



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

AOS PROTOCOL AND PROCEDURE: AMC – AQUATIC MICROBIAL SAMPLING

PREPARED BY	ORGANIZATION	DATE
Stephanie Parker	AQU	01/04/2024
Heather Adams	AQU	06/07/2013
Keli Goodman	AQU	05/08/2015

APPROVALS	ORGANIZATION	APPROVAL DATE
Kate Thibault	SCI	01/04/2024

RELEASED BY	ORGANIZATION	RELEASE DATE
Tanisha Waters	CM	01/04/2024

See configuration management system for approval history.

The National Ecological Observatory Network is a project solely funded by the National Science Foundation and managed under cooperative agreement by Battelle. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
A	01/21/2016	ECO-03455	<ul style="list-style-type: none"> Initial release Amalgamation of NEON.DOC.001200 and NEON.DOC.001201 Updated lake sampling depth guidelines
B	02/08/2017	ECO-04359	<ul style="list-style-type: none"> Update NEON template Update sample ID template Update field and lab sterilization SOP Lengthen holding time on cell count samples
C	02/13/2018	ECO-05326	<ul style="list-style-type: none"> Add barcode labels Change formaldehyde concentration Update shipping hold times Add benthic large substrate sampler Metagenomics labeling for mid-summer samples Move datasheets to appendix Update D14 bout dates Cell count packaging
D	12/19/2018	ECO-05967	<ul style="list-style-type: none"> Update D17 bout windows Update barcode info and overnight shipping Add contingencies and rules for stream drying Update benthic sample ID format Add Van Dorn
E	12/15/2021	ECO-06707	<ul style="list-style-type: none"> Updated to new template (NEON.DOC.050006 Rev K) Add separate metagenomics sample in Bout 2 Add contingency for broken DI system Add cryostorage box for ARC filters
F	01/04/2024	ECO-07048	<ul style="list-style-type: none"> Clarify rescheduling, sampling impractical, and biophysical criteria Updated Kemmerer and Van Dorn language for rivers Change 10% buffered formaldehyde wording to 10% buffered formalin and added clarification to equipment list Filtered formalin storage requirements Removed sample timing from appendix Removed part numbers from equipment lists



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

			<ul style="list-style-type: none">• New cell count container, sample volume, and preservative volume• Updated to new template Rev L• Updated NEON logo
--	--	--	--



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

TABLE OF CONTENTS

1 OVERVIEW 1

1.1 Background 1

1.2 Scope 1

1.3 Acknowledgments 2

2 RELATED DOCUMENTS AND ACRONYMS 3

2.1 Applicable Documents 3

2.2 Reference Documents 3

2.3 Acronyms 4

2.4 Definitions 4

3 METHOD 7

4 SAMPLING SCHEDULE 9

4.1 Sampling Frequency and Timing 9

4.2 Criteria for Determining Onset and Cessation of Sampling 10

4.3 Timing for Laboratory Processing and Analysis 11

4.4 Sampling Timing Contingencies 11

4.5 Missed or Incomplete Sampling 12

4.6 Estimated Time 15

4.7 Criteria for Permanent Reallocation of Sampling within a Site 15

4.8 Sampling Specific Concerns 16

5 SAFETY 18

6 PERSONNEL 19

6.1 Training Requirements 19

6.2 Specialized Skills 19

7 STANDARD OPERATING PROCEDURES 20

SOP A PREPARING FOR SAMPLING 21

A.1 Preparing for Data Capture 21

A.2 Microbe Sampling Preparation 21

A.3 Labels and Identifiers 24

A.4 Field Sterilization and Storage 27

SOP B FIELD SAMPLING: SURFACE WATER MICROBES 28



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

B.1	Spatially and Temporally Linked Protocols	29
B.2	Wadeable Stream Collection	29
B.3	Lakes and River Collection	30
B.4	Surface Water Processing	33
B.5	Surface Water Sample Preservation	37
SOP C	FIELD SAMPLING: BENTHIC MICROBES (WADEABLE STREAMS ONLY)	38
C.1	Spatially and Temporally Linked Protocols	38
C.2	Benthic Substrata Selection	38
C.3	Epilithon (Rock Scrubs) and Epixylon (Wood Scrubs)	40
C.4	Epilithon (Rock Scrubs) and Epixylon (Wood Scrubs) – Large Substrates.....	44
C.5	Epiphyton (Aquatic Plant Surfaces).....	47
C.6	Epipsammon (Sand) and Epipelon (Silt).....	50
C.7	Benthic Sample Preservation	51
SOP D	POST-FIELD SAMPLING TASKS	52
D.1	Laboratory Sampling and Analysis	52
D.2	Ending the sampling day.....	52
SOP E	DATA ENTRY AND VERIFICATION	54
SOP F	SAMPLE SHIPMENT	55
8	REFERENCES	56
APPENDIX A	QUICK REFERENCES	57
APPENDIX B	REMINDERS	58
APPENDIX C	ESTIMATED DATES FOR ONSET AND CESSATION OF SAMPLING	59
APPENDIX D	SITE-SPECIFIC INFORMATION	60
APPENDIX E	EQUIPMENT	61

LIST OF TABLES AND FIGURES

Table 1. Sampling frequency for Aquatic Microbial Sampling procedures on a per SOP per site type basis.	10
Table 2. Contingent decisions.....	11
Table 3. Guidance for responding to delays and cancellations encountered during implementation of the Aquatic Microbe protocol.....	14

Table 4. Protocol-specific Sampling Impractical reasons entered in the Fulcrum application. In the event that more than one is applicable, choose the dominant reason sampling was missed..... 15

Table 5. Estimated staff and labor hours required for implementation of the Aquatic Microbial Sampling protocol..... 15

Table 6. Contingent decisions for benthic sampling in a wadeable stream. 16

Table 7. Barcode requirements for sample types generated by the Aquatic Microbes protocol. 26

Table 8. Site-specific sampling locations and habitats. 60

Table 9. Equipment List – Prep/General Equipment. 61

Table 10. Equipment List – Lab Sterilization. 62

Table 11. Equipment List – Surface Water Microbes. 63

Table 12. Equipment List – Benthic Microbes. 65

Table 13. Equipment List – Sample processing and preservation. 67

Table 14. Equipment List – Sample Shipment. 67

Figure 1. Generic site layouts with microbe sampling locations in red. 8

Figure 2. The documentation to account for a Missed Sampling event depends on the situation. 14

Figure 3. A high level workflow diagram that visually shows how the separate SOPs are sequentially connected. 20

Figure 4. Filter adapter for peristaltic pump, you may put the C-flex tubing inside or outside (cut off top) of the 3 mL syringe..... 21

Figure 5. Microbe sample containers. 23

Figure 6. Example NEON microbes label. 25

Figure 7. An expanded diagram of the workflow for surface water microbe sampling..... 28

Figure 8. Surface water microbe sampling locations in rivers and lakes..... 30

Figure 9. Vertical Kemmerer water sampler in the a) open or cocked position and b) closed (after dropping the messenger) position. c) Horizontal Van Dorn in the open position..... 32

Figure 10. Schematic for surface water sample partitioning. You may also use a syringe to directly collect surface water in some systems..... 33

Figure 11. Barcode label scanning. 34

Figure 12. Pump sample water out of the 4-gallon HDPE jug, through the peristaltic pump, the through the Sterivex filter, and into a graduated cylinder to measure the volume filtered..... 35

Figure 13. Capsule (Sterivex) filter and luer lock closures..... 36

Figure 14. An expanded diagram of the workflow for benthic microbe sampling..... 38

Figure 15. Template placement for epilithic microbe sampling protocol. 41

Figure 16. Schematic for benthic biofilm sampling via scrub slurry..... 44

Figure 17. Schematic of PVC periphyton sampler. 46

Figure 18. Example of epiphytes growing on bulrush in a Colorado stream. The red circle shows thick epiphyton on the surface of a submerged plant. 48

Figure 19. 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..... 49



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

Figure 20. Cryogenic storage box for ARC samples. Use these boxes rather than zip-top bags for shipping to the Biorepository..... 55

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

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 with surface water chemistry (surface water microbes) or benthic algae (benthic microbes) 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 inflow and outflow of a lake. The potentially large impact of stream flow on microbial communities has been incorporated into the NEON Aquatic Sample Strategy (RD[07], RD[20]). 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]).



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

1.3 Acknowledgments

Collection techniques have been standardized to be consistent with the NEON water chemistry protocol and stream algae 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.004104	NEON Science Data Quality 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.002652	NEON Data Products Catalog
RD[04]	NEON.DOC.001271	OS Protocol and Procedure: DMP – Data Management
RD[05]	NEON.DOC.003041	Datasheets for AOS Protocol and Procedure: Aquatic Microbial Sampling
RD[06]	NEON.DOC.001646	NEON General AQU & GAG Field Data Sheet
RD[07]	NEON.DOC.001152	NEON Aquatic Sample Strategy
RD[08]	NEON.DOC.004257	Standard Operating Procedure: Decontamination of Sensors, Field Equipment, and Field Vehicles
RD[09]	NEON.DOC.003045	AOS Protocol and Procedure: ALG – Periphyton and Phytoplankton Sampling
RD[10]	NEON.DOC.002905	AOS Protocol and Procedure: SWC – Water Chemistry Sampling in Surface Waters and Groundwater
RD[11]	NEON.DOC.003162	AOS Protocol and Procedure: Wadeable Stream Morphology
RD[12]	NEON.DOC.014048	TOS Protocol and Procedure: SLS – Soil Physical, Chemical, and Microbial Measurements
RD[13]	NEON.DOC.003046	AOS Protocol and Procedure: INV – Aquatic Macroinvertebrate Sampling
RD[14]	NEON.DOC.003039	AOS Protocol and Procedure: APL – Aquatic Plant, Bryophyte, Lichen, and Macroalgae Sampling
RD[15]	NEON.DOC.001197	AOS Protocol and Procedure: BAT – Bathymetry and Morphology of Lakes and Non-Wadeable Streams
RD[16]	NEON.DOC.002191	Datasheets for Secchi Depth and Depth Profile Sampling
RD[17]	NEON.DOC.002792	AOS Protocol and Procedure: DEP – Secchi Disk and Depth Profile Sampling in Lakes and Non-wadeable Streams
RD[18]	NEON.DOC.003282	NEON Protocol and Procedure: SIM – Site Management and Disturbance Data Collection

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

RD[19]	NEON.DOC.005224	NEON Protocol and Procedure: SCS – Shipping Ecological Samples and Equipment
RD[20]	NEON.DOC.003600-NEON.DOC.003618	Aquatic Site Sample Design – NEON Domain ##
RD[21]	NEON.DOC.005346	OS Standard Operating Procedure: FRZ – Preparation and Use of Dry Ice Alternative Freezing Materials

2.3 Acronyms

Acronym	Definition
°C	Degrees Celsius
DI	De-ionized water
DNA	Deoxyribonucleic acid
DSF	Domain Support Facility
ETOH	Ethanol
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
v/v	Volume concentration of a solution (volume per volume)

2.4 Definitions

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

Clean technique: Procedures to minimize the introduction of chemical or biological contaminants into a sample. Contamination can result from dust particles, non-purified water, sweat, hair, and other environmental sources.

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.

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

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 and measured using a Secchi disk.

Field sterile technique: Procedures to minimize the introduction of microbial/DNA contaminants into a sample, such as human microbiota or DNA from a different source material or habitat. Field sterilization is done at the sampling site and may include priming and rinsing with native water rather than DI, and using ethanol wipes instead of sterile washes.

Fulcrum: Software tool used to create NEON electronic data entry applications.

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 (m³) 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. This term encompasses epilithon, epixylon, epipsammon, epipelon, and epiphyton.

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, causing fine sediments to 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.

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

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

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

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.

ServiceNow: Software tool used for problem/incident tracking and resolution.

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

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

Sterile technique: Procedures to minimize the introduction of microbial/DNA contaminants into a sample, such as human microbiota or DNA from a different source material or habitat.

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 $^{\circ}\text{C}$ per meter. The denser and cooler layer below the thermocline is the hypolimnion, warmer upper layer is termed the epilimnion.

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

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. In wadeable streams, microbes are collected in both surface water and benthic environments. In lakes and rivers, microbe samples are collected only in surface water.

Surface water microbes: Surface water microbes are collected 12 times per year in wadeable streams and 6 times per year in lakes and rivers, at the same time and location as standard recurrent water chemistry samples (RD[10]). Sample timing is based on statistical analysis of environmental parameters. Details on sampling locations and timing are provided in the Aquatic Site Sample Designs (RD [20]). Surface water microbe 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 C, Parker and Utz 2022). Benthic microbes are not collected in lakes and rivers. Benthic biofilms are collected in wadeable streams during periods of stable stream flow (e.g., when the stream is not flooding, defined as >3x historic median discharge of all released NEON data from a site, Clausen and Biggs 1997, Stevenson and Bahls 1999) using a series of scrubbing procedures, depending on habitat and substratum type (Moulton et al. 2002). Microbial samples are collected either as whole sample (plant or sediment grab) and on filters for archive and analysis, depending on the substratum type at the site.

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 following these steps, it is critical that technicians document the problem and enter it in NEON’s problem tracking system.

Quality assurance is performed on data collected via these procedures according to the NEON Science Data Quality Plan (AD[05]).

