	ORGANIZATION	RELEASE DATE
Jennifer DeNicholas	CM	06/01/2015

See configuration management system for approval history.

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Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
A	02/10/2014	ECO-01179	Initial release
B	08/29/2014	ECO-02210	Minor updates based on feedback from the field
C	01/09/2015	ECO-02621	Migration to new protocol template
D	06/01/2015	ECO-02697	Minor updates to equipment, updates to shipping and labeling, flash-freezing filters in the field, removal of RNAlater preservation, and the addition of sampling dates to appendix.

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1 OVERVIEW

1.1 Background

This document describes the required protocols for conducting field sampling of microbes in wadeable streams. 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 and so concurrent sampling ensures comparison between drivers and effects. Microbes also form biofilms in stream beds 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, and large storm events can increase the similarity of microbial communities between sampling sites. The potentially large impact of stream flow on microbial communities has been incorporated into the NEON Aquatic Sample Strategy (RD [04]). Within the stream bed, there is heterogeneity in community composition, particularly where stream 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 technique 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 stream water chemistry protocol and stream algal protocol which is 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), Arctic Streams LTER program (Slavik et al. 2004), and the Methods in Stream Ecology text (Lowe and LaLiberte 2006), 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. 002198	Datasheets for AOS Protocol and Procedure: Microbes in Wadeable Streams
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.000691	AOS Protocol and Procedure: Periphyton and Seston Sampling in Wadeable Streams
RD[10]	NEON.DOC.000694	AOS Protocol and Procedure: Surface Water Chemistry Sampling in Wadeable Streams
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.000690	AOS Protocol and Procedure: Macroinvertebrate Sampling in Wadeable streams
RD[15]	NEON.DOC.000692	AOS Protocol and Procedure: Aquatic Plant, Bryophyte, Lichen, and Macroalgae Sampling in Wadeable Streams

2.3 Acronyms

Acronym	Definition
^{o}C	Degrees Celsius
DI	De-ionized water
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
ft	Foot
HDPE	High-density polyethylene
L	Liter
LTER	Long Term Ecological Research
m	Meter
mL	Milliliter
µm	Micrometer
mm	Micrometer
mRNA	Messenger RNA
qPCR	Quantitative polymerase chain reaction
qt	Quart
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
s	Second
USGS	US Geological Survey

2.4 Definitions

Bryophyte: Aquatic moss, liverwort, or hornwort lacking true vascular tissues.

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

Epixylon: Colonizing woody substrate.

Epilithon: Colonizing rock substrates.

Epipelon: Colonizing silt substrates.

Epiphyton: Colonizing surface of aquatic plants.

Epipsammon: Colonizing sand substrates.

Macrophyte: Aquatic plant with vascular tissues.

Pool: Areas of slow moving, deep water relative to rest of channel.

Riffle: Shallow, swiftly moving water, characterized by a choppy water surface.

Run: Flowing water, typically deeper than riffles, water surface remains smooth due to relatively uniform flow.

S1 and S2: Locations of NEON aquatic sensors.

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

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.

Thalweg: The line of least resistance to water flow in a stream, often the line of maximum water velocity.

Woody Snag: Woody debris that catches on the stream bottom (or stream bank), and collects additional debris from the stream flow. Snags often create a refuge for organisms from the stream flow, as well as increased food sources.

3 METHOD

The field protocol used by NEON for collecting aquatic microbe samples in wadeable streams in both surface waters and benthic biofilms follows the procedure for periphyton sampling (RD [08]), modified to include sterile sampling technique, the Arctic LTER techniques for surface water sampling, and following LaRouche et al. (2012) for epipsammic and epipelic sampling of microbes. Samples are taken at the same time and location as water chemistry (surface water microbes) and algal samples (benthic microbes); sample timing and location are determined by the size and depth of the wadeable streams. At each location, aquatic microbe sampling occurs 3 to 12 times per year on the same day as water chemistry and periphyton sampling; sample timing is based on statistical analysis of environmental parameters. If water chemistry and algal periphyton samples are not collected on the same day, surface water microbe samples are collected with water chemistry samples while benthic biofilm samples are collected with algal periphyton. Details on sampling location are provided in the Surface Water Chemistry Sampling in Wadeable Streams (RD [09]) and Periphyton and Seston Sampling in Wadeable Streams (RD [08]) protocols. Benthic biofilms are collected during periods of stable stream flow (Biggs et al. 1999, Stevenson and Bahls 1999) using a series of scrubbing procedures, depending on habitat and substrate type (Moulton et al. 2002). Microbial samples are collected both as whole sample (water or substratum) and on filters for archive and analysis.

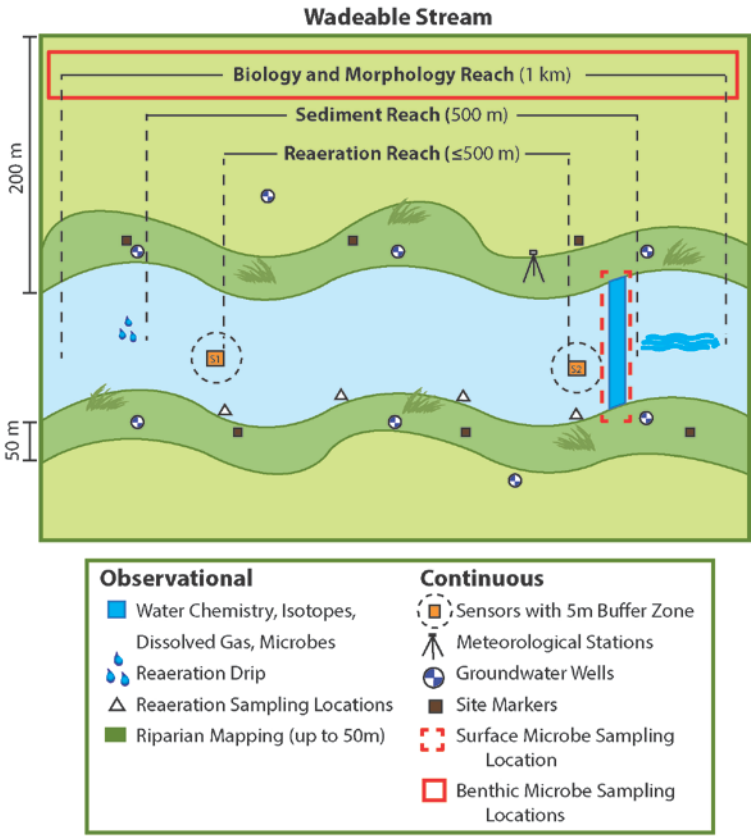

Figure 1. A generic wadeable stream site layout example with microbe sampling locations

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 in wadeable streams will be collected up to 12 times each year at the same time and location as monthly water chemistry samples (RD[10]). Microbial samples may be collected either before or after chemistry sample collection as long as the water column remains undisturbed. 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 or after periphyton sampling as long as they are collected from undisturbed substrate. Sampling must occur within 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.

