



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

AOS PROTOCOL AND PROCEDURE: INV – AQUATIC MACROINVERTEBRATE SAMPLING

PREPARED BY	ORGANIZATION	DATE
Stephanie Parker	AQU	8/29/2019

APPROVALS	ORGANIZATION	APPROVAL DATE
Kate Thibault	SCI	03/16/2022

RELEASED BY	ORGANIZATION	RELEASE DATE
Tanisha Waters	CM	03/16/2022

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.



Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
A	01/22/2016	ECO-03470	Initial release, supersedes NEON.DOC.000690 and NEON.DOC.001204.
B	02/08/2017	ECO-04359	Update NEON template; Separate and rename snag sampling SOPs; Update sample ID template; Add DNA metabarcode SOP
C	01/19/2018	ECO-03046	Tape jar lids for shipping, remove mini surber, move datasheets to appendix
D	12/19/2018	ECO-05968	Remove stream ponar sampling, update habitat and sampler preference, add contingencies and rules for stream drying, clarify DNA sterilization, lab processing, and storage, reorder sampling sections
E	10/22/2019	ECO-06244	Change lab processing time to 24 hours for DNA samples, individually bag DNA samples, new template
F	03/16/2022	ECO-06781	<ul style="list-style-type: none">Update to reflect change in terminology from relocatable to gradient sites



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

TABLE OF CONTENTS

1 OVERVIEW 1

1.1 Background..... 1

1.2 Scope 2

1.2.1 NEON Science Requirements and Data Products..... 2

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.....10

4 SAMPLING SCHEDULE.....13

4.1 Sampling Frequency and Timing13

4.2 Criteria for Determining Onset and Cessation of Sampling14

4.3 Timing for Laboratory Processing and Analysis14

4.4 Sample Timing Contingencies14

TABLE 2. CONTINGENT DECISIONS.....14

4.5 Missed or Incomplete Sampling15

4.6 Estimated Time.....18

5 SAFETY.....19

6 PERSONNEL.....20

6.1 Training Requirements.....20

6.2 Specialized Skills20

7 STANDARD OPERATING PROCEDURES21

SOPA PREPARING FOR SAMPLING.....23

SOPB DETERMINING SAMPLING LOCATIONS AND SAMPLER TYPE.....27

SOPC FIELD SAMPLING31

SOPD MACROINVERTEBRATE DNA METABARCODE FIELD COLLECTION54

SOPE POST-FIELD SAMPLING TASKS56

SOPF LABORATORY SAMPLING AND ANALYSIS.....58



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

SOPG DATA ENTRY AND VERIFICATION.....60

SOPH SAMPLE SHIPMENT.....61

8 REFERENCES63

APPENDIX A QUICK REFERENCES65

APPENDIX B REMINDERS.....67

APPENDIX C ESTIMATED DATES FOR ONSET AND CESSATION OF SAMPLING.....69

APPENDIX D SITE-SPECIFIC INFORMATION: HABITAT AND SAMPLER RECOMMENDATIONS70

APPENDIX E EQUIPMENT.....71

LIST OF TABLES AND FIGURES

Table 1. Sampling frequency for macroinvertebrate procedures on a per SOP per site type basis.13

Table 2. Contingent decisions.14

Table 3. Protocol-specific Sampling Impractical reasons entered in the Fulcrum application17

Table 4. Contingent decisions for sampling in a wadeable stream.17

Table 5. Estimated staff and labor hours required for implementation of Macroinvertebrate Collection.18

Table 6. Sample types and barcodes used.....25

Table 7. Equipment list – General equipment.....71

Table 8. Equipment list – Macroinvertebrate Samplers.....72

Table 9. Equipment list – Macroinvertebrate field elutriation and preservation.73

Table 10. Equipment list – Laboratory processing: preservative replenishment.....74

Figure 1. Mayflies (Ephemeroptera: Heptageniidae) are scrapers that are adapted to feed most efficiently on attached periphyton in flowing waters..... 1

Figure 2. Caddisflies (Trichoptera) often build cases out of small pebbles, sand, or pieces of small woody debris..... 5

Figure 3. Black fly (Diptera: Simuliidae) larva, an invertebrate that uses fanlike mouthparts to strain particulates from moving water. 5

Figure 4. Freshwater mussels are often found in streams. Pictured here, lampmussels (*Lampsilis virescens*) from Alabama..... 7