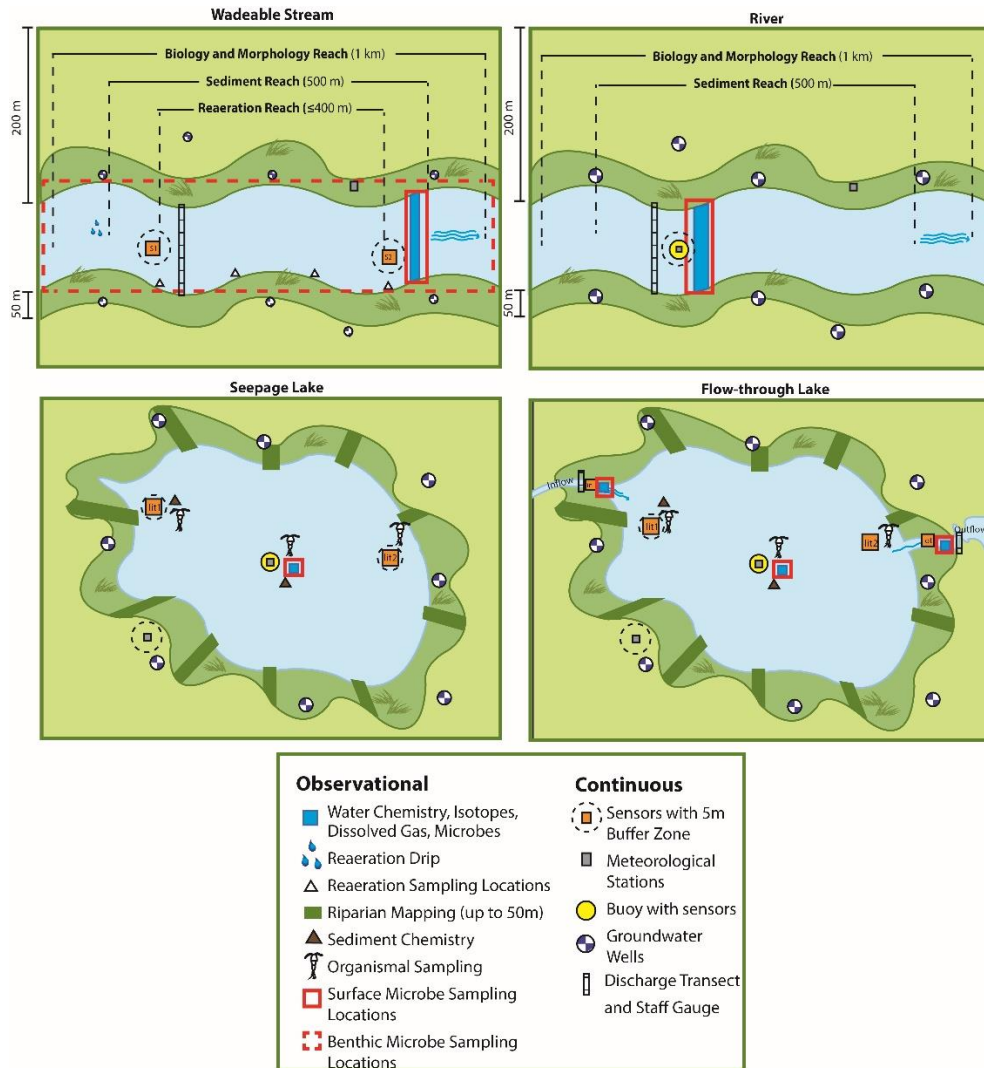


Figure 1. Generic site layouts with microbe sampling locations in red. Seepage lakes have no true inlet or outlet stream. In flow-through streams, inflow and outflow infrastructure are located in the inlet or outlet stream channel.



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

4 SAMPLING SCHEDULE

4.1 Sampling Frequency and Timing

Surface water microbes

Surface water microbes are collected 12 times per year in wadeable streams and 6 times per year in lakes and rivers. Sampling will occur at the same time, location and sampling depth as standard recurrent surface water chemistry samples collected on the same sampling day (RD[10]). If the standard recurrent (monthly) water chemistry is rescheduled or canceled, surface microbes may be rescheduled to another water chemistry sampling day during the same month, with science approval. Microbial samples may be collected either just before or just after chemistry sample collection as long as the water column remains undisturbed.

Field blanks are collected once per site per year. Blanks may be scheduled during any bout, however, Science suggests scheduling during metagenomics (MET) collection to better keep track of once-per-year tasks.

Synchronized protocols and SOPs include:

- AOS Protocol and Procedure: SWC – Water Chemistry Sampling in Surface Waters and Groundwater (RD[10])
- AOS Protocol and Procedure: DEP – Secchi Disk and Depth Profile Sampling in Lakes and Non-wadeable Streams (RD[17])

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 on the same day as algae sampling within the first 21 days of the 1-month biological sampling bout window specified in Appendix C, with a minimum of two weeks between sampling dates. If contingent decisions are followed and sampling is rescheduled, observe a minimum of two weeks between the sampling date and the start of the next scheduled bout window for Bouts 1 and 2. For Bout 3, allow for 30 days past the end date of the sampling window to reschedule missed sampling. 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. See the Aquatic Site Sampling Design (RD[20]) for your domain for additional details and scheduling preferences. Use NEON’s problem reporting system to seek guidance and report sampling efforts that take place outside of the defined sampling window.

Synchronized protocols and SOPs include:

- AOS Protocol and Procedure: Periphyton and Phytoplankton Sampling (RD[09])

Table 1. Sampling frequency for Aquatic Microbial Sampling procedures on a per SOP per site type basis.

SOP	AOS Site Type	Location	Bout Duration	Bouts Per Year	Remarks
SOP B	Lake/river	Buoy	1 day	6	Sample collected with water chem and depth profile
	Stream	S2	1 day	12	Sample collected with water chem
SOP C	Stream	Reach	1 day	3	Samples collected with periphyton

Scheduling Considerations

- All samples for a bout at a site must be collected during the same day.

Field Work and Laboratory Processing:

- Cell counts: 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 60 days before shipping.
- DNA, ARC, and MET samples: Sterivex filters, sediment grabs, and plant grabs for DNA, ARC, and MET analysis must be flash-frozen in the field and may be held at the domain lab at -80 °C until shipping.

4.2 Criteria for Determining Onset and Cessation of Sampling

Surface water microbes

At **wadeable stream** sites, sampling is scheduled with recurrent monthly surface water chemistry sampling for a total of 12 sampling dates per year, including dates that are under ice following the criteria for surface water chemistry sampling in RD[10]. Water chemistry cancellations may result in rescheduling microbes with water chemistry sampling later in the month to maintain the monthly sampling schedule. Follow scheduling and cancellation guidelines in the water chemistry protocol.

At **lake and river** sites, sampling is scheduled every-other month with recurrent monthly surface water chemistry sampling for a total of 6 sampling dates per year, including dates that require sampling under the ice at northern sites. Water chemistry cancellations may result in rescheduling microbes with water chemistry sampling later in the month to maintain the sampling schedule. Follow scheduling and cancellation guidelines in the water chemistry protocol (RD[10]).

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 C). Benthic microbes are collected with benthic algae samples (RD[09]) during periods of stable stream flow (Stevenson and Bahls 1999). Follow guidelines for rescheduling in

the Periphyton and Phytoplankton protocol (RD[09]), benthic microbes would not be collected without periphyton samples.

4.3 Timing for Laboratory Processing and Analysis

If samples are not processed in the field (filtered and/or subsampled for cell counts), processing must be completed within 4 hours of collection at the Domain Support Facility.

1. Cell count samples and cell count blanks must be preserved in formalin in the field or within 4 hours of collection, and may be held at the domain lab at 4 °C for up to 60 days before shipping.
2. DNA, ARC, and MET samples and blanks (filters and grabs) must be filtered within 4 hours of collection and flash-frozen immediately after processing. These samples must be stored at the domain lab at -80 °C until shipping.

4.4 Sampling Timing Contingencies

All samples from this protocol from one sampling bout must be collected within one day (i.e., all samples per site as detailed in this protocol) because of the fluctuating nature of aquatic habitats. Spreading sample collection over multiple days increases variability among samples. A minimum of 2 weeks between sample dates from consecutive bouts shall be observed. If rescheduling > 3 days past the end of the bio bout window or rescheduling after fish sampling, submit a schedule change request in ServiceNow. If sampling is cancelled and will not be rescheduled, submit an incident ticket.

Table 2. Contingent decisions.

Delay/ Situation	Action	Outcome for Data Products
Hours	If weather conditions deteriorate and conditions become unsafe (e.g., approaching thunderstorm or rapid increase of water level), or the lake/river becomes too windy (>35 km hr ⁻¹ (21.7 mph)) 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, discard samples, 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 may be flagged.
	If field conditions cause reduced sampling (see Table 6), collect a minimum of 3 samples in a single habitat type. If you are not able to collect 3 samples in a single habitat type, re-attempt sampling on another day.	Reduced sample replication

14 Days	Benthic samples: If flooding occurs on or <14 days prior to the targeted sampling date in a wadeable stream (>3x median historic discharge of released NEON data; 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 to allow biofilm to 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 may be flagged
	If a stream has dried and enough water (>100 m length) to sample returns to the channel, wait for the channel to be wet for 14 days of recolonization before sampling.	None as long as samples are collected within the pre-determined sampling window. If waiting for water to return causes sampling to occur outside of the sampling window, data may be flagged.
60 Days	Surface samples: Preserved cell count samples may be held for a maximum of 60 days at 4 °C in the domain lab if circumstances do not allow shipping to the external lab.	Sample degradation is possible during the 60-day window, however preliminary studies at the external lab show that samples are relatively stable until 60 days. Data shipped within this window are not flagged.
6 months	Frozen samples (filters, sediment, and plant material) may be held for up to 6 months at -80 °C in the domain lab if circumstances do not allow shipping to the external lab.	Holding samples longer than expected affects external lab schedules, staffing, and budgets and delays data release on the NEON portal. However, sample integrity is not affected.

4.5 Missed or Incomplete Sampling

Sampling according to the schedule is not always possible, and multiple factors may impede work in the field at one or more plots or sampling locations in a given bout. For example:

- Logistics – e.g., insufficient staff or equipment
- Environment – e.g., deep snow, flooding, inclement weather, or
- Management activities – e.g., controlled burns, pesticide application

Instances such as those listed above must be documented for scheduling, tracking long-term plot suitability, and informing end users of NEON data availability. Some types of missed sampling are due to events that should be recorded in the Site Management App; refer to the Site Management and Disturbance Reporting Protocol for more detail (RD[18]).

Missed or Incomplete Sampling Terms

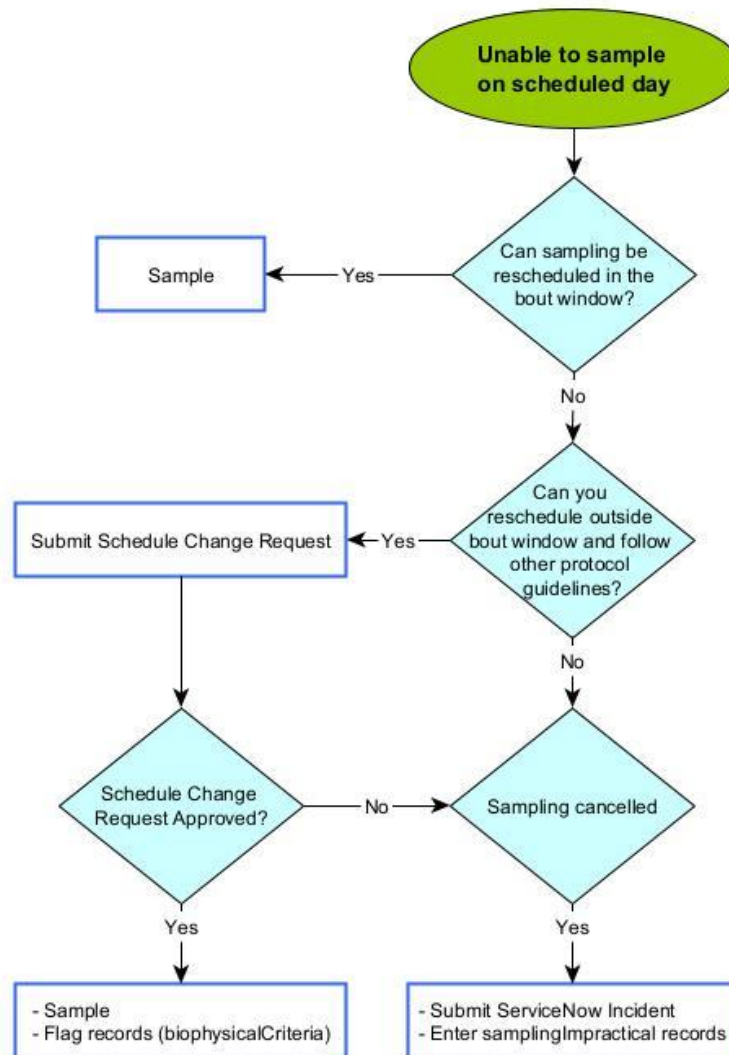
Terms that inform Missed or Incomplete Sampling include:



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

- **Canceled Sampling:** Incidence of *scheduled sampling* that did not, and will not, occur. Canceled Sampling is recorded at the same resolution as data that are ordinarily recorded (e.g., each missed macroinvertebrate sample gets its own record).
- **Sampling Impractical:** The field name associated with a controlled list of values that is included in the data product to explain a Missed Sampling event – i.e., why sampling did not occur.
- **Biophysical Criteria:** This field name associated with a controlled list of values that is included in the data product to explain changes to the schedule.
- **Rescheduled:** Missed Sampling is rescheduled for another time according to one of the scenarios documented in **Figure 2**, resulting in no change to the total number of sampling events per year.

The documentation that must accompany missed sampling depends on the timing, subsequent action, and the audience appropriate for numerous scenarios (**Figure 2**).



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

Figure 2. The documentation to account for a Missed Sampling event depends on the situation. Diamonds represent decision points and boxes describe the required action. Required actions may include: a) Submitting a ServiceNow incident, b) creating a Sampling Impractical record, c) creating a data Flag, d) creating a Site Management record, or e) some combination of (a) – (d).

To Report Missed or Incomplete Sampling:

1. Canceled or rescheduled sampling must be communicated to Science by a Service Now Incident or Schedule Change Request.
 - a. The lead Field Ecologist should consult the [Delayed or Cancelled Activities table](#) to best determine when reporting is required (**Figure 2**).
2. Canceled sampling: For each missed sample, the **Sampling Impractical** field must be populated in the mobile collection device (Table 4). One record per missed sample location.
 - a. A minimum of three benthic field samples from the one habitat type should be collected during each sampling event if following contingent decisions (**Table 6**, else sampling should be re-attempted.
3. **Rescheduled** benthic sampling events that occur within the biology bout window, sample and record data as normal. Rescheduling within the bout window does not need to be reported to science unless sampling will occur after fish sampling or another disturbance event.
4. **Rescheduled** sampling events that occur outside of the defined AOS water chemistry or biology sampling bout window must be approved by Science in a Schedule Change Request.
5. Sampling events that are rescheduled ± 3 days outside the sampling bout require an entry in **Biophysical Criteria**.
 - a. biophysicalCriteria – An indicator of whether sampling coincided with the intended biophysical conditions (i.e., within the AOS biology sampling bout window)

Table 3. Guidance for responding to delays and cancellations encountered during implementation of the Aquatic Microbe protocol.

Activity Name	Days Delayed from Schedule	Delay Action	Cancellation Action
Surface microbes sampling	>14 days of protocol sampling date or >5 days of scheduled sampling date.	IS/OS Schedule Change Request	Submit incident ticket
Benthic microbes sampling	> 3 days outside bio bout window or rescheduling after fish (streams only)	IS/OS Schedule Change Request	Submit incident ticket

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

Table 4. Protocol-specific Sampling Impractical reasons entered in the Fulcrum application. In the event that more than one is applicable, choose the dominant reason sampling was missed.