4.2 Criteria for Determining Onset and Cessation of Sampling

A range of dates for each site were determined *a priori*, based on historical data including streamflow, the accumulation of degree days, weather, and riparian phenology (Appendix D). Periphyton will be collected during periods of stable stream flow (Stevenson and Bahls 1999).

4.3 Timing for Laboratory Processing and Analysis

Filtered samples 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 ^{o}C for up to 7 days before shipping. For additional storage and shipping timelines see SOP G.

4.4 Sampling Timing Contingencies

All samples from one sampling bout must be collected within one day (i.e., all 9 samples per stream 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 circumstances occur that impede sampling (e.g., wildlife, weather), discard samples and start over the next day that conditions permit.	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 requirements below, continue to collect samples.	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 flooding (>1.5x above baseflow) or unsafe wading conditions occur (Lane and Fay 1997) occurs before all samples have been collected for the day, return samples already collected to the stream and start over on the next appropriate day (see 14 day rule below).	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.
14 Days	If flooding occurs on or prior to the targeted sampling date (>1.5x above baseflow) or unsafe wading conditions occur (Lane and Fay 1997) wait a minimum of 14 days until the water level drops so the periphyton community can recolonize (Biggs et al. 1999; maximum wait = 1 month).	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.

4.5 Sampling Specific Concerns

1. Sampling too soon after a disturbance event (e.g., flooding or wildlife crossing the stream) can dramatically decrease biomass and diversity. Be sure to wait at least 14 days for recolonization to occur.
2. Should it begin to rain during microbial sampling of surface waters, collect surface water samples in two 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.
3. Care must be taken to avoid contaminating the sample with re-suspended bed sediment. Such contamination may be minimized by entering the stream downstream of the sampling location.
    a. The sampling location should be located in a flowing section of the stream that is deep enough to sample without disturbing the stream sediments.
    b. If sediments are disrupted, wait until the area has visually cleared before sampling.
4. 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.
5. Take care to keeping 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.

6. 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 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.

Activities in 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). See Section 10 in the NEON Operations Field Safety and Security Plan (AD 02]).

Technicians should wear protective nitrile gloves when handling microbial samples to prevent contamination and to protect the technician from chemicals used in preservation. All chemicals shall be stored, transported, used and disposed of according to AD[03]. Safety Data Sheets (SDS) shall be readily available and reviewed for all chemicals used during this task.

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]	R	Site-specific Stream Morphology Map	Determining sampling locations	1	N
	R	Clipboard	Recording data	1	N
	R	Cooler (9-28 qt)	Field sample storage; use size appropriate to samples being collected	2	N
	R	Waders (hip or chest)	Wading	1 pair per person	N
	R	Cryogenic gloves	Handling dry ice	1 pair	N
Consumable items					
RD[06]	R	Aquatic Field Metadata Sheet (RD[06])	Recording metadata	1	N
RD[05]	R	Field data sheets (all-weather paper, RD[05])	Recording data	1	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
	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 filters in the field	0.5-1 kg	Y
	R	Nitrile gloves, powderless	Keeping collection method sterile	10	N
	R	Resealable bags, gallon	Keeping collection method sterile	20	N

R/S=Required/Suggested

Table 3. Equipment list – Lab sterilization equipment

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
	R	Wash basin	Container for sterilization solutions	1	N
	R	Clean bench paper or paper towels	Storing equipment	1 roll	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
	R	Pieces of C-flex tubing, 4 feet and 2 feet in length	Filtering water with peristaltic pump	2	N
	R	Hose 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	ETOH, 70%	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	0.2 µm Sterivex SVGP® 010 50 capsule filter	Filtering DI	2	N
	R	Deionized water	Rinsing equipment, preparing filtered DI	4 gallons	N
	R	Gallon zip top bags	Storing equipment	5	N

R/S=Required/Suggested

Table 4. Equipment list – Surface water sampling equipment

Item No.	R/S	Description	Purpose	Quantity	Special Handling
Durable items					
	R	Sterile 4 L HDPE jug	Collecting samples	2	N
	R	Pieces of C-flex tubing, 4 feet and 2 feet in length	Filtering water with peristaltic pump	2	N
	R	Hose connector	Attaching C-flex to peristaltic tubing	1	N
	S	140 mL syringes	Alternative to peristaltic pump	3	N
	R	Filter adapters for tubing (cut-off 3 mL syringe)	Connecting peristaltic tubing and C-flex tubing	2	N

Item No.	R/S	Description	Purpose	Quantity	Special Handling
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
Consumable items					
	R	Zip tie, small or small hose clamp	Attaching C-flex tubing to hose connector	1	N
	R	10 mL pipette tips	Pipetting cell count samples into vials	20	N
MX106249	R	20 mL scintillation vials (glass) with caps	Cell count sample container	1	N
MX107386	R	0.2 µm Sterivex SVGP® 010 50 capsule filter	DNA and RNA sample filters	2	N
	R	Luer lock male closures	Capping the Sterivex® filters	4	N
	R	Luer lock female closures	Capping the Sterivex® filters	4	N

R/S=Required/Suggested

Table 5. Equipment list – Benthic sampling equipment

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
Consumable items						
	R	Filtered DI (0.2 µm filter)	Field-sterilization and rinsing	All	1-4 L	N
	R	Alcohol wipes, individually-wrapped	Field-sterilization	All	20	N
	R	Nylon toothbrushes for each habitat type	Epilithon and epixylon scrubbing	Cobbles and	≤8	N

Item No.	R/S	Description	Purpose	Conditions Used	Quantity	Special Handling
		(new toothbrush for each sample)		wood		
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
MX106241	R	0.2 µm Sterivex SVGP® 010 50 capsule filter	DNA and RNA 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						
	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
	R	Shipping inventory (RD[13])	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]).

Personnel will be trained in field protocols associated with this document, trained in pipetting skills, and trained in safe working practices for stream field work. Technicians must also be trained in safe handling of formalin (AD[03]). A Safety Data Sheet (SDS) should be readily available to employees for each chemical used in sampling process.