Figure 5. A mayfly predator feeding on another aquatic invertebrate. 7

Figure 6. Mayfly scrapers feeding on a rock surface. 8

Figure 7. A stonefly shredder feeding on plant detritus in a stream. 8

Figure 8. *Elimia* is a genus of snail often found in rivers and streams. 9

Figure 9. Step pools occur in high-gradient streams where there are channel-spanning pools separated by boulder/cobble steps. 9



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

Figure 10. Generic site layouts with macroinvertebrate sampling locations in red. Seepage lakes have no true inlet or outlet stream. In flow-through streams, inlet and outlet infrastructure are located in the inlet or outlet stream channel.....11

Figure 11. The documentation to account for a Missed Sampling event depends on the situation for each plot of each bout that is not sampled.....16

Figure 12. A high level workflow diagram that visually shows how the separate SOPs are sequentially connected.....21

Figure 13. Macroinvertebrate sample suite, including 5 dominant INV-tax, 3 subdominant INV-tax, and 3 dominant INV-dna bottles with human readable and barcode labels22

Figure 14. Example of adhesive jar labels for macroinvertebrate sampling.23

Figure 15. Example of write in the rain paper internal labels for macroinvertebrate taxonomy samples.24

Figure 16. Example of adhesive barcode labels, Type I.....24

Figure 17. Workflow for habitat and sampler selection (SOP B) for macroinvertebrate collection.27

Figure 18. Workflow for macroinvertebrate collection with key points for each sampler type (SOP C). ...31

Figure 19. Diagram of Surber sampler set-up32

Figure 20. Hold the Surber net on the stream bottom, and keeps the bucket nearby for depositing rocks from the base of the Surber.....33

Figure 21. Cobble selection at Surber edges.....33

Figure 22. Scrub rocks from the Surber sample in the 3 gallon bucket.....34

Figure 23. Removing the tip of the 500 mL wash bottle helps makes rinsing easier and increases water pressure.....35

Figure 24. Hand-corer assembly: A) Disassembled corer36

Figure 25. Push the hand corer through the water and into the sediments at a 90° angle37

Figure 26. Example of modified kicknet setup.38

Figure 27. Hess sampler with collection net and dolphin bucket.40

Figure 28. Sites such as D08 Mayfield Creek are well-suited to snag sampling with their sandy/silty substrate and abundance submerged large woody debris.....42

Figure 29. Example of a petite ponar setup.....44

Figure 30. From Angradi et al. 2006, example of snag sampling in a large river.....46

Figure 31. Example of Riparian Stations splitting the lake into sections at D03 Lake Barco.47

Figure 32. D-frame net, used for sweep samples in vegetation and silty habitats.....48

Figure 33. D-frame sweep technique in a littoral area; the D-frame net must remain submerged and close to the substrate throughout the entire sweep.....49

Figure 34. Process of elutriation (Dates and Byrne 1997).50

Figure 35. Carefully pours water from the 3 gallon bucket into the sieve.....51

Figure 36. Rinse the sample from the sieve into the open container with the 500 mL wash bottle.52

Figure 37. Barcode label scanning.53

Figure 38. An expanded diagram of the lab workflow for the macroinvertebrate lab SOP.58



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

1 OVERVIEW

1.1 Background

Aquatic macroinvertebrates are a diverse and ubiquitous group of organisms (Hauer and Resh 2006). Streams and rivers harbor most major taxa of aquatic organisms, however some invertebrates occur only in lakes and ponds (Hynes 2001). Because of their presence in nearly all bodies of freshwater, aquatic macroinvertebrates are excellent study organisms to address questions of biodiversity. Aquatic invertebrates are easily sampled, common in all but the most polluted waters, and strongly affected by water quality.

Aquatic macroinvertebrates are an important part of the aquatic community. Many taxa spend their larval life stage in water then emerge to inhabit the terrestrial environment. Most benthic macroinvertebrates are primary consumers and feed on autotrophic (algae, plants) and heterotrophic (leaf litter, dissolved organic carbon) production, while some benthic macroinvertebrates are predators. In general, benthic macroinvertebrates are important vectors of energy transfer from one trophic level to the next (e.g., from sunlight + nutrients → primary producers → consumers → predators) and from the aquatic environment to terrestrial upon emergence. Macroinvertebrates can be classified into functional feeding groups based on morphological and behavioral mechanisms for food acquisition (Merritt and Cummins 2006), including scrapers (feed on algae; **Figure 1**), shredders (feed on leaf litter), collector-gatherers (feed on organic matter and other material), filter feeders (filter fine particles from the water column), and predators (feed on other macroinvertebrates).