Field name	Dropdown list option	Description
Sampling Impractical	Location dry	Location dry
Sampling Impractical	Location frozen	Location frozen
Sampling Impractical	Location snow covered	Location snow covered
Sampling Impractical	High water velocity	Water velocity too high to sample in stream or river
Sampling Impractical	Logistical	Site or plot access compromised, staffing issues, errors (e.g., equipment not available in the field)
Biophysical Criteria	OK – within bout window	Sampling occurred within the bout window, no known issues
Biophysical Criteria	conditions not met: sampled after fish	Sampling does not reflect the target biophysical conditions, benthic sampling occurred after benthos was disturbed during seasonal fish sampling
Biophysical Criteria	conditions not met: outside bout window	Sampling was conducted outside of the AOS sampling window

4.6 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. Please note that if sampling at particular locations requires significantly more time than expected, Science may propose to move these sampling locations.

Table 5. Estimated staff and labor hours required for implementation of the Aquatic Microbial Sampling protocol.

SOP	Estimated time	Suggested staff	Total person hours
SOP A: Preparing for sampling (includes sterilizing equipment)	30-45 min	1	0.5-0.75
SOP B: Field Sampling – Surface Water	3 hours in addition to water chem	2	3
SOP C: Field Sampling - Benthic	3 hours in addition to algae	2	3
SOP D: Post Field Sampling Tasks	30-45 min	1	0.5-0.75
SOP E: Data Entry and Verification	0.5	2	0.5

4.7 Criteria for Permanent Reallocation of Sampling within a Site

Surface water microbes: Collect with surface water chemistry suite, follow instructions for moving sampling in RD[10]. If microbe sampling is missed during the monthly sample, you may reschedule to another surface water chemistry bout in the same month.

Benthic microbes: Collect with stream benthic algae samples, follow instructions for moving sampling in RD[09] (Table 6).

Table 6. Contingent decisions for benthic sampling in a wadeable stream.

Situation	Action	Outcome for Data Products	Considerations
<u>Wadeable stream</u> site with <500 m aboveground stream length due to stream size, permitting restrictions, seasonal drying or other environmental factors	Habitat available may be insufficient to accommodate all 8 samples without causing harm to the stream. Reduce sampling using the following options depending on how much habitat is available and enter sampling impractical records for all missing samples: <ol style="list-style-type: none"> 1. 3 samples dominant, 3 samples subdominant (D06 MCDI normal sample design) 2. 5 samples in the 1 remaining habitat type (usually the subdominant habitat) 3. 3 samples in the 1 remaining habitat type (usually the subdominant habitat). 	Lower resolution for diversity metrics, less standardization of the dataset.	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[13], RD[14]). The remaining habitat type should also be one of the regularly sampled habitats at the site. Contact Science with questions.
<u>Wadeable stream</u> site with seasonal drying such that there is <100 m of wetted channel	If the stream dries such that is <100 m of wetted channel, there is not enough habitat left to sample and sampling is considered impractical. Wait for water to return or contact Science.	Missing data points	Mark as “sampling impractical” for each sample missed in the field data.

4.8 Sampling Specific Concerns

1. Care must be taken to avoid contaminating samples with re-suspended bed sediments.
 - 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.



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

- b. In **lakes and rivers**, 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. All reusable equipment must be cleaned and sterilized using the lab sterilization process (see SOP A.2), and any equipment used for multiple samples in the field must be field-sterilized during sampling. Disposable nitrile gloves, cleaned with an ethanol wipe, 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 important for conversion to higher data products. Use a graduated cylinder, automatic pipette, or syringe that has been calibrated as the markings on syringes are not always accurate.
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. **Surface microbe** samples may be collected in 4-L containers and returned on ice to the lab or base-camp where samples can be subsampled and filtered within a maximum of 4 hours of sample collection. If you are not able to complete processing within 4 hours of collection, plan to subsample and filter in the field.
 - a. Water jugs must be shaken for 15 seconds 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 after water levels drop below 3x median discharge for recolonization to occur. This rule is the same for periphyton sampling (RD[09]).
7. When making contingent decisions (**Table 2** and **Table 6**), a suite of 3 benthic samples collected using the same sampler and habitat type is the minimum number of samples in order to do statistics on the data. Do not collected fewer than 3 samples unless sampling is cancelled.

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

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.

Field staff must wear protective nitrile gloves when handling microbial samples to prevent cross-contamination and to protect from chemicals used in preservation. Safety Data Sheets (SDS) shall be made available for all chemicals used in this work (ethanol, formalin). Whenever chemicals are used, follow requirements of the site-specific Chemical Hygiene and Biosafety Plan (AD[03]) for laboratory safety and NEON EHSS Policy, Program and Management Plan (AD[01]).

See Section 8 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 $\geq 10 \text{ ft}^2/\text{s}$ ($0.93 \text{ m}^2/\text{s}$; Lane and Fay 1997).
2. In lakes and rivers, site-specific hazards may be encountered that necessitate sampling from the boat, without dismounting from the vessel. In addition, use extra caution in waters where alligators are present and maintain a safe distance from hazards.
3. When working around ice, refer to (AD[02], Section 10.3 Winter Water Safety). Do not attempt to walk on frozen lake if 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 field operations managers should be consulted regarding work on ice, prior to deploying employees and equipment.
4. All personnel must be wearing a personal flotation device (PFD) prior to entering a boat.
5. All personnel shall have access to a form of communication with other team members such as a two-way radio.
6. 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.).

<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

6 PERSONNEL

6.1 Training Requirements

All technicians must complete protocol-specific training as required in the Field Operations Job Instruction Training Plan (AD[04]). Additional protocol-specific required skills and safety training are described here.

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. Personnel must also be trained in safe handling of formalin as documented in the Domain Chemical Hygiene Plan and Biosafety Manual (AD[03]).

6.2 Specialized Skills

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



7 STANDARD OPERATING PROCEDURES

SOP Overview

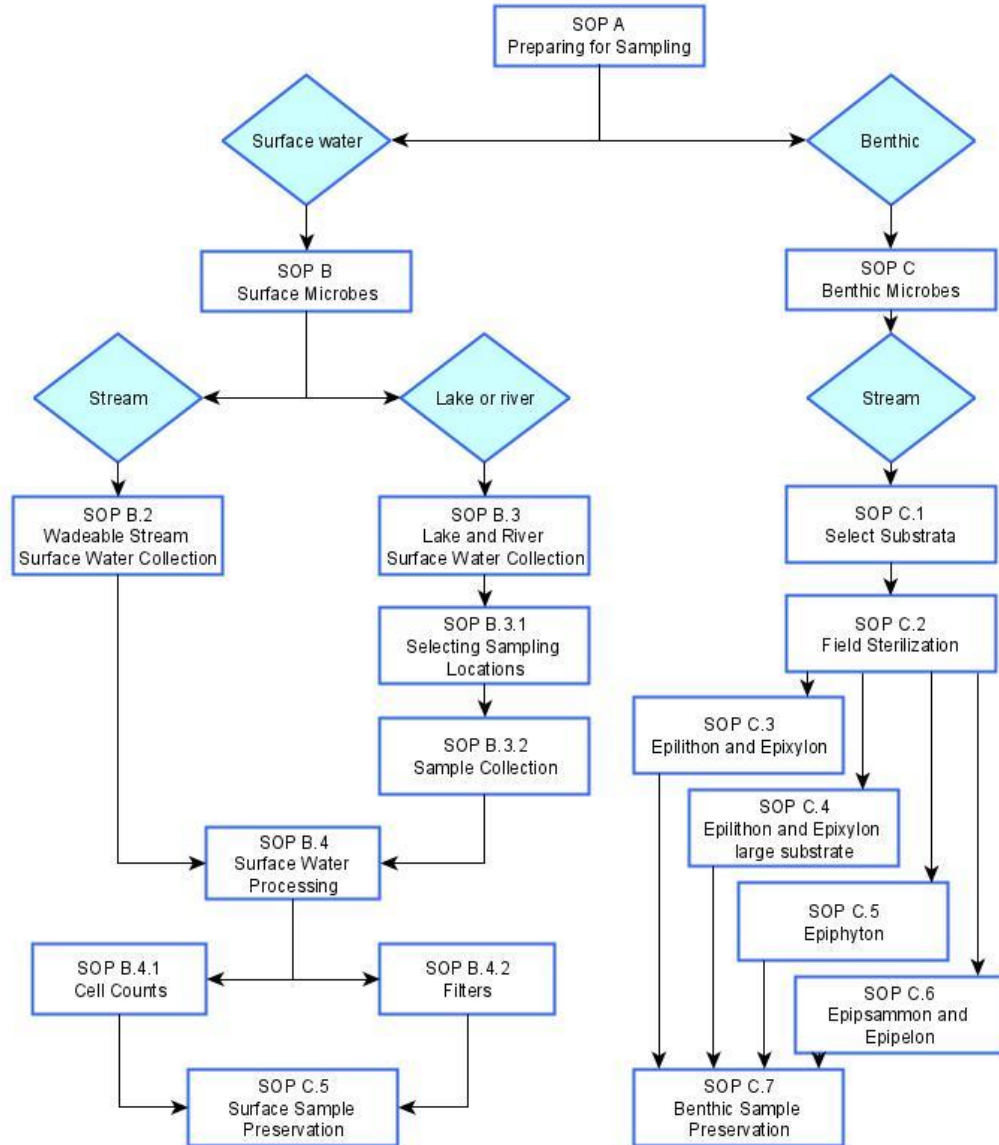


Figure 3. A high level workflow diagram that visually shows how the separate SOPs are sequentially connected.

- **SOP A:** Preparing for Sampling
- **SOP B:** Field Sampling: Surface Water Microbes
- **SOP C:** Field Sampling: Benthic Microbes (Wadeable Streams Only)
- **SOP D:** Post-Field Sampling Tasks
- **SOP E:** Data Entry and Verification

SOP A Preparing for Sampling

A.1 Preparing for Data Capture

Mobile applications are the preferred mechanism for data entry. Mobile devices should be fully charged and synced at the beginning of each field day.

However, given the potential for mobile devices to fail under field conditions, it is imperative that paper datasheets are always available to record data. Paper datasheets should be carried along with the mobile devices to sampling locations at all times.

A.2 Microbe Sampling Preparation

1. Make sure all supplies are packed, 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 of a 3 mL syringe (**Figure 4**).
 - b. Connect 3/8" inner diameter C-flex tubing to the top of the 3mL syringe.

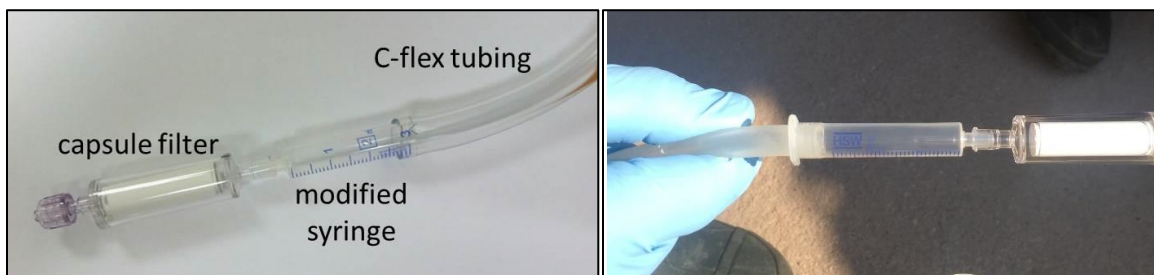


Figure 4. Filter adapter for peristaltic pump, you may put the C-flex tubing inside or outside (cut off top) of the 3 mL syringe.

3. Prepare filtered DI for rinsing.
 - a. Wear clean nitrile gloves.
 - b. Filter DI using the peristaltic pump setup (with sterilized tubing) and a 0.22 μm Sterivex filter.
 - c. 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 or does not come from the manufacturer in sterile packaging.
 - a. Wear clean nitrile gloves.
 - b. Equipment to be sterilized includes:
 - i. **Surface microbes (all sites):**
 - 1) 4 L jug, 100-200 mL syringe





Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

- 2) graduated cylinder (if using prior to filtering)
- 3) modified 3 mL syringe (**Figure 4**)
- 4) pump tubing
- 5) Kemmerer (lakes and rivers only).
- 6) 2 pipette tips (1 plus 1 backup)
- 7) male and female luer lock caps (1 each per Sterivex filter)
- 8) 50 mL conical tube for cell counts
- 9) 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):**

- 1) 125 mL wash bottle
- 2) graduated cylinder
- 3) brushes
- 4) slide template
- 5) larval tray
- 6) spatula
- 7) scissors
- 8) male and female luer lock caps (1 each per Sterivex filter)
- 9) 15 mL Falcon tubes
- 10) 2 – 100-200 mL syringes

c. Wash in mild detergent solution.

- i. Prepare a 0.2% (v/v) solution of detergent and DI water in a wash basin (i.e., 2 mL detergent in 1L water).
- ii. Wearing ETOH-cleaned nitrile gloves, submerge equipment thoroughly in detergent solution. Use bottle brush and/or hands to clean equipment.

d. Triple rinse with DI water.

e. Follow last DI rinse with 0.2 μm filtered (“sterile”) DI water.

f. Wipe down drying racks with 95% ethanol and use to completely dry all equipment.



- i. If drying racks are not available, clean the benchtop with 95% ethanol and place items directly on the sterilized benchtop to dry. The drying rack is strongly preferred.
- g. When dry, cap bottles and place equipment in clean gallon Ziploc bags for storage or transport to the field.

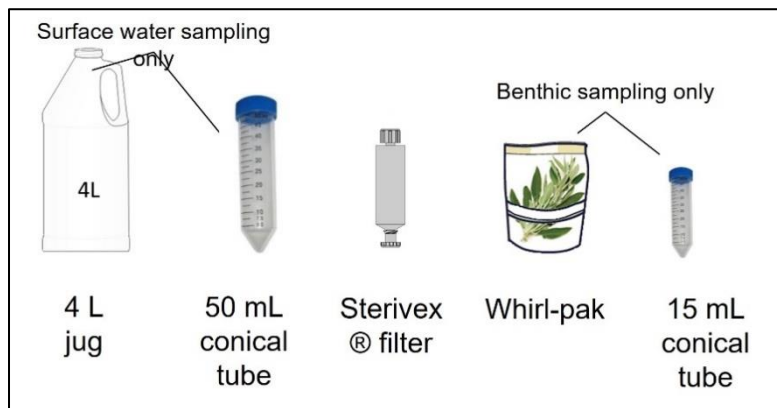


Figure 5. Microbe sample containers.