See Section 10 in the NEON Operations Field Safety and Security Plan (AD[02]) for aquatic-specific field safety requirements.

6.3 Specialized Skills

N/A

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 ensure peristaltic pump batteries are charged and the pump is in good working condition.
2. Sterilize any equipment that is reused between sampling bouts.
   a. Equipment to be sterilized includes: 4 L jug, 140 mL syringe, 125 mL wash bottle, graduated cylinder, 3/8" pump tubing adapter, brushes, slide template, larval tray, spatula, scissors.
      1) Having an additional piece of peristaltic pump tubing, hose connector, and C-flex tubing that can be dedicated to microbe sampling (i.e., not used for water chemistry) may be easier for logistics.
   b. Wash in detergent solution.
      1) Prepare a 0.2% (v.v) solution of detergent and DI water in a wash basin.
      2) Wearing nitrile gloves, submerge equipment thoroughly in detergent solution. Use bottle brush and/or hands to clean equipment.
   c. Rinse with DI water.
      1) Remove equipment from detergent solution and rinse thoroughly in DI. Place on clean bench top paper.
   d. Rinse with 70% ETOH.
      1) Dilute 95% ETOH to 70%. Rinse all equipment in ETOH and allow to thoroughly dry (minimum drying time 5-10 minutes).
   e. When dry, cap bottles and place all other equipment in clean gallon Ziploc bags to transport to the field.
3. Prepare filtered DI for rinsing in the field.
   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. Attach pre-printed labels (Figure 3, RD[05]) to bottles and capsule filters (Figure 2), use permanent marker to fill out labels as necessary before going into the field.
   a. Site = 4-letter site code
   b. Date = YYYYMMDD
   c. Sample type = 'seston', 'epilithon', 'epipelon', 'epipsammon', 'epipelon', or 'epiphyton'
   d. Replicate = '1-3' or '1-5'
   e. Sample ID = SITE.DATE.'amc'.sample type.replicate
5. 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.
6. Prepare coolers, frozen ice packs or water ice, and dry ice.

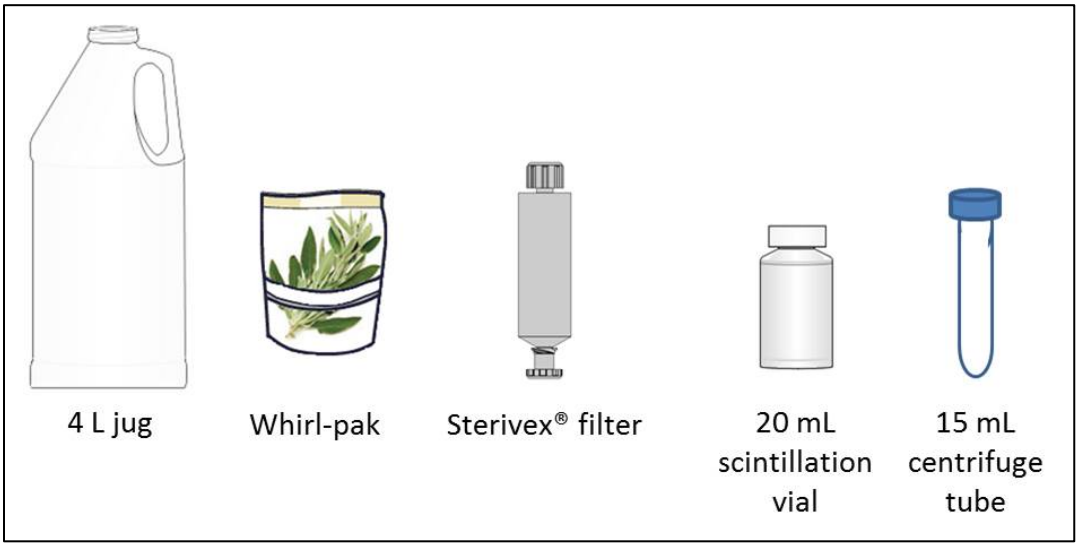

Figure 2. Sample containers.

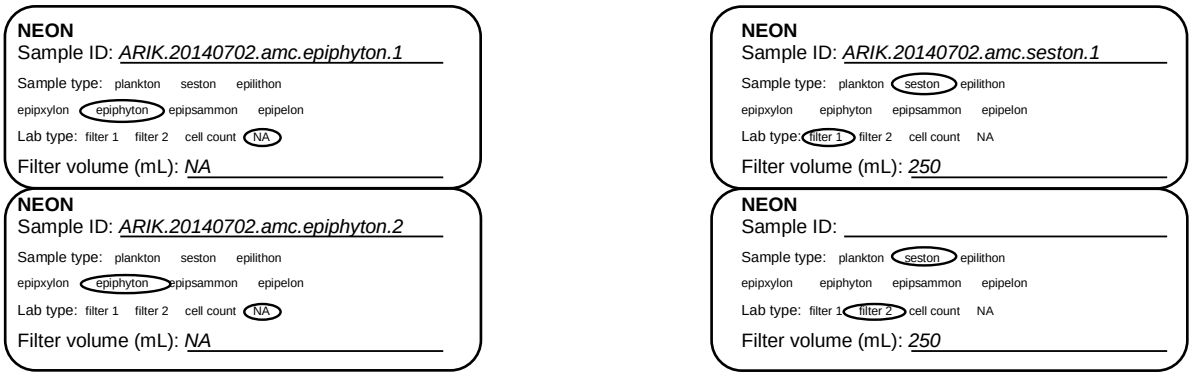

Figure 3. Example of 1”x2” NEON microbe labels.

SOP B Field Sampling

1. Always wear nitrile gloves.
2. Do not sample anywhere you or other field technicians have walked in the reach or locations that appear recently disturbed (e.g., overturned rocks, footprints, dislodged plants, other evidence of wildlife, cattle, humans, etc.). Move collection upstream of disturbance, or laterally if upstream is not possible.
3. Do not sample within a 5 m radius of the aquatic instrumentation.
4. Surface water samples (SOP C) should be collected at the same time and location as water chemistry samples, prior to or upstream of benthic biofilm sampling (SOP D).
5. For benthic biofilm samples, 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. Spread samples out along the reach. For example, do not collect all 5 rocks for rock scrubs within the same riffle, collect one rock from each of 5 different riffles along the reach (if possible).
6. Benthic biofilm will be collected during periods of stable stream flow using a series of scrubbing procedures, depending on habitat and substrate type.
7. Determine dominant habitat and substratum types available within the sampling reach based on Stream Morphology Maps (RD [10]).
8. The order of preference for sampling benthic substrata is as follows (Porter et al. 1993):
   a. Epilithon (rock substrata)
   b. Epixylon (wood substrata)
   c. Epiphyton (plant substrata)
   d. Epipsammon (sand substrata)
   e. Epipelon (silt substrata)
9. Complete the necessary parts of the field datasheet (RD[05]).
10. Fill out general aquatic field metadata sheet (RD[06]) upon every field sampling visit.