Figure 1. Mayflies (Ephemeroptera: Heptageniidae) are scrapers that are adapted to feed most efficiently on attached periphyton in flowing waters.

Freshwater benthic macroinvertebrate communities are strongly affected by abiotic factors such as disturbance, shifts in nutrient concentrations, pollutants, temperature, and pH, as well as environmental factors such as scouring, freezing, flooding, drought, and biological factors such as vegetation structure and predation (Allan 1995, Wetzel 2001). Community composition, abundance, and diversity are often seasonal as many macroinvertebrate taxa spend only a portion of their life history in the aquatic environment. Such sensitivity to environmental conditions makes benthic invertebrates ideal for use in



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

monitoring programs such as the US Environmental Protection Agency Rapid Bioassessment Protocol (EPA RBP; Barbour et al. 1999) and US Geological Survey National Water-Quality and Assessment Program (USGS NAWQA; Moulton et al. 2002). Members of the Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) are often thought to be the most sensitive taxa and when present in the aquatic habitat, can be indicators of good to excellent water quality. However, other taxa such as members of the family Chironomidae (midges, non-biting flies) and oligochaetes are ubiquitous and highly tolerant, and can be indicators of poorer water quality. Sampling stream and lake benthic communities over the time span of the NEON Observatory will help researchers determine changes in presence/absence of taxa, taxa diversity and richness, community structure, and species loss.

1.2 Scope

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

1.2.1 NEON Science Requirements and Data Products

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

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

1.3 Acknowledgments

Benthic invertebrate protocols for **wadeable streams** are informed by the US Geological Survey (USGS) National Water Quality Assessment (NAWQA) program (Moulton et al. 2002), the US Environmental Protection Agency (EPA) Rapid Bioassessment Protocols (Barbour et al. 1999), and Arctic Long-Term Ecological Research (LTER) Protocols (Slavik et al. 2004). Benthic invertebrate protocols for **lakes and rivers** are based on those of the US Environmental Protection Agency (USEPA) Environmental Monitoring and Assessment Program (EMAP) Program for Surface Waters (Baker et al. 1997), Standard Operating Procedure for Benthic Invertebrate Field Sampling (USEPA 2002), and the USEPA Great River Ecosystems Field Operations Manual (Angradi et al. 2006).



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

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	NEON EHSS Policy, Program and Management Plan
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 Level 1, Level 2, Level 3 Data Products Catalog
RD[04]	NEON.DOC.001271	NEON Protocol and Procedure: Manual Data Transcription
RD[05]	NEON.DOC.003043	Datasheets for AOS Protocol and Procedure: Aquatic Macroinvertebrate Sampling
RD[06]	NEON.DOC.001646	General AQU Field Metadata Sheet
RD[07]	NEON.DOC.001152	NEON Aquatic Sample Strategy Document
RD[08]	NEON.DOC.004257	NEON Standard Operating Procedure (SOP): Decontamination of sensors, field equipment and field vehicles
RD[09]	NEON.DOC.003162	AOS Protocol and Procedure: Wadeable Stream Morphology
RD[10]	NEON.DOC.014050	TOS Protocol and Procedure: Ground Beetle Sampling
RD[11]	NEON.DOC.002494	Datasheets for AOS Sample Shipping Inventory
RD[12]	NEON.DOC.003045	AOS Protocol and Procedure: Periphyton and Phytoplankton Sampling
RD[13]	NEON.DOC.003039	AOS Protocol and Procedure: Aquatic Plant, Bryophyte, Lichen, and Macroalgae Sampling
RD[14]	NEON.DOC.003044	AOS Protocol and Procedure: Aquatic Microbial Sampling
RD[15]	NEON.DOC.001195	AOS Protocol and Procedure: Riparian Habitat Assessment in Lakes and Non-wadeable Streams
RD[16]	NEON.DOC.001197	AOS Protocol and Procedure: Bathymetry and Morphology of Lakes and Non-wadeable Streams
RD[17]	NEON.DOC.001194	AOS Protocol and Procedure: Zooplankton Sampling in Lakes
RD[18]	NEON.DOC.003282	NEON Protocol and Procedure: Site Management and Disturbance Data Collection
RD[19]	NEON.DOC.003600- NEON.DOC.003618	Aquatic Site Sample Design – NEON Domain ##
RD[20]	NEON.DOC.005224	Shipping Ecological Samples, Sensors and Equipment