5. Contingency for DI water: DI water is required for sampling. However, if DI systems at the Domain Support Facility are broken, Ultrapure or clean, unopened distilled water filtered through a 0.22 μm Sterivex filter are acceptable substitutes. Contact Science to confirm if you are in this situation.
6. Check the volume of the 125 mL wash bottle using a graduated cylinder. If it is within 5 mL, use the “fill line.” If the difference is >5 mL, create a new “fill line.”
7. Use the Acrodisc® 0.2 μm filter attached to a sterile 3 mL syringe, add filtered 10% buffered formalin preservative to sterile 50 mL conical tubes for cell counts (2.8 mL per 25 mL sample) making sure to wear gloves.
 - a. Formalin may be filtered up to 30 days in advance of sampling, but not longer due to the formalin shelf life and storage time from collection to lab analysis. Preference is to filter within the week of sampling.
8. Prepare coolers with frozen ice packs or water ice for cell counts and dry ice for DNA, ARC, and MET) filters and grab samples.
 - a. If dry ice is not available at your domain due to supply chain shortages, refer to the alternative freezing methods in RD[21]. Alternative freezing methods are only used for field storage, not for shipping.
 - b. Using -20 °C ice packs for DNA, ARC and MET sample field storage is not acceptable.
 - c. Select the appropriate sample prep method in the mobile app for DNA, ARC, and MET samples to indicate whether dry ice or an alternative method is used.



9. Fill out the General Aquatic Field Metadata mobile app once per day upon every field sampling visit, and Secchi and Depth Profile mobile app in lakes and rivers. If other protocols are done in the same day, one record for field metadata and one record for Secchi and depth profile are sufficient.



10. **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, and use new zip-top bags to contain cleaned items. Plastic containers or organizers work well.
11. Be prepared for the extra MET sample during your mid-summer bout.
12. Schedule blank collection one per year per site. Science suggests scheduling with MET collection to keep on-per-year tasks together.

A.3 Labels and Identifiers

1. Prepare labels (**Figure 6**) for bottles and Sterivex filters, print as much label information as possible using a laser printer before going into the field. Be aware: labels printed with permanent marker or pencil are prone to smudging during storage and shipping.
 - a. Do not adhere labels to filters until you are in the field as this compromises the sterility of the filter.
 - b. Add adhesive labels to cell count tubes prior to going in the field and getting the container wet.
 - c. Note that Sterivex filters do have expiration dates set by the manufacturer. Per NEON standards, do not use expired filters for samples.
 - d. Surface water, number of samples and labels (**Figure 5**)
 - i. Wadeable streams surface water: 1 sample
 - ii. Seepage Lakes (no inlet or outlet stream) surface water: 1-2 samples, depending on stratification
 - iii. Flow-through Lakes (have true inlet and outlet stream) surface water: 3-4 samples, depending on stratification
 - iv. Rivers surface water: 1-2 samples, depending on stratification
 - e. Benthic samples (**Figure 5**)
 - i. Wadeable streams benthic, dominant habitat: 5 samples
 - ii. Wadeable streams benthic, subdominant habitat: 3 samples
 - iii. Starting in 2024, periphyton samples are composited by habitat type after sampling (RD[09]). Benthic microbe samples stay separate and are not composited.



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

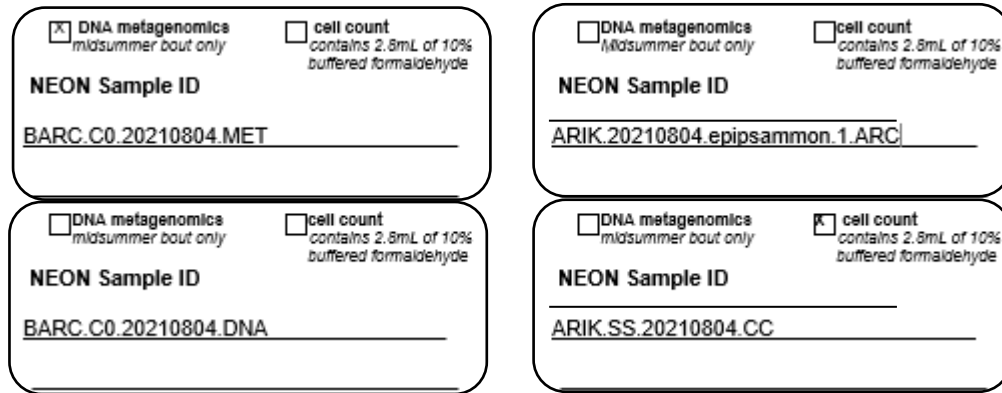


Figure 6. Example NEON microbes label. Adhere right side of label to filter or vial first, label will overlap on filters. Note that surface water samples include the location code in the sample ID. Metagenomics samples collected in midsummer (July or August surface water sample or Bout 2 benthic sample) is denoted by checking the metagenomics checkbox to help the external lab as well as “.MET” in the sampleID.

2. Add Type II adhesive barcode labels to the sample containers and scan into the mobile app. Add labels to HDPE bottles at least 30 minutes prior to going in the field and getting the bottle wet.
 - a. Keep a human-readable label on each container or filter with a minimum of the sample ID printed to assist with organization and shipping.
 - b. Use Type I barcodes for cell counts, and Type II barcodes for flash-frozen samples. All barcodes need to be applied to dry containers for 30 mins before use.
 - c. Barcodes for Sterivex filters are applied to the inside of the Whirl-Pak that contains the filter. Do not apply to the Sterivex filter as they will fall off. Apply barcodes approximately 30 minutes prior to sampling to ensure adherence.
 - d. Barcodes are required on all samples that are shipped to an external lab or to the biorepository.
3. Human-readable sample IDs are generated by the mobile app as follows (**Table 7**). Sample IDs written on the physical sample label must match the sample ID generated by the mobile app.

Table 7. Barcode requirements for sample types generated by the Aquatic Microbes protocol.

Sample Type	Description	Example Identifier	Fulcrum App	Container Type	Barcode Used	Barcode Required?	Barcode Qty
Surface water cell counts	Surface water grabs, unfiltered	REDB.SS.20161123.CC	(AOS)_ Water Chemistry	50 mL conical tube	Type I	Always required	1 per sample
Surface water microbes	Surface water grabs, filtered: DNA, ARC, MET	REDB.SS.20161123.DNA REDB.SS.20161123.ARC REDB.SS.20161123.MET	(AOS)_ Water Chemistry	Sterivex filter + Whirl-Pak	Type II	Always required (apply inside Whirl-Pak)	1 per sample
Benthic epilithon (stream only)	Cobble scrubs, filtered: DNA, ARC, MET	REDB.20161123.epilithon.1.DNA REDB.20161123.epilithon.1.ARC REDB.20161123.epilithon.1.MET	(AOS) Algae - Field	Sterivex filter + Whirl-Pak	Type II	Always required (apply inside Whirl-Pak)	1 per sample
Benthic epixylon (stream only)	Wood scrubs, filtered: DNA, ARC, MET	REDB.20161123.epixylon.1.DNA REDB.20161123.epixylon.1.ARC REDB.20161123.epixylon.1.MET	(AOS) Algae - Field	Sterivex filter + Whirl-Pak	Type II	Always required (apply inside Whirl-Pak)	1 per sample
Benthic epipsammon (stream only)	Sand grab: DNA, MET	REDB.20161123.epipsammon.1.DNA REDB.20161123.epipsammon.1.MET	(AOS) Algae - Field	15 mL conical tube	Type II	Always required	1 per sample
Benthic epipelon (stream only)	Silt grab: DNA, MET	REDB.20161123.epipelon.1.DNA REDB.20161123.epipelon.1.MET	(AOS) Algae - Field	15 mL conical tube	Type II	Always required	1 per sample
Benthic epiphyton (stream only)	Plant grab: DNA, MET	REDB.20161123.epiphyton.1.DNA REDB.20161123.epiphyton.1.MET	(AOS) Algae - Field	Whirl-Pak	Type II	Always required	1 per sample
Benthic epilithon largeSubstrate (stream only)	Large cobble scrubs, filtered: DNA, ARC, MET	REDB.20161123.epilithon.1.DNA REDB.20161123.epilithon.1.ARC REDB.20161123.epilithon.1.MET	(AOS) Algae - Field	Sterivex filter + Whirl-Pak	Type II	Always required (apply inside Whirl-Pak)	1 per sample
Benthic epixylon largeSubstrate (stream only)	Large wood scrubs, filtered: DNA, ARC, MET	REDB.20161123.epixylon.1.DNA REDB.20161123.epixylon.1.ARC REDB.20161123.epixylon.1.MET	(AOS) Algae - Field	Sterivex filter + Whirl-Pak	Type II	Always required (apply inside Whirl-Pak)	1 per sample



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

A.4 Field Sterilization and Storage

1. Any equipment that is not sterile from the manufacturer upon use or is reused from sample to sample or bout to bout, must be field-sterilized and primed. Any equipment used from bout to bout must also be cleaned appropriately in the domain support facility prior to sampling.
2. At each location prior to collecting a sample, prime sterile equipment and gloves with native water by rinsing ≥ 3 times.
3. After sample collection at a location, wipe equipment and gloves with an ethanol wipe. You may spray with an ethanol sprayer; however the surface of the equipment still needs to be wiped off with an ethanol wipe to remove particles. If using both the sprayer and the wipe, you may prolong the life the wipe by folding over to a new side and storing in a zip-top bag until the next sampling location.
 - a. For equipment that is not easily cleaned with an ethanol wipe such as the Kemmerer or tubing, prime well (triple rinse) with source water at each sampling location within the same site.
4. Store smaller ethanol-cleaned equipment in a secondary containment zip-top bag for transport to the next sampling location. Keep domain lab-cleaned equipment in a separate bag. Larger equipment such as the Kemmerer sampler at lakes should be cleaned as well as possible with ETOH wipes, rinsed with sterile DI, then closed until reaching the sampling location.



SOP B Field Sampling: Surface Water Microbes

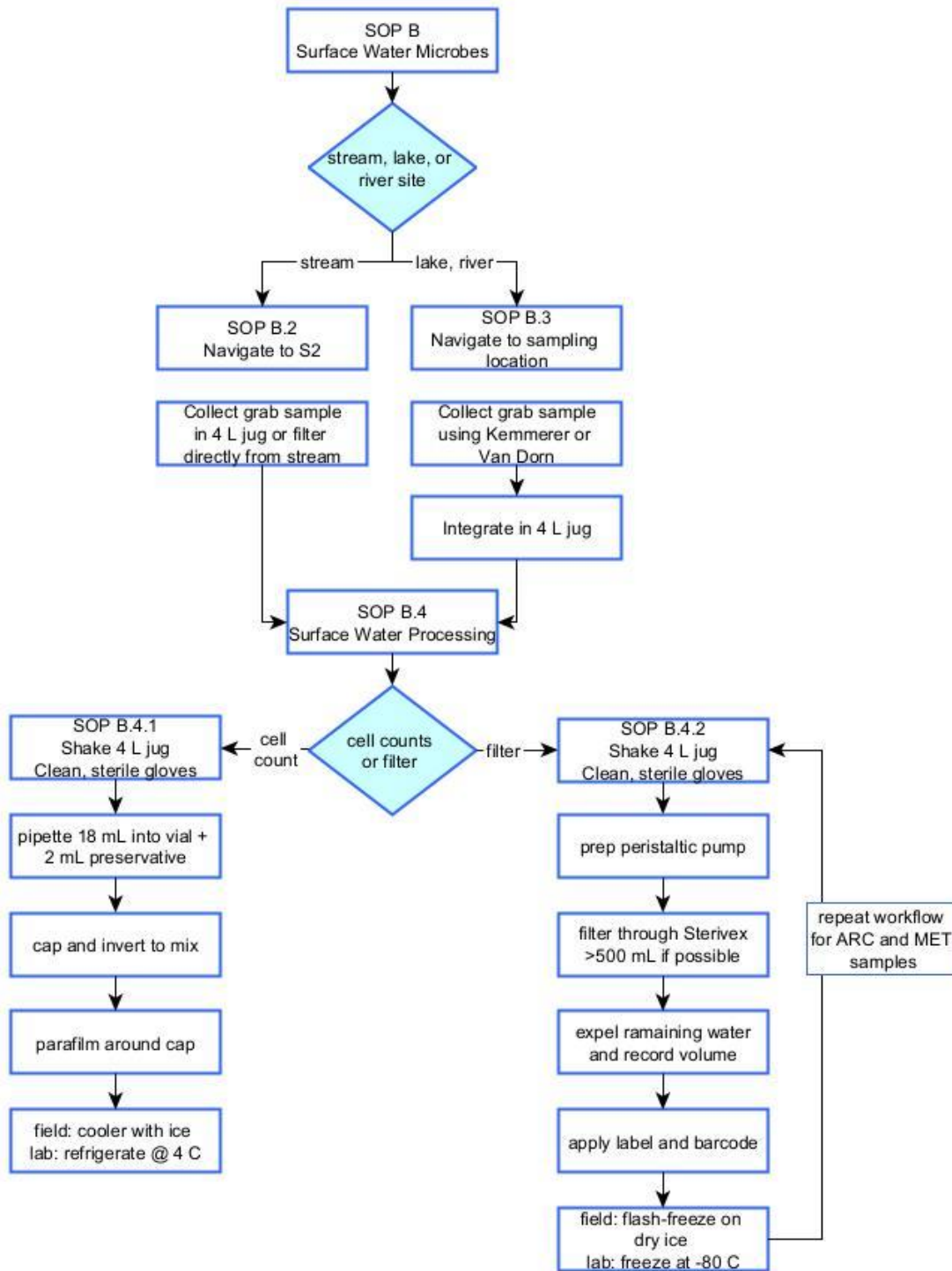


Figure 7. An expanded diagram of the workflow for surface water microbe sampling.




B.1 Spatially and Temporally Linked Protocols

Surface water chemistry and algae protocols are implemented in the same space and at the same time as this protocol.

Surface Water Chemistry

- Data are collected in the (AOS) Water Chemistry app
- Surface water microbes are collected at the same time and location as surface water chemistry monthly sampling.
 - Sampling decisions for microbes follow any decisions made during the surface water sampling on the same day, including timing or changing location due to contingencies.

B.2 Wadeable Stream Collection

1. Samples are collected at one location near the wadeable stream S2 sensor set (ss) along with surface water chemistry sampling.
 - a. Collect surface water samples prior to or upstream of any benthic sampling. Confirm that no one is wading upstream during sampling.
2.  **Wearing ETOH cleaned nitrile gloves**, prime (rinse) both 4 L HDPE bottle and cap 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.
 - a. When water temperatures are extremely cold, you may opt to add insulated PVC-coated gauntlet or shoulder-length gloves. When using additional gloves for warmth, always add a pair of cleaned extra-large disposable nitrile gloves as an outside layer to maintain field sterility during sampling.
 - b. If the sampling location is too shallow to use 4 L jugs, use either the peristaltic pump and tubing, or 100-200 mL syringe to pump water directly out of the stream.
 - i. If using the pump and tubing, prime the pump tubing by pumping at least 100 mL through the tubing. Allow water to rinse the end of the tubing as well.
 - ii. If using the 100-200 mL syringe, prime (rinse) 3 times with stream water and discard downstream.
3. 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.
 - a. If the sampling location is too shallow, and you are using the pump and tubing or the 100-200 mL syringe, you may pump directly into the syringe or into a primed 4 L jug. See SOP B.4 for Surface Water Processing.
4. Return to the stream bank and proceed to SOP B.4 for Surface Water Processing.