B.1 Contingent decisions

Table 8. Contingent decisions for sampling.

Situation	Action	Outcome for Data Products	Considerations
Wadeable stream site with <200 m aboveground stream length due to stream size or seasonal drying	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]).

SOP C Surface Water Microbe Sampling

1. Collect whole water samples in containers.
   a. Sample in the same location where as water chemistry samples are collected. Confirm that no one is wading upstream during sampling.
   b. **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.
   c. Standing in the thalweg, hold 4 L bottles 10 cm 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.
   d. Return to the stream bank to process samples.
      1) **Cell counts**: Use pipette with new sterile tips (if tips are not sterile, transport clean tips from the lab to the field in clean Ziploc bags) to add 18 mL of sample to 20 mL glass scintillation vial containing preservative. Recap bottle and invert 5 times to mix. Immediately preserve samples with formalin in the field.
      2) Place cell count vial in cooler with ice packs, do not freeze.

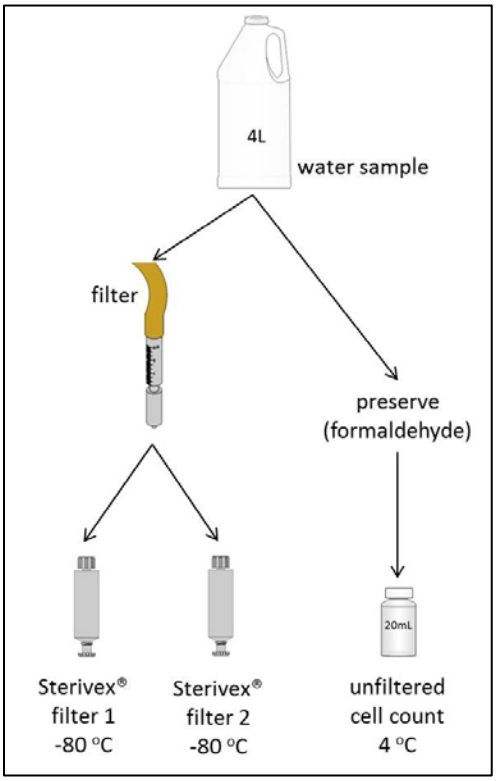

Figure 4. Schematic for surface water sample partitioning

   e. Fill out field data sheet in pencil (RD[05]).

2. Filtered water samples *(Note: filtering can be conducted in the domain lab in cases of inclement weather within 3-4 hours of collection provided 4L jugs are appropriately labeled)*:
   a. Set-up filter apparatus on stream bank or other relatively level surface:
      1) **WEAR GLOVES**, the same gloves can be worn while collecting the sample.
      2) Set peristaltic pump speed below manufacturer specifications (45 psi for Millipore Sterivex SVGP® filter). If pump speed is set too high, the filter can blow out.
   b. Check that the 3 mL syringe adapter is in place to connect pump tubing to the capsule filter (Figure 5).
   c. Label capsule filter with an adhesive label marked in permanent marker.
   d. 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.
   e. Rinse tubing by pumping 100 mL of sample water completely through the tube.
   f. When tubing is flushed with sample water after rinsing, attach filter and 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.
      1) A clean (sterilized and rinsed three times in filtered DI) 140 mL syringe maybe be used, connected directly to the capsule filter, if peristaltic pump is unavailable.
   g. Filter >500 mL (1 L or more if possible) of surface water through the filter, discarding the filtrate. Keep track of volume filtered with the 500 mL plastic graduated cylinder. Stop filtering when filter clogs with sample. Only filter a maximum of 2 L each for the Millipore Sterivex SVGP® capsule filter. *Note that filtering the second 4 L bottle may be required for very clear streams to collect all samples.*
      1) Filter two capsule filters per site for each parent sample (Figure 4). Filters should remain in original packaging until filtration.
      2) When finished, remove filter from set up and push air gently through the filter with 140 mL syringe until dry.
      3) Cap ends of filter with luer lock male and female end caps (Figure 6).
   h. Record volume of filtration on field data sheet (RD[05]) for each sample.
   i. 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.

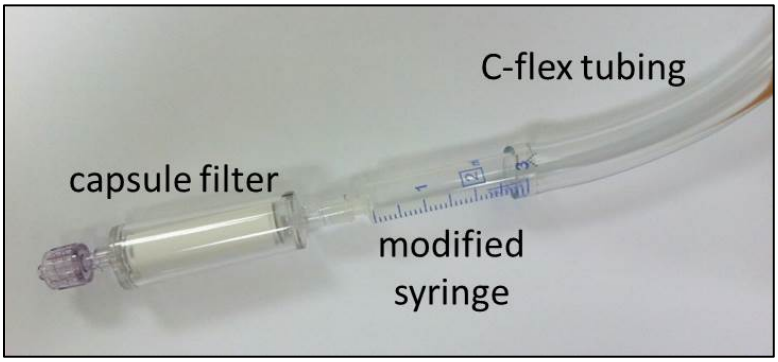

Figure 5. Water filtration setup

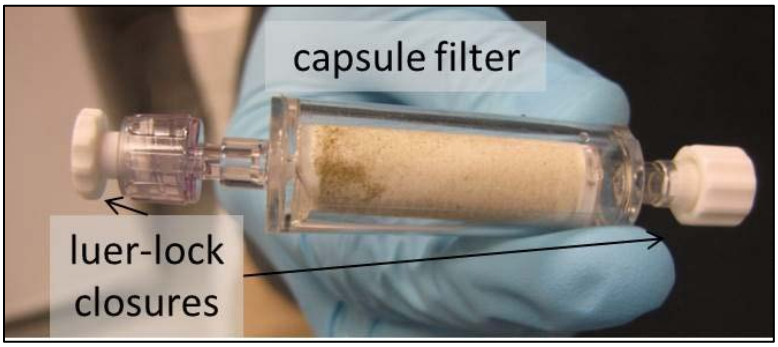

Figure 6. Capsule (Sterivex®) filter and luer lock closures

SOP D Benthic Biofilm Sampling

Benthic biofilm microbial samples are collected in conjunction with algal periphyton samples (see RD [09]), but collected independently, e.g., from different cobbles within the same habitats. Benthic microbes do not need to be collected on the same day as surface water microbes.