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

2.3 Acronyms

Acronym	Definition
cm	centimeter
DNA	Deoxyribonucleic acid
EMAP	Environmental Monitoring and Assessment Program
FFG	Functional feeding group
ft	Foot
GHS	Globally Harmonized System
GPS	Global Positioning System
hr	Hour
Km	kilometer
LTER	Long Term Ecological Research Program
m	Meter
µm	micrometer
mL	milliliter
mm	millimeter
NAWQA	National Water-Quality Assessment (USGS)
NEON	National Ecological Observatory Network
NLA	National Lakes Assessment
PFD	Personal Flotation Device
qt	Quart
RBP	Rapid Bioassessment Protocol (US EPA)
s	second
USEPA	US Environmental Protection Agency
USGS	US Geological Survey

2.4 Definitions

Autotroph: (Primary producers) Organisms that acquire energy from non-living inorganic sources.

Benthic: The region in or near the sediments or bed of a body of water (e.g., bottom of the stream).

Caddisflies: Aquatic invertebrates in the Phylum Insecta, Order Trichoptera. Larvae of most species build a case out of sand or small pieces of wood around the soft, larval body (**Figure 2**), or spin a net to capture suspended particles in the water column.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F



Figure 2. Caddisflies (Trichoptera) often build cases out of small pebbles, sand, or pieces of small woody debris.

Clams: Benthic mollusks sometimes found in the sediments of streams and lakes, with a shell composed of two hinged shell, thus “bivalves” and similar to mussels. Clams are filter feeders.

Collector-gatherer: (Functional feeding group) Invertebrates that are morphologically adapted to feed on a variety of items, including coarse detritus and periphyton.

Consumers: (Trophic level) Mid-levels of the food chain, organisms that consume primary producers and detritus.

D-frame net: Collecting net with handle. The net frame is shaped like a ‘D’, allowing the net to be placed against a stream or lake bottom. The D-frame net has a 30 cm wide opening and 243 μ m Nitex mesh collection net with handle.

Filter feeder: (Functional feeding group) Invertebrates that are morphologically adapted to feed by straining suspended particles from the water column (**Figure 3**). In streams, filter feeders are typically attached to the substratum (e.g., blackflies, mussels).



Figure 3. Black fly (Diptera: Simuliidae) larva, an invertebrate that uses fanlike mouthparts to strain particulates from moving water.



Functional feeding group (FFG): Benthic invertebrate groupings based on morphological and behavioral mechanisms for food acquisition (includes scrapers, filter-feeders, collector-gatherers, predators, and shredders).

Hand corer: Benthic sampler for sand- and silt-bottomed streams (**Figure 24**). Sampler consists of an inner PVC tube and an outer, stainless steel housing for pushing the corer into the substratum. The stainless steel barrel is 5 cm in diameter by 50 cm high with a handle on top.

Hess sampler: Benthic sampler for gravel-bottomed streams (**Figure 27**). Sampler consists of a stainless steel drum fitted with a flow-through collection net. The drum is 33 cm in diameter by 40 cm high, with a 243 μm Nitex mesh net.

Heterotroph: Organisms that acquire energy from living or non-living sources of organic matter.

Invertebrate: Animal without a backbone. Most stream invertebrates have an exoskeleton, including mussels, snails, crayfish, insects, and zooplankton.

Large woody debris: Downed woody debris submerged in the stream/river that is ≥ 0.3 m (6 inches) in diameter at the large end and ≥ 5 m long (Angradi et al. 2006).

Leaf litter: Terrestrial leaves that have fallen into the stream channel, where they are colonized by bacteria and fungi.

Littoral: 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.

Macroinvertebrate: Typically refers to benthic invertebrates that are >500 μm in length. For the purposes of this protocol, benthic invertebrates >250 μm will be considered macroinvertebrates.

Modified kicknet: Benthic sampler used for faster and deeper water (runs). The modified kicknet has a 33 x 5- cm frame with 243 μm Nitex mesh net attached, and a long handle for holding the net in fast current (**Figure 26**).

Mollusks: A large phylum of invertebrates, freshwater mollusks often have a soft body surrounded by a shell. This group includes snails, mussels, and clams in freshwaters.

Mussels: Benthic mollusks often found in the sediments of streams and lakes, with a shell composed of two hinged shell, thus “bivalves” (**Figure 4**). Mussels are filter feeders.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F



Figure 4. Freshwater mussels are often found in streams. Pictured here, lampmussels (*Lampsilis virescens*) from Alabama.