B.3 Lakes and River Collection

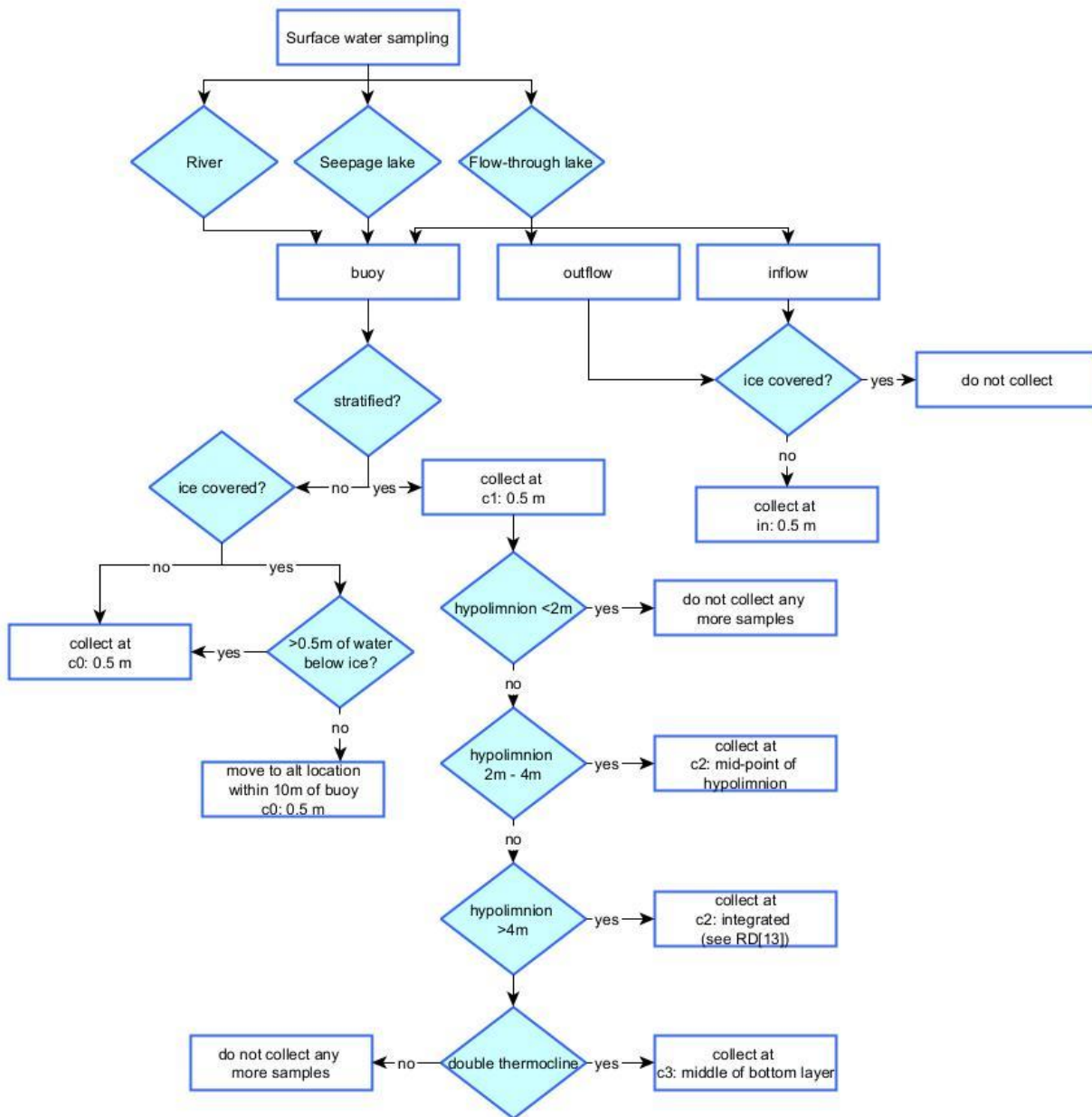


Figure 8. Surface water microbe sampling locations in rivers and lakes.

1. Select Sampling Locations (Figure 8)

- a. **Rivers** are sampled at the buoy ('c0' - non-stratified, or 'c1', 'c2', 'c3' - stratified) location at the same time as water chemistry sampling.
 - i. Sample depths are dependent on stratification conditions on the day of sampling.



- b. **Lakes** are sampled at up to three locations at the same time as water chemistry sampling.
 - i. Seepage lakes are only be sampled at buoy (c0, c1, c2, c3)
 - ii. Flow-through lakes are sampled at the inflow (in), outflow (ot) and buoy (c0, c1, c2, c3)
 - iii. Sample depts at the buoy depend on stratification conditions on the day of sampling.
 - c. Refer to Section B.4 in Water Chemistry Sampling in Surface Waters and Groundwater (RD[10]).
 - d. Determine the stratification conditions at the buoy from the Secchi Disk and Depth Profile Sampling in Lakes and Rivers (RD[17]), Section 7, SOP B.3.
 - e. Sample in the same locations and depths where water chemistry samples are collected (RD[10]).
2. 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. Use a bow anchor line 2 times the depth to prevent contamination from sediments.
 - b. Allow ~5 minutes for sediments to settle after lowering the anchor. You can use this time to prepare the sampling equipment.
 3. 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 into the wind or current.
 4. Rinse sample bottles (4 L jug) 3 times with source surface water at each location.
 - a. Hold the bottle cap in a gloved hand, setting the cap down increases the risk of contamination.
 - b. Discard rinse water away from the location where you are sampling.
 5. The Kemmerer is the accepted sampler for lake/river surface water sampling. At select river sites with fast flow, a Van Dorn may be used in place of the Kemmerer.
 - a. If a Kemmerer is used, it is suggested to drop the Kemmerer slightly up current so that when the Kemmerer gets to the correct sampling depth, the messenger can be dropped as it is passing by the sampling location.
 6. **Wearing ETOH-cleaned gloves**, prepare the sampler (**Figure 9**). 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.
7. Tie the free end of the sampler line to a cleat on the boat to prevent losing the sampler.
8. Carefully lower the sampler over the side of the boat into the water. Hold the messenger in one hand. Ensure it is secured properly to the line.
9. Prime (rinse) the sampler with surface water 3 times. Allow rinse water to drain through the spigot to prime the spigot too.
10. Continue to lower the sampler until it reaches the desired depth by using the depth markings on the line attached to the sampler.
11. 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.
12. Pull the sampler back into the boat and dispense sample water into the collection bottles through the spout (**Figure 9**).

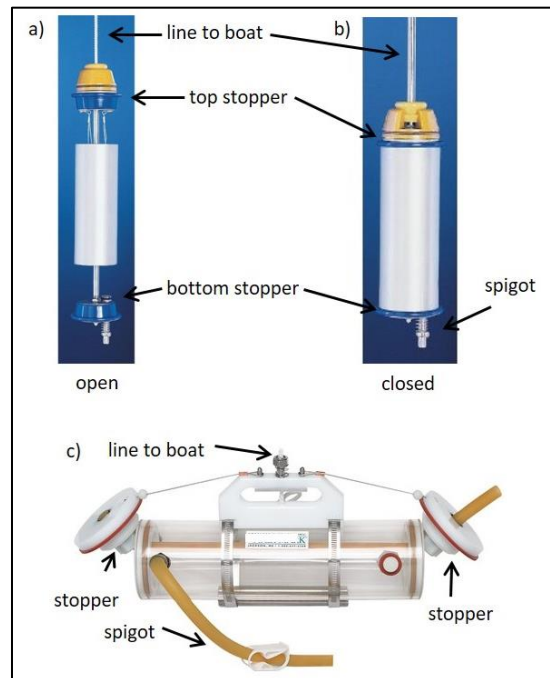


Figure 9. Vertical Kemmerer water sampler in the a) open or cocked position and b) closed (after dropping the messenger) position. c) Horizontal Van Dorn in the open position.

13. Allow at least 100 mL to drain through the spigot to rinse and residual water, discarding away from where you are sampling. Wipe the end of the spigot with an ETOH wipe.
14. Collect samples to be integrated in 4 L jug.
15. Repeat for each sample, priming the Kemmerer with source water at each new location.

16. Proceed to SOP B.4 for Surface Water Processing.

B.4 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 10**).

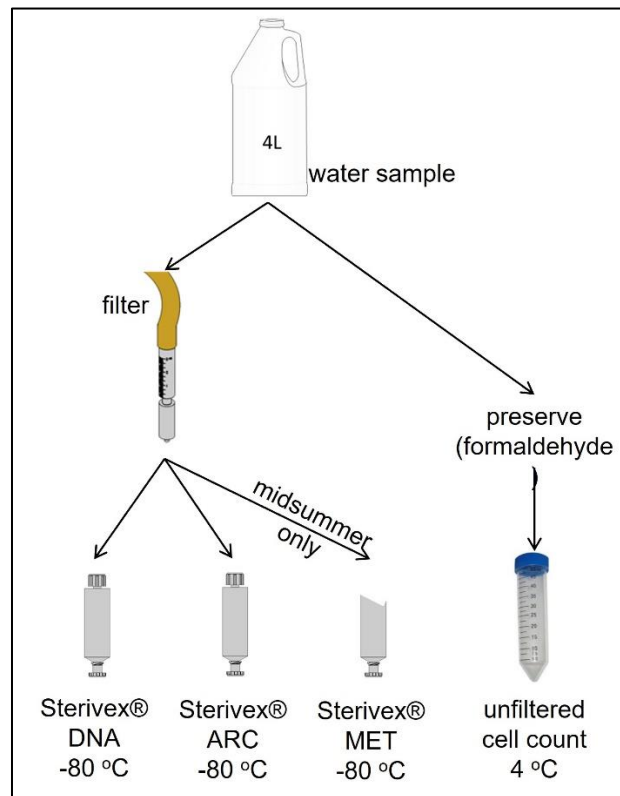


Figure 10. Schematic for surface water sample partitioning. You may also use a syringe to directly collect surface water in some systems.

B.4.1 Cell Counts

1. Shake 4 L jug or 100-200 mL syringe to mix sample.
2. Use adjustable pipette with new sterile tips to transfer 25 mL of sample from 4 L jug to 50 mL conical tube containing 2.8 mL buffered, filtered 10% formalin preservative. Samples must be immediately preserved with formalin.
 - a. If using the 100-200 mL syringe, transfer sample directly to the conical tube using the syringe.
3. Recap conical tube and invert at least 3 times to mix.



4. Wrap parafilm around the lid to prevent leaks.
5. Place cell count vial in cooler with ice packs, do not freeze.
6. Enter data in the mobile app.
 - a. Scan the barcode label with the tablet (**Figure 11**).
 - b. Ensure that the human-readable sample ID matches the sample ID generated by the mobile app.

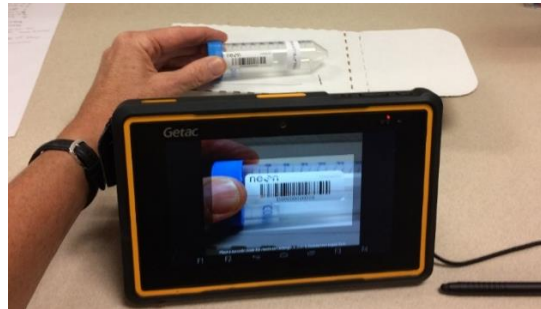


Figure 11. Barcode label scanning.

B.4.2 Filters



1. **WEAR ETOH-CLEANED GLOVES**, the same gloves can be worn while collecting the sample.
2. If using the peristaltic pump
 - a. Set-up filter apparatus on stream bank, boat, or other relatively level surface.
 - b. 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.
 - c. Check that the 3 mL syringe adapter is in place to connect pump tubing to the capsule filter (**Figure 4**).
 - d. Attach 3/8" inner diameter C-flex tubing to the peristaltic pump. Use a zip tie, small hose clamp, or Aquaseal to firmly attach the tubing to the hose connector, if needed. Rinse tubing by pumping ~100 mL of sample water completely through the tube.
 - e. After tubing is flushed with sample water, open Sterivex filter and attach to the luer lock end of 3 mL syringe/filter adapter. 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.
3. If using the 100-200 mL syringe:
 - a. Connected syringe directly to the Sterivex filter using the Luer lock end of the filter and syringe.



- b. You may need to fill the syringe multiple times to have enough water for all filters and subsamples.
4. 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 12**), or pump water out of the graduated cylinder into the filter (this requires sterilization of the graduated cylinder).
 - b. Use this step even if you are using the 100-200 mL syringe as large syringes are not necessarily accurate for measuring volume.
 - c. Filter **<500 mL** if filter becomes clogged and record the filter volume.



Figure 12. Pump sample water out of the 4-gallon HDPE jug, through the peristaltic pump, the through the Sterivex filter, and into a graduated cylinder to measure the volume filtered.

5. Label capsule filter with an adhesive label marked in permanent marker (**Figure 6**). Place in a small Whirl-pak with a Type II barcode label adhered to the inside of the Whirl-Pak.
 - a. Helpful tip – writing the sample ID on the outside bottom or side of the Whirl-pak, in addition to the labeling requirements above, helps expedite inventory with frozen samples.
6. Filter two Sterivex filters per site for each parent sample (**Figure 10**) for most sampling bouts. During midsummer (July or August), collect 3 filters per site (DNA, ARC, and MET). Filters should remain in original packaging until filtration.
7. When finished, remove filter from set up. Using the 100-200 mL syringe, draw air into the syringe and push air gently through the filter with 100-200 mL syringe to remove excess water



from filter (about 1-2 syringes of air should be enough). If measuring water volume after it has been filtered, include this water in the sample volume as it has passed through the filter.

8. Cap ends of filter with luer lock male and female end caps (**Figure 13**).

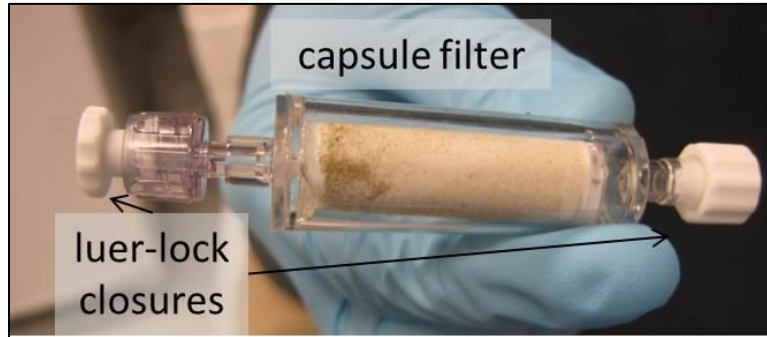


Figure 13. Capsule (Sterivex) filter and luer lock closures.