D.1 Decision Tree: Determining Habitat for Benthic Samples

1. Determine percent cover of habitat types throughout the sampling reach using the Stream Morphology Map (RD [10]).
    a. The habitat type chosen should be present during all sampling bouts. If the site is a STREON site, the same habitat types should be sampled in both the Aquatic and the STREON reaches.
    b. 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) causes significant changes to the stream channel.
    c. Targeted habitat types (see Definitions, Section 2.4):
        1) Riffle
        2) Run
        3) Pool
2. Does habitat type account for >20% of the reach throughout the year?
    a. If YES, go to Step 3.
    b. If NO, ignore this habitat type.
3. Determine dominant substratum type in habitat (i.e., highest percent cover of habitat and/or where visible algal community is attached) and secondary habitat (i.e., second-highest percent cover and/or second-most heavily colonized) and proceed to Step 1.
    a. Targeted substratum types, in order of sampling preference (see Definitions, Section 2.4):
        1) Cobble (epilithon)
        2) Woody snag (epixylon)
        3) Plant surface (epiphyton)
        4) Sand (epipsammon)
        5) Silt (epipelon)
    b. Dominant habitat/substratum type = 5 replicate samples
    c. Secondary habitat/substratum type = 3 replicate samples
4. Is dominant/secondary substratum cobble?
    a. If YES, follow sampling procedure for epilithon (SOP D.2).
    b. If NO got to Step 5.
5. Is dominant/secondary substratum sand or silt?
    a. If YES, follow sampling procedure for epipsammon and epipelon (SOP D.3).
    b. If NO, go to Step 6.
6. Is dominant/secondary substratum type woody debris?
    a. If YES, follow sampling procedure for epixylon (SOP D.2).

b. If NO, go to Step 7.

7. Is dominant/secondary substratum type aquatic plants?
   a. If YES, follow sampling procedure for epiphyton (SOP D.4).
   b. If NO, return to Step 3 and reassess substratum types.

D.2 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 3).
   a. Sample type = “epilithon” or “epixylon”
   b. Sample ID = SITE.DATE.“amc”.sample type.replicate (where “amc” = aquatic microbes module)
2. 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.
3. **Wear gloves.** Replace gloves between habitat types.
   a. Field-sterilize gloves with alcohol wipes between samples.
4. 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 the following requirements:
   a. Located in flowing water. Avoid cobbles that are directly downstream of large boulders (i.e., not in flowing water because of the effects of the boulder). Also avoid cobbles that are close to the stream bank.
   b. Representative of the periphyton cover of the reach (i.e., not extremely dense or extremely sparse cover relative to nearby substrata).
   c. Stable in the stream bed (i.e., have not recently tumbled).
   d. Larger than the scrubbing template (i.e., > 2 inches in diameter).
   e. Not heavily colonized with aquatic plants, bryophytes, invertebrates. You may brush some invertebrates off the surface using gloved hands.
   f. Do not have leaf litter clinging to the surface (see Definitions, Section 2.4 for details on identifying macrophytes and bryophytes).
   g. Have not noticeably tumbled or been recently disturbed.
   h. Avoid cobbles/woody debris that you or other observers may have recently stepped on.
   i. Do not choose all of the cobbles/woody debris from the same location. Collect samples from several different riffles (if possible), or from different portions of the same riffle.
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, where you sample.
   a. Keep remaining cobbles/woody debris moist with stream water until scrubbing.
6. Field sterilize and prepare equipment at stream bank.

a. Wearing gloves, rinse the 140 mL syringe and 125 mL wash bottle 3 times in filtered DI and discard rinse water.
b. Wipe surfaces of all reusable equipment (e.g., larval tray, scrubbing template) with new alcohol wipes. After cleaning, rinse with filtered DI.
c. Fill the 125 mL wash bottle to the "fill line" (as marked on bottle).
d. **Since this is a volume-based sampling technique, it is important to keep the sample volume consistent and record the volume of water used.**

7. Holding cobble/woody debris underwater, briefly rinse any leaves and/or large invertebrates from surface (you can use your hand to gently sweep insects off the cobble/woody debris surface).
   a. If there are more than 10 insects within your template, discard and choose a new cobble/piece of woody debris.
   b. If there is growth of aquatic plants on the surface that will fall within your template, discard and choose a new cobble.
8. 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 periphyton).
   a. Recheck cobble, if there are more than 10 invertebrates 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. 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.
9. Place white slide template on top of cobble/woody debris (surface that was exposed to light at the stream bottom; Figure 7). Check cobble/woody debris again for colonization of invertebrates, bryophytes, or plants.
10. 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 7).
    d. Scrubbing should be similar to brushing your teeth.

Figure 7. Template placement for epilithic microbe sampling protocol.

e. Periodically rinse the inside area of the template using the 125 mL wash bottle **while holding the template in place**. Allow water to run into the white tray – **DO NOT DISCARD** rinse-water.

f. Continue scrubbing until the inside of the template is clean (scrub cobble longer than woody debris).

g. Remove template. There should be a clean rectangle left from your scrubbing. This is harder to see on woody substrates.

h. 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.

i. 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.**

11. Discard cobble/woody debris on the stream bank. A composite of three cobbles/pieces of woody debris is not required for this protocol.
12. Carefully swirl contents of tray (scrubbate + rinse-water) to re-suspend the sample.
13. 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.

14. Attach Sterivex® filter to 140 mL syringe. Filter ~50 mL of sample, or until filter becomes clogged.
15. After filtering the sample, push air through the filter to dry the filter.
16. Cap both ends of the capsule filter tightly with luer lock caps, place in a Whirl-pak® bag and flash-freeze on dry ice.
17. Repeat Steps 14-16 for second Sterivex® filter.
18. Fill out field data sheet in pencil (Figure 8, RD[05]).