Petite ponar: Benthic sampler used for soft sediments in deep and slow-moving water. The Ponar consists of 2 steel halves that close when the sampler reaches the sediment, grabbing a 6" x 6" area of sediment (**Figure 29**).

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

Predator: (Functional feeding group and trophic level) Animals that eat other animals. In stream communities, predators include insects, fish, and birds; higher level of the food chain (**Figure 5**).



Figure 5. A mayfly predator feeding on another aquatic invertebrate.

Primary producers: (Trophic level) Lowest level of the food chain, organisms that use sunlight and nutrients to create energy.

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.

Scraper: (Functional feeding group) Invertebrates morphologically adapted to feed by scraping algae and periphyton off surfaces (e.g., rocks or plant surfaces; **Figure 6**).



Figure 6. Mayfly scrapers feeding on a rock surface.

Shredder: (Functional feeding group) Invertebrates morphologically adapted to feed by shredding leaf litter, aquatic plants, and coarse organic matter (**Figure 7**).



Figure 7. A stonefly shredder feeding on plant detritus in a stream.

Snag (woody snag): Submerged woody debris in the stream channel that often provides substratum and refuge for macroinvertebrates, often associated with other organic matter such as leaf packs (Moulton et al. 2002). For the purposes of this protocol, woody snags are considered to be smaller than large woody debris (LWD) and are <0.3 m in diameter and 5 m in length.

Snails: Mollusks, members of the class Gastropoda (literally “stomach foot”), snails typically have a coiled shell around a soft body (**Figure 8**). Aquatic snails are often found in streams or lakes.



Figure 8. *Elimia* is a genus of snail often found in rivers and streams.

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. (**Figure 9**).



Figure 9. Step pools occur in high-gradient streams where there are channel-spanning pools separated by boulder/cobble steps.

Surber sampler: Benthic sampler for flowing waters. A standard Surber sampler consists of a 30 cm x 30 cm frame and a long 243 μ m Nitex mesh net that collects organisms (**Figure 19**).

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.

Trophic level: Step in the transfer of energy within a food chain or food web.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

3 METHOD

The goal of benthic invertebrate sampling is to determine taxa diversity, the number of species present (richness), biomass, and to enable DNA analysis for benthic invertebrates at each aquatic sampling site. These variables, especially diversity and biomass, will be tracked over time to detect changes in species loss, changes in community structure and function, as well as the introduction and the spread of invasive taxa (presence/absence).

Benthic invertebrates at NEON aquatic sites are sampled using a percent-based macrohabitat approach (after Moulton et al. 2002). Habitats sampled focus on riffles, runs, and pools depending on the percent cover of each habitat within each 1 km-long NEON Aquatic wadeable stream site (NOTE: some NEON sites may be less than 1 km due to permitting restrictions), and pelagic and littoral samples in lakes and non-wadeable streams (or rivers). Five samples for morphological taxonomy are collected in the dominant habitat type (wadeable stream) or littoral area (lake and river), and three samples for morphological taxonomy are collected in the sub-dominant habitat type (wadeable streams) or pelagic area (lakes and rivers). An additional three samples for DNA metabarcoding will be collected in the dominant habitat type, for a total of eight taxonomy samples plus three DNA samples (11 samples total) on a given sampling date at a site. Field protocols differ depending on the habitat and substrate being sampled. However, all samples are collected from the surface of the natural substratum in each habitat. In wadeable streams, the majority of the invertebrate community is likely to colonize riffles and runs, while in sandy and silty habitats and in pools, invertebrates are likely to be most diverse on woody debris. In lakes and rivers, macroinvertebrates are likely to be most diverse in littoral aquatic plant beds. Appropriate site-specific sampling procedures will be determined prior to sampling and documented in the Site-Specific Sampling Design document. Samples are collected by field personnel then preserved and sent to taxonomists for identification.

Macroinvertebrate sampling occurs three times per year. Timing of sampling is site-specific and determined based on historical hydrological and meteorological data. Specific details on sample dates are provided in the NEON Aquatic Sample Strategy Document (RD[07]) and Appendix C. Sample bout 1 is an early-season date, representing a period of rapid biomass accumulation after winter, typically prior to leaf out or ice-off where applicable. Sample bout 2 targets mid-summer low-flow conditions and sample bout 3 represents the late growing season (typically autumn) during leaf-fall where applicable. These dates will differ on a site-by-site basis, but should always occur at, or near, normal flow conditions within