9. During midsummer, collect a separate filter for metagenomics, with human-readable sampleID ending in “MET”. Check the “metagenomics” box on MET filter labels. Every site should have 1, and only 1, set of surface water samples and 1 set of benthic samples designated for metagenomics annually.
 - a. Metagenomics filters
 - i. Streams: Collect metagenomics in July.
 - ii. Lakes and rivers: Collect metagenomics in July if that is a normally scheduled sample. If working at a lake/river where no sample is collected in July, use an August sample.
 - iii. The mobile app will help you determine whether you need a metagenomics sample.
 - iv. At D14 SYCA, collect metagenomics during Bout 3 to align with peak greenness.
10. Record filter volume in the mobile app (**Figure 9**) for each sample.
 - a. Scan the barcode label with the tablet.
 - b. Ensure that the human-readable sample ID matches the sample ID generated by the mobile app.
11. Separate labeled filters by DNA, MET, or ARC in zippered plastic bags or Whirl-Paks and flash-freeze on dry ice or dry ice alternative (RD[21]) in the field. Ensure that samples remain frozen until returning to the domain lab.
 - a. Reminder that ice packs from the -20 °C freezer are not acceptable.
12. Proceed to Sample Preservation (SOP B.5).



B.5 Surface Water Sample Preservation

1. Cell Count Samples

- a. Maximum time to process and preserve cell counts = 4 hours.
- b. If filtered formalin was not already added to the 50 mL conical tubes in the lab (SOP A), add 2.8 mL of 0.2 µm (Acrodisc® filter) filtered 10% buffered formalin to cell counts samples using a 3 mL syringe or pipet for every 25 mL of sample.
- c. Seal cap with parafilm to prevent leaks.
- d. Once capped, invert to mix at least 3 times.
- e. Put samples in cooler with ice packs. Do not freeze. Chill sample at 4°C upon return to lab. Samples may be stored at 4°C for a maximum of 60 days to allow for bundling of two months-worth of samples into one shipment (streams only as lakes/river sample every other month).

2. Sterivex Filters: DNA, ARC, and MET

- a. Flash-freeze capped Sterivex filters on dry ice or dry ice alternative (RD[21]) in the field immediately after collection.
- b. Store filters at -80 °C upon return to lab. Filters may be held at -80 °C until shipping.

SOP C Field Sampling: Benthic Microbes (Wadeable Streams Only)

It is recommended, but not required, that three people 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

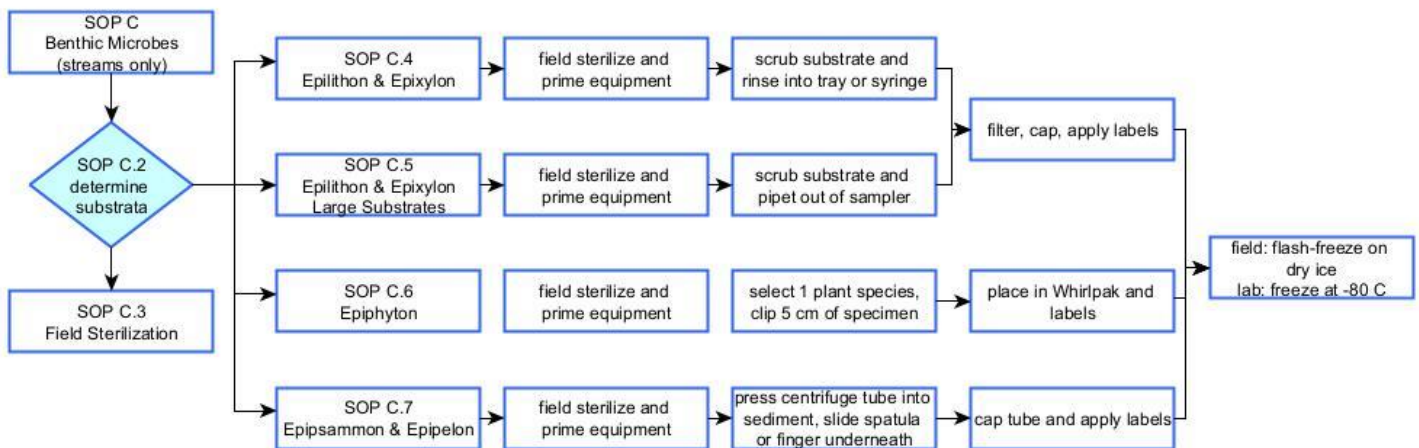


Figure 14. An expanded diagram of the workflow for benthic microbe sampling.

C.1 Spatially and Temporally Linked Protocols

Periphyton and phytoplankton

- Data are collected in the (AOS) Algae – Field app
- 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.
 - Sampling decisions for microbes follow any decisions made during stream periphyton sampling on the same day, including timing or changing location due to contingencies.
- Benthic microbes do not need to be collected on the same day as surface water microbes.

C.2 Benthic Substrata Selection

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 Rapid Habitat Assessment (RD[11]) for the site. Habitat suggestions for NEON sites are provided in 8Appendix D.
 - b. The habitat type chosen should be present during all sampling bouts.



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

- c. All 5 (or 3) 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, section 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. Boulder/bedrock (epilithon large substrate) → SOP C.4
 - d. Large woody debris (epixylon large substrate) → SOP C.4
 - e. Plant surface (epiphyton) → follow sampling procedure SOP C.5
 - f. Sand (epipsammon) → follow sampling procedure SOP C.6
 - g. Silt (epipelon) → follow sampling procedure SOP C.6
 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) and representative of the light regime within the reach (i.e., do not sample in the one sunny patch of a heavily canopied stream).
 5. Do not collect samples within a 5 m radius of the aquatic instrumentation.
 6. Do not sample substrata where you or other field people/animals 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/riverbank or lakeshore 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 samples from the same location.
 - a. In wadeable streams, collect each sample from a different habitat unit (i.e., 5 riffle samples should be collected from 5 separate riffles).
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.3 Epilithon (Rock Scrubs) and Epixylon (Wood Scrubs)

1. Prepare 1"x2" adhesive labels and barcode labels, plan for two Sterivex filters for each parent sample during Bouts 1 and 3, and 3 per sample in Bout 2 (**Figure 16**).
 - a. Bouts 1 and 3
 - i. Dominant habitat: 10 filters/labels (DNA, ARC)
 - ii. Subdominant habitat: 6 filters/labels (DNA, ARC)
 - b. Bout 2 (midsummer)
 - i. Dominant habitat: 15 filters/labels (DNA, ARC, MET)
 - ii. Subdominant habitat: 9 filters/labels (DNA, ARC, MET)
2. **Wear nitrile gloves.** Replace gloves between habitat types.
 - a. Field-sterilize gloves with ethanol wipes after sampling at a location and before moving to the next location in the same habitat type.
 - b. Field-sterilized materials can be contained in a specified zip-top bag for field-cleaned items, and not mixed with lab-cleaned or sterile items.
3. A clean, sterile tray or tote is recommended for setting equipment on the stream bank (**Figure 15**).
4. Prime all field equipment with stream water and set in a clean location (e.g., tray, tote, or new plastic bag).
5. Select one cobble and/or piece of woody debris from the dominant habitat at each of five locations in the dominant habitat type, or three from the subdominant habitat. Select cobbles and/or pieces of woody debris that meet both the requirements in SOP C.2 AND the following:
 - a. Larger than the scrubbing template (i.e., > 2 inches in diameter).



- 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.**

11. 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.
12. 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.
13. Place white slide template on top of cobble/woody debris (surface that was exposed to light at the stream bottom; **Figure 15**). Check cobble/woody debris again for colonization of invertebrates, bryophytes, or plants.
 - a. While still in the tray to contain the sample, hold the template firmly in place on the cobble/woody debris, begin scrubbing inside the template (scrub gently if woody debris). Both cobbles and woody debris are scrubbed using a sterile or individually wrapped toothbrush for this protocol. Prime toothbrush with stream water prior to sampling by working stream water into the bristles using your fingers.
 - 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 15**).
 - d. Scrubbing should be similar to brushing your teeth.
14. Periodically rinse the inside area of the template into the tray using the 0.2 μm filtered DI 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 with a graduated cylinder.
 - a. Keep total volume <the maximum volume the syringe can hold.





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 primed 100-200 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 100-200 mL syringe. Filter ~50 mL of sample, or until filter becomes clogged. If you notice the sample settling, shake the syringe periodically to homogenize the sample while filtering.
 - a. You do not need to filter all of the material in the syringe.
 - b. Keep track of the volume filtered.
 - c. Do not allow material to get sucked back up into the syringe from the filter.
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 1-2 passes 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.
 - a. If dry ice is not available at your domain due to supply chain shortages, refer to the alternative freezing methods in RD[21]. Alternative freezing methods are only used for field storage, not for shipping.
 - b. Using -20 °C ice packs for DNA and ARC sample field storage is not acceptable.
 - c. Select the appropriate sample prep method in the mobile app for DNA, ARC, and MET samples to indicate whether dry ice or an alternative method is used.
25. Repeat Steps 22-24 for second Sterivex filter (**Figure 11**). During Bout 2, also create third filter for the metagenomics sample.

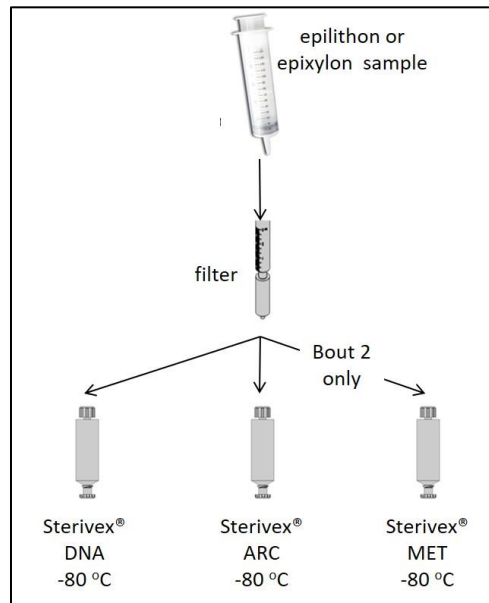


Figure 16. Schematic for benthic biofilm sampling via scrub slurry.

26. Enter data in the mobile app.
 - a. Scan the barcode label with the tablet.
 - b. Ensure that the human-readable sample ID matches the sample ID generated by the mobile app.
27. Rinse all equipment well with stream water prior to moving to the next sampling location.
28. Repeat above steps until all locations have been sampled.
 - a. Dominant habitat: 5 parent samples (10 filters for Bouts 1 and 3, 15 filters for Bout 2)
 - b. Subdominant habitat: 3 parent samples (6 filters for Bouts 1 and 3, 9 filters for Bout 2)

C.4 Epilithon (Rock Scrubs) and Epixylon (Wood Scrubs) – Large Substrates

This section is for epilithon and epixylon on substrates that you are not able to pick up to sample, e.g., boulder, bedrock, and large woody debris. Note that this sample is NOT a composite.

1. Prepare 1"x2" adhesive labels and barcode labels, plan for two capsule filters for each parent sample during Bouts 1 and 3, and 3 per sample in Bout 2 (**Figure 16**).
 - a. Bouts 1 and 3
 - i. Dominant habitat: 10 filter/labels (DNA, ARC)
 - ii. Subdominant habitat: 6 filters/labels (DNA, ARC)
 - b. Bout 2 (midsummer)



- i. Dominant habitat: 15 filters/labels (DNA, ARC, MET)
 - ii. Subdominant habitat: 9 filters/labels (DNA, ARC, MET)
2. **Wear clean nitrile gloves.** Replace gloves between habitat types.
 - a. Field-sterilize gloves with ethanol wipes after sampling at a location and before moving to the next location in the same habitat type.
 - b. Field-sterilized materials can be contained in a specified zip-top bag for field-cleaned items, and not mixed with lab-cleaned or sterile items.
3. A clean, sterile tray or tote is recommended for setting equipment on the stream bank (**Figure 15**).
4. Use the large substrate sampler and modified toothbrush for sampling. Wipe the inside of the sampler with an ethanol wipe prior to sampling. Use a new toothbrush for each sample.
 - a. Create a long-handled t-shaped brush by cutting the brush head off a toothbrush. Make sure the handle is longer than the PVC tube of the sampler.
 - b. There are multiple ways to make the t-shaped brush. Recommended materials include a wooden dowel for the handle and an aquarium epoxy stick to adhere the brush head to the dowel.
5. Prime all field equipment, including the turkey baster, with stream water and set in a clean location (e.g., tray, tote, or new plastic bag).
6. Rinse the inside of the amber wide-mouth HDPE sample bottle with native water. Fill bottle ~1/4 full, cap, and shake vigorously. Discard rinse water into stream or onto the bank. Rinse each bottle 3 times. Recap bottle and set aside.
7. Select a sampling location that is shallower than the PVC tube on the sampler. Choose substratum that is free of plants/bryophytes and minimal macroinvertebrate colonization and is relatively smooth (this will help with sealing the sampler).
8. Fill the 125 mL wash bottle to the calibrated (as tested by field science) fill line with DI.
 - a. You may use as much rinse water as you need, as long as you keep track of the volume used and record in the mobile application.
9. Place the PVC periphyton sampler tightly on the substratum to be sampled. Hold the sampler as allowing it to slip will affect the benthic area sampled and allowing water into the PVC will affect the sample volume. You may need to push firmly down onto the substrate.
10. Use the turkey baster to remove existing water from the PVC column prior to scrubbing. Ensure that you have a good seal on the substrate.
11. Using a modified toothbrush (**Figure 17A**), scrub the substratum within the PVC tube.

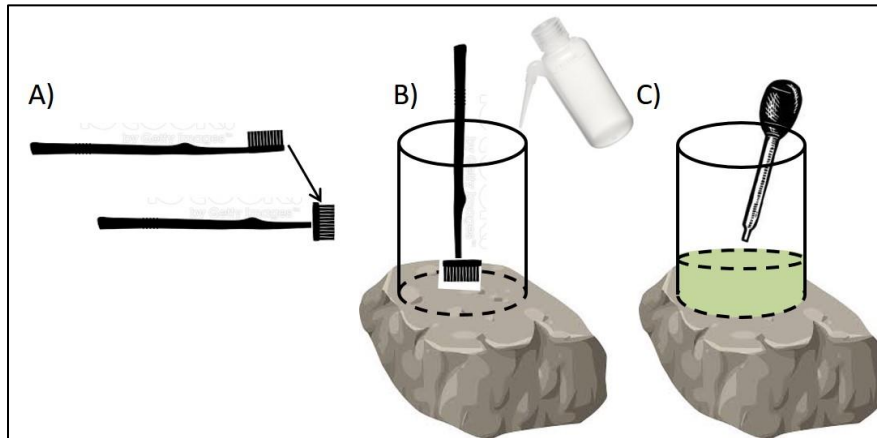


Figure 17. Schematic of PVC periphyton sampler. A) Cut off the head of a toothbrush and re-adhere it horizontally. B) Set the PVC sampler on the substrate and scrub the substrate surface using the modified toothbrush. Rinse rock surface. C) Remove water + algae sample with a turkey baster or disposable pipet.