NEON Microbe Collection
Wadeable Streams

Site (4-letter code): *ARIK* | **Recorded by:** *sparker*
Date (YYYYMMDD): *20140702* | **Collected by:** *kgoodman*
Local time (HH:MM): *0845* | **Sampling protocol & Rev.:** *NEON.DOC.001201vA*

Location ID	Habitat	Sample type	Lab type	Replicate	Sample ID	Habitat percent	Substratum size class	Sample volume (mL)	Filter volume (mL)
S2	pool	seston	DNA	1	ARIK.20140702.amc.seston.1	NA	NA	NA	500
S2	pool	seston	RNA	1	ARIK.20140702.amc.seston.1	NA	NA	NA	500
S2	pool	seston	cell count	1	ARIK.20140702.amc.seston.1	NA	NA	20	NA
DS of sensors	pool	epipsammon	NA	1	ARIK.20140702.amc.epipsammon.1	20%	sand	NA	NA
DS of sensors	pool	epipsammon	NA	2	ARIK.20140702.amc.epipsammon.2	20%	sand	NA	NA
US of sensors	pool	epipsammon	NA	3	ARIK.20140702.amc.epipsammon.3	20%	sand	NA	NA

Figure 8. Example of field data sheet for microbe sampling

19. Rinse tray, brush, and template with filtered DI and field sterilize with alcohol wipes before starting next sample.
20. Process remaining cobbles/woody debris one at a time.
21. Replace cobble/woody debris in stream before you leave the site.
22. Recheck labels and place all sample filters on dry ice. Use resealable bags to contain all samples from the same habitat.

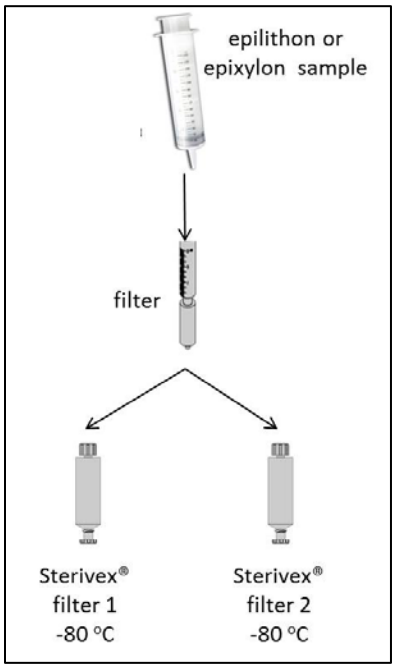

Figure 9. Schematic for benthic biofilm sampling via scrub slurry (SOP D).

D.3 Epipsammon (Sand) and Epipelon (Silt) Microbes

1. Using adhesive labels (RD[05]), label five (or three for secondary habitat) sterile 15 mL plastic tubes Figure 3). **NOTE:** tubes may NOT be reused from the last sampling trip.
2. **Wear gloves.** Replace gloves between habitat types.
   a. Field-sterilize gloves using alcohol wipes between samples.
3. Choose sampling locations with relatively shallow (<1 m) water that appears to be historically wetted (i.e. is usually underwater). Avoid areas that have been recently dried (e.g., extremely shallow areas).
4. Select five locations if this is the dominant habitat and three if this is the secondary habitat. Select locations to sample that meet the following requirements:
   a. Representative of the epipsammon/epipelon habitat and periphyton cover of the reach (i.e., not extremely dense or extremely sparse periphyton cover).
   b. Exposed to ambient light (e.g., not under a log or under a cut bank).
   c. Not heavily colonized with aquatic plants, invertebrates, or have leaf litter covering the surface.
   d. Have not been recently disturbed (e.g., stepped on or sampled for another protocol).
   e. Do not take all of the samples from the same location. Collect samples from several different runs/riffles/pools (if possible), or from different sections of the same run/riffle/pool.

5. Rinse and fill 125 mL wash bottle with 0.2 µm filtered DI.
6. Wipe surfaces of all reusable equipment (e.g., spatula) with new alcohol wipes. After cleaning, rinse with filtered DI.
7. 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.
8. 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.
9. Hold spatula + sample + tube over white sampling tray and invert tube.
10. 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.
11. Place in Whirl-pak® bags and flash-freeze on dry ice.
12. Fill out field data sheet in pencil (RD[05]).
13. Field-sterilize and rinse spatula with filtered DI before starting next sample.
14. Repeat steps 1-13 until samples have been collected from all selected locations.
15. Recheck labels and place all sample bottles in cooler with ice packs. Using secondary resealable bags to group tubes from same habitat.
16. 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]).

D.4 Epiphytic (Aquatic Plant Surfaces) Microbes

1. Label five (or three if secondary habitat) Whirl-paks® with domain, date, site, sample number, location, habitat type, and type of sample (e.g. epiphytic microbes), and collector's name (Figure 3).

2. **Wear gloves.** Replace gloves between habitat types.
    a. Field sterilize gloves with alcohol wipes between samples.
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 10) and that meet the following requirements:
    a. The plant species should be common (i.e., accounts for >50% of the aquatic plants) in the reach throughout the year.
    b. Exposed to ambient light (e.g., not under a log or cut bank).
    c. Plants should not covered by sediments.
    d. Have not been recently disturbed or trampled.

e. Do not take all of the samples from the same location. Collect samples from several different runs/riffles/pools (if possible) or from different sections of the same run/riffle/pool.

4. Wearing clean gloves, and 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 in the same habitat type. Field-sterilize with alcohol pads and rinse with filtered DI between samples.
   c. Be gentle with the plants as epiphytes may be easily dislodged.

Figure 10. 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. Close Whirl-pak® bag and flash-freeze on dry ice.
   a. Close the Whirl-pak® by holding the wire tabs at either side of the bag (Figure 11), then whirl the bag at least 3 complete revolutions to form leakproof 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.
   b. Place Whirl-paks® inside a zip top bag so they are not damaged by the dry ice.

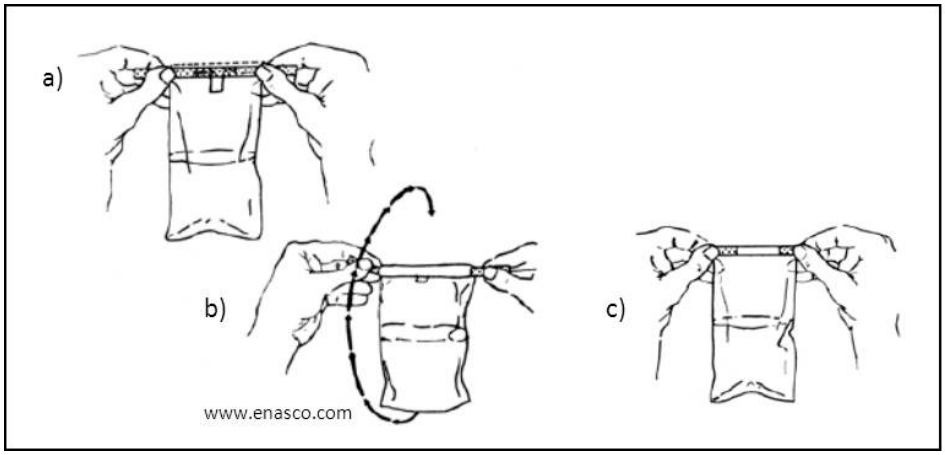

Figure 11. How to close a Whirl-pak® bag: a) hold the wire tabs; b) whirl the bag 3 complete revolutions (or fold the top over); and c) fold the wire ends over to close

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

D.5 Sample Preservation

Cell count samples:

1. Cell counts must be preserved immediately in the field by adding 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.
4. Chill sample at 4°C upon return to lab. Samples can be held for 7 days before shipping.