12. When finished scrubbing, use the turkey baster to pipet the sample out of the PVC tube. Place the sample directly into the sample bottle. It's helpful to have a second person to hold materials.
13. Pipet all of the scrubbed sample out of the PVC tube. Before moving the tube, rinse the sides of the tube and the substratum at least once with the wash bottle, and pipet into the sample bottle. Continue to rinse and pipet until it appears that the water is relatively clear and free of scrubbed algae.
14. When finished removing the sample from the PVC tube, remove the tube from the stream.
15. Ensure that the amount of water in the sample bottle is known. You may add the remainder of the wash bottle directly to the sample bottle if necessary.
16. Carefully pour sample water into primed 100-200 mL syringe with a luer lock end cap attached.
17. Attach Sterivex filter to end of 100-200 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 using a graduated cylinder.
18. 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 1-2 passes of air from the syringe.
19. 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.



Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

- a. If dry ice is not available at your domain due to supply chain shortages, refer to the alternative freezing methods in RD[21]. Alternative freezing methods are only used for field storage, not for shipping.
 - b. Using -20 °C ice packs for DNA and ARC sample field storage is not acceptable.
 - c. Select the appropriate sample prep method in the mobile app for DNA, ARC, and MET samples to indicate whether dry ice or an alternative method is used.
20. Repeat steps above for second Sterivex filter (**Figure 11**). During Bout 2, also create third filter for the metagenomics sample.
21. Enter data in the mobile app.
22. Scan the barcode label with the tablet.
23. Ensure that the human-readable sample ID matches the sample ID generated by the mobile app.
24. Rinse all equipment well with stream water prior to moving to the next sampling location.
25. Repeat above steps until all locations have been sampled.
- a. Dominant habitat: 5 parent samples (10 filters for Bouts 1 and 3, 15 filters for Bout 2)
 - b. Subdominant habitat: 3 parent samples (6 filters for Bouts 1 and 3, 9 filters for Bout 2)

C.5 Epiphyton (Aquatic Plant Surfaces)

1. Prepare 1"x2" adhesive labels and barcode labels, plan for 1 Whirl-Pak for each epiphyton plant grab sample during Bouts 1 and 3 and Whirl-Paks per sample in Bout 2 (**Figure 16**).
 - a. Bouts 1 and 3
 - i. Dominant habitat: 5 Whirl-Paks labels (DNA)
 - ii. Subdominant habitat: 3 Whirl-Paks/labels (DNA)
 - b. Bout 2 (midsummer)
 - i. Dominant habitat: 10 Whirl-Paks/labels (DNA, MET)
 - ii. Subdominant habitat: 6 Whirl-Paks labels (DNA, MET)
2. **Wear clean nitrile gloves.** Replace gloves between habitat types.
 - a. Field-sterilize gloves with ethanol wipes after sampling at a location and before moving to the next location in in the same habitat type.
 - b. Field-sterilized materials can be contained in a specified zip-top bag for field-cleaned items, and not mixed with lab-cleaned or sterile items.



3. Select one plant from the dominant habitat at each of five locations or three from the subdominant habitat. Select plants for sampling that are well-colonized with epiphytes (**Figure 18**) and that meet both the requirements in SOP C.2 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 be 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 after sampling and prime with local stream water at the next sampling location.
 - c. Be gentle with the plants as epiphyton/sample material may be easily dislodged.



Figure 18. Example of epiphytes growing on bulrush in a Colorado stream. The red circle shows thick epiphyton on the surface of a submerged plant.

5. Place plant material in a Whirl-pak®. Do not add water to sample.
6. Label outside of Whirl-pak® with a human-readable label and a Type II barcode label.

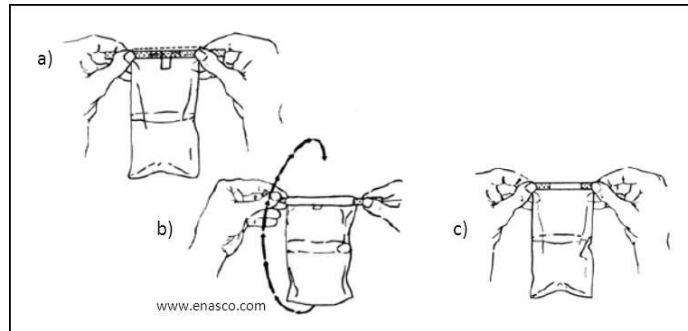


Figure 19. How to close a Whirl-Pak bag: a) hold the wire tabs; b) whirl the bag 3 complete revolutions (or fold the top over); and c) fold the wire ends over to close.

7. 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 19**), 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.
 - b. If dry ice is not available at your domain due to supply chain shortages, refer to the alternative freezing methods in RD[21]. Alternative freezing methods are only used for field storage, not for shipping.
 - c. Using -20 °C ice packs for DNA and ARC sample field storage is not acceptable.
 - d. Select the appropriate sample prep method in the mobile app to indicate whether dry ice or an alternative method is used.
8. Enter data in the mobile app.
 - a. Scan the barcode label with the tablet.
 - b. Ensure that the human-readable sample ID matches the sample ID generated by the mobile app.
9. Rinse all equipment well with stream water prior to moving to the next sampling location.
10. Repeat above steps until all locations have been sampled.
 - a. Dominant habitat: 5 grab samples in Bouts and 3, 10 grab samples in Bout 2
 - b. Subdominant habitat: 3 grab samples in Bouts 1 and 3, 6 grab samples in Bout 2
11. No further filtering or processing occurs at the domain lab. Freeze samples at -80 °C and send to external lab for extraction.



C.6 Epipsammon (Sand) and Epipelon (Silt)

1. Using 1"x2" adhesive labels, label one sterile 15 mL plastic tube per sample (**Figure 5**). Adhere barcode labels to sample containers. **NOTE:** Tubes may NOT be reused from the last sampling trip.
 - a. Bouts 1 and 3
 - i. Dominant habitat: 5 tubes/labels (DNA)
 - ii. Subdominant habitat: 3 tubes/labels (DNA)
 - b. Bout 2
 - i. Dominant habitat: 10 tubes/labels (DNA, MET)
 - ii. Subdominant habitat: 6 tubes/labels (DNA/MET)
2. **Wear clean nitrile gloves.** Replace gloves between habitat types.
 - a. Field-sterilize gloves with ethanol wipes after sampling at a location and before moving to the next location in in the same habitat type.
 - b. Field-sterilized materials can be contained in a specified zip-top bag for field-cleaned items, and not mixed with lab-cleaned or sterile items.
3. Select five locations from the dominant habitat or three from the subdominant habitat. Select locations to sample that meet the requirements in SOP C.2.
4. Note the dominant substratum size class (silt or sand) at the sampling location in the mobile app.
5. Fill the 125 mL wash bottle with 0.2 μ m filtered DI if needed for rinsing.
6. Uncap sterile conical (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. Prime spatula and/or gloves with source water.
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 (<25%) sample is lost.
 - a. You may also use a clean, gloved hand instead of the spatula.
9. Invert tube, 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. Enter data in the mobile app.
11. Rinse all equipment well with stream water prior to moving to the next sampling location.
12. Repeat above steps until all locations have been sampled.
 - a. Dominant habitat: 5 grab samples in Bouts and 3, 10 grab samples in Bout 2
 - b. Subdominant habitat: 3 grab samples in Bouts 1 and 3, 6 grab samples in Bout 2
13. No further filtering or processing occurs at the domain lab. Freeze samples at -80 °C and send to external lab.

C.7 Benthic Sample Preservation

1. Sterivex Filters: epilithon or epixylon
 - a. Flash-freeze capped Sterivex filters on dry ice or dry ice alternative in the field immediately after collection.
 - b. Store filters at -80 °C upon return to lab and hold at -80 °C until shipping.
2. Grab samples: epipsammon, epipelon, or epiphyton
 - a. Flash-freeze whole samples on dry ice or dry ice alternative in the field immediately after collection.
 - b. Store whole samples at -80 °C upon return to the lab, and hold at -80 °C until shipping.

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

SOP D Post-Field Sampling Tasks

D.1 Laboratory Sampling and Analysis

Surface water microbes: If inclement weather prevents field filtering and preservation, surface water samples may be collected in bulk (sterilized 4 L jugs from the field) and processed back at the Domain Support Facility following the field standard operating procedure outlined in SOPB.4. Samples must be processed within 4 hours of collection.

All microbe samples: Samples should be stored as outlined in preservation, SOP B.5 and SOP C.7.

D.2 Ending the sampling day

1. Sterilize and air-dry equipment and place in new, clean zip-top bags for the next sampling bout following instructions in SOP A.2.
 - a. Having a microbe-dedicated set of peristaltic pump tubing, connector, and c-flex tubing may be logistically helpful, or you can sterilize with ETOH 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 Appendix E.
 - 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.
4. Data QA/QC
 - a. Required checks
 - i. Check that the sample IDs generated by the mobile application(s) match the sample IDs written on the sample bottles/packets.
 - ii. Check that the barcode labels in in the mobile application(s) match the barcode labels adhered to the samples. At a minimum, check the last few numbers of the barcode.
 - b. Nice to check
 - i. Site ID, collect date, sampling protocol version



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

ii. Filter volume

<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

SOP E Data Entry and Verification

Mobile applications are the preferred mechanism for data entry. Data should be entered into the protocol-specific application as they are being collected, whenever possible, to minimize data transcription and improve data quality. Mobile devices should be synced at the end of each field day, where possible; alternatively, devices should be synced immediately upon return to the Domain Support Facility.

However, given the potential for mobile devices to fail under field conditions, it is imperative that paper datasheets are always available to record data. Paper datasheets should be carried along with the mobile devices to sampling locations at all times. 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.

Data and sample IDs must be entered digitally, and quality checked prior to shipping samples to an external lab.



SOP F Sample Shipment

1. Different types of samples are shipped to different labs
 - a. Cell counts (do not freeze)
 - b. DNA (-80 °C)
 - c. MET (-80 °C)
 - d. ARC (-80 °C) – store and ship in cryogenic storage boxes to the Biorepository (**Figure 20**).
2. All samples shipped to external labs and the Biorepository are required to have a barcode label.
3. See Shipping Ecological Samples, Sensors, and Equipment for instructions on shipping samples to external laboratories (RD[19]) for more details.



Figure 20. Cryogenic storage box for ARC samples. Use these boxes rather than zip-top bags for shipping to the Biorepository.

8 REFERENCES

- Crump, B.C., H.E. Adams, J.E. Hobbie, and G. W. Kling. 2007. Biogeography of freshwater bacterioplankton in lakes and streams of an Arctic tundra catchment. *Ecology* 88:1365-1378.
- Clausen, B. and B. J. F. Biggs. 1997. Relationships between benthic biota and hydrological indices in New Zealand streams. *Freshwater Biology*. 38: 327-342.
- Didan, K. 2015. MOD13Q1 MODIS/Terra Vegetation Indices 16-Day L3 Global 250m SIN Grid V006. NASA EOSDIS Land Processes DAAC. <https://doi.org/10.5067/MODIS/MOD13Q1.006>.
- Hobbie, J.E., J. Daley, and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Applied Environmental Microbiology* 33:1225-1228.
- LaRouche, J.R., W.B. Bowden, R. Giordano, M.B. Flinn, and B.C. Crump. 2012. Microbial biogeography of arctic streams: exploring influences of lithology and habitat. *Frontiers in Microbiology* 3 (309):1-9.
- Lisle J.T. and J.C. Priscu. 2004. The occurrence of lysogenic bacteria and microbial aggregates in the lakes of the McMurdo Dry Valleys, Antarctica. *Microbial Ecology* 47(4):427-439.
- Lowe, R.L. and G.D. LaLiberte. 2006. Benthic Stream Algae: Distribution and Structure. Pages 327-356 in F.R. Hauer and G.A. Lamberti, editors. *Methods in Stream Ecology, Second Edition*. Academic Press, Boston, MA.
- Moulton, S.R., II, J.G. Kennen, R.M. Goldstein, and J.A. Hambrook. 2002. Revised protocols for sampling algal, invertebrate, and fish communities as part of the National Water-Quality Assessment Program. Open-File Report 02-150. U.S. Geological Survey, Reston, VA.
- Parker, S. M. and R. M. Utz. 2022. Temporal design for aquatic organismal sampling across the National Ecological Observatory Network. *Methods in Ecology and Evolution*. 00: 1-15. <https://doi.org/10.1111/2041-210X.13944>.
- Slavik, K., B. J. Peterson, L. A. Deegan, W. B. Bowden, A. E. Hershey, and J. E. Hobbie. 2004. Long-term responses of the Kuparuk River ecosystem to phosphorus fertilization. *Ecology* 85:939-954.
- Stevenson, R. J., and L. L. Bahls. 1999. Periphyton Protocols. Pages 6.1-6.93 in M. T. Barbour, J. Gerritsen, B. D. Snyder, and J. B. Stribling, editors. *Rapid Bioassessment Protocols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish, 2nd Edition*. EPA 841-B-99-002. U.S. Environmental Protections Agency, Office of Water, Washington, D.C.



APPENDIX A QUICK REFERENCES

Step 1 – Check the microbe field sampling kit and ensure equipment has been sterilized in the lab or was received in sterilized packaging from the manufacturer.

Step 2 – Prepare adhesive labels and barcode labels.

Step 3 – Ensure the General AQU Field Metadata app is completed per field site visit and Secchi and Depth Profile app in lakes and rivers (RD[17]).

Step 4 – Determine habitat sampling locations and sampler type, continue with the same habitats and sampler types as previous years and bouts at a site.

1. Surface water - wadeable streams:

- a. Sample **surface water** near the S2 sensor, same location as water chemistry.
- b. Wear gloves and maintain field sterile conditions.
- c. Collect in the thalweg using 4 L jugs.
- d. Subsample for cell counts. Filter for DNA and ARC. If July or August, also collect a MET filter.
- e. Preserve and keep cell count samples cool.
- f. Flash-freeze and store Sterivex filters at -80 °C

2. Surface water - lakes and rivers:

- a. Sample **surface water** at the same sample locations and water depths as water chemistry.
- b. Wear gloves and maintain field sterile conditions.
- c. Collect samples using the Kemmerer (or Van Dorn and integrate the samples in a 4 L jug.
- d. Subsample for cell counts. Filter for DNA and ARC. If July or August, also collect a MET filter.
- e. Preserve and keep cell count samples cool.
- f. Flash-freeze and store Sterivex filters at -80 °C

3. Benthic microbes – wadeable streams:

- a. Locate habitats and substrate type consistent with prior sampling bouts
- b. **Wear gloves** and field sterilize and prime all re-usable sampling equipment prior to each sample.
- c. Epilithon and epixylon scrub samples – create 2-3 Sterivex filters per bout (DNA, ARC, MET)
- d. Epiphyton, epipsammon, epipelon grabs – create 1-2 grab samples per bout (DNA, MET)
- e. Flash freeze and store samples at -80 °C.

Title: AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		Date: 01/04/2024
NEON Doc. #: NEON.DOC.003044	Author: S. Parker	Revision: F

APPENDIX B REMINDERS

Before heading into the field:

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

Surface microbe collection:

- 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 microbe collection 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]) or Rapid Habitat Assessment. Sample in same locations as periphyton. Be consistent with habitats and sampler types as in prior sampling bouts.
- Start sampling at the bottom of the reach, working upstream so as not to decrease visibility and disrupt benthic biofilm communities.
- Spread 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 people/animals have walked, or locations that appear recently disturbed.

Sample processing:

- Field filtering: Flash-freeze filters and grab samples on dry ice or dry ice alternative in the field.
- Lab filtering: Filtering for surface microbes can be conducted in the DSF 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.

Data QA/QC:

- *Required checks:*
 - Check that the sample IDs generated by the mobile application(s) match the sample IDs written on the sample containers/filters.
 - Check that the barcode labels in the mobile application(s) match the barcode labels adhered to the samples. At a minimum, check the last few numbers of the barcode.
- *Nice to check:*
 - Site ID, collect date, sampling protocol version
 - Filter volumes



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

APPENDIX C ESTIMATED DATES FOR ONSET AND CESSATION OF SAMPLING

Preliminary date ranges for benthic biological sampling bouts are based on the NEON temporal sampling strategy (Parker and Utz 2022). Refer to the Aquatic Site Sampling Design for your domain (RD[20] for bout window start and end dates. Benthic samples collected during Bout 2 will produce a separate sample for metagenomics analysis.

APPENDIX D SITE-SPECIFIC INFORMATION

Table 8. Site-specific sampling locations and habitats.

Domain	Site	Benthic habitat 1 (5 reps)	Benthic habitat 2 (3 reps)	Surface water
D01	HOPB	Riffle (epilithon)	Pool (epipsammon)	ss (S2)
D02	LEWI	Run (epipelon)	Riffle (epilithon)	ss (S2)
D02	POSE	Riffle (epilithon)	Pool (epipelon)	ss (S2)
D03	FLNT	NA	NA	c0
D03	BARC	NA	NA	c0
D03	SUGG	NA	NA	c0
D04	GUIL	Riffle (epilithon)	Pool (epilithon)	ss (S2)
D04	CUPE	Riffle (epilithon)	Run (epilithon)	ss (S2)
D05	CRAM	NA	NA	c1, c2
D05	LIRO	NA	NA	c1, c2
D06	KING	Riffle/run (epilithon)	Pool (epilithon)	ss (S2)
D06	MCDI	Riffle (epilithon)	Pool (epilithon)	ss (S2)
D07	LECO	Riffle (epilithon)	Pool (epipsammon)	ss (S2)
D07	WALK	Riffle (epilithon)	Run (epixylon)	ss (S2)
D08	MAYF	Riffle/run(epixylon)	Run (epipsammon)	ss (S2)
D08	BLWA	NA	NA	c0
D08	TOMB	NA	NA	c0
D09	PRLA	NA	NA	c0
D09	PRPO	NA	NA	c0
D10	ARIK	Run (epiphyton)	Pool (epipsammon)	ss (S2)
D11	PRIN	Run (epipsammon)	Riffle (epilithon)	ss (S2)
D11	BLUE	Run (epilithon)	Riffle (epilithon)	ss (S2)
D12	BLDE	Riffle (epilithon)	Run (epilithon)	ss (S2)
D13	COMO	Riffle (epilithon)	Run (epipsammon)	ss (S2)
D13	WLOU	Riffle (epilithon)	Pool/step pool (epipsammon)	ss (S2)
D14	SYCA	Run (epipsammon)	Riffle (epilithon)	ss (S2)
D15	REDB	Step pool (epipelon)	Run (epilithon)	ss (S2)
D16	MCRA	Step pool/Riffle (epilithon)	Step pool/Pool (epilithon)	ss (S2)
D16	MART	Riffle (epilithon)	Pool (epilithon)	ss (S2)
D17	TECR	Riffle (epilithon)	Riffle (epilithon large substrate)	ss (S2)
D17	BIGC	Run (epipsammon)	Riffle (epilithon)	ss (S2)
D18	OKSR	Run (epilithon)	Riffle (epilithon)	ss (S2)
D18	TOOK	NA	NA	c1, c2, in, ot
D19	CARI	Run (epilithon)	Riffle (epilithon)	ss (S2)

APPENDIX E 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 9. Equipment List – Prep/General Equipment.

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
RD[11]	Y	Site-specific Stream Morphology, Rapid Habitat Assessment, or Bathymetry Map	Determining sampling locations	1
	N	Handheld GPS unit (with batteries, ± 4 m accuracy) or Humminbird™	Navigating to sampling locations	1
	N	Clipboard	Recording Data	1
	N	Cryogenic gloves	Handling dry ice	1 pair
	N	Cooler (9-28 qt)	Field sample storage; use size appropriate for number of samples	2
	N	Waders (hip or chest) or knee boots	Boating or wading	1 pair per person
Consumable items				
RD[06]	Y	Aquatic Field Metadata Sheet	Recording metadata in case tablet fails	1
	N	Field Datasheets (all-weather paper)	Recording data in case tablet fails	1
	N	Mobile data entry tablet	Field data entry	1
	N	Pre-printed adhesive labels	Labeling samples	20
	Y	Adhesive barcode labels	Labeling sample bottles with barcode-readable	1 sheet
	N	Pencils	Recording data if tablet fails	2
	N	Permanent markers	Labeling samples	2

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
	N	Ice or chemical ice packs	Keeping cell count samples cool	2
	N	Dry ice	Flash-freezing samples in the field	0.5-1 kg
	N	Nitrile gloves, powderless	Sterile collection methods	10
	N	Resealable bags, gallon	Containing sterilized equipment	20

Table 10. Equipment List – Lab Sterilization.

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
	N	Wash basin	Container for sterilization solutions	1
Cole-Parmer/ HV0751802	Y	Peristaltic pump head	Filtering DI water	1
Grainger, W.W./ 3HFV9	Y	18V drill pump	Filtering DI water	1
	N	Peristaltic pump tubing	Filtering DI water with peristaltic pump	1
Fisher Scientific Company, LLC / 141697G	N	Flexible tubing, 3/8" inner diameter	Filtering water with peristaltic pump	2
Fisher Scientific Company, LLC / 1531528C	N	Tubing connector	Attaching C-flex to peristaltic tubing	1
	N	1 L HDPE bottle or 4 L HDPE jug with lid, sterilized	Container for filtered DI	1

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
	N	Lab drying rack	Drying equipment	1
Consumable items				
	N	ETOH, 95%	Sterilizing lab bench	1 L
	N	Mild detergent (Alconox or Liquinox)	Sterilizing equipment	<1 gallon
	N	0.22 µm Sterivex SVGP® L10RC capsule filter with luer lock outlet	Filtering DI	2
	N	Deionized water	Rinsing equipment, preparing filtered DI	4 gallons
	N	New gallon zip top bags	Storing sterilized equipment	5

Table 11. Equipment List – Surface Water Microbes.

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
	N	Kemmerer sampler with rope and messenger	<u>Lakes and rivers</u> , collecting water	1
	N	Horizontal Van Dorn sampler with rope and messenger	Sample collection in <u>rivers</u> with fast flow	1
	N	Ice auger	<u>Lake</u> sampling under ice	1
	N	Sterile 4 L HDPE jug	Collecting or integrating samples	2-6
	N	Flexible tubing, 3/8" inner diameter	Filtering water with peristaltic pump	2
	N	Tubing connector	Attaching C-flex to peristaltic tubing	1

Supplier/ Item No.	Exact Brand	Description	Purpose	Quan- tity
Thomas Scientific, Inc. / 1236A26	Y	Filter adapters for tubing (3 mL syringe)	Connecting peristaltic tubing and C-flex tubing	2
Cole-Parmer / HV0751802	Y	Peristaltic pump head	Filtering water	1
	Y	18V drill pump	Filtering water	1
	N	Peristaltic pump tubing	Filtering water with peristaltic pump	1
	N	500 mL plastic graduated cylinder	Measuring filtered water volume	1
	N	10 mL adjustable pipette	Pipetting cell count samples into vials	KB0012 922
	N	100 - 200 mL syringes (140 mL suggested)	Alternative to peristaltic pump, must have luer-lock connection	2
Consumable items				
	N	Small zip tie or small hose clamp	Attaching flexible tubing to hose connector	1
	N	10 mL pipette tips, sterile	Pipetting cell count samples into vials	1 per station
	N	50 mL sterile conical polypropylene tube with cap – Falcon brand preferred	Cell count sample container	1 per station
	N	0.22 µm Sterivex SVGP® L10RC capsule filter with luer lock outlet	Microbe sample filters	2 per station
	N	Luer lock male closures, sterile	Capping the Sterivex filters	4 per station
	N	Luer lock female closures, sterile	Capping the Sterivex filters	4 per station
	N	Filtered DI (0.2 µm filter)	Field-sterilization and rinsing for multiple stations	1-4 L

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
	N	Sterile 70% ethanol wipes (e.g., www.soscleanroom.com item TX3044P pre-wetted wipe OR TX3215 dry wipe)	Field-sterilization for multiple stations and gloves	20

Table 12. Equipment List – Benthic Microbes.

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
B&H / GESM7001	Y	Template (35 mm plastic slide cassette)	Epilithon/epixylon: Sampling area template	1
	N	PVC large substrate sampler: <ul style="list-style-type: none"> 2" inner diameter PVC Rubber seal or O-ring	Epilithon, epixylon large substrate : Sampling area template	1
	N	Turkey baster	Epilithon/epixylon large substrate: Sample removal	1
	N	Larval insect tray, plastic (may have pouring lip)	Epilithon/epixylon: Container to scrub substrate in	1
	N	Wash bottle, unitary, 125 mL	Epilithon/epixylon: Rinsing substrate and larval tray into syringe	1
	N	100 - 200 mL HDPE syringe (140 mL suggested)	Epilithon/epixylon and epilithon/epixylon large substrate: Filtering samples, must have luer-lock connection	2
	N	Spatula (metal, flat)	Epipsammon/epipelon: Sample collection	1
	N	Sterilized scissors	Epiphyton: Sample collection	1
	N	ETOH rinse or spray bottle	Field sterilization	1
Consumable items				

Supplier/ Item No.	Exact Brand	Description	Purpose	Quantity
	N	Filtered DI (0.2 µm filter)	Field sterilization and rinsing	1-4 L
	N	Sterile 70% ethanol wipes (e.g., www.soscleanroom.com/content/texwip_e_pdf/3044p.pdf)	Field sterilization	20
	N	ETOH, 95%	Field sterilization, refill ETOH wash/spray bottle above	500 mL
	N	Nylon toothbrushes for each habitat type (new toothbrush for each sample)	Epilithon/epixylon: Scrubbing substrate	≤8
	N	Disposable pipets	Epilithon/epixylon large substrate: Sample removal	5
	N	15 mL plastic conical (centrifuge) tubes with lids, sterile	Epipsammon/epipelon: Sample container	≤8
	N	Whirl-Paks, 24 oz.	Epiphyton: sample container	≤8
	Y	0.22 µm Sterivex SVGP® L10RC capsule filter with luer lock outlet	Epilithon/epixylon and epilithon/epixylon large substrate: Microbe sample filters	≤16
	N	Luer lock male closures, sterile	Epilithon/epixylon and epilithon/epixylon large substrate: Capping the Sterivex filters	≤16
	N	Luer lock female closures, sterile	Epilithon/epixylon and epilithon/epixylon large substrate: Capping the Sterivex filters	≤16
	N	Aquarium epoxy stick	Epilithon/epixylon large substrate: Adhesive for modified t-shaped brush	1
	N	Whirl-Paks, small	Enclosing the capped Sterivex filter	2-8

Table 13. Equipment List – Sample processing and preservation.

Supplier/ Item No.	Exact Brand	Description	Purpose	Quan- tity
Durable items				
	N	3 mL HDPE syringe, luer lock end	Filtering formalin for Surface water, epilithon, and epixylon	1
	N	Freezer (-80 °C)	Sample storage for Sterivex filters, whole sample grabs	1
Consumable items				
	N	10% formalin, buffered, colorless (sodium borate or phosphate buffer, 3.7–4% formaldehyde) – note that manufacturers label the same product differently, this is equivalent to 10% buffered formaldehyde and is the same product used in the NEON fish protocols	Preserving cell count samples	1 L
	N	Parafilm	Sealing cell count vial lids prior to shipping	1 roll
	Y	0.2 µm Acrodisc® filter (sterile not required)	Filtering formalin	2

Table 14. Equipment List – Sample Shipment.

Supplier/ Item No.	Exact Brand	Description	Purpose	Quan- tity
Durable items				
	N	Dry ice shipping container	Shipping filters	1
	N	Cooler, 9 qt., Styrofoam-lined box, or small insulated shipper	Shipping cell count samples	1
Consumable items				
	N	Vermiculite, Grade 2	Absorbing liquid leaks and cushioning shipment	As needed



<i>Title:</i> AOS Protocol and Procedure: AMC – Aquatic Microbial Sampling		<i>Date:</i> 01/04/2024
<i>NEON Doc. #:</i> NEON.DOC.003044	<i>Author:</i> S. Parker	<i>Revision:</i> F

Supplier/ Item No.	Exact Brand	Description	Purpose	Quan- tity
	N	Trash bag to line cooler (~13-gallon size)	Protecting against leaks	1
	N	Resealable plastic bags (gallon and quart size)	Protecting against leaks	6
	N	Dry Ice	Shipping filters	As needed
	N	Ice or chemical ice packs	Shipping cell counts	As needed
	N	Cryovial storage box, 13.2 x 13.2 x 5.1 cm	Box for storing and ARC filters to the Biorepository. Dividers not needed.	1