Sterivex® filters:

1. Flash-freeze filters on dry ice in the field immediately after collection.
2. Freeze capped Sterivex® 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 samples on dry ice in the field immediately after collection.

Freeze whole samples at -80 ^{o}C upon return to the lab. Samples may be held for up to 30 days before shipping.

D.6 Ending the Sampling Day

1. Sterilize equipment and place in 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.
   b. Restock the sampling kit preservation vials, nitrile gloves, filters, luer locks, resealable bags, toothbrushes, etc. Refer to equipment list in Section 6.1.
2. Equipment maintenance, cleaning and storage
   a. Charge drill pump batteries.
   b. Wash all equipment that has come in contact with stream water and does not need to be sterilized according to the NEON Aquatic Decontamination Protocol (RD[08]).
   c. Dry all items completely before storing.
   d. Discard and replace any broken templates.

SOP E Laboratory Sampling and Analysis

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 D.5. Samples must be processed within 4 hours of collection.

Samples should be stored as outlined in preservation, SOP D.5.

SOP F 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.

SOP G 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 CLA's NEON intranet site.

Shipments are to include a hardcopy of the "per sample" tab of the shipping inventory 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.

G.1 Cell counts

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., take note of 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 ground on ice or ice packs.

G.2 Filters, Plant, and Sediment Samples

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 AD[03].

G.3 Handling Hazardous Material

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

G.4 Supplies/Containers

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

SOP H Timelines and Conditions

1. **Cell counts**: Formalin-preserved samples may be stored at 4 °C for up to 7 days. Do not freeze.
2. **Sterivex® filters**: Filters may be stored at -80 °C for up to 30 days.
3. **Whole samples (plants, sediment):** Samples may be stored at -80 °C for up to 30 days.

H.1 Grouping/Splitting Samples

Group samples by site per bout.

H.2 Return of Materials or Containers

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

H.3 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.

H.4 Laboratory Contact Information and Shipping/Receipt Days

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

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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. 002198	Datasheets for AOS Protocol and Procedure: Microbes in Wadeable Streams
NEON.DOC.001646	General AQU Field Metadata Sheet
NEON.DOC.002494	Datasheets for AOS Sample Shipping Inventory

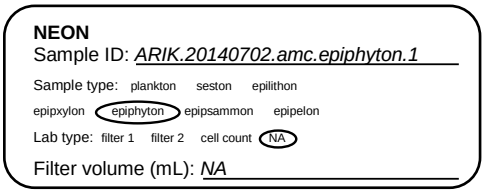
These datasheets can be found in Agile or the NEON Document Warehouse.

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 – Prepare labels (2" * 4").



Step 3 – Ensure the General AQU Field Metadata Sheet (RD[06]) is completed per field site visit.

Step 4 – Determine habitat sampling locations from the Stream Morphology Map (RD[11]) and water chemistry or periphyton sampling locations.

Step 5 – Determine sampler type based on the habitats present and the order of preference for sampling substratum.

B.2 Steps for Surface Water Microbe Sampling

Step 1 – Collect samples in the thalweg using the 4 L jugs.

Step 2 – Process the samples

1. Cell counts:
   a. Collect in 20 mL glass 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.
2. Sterivex® filters (2 replicates):
   a. Filter >500 mL of stream water through each capsule filter (2 filters total).
   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

Step 1 – Determine habitat and sampling method.

Step 2 – Collect samples:

1. 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:
      1) Filter ~50 mL through each capsule filter (2 filters total).
      2) Flash-freeze on dry ice in the field.
      3) Freeze at -80 °C upon returning to the lab.
      4) Ship on dry ice overnight.
2. 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.
3. 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.

B.4 Determining Habitat to Sample

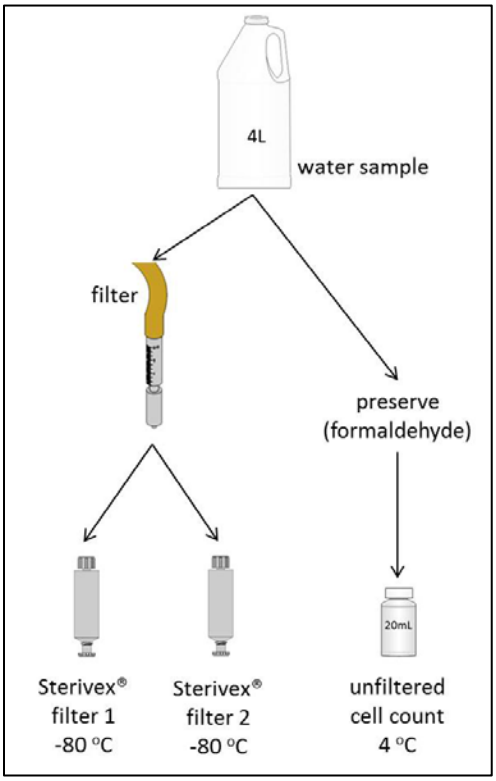
1. Determine percent cover of habitat types throughout the sampling reach using the Stream Morphology Map (RD [10]).
   a. Targeted habitat types (see Definitions, Section 2.4):
      1) Riffle
      2) Run
      3) Pool
2. Does habitat type account for >20% of the reach?
   a. If YES, go to Step 3.
   b. If NO, ignore this habitat type.
3. Determine dominant substratum type in habitat (i.e., highest percent cover of habitat and/or where visible algal community is attached) and secondary habitat (i.e., second-highest percent cover and/or second-most heavily colonized) and proceed to Step 1.
   a. Targeted substratum types, in order of sampling preference (see Definitions, Section 2.4):
      1) Cobble (epilithon)
      2) Woody snag (epixylon)
      3) Plant surface (epiphyton)
      4) Sand (epipsammon)
      5) Silt (epipelon)
   b. Dominant habitat/substratum type = 5 replicate samples
   c. Secondary habitat/substratum type = 3 replicate samples
4. Is dominant/secondary substratum cobble?
   a. If YES, follow sampling procedure for epilithon (SOP D.2).
   b. If NO got to Step 5.
5. Is dominant/secondary substratum sand or silt?
   a. If YES, follow sampling procedure for epipsammon and epipelon (SOP D.3).
   b. If NO, go to Step 6.
6. Is dominant/secondary substratum type woody debris?
   a. If YES, follow sampling procedure for epixylon (SOP D.2).
   b. If NO, go to Step 7.
7. Is dominant/secondary substratum type aquatic plants?
   a. If YES, follow sampling procedure for epiphyton (SOP D.4).
   b. If NO, return to Step 3 and reassess substratum types.

B.5 Order of Preference for Sampling Substrata

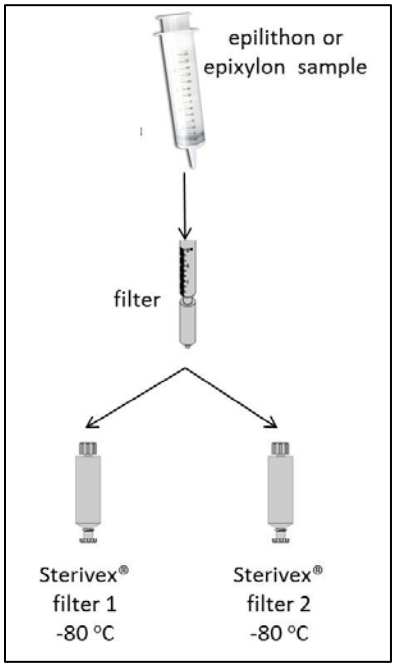
The order of preference for sampling benthic substrata is as follows (Porter et al. 1993):

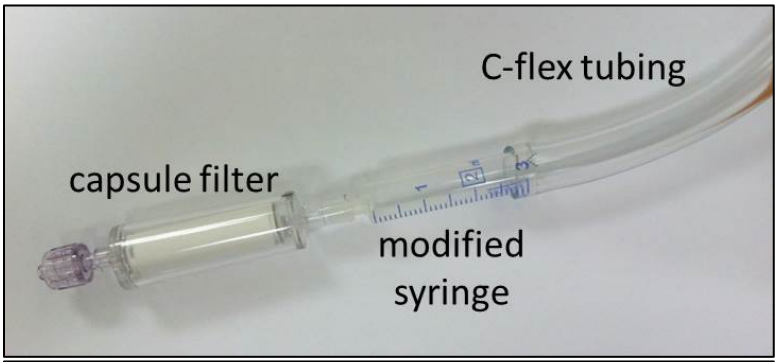
1. Epilithon (rock substrata)
2. Epixylon (wood substrata)
3. Epiphyton (plant substrata)
4. Epipsammon (sand substrata)
5. Epipelon (silt substrata)

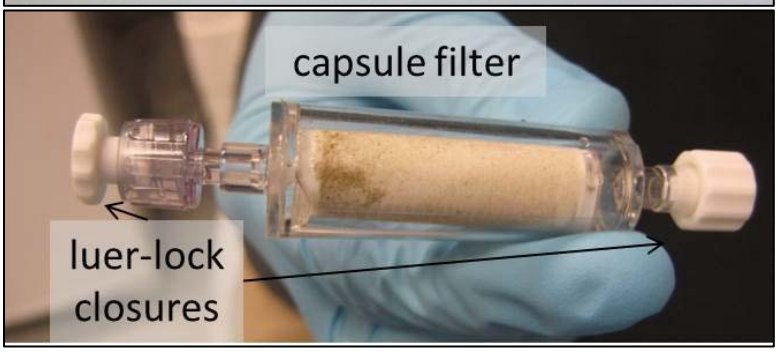
B.6 Schematic for Surface Water Microbe Sampling



B.7 Schematic for Benthic Microbe Sampling (Epilithon, Epixylon)







APPENDIX C REMINDERS

Before heading into the field: Make sure you…

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

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

- ☑ 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 benthic biofilm sampling, 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 3-4 hours of collection provided 4L jugs are appropriately labeled.
- ☑ Keep track of the volume of sample filtered.
- ☑ DO NOT FREEZE cell count samples.

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 AQU's NEON intranet site.

Domain	Site	Bout 1	Bout 2	Bout 3
D01	West Branch Bigelow Brook*	11Apr-9May	9Jul-6Aug	3Oct-31Oct
D01	Sawmill Brook	8Apr-6May	9Jul-6Aug	9Oct-6Nov
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
D05	Pickerel Creek*	20Apr-18May	5Jul-2Aug	13Sep-11Oct
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	Bozeman Creek	11Apr-9May	7Jul-4Aug	6Sep-4Oct
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	Planting 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

APPENDIX E SITE-SPECIFIC INFORMATION: HABITAT AND SUBSTRATA RECOMMENDATIONS FOR WADEABLE STREAMS

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

Domain	Site	Habitat 1	Habitat 2
D01	West Branch Bigelow Brook	Pools (epipsammon)	Pools (epixylon)
D01	Sawmill Brook	*	*
D02	*Site to be determined*	*	*
D02	Posey Creek	Riffles (epilithon)	Pools (epipelon)
D04	Rio Guilarte	Riffles (epilithon)	*
D04	Rio Cupeyes	Riffles (epilithon)	*
D05	*Site to be determined*	*	*
D06	Kings Creek	*	*
D06	McDowell Creek	*	*
D07	Leconte Creek	*	*
D07	Walker Branch	*	*
D08	Mayfield Creek	Runs (epixylon)	Pools (epipsammon)
D10	Arikaree River	Runs (epiphyton)	Runs (epipsammon)
D11	Pringle Creek	*	*
D12	Bozeman Creek	Riffles (epilithon)	Riffles/Runs (epixylon)
D12	Blacktail Deer Creek	Riffles (epilithon)	Riffles/Runs (epixylon)
D13	Como Creek	Riffles (epilithon)	Pools (epipsammon)
D13	*Site to be determined*	*	*
D14	Sycamore Creek	*	*
D15	Red Butte Creek	Riffles (epilithon)	*
D16	McRae Creek	Riffles (epilithon)	*
D16	Planting Creek	Riffles (epilithon)	*
D17	*Site to be determined*	*	*
D17	*Site to be determined*	*	*
D18	Oksrukuyik Creek	Riffles (epilithon)	Pools (epipelon)
D19	Caribou Creek	Riffles (epilithon)	*

*To be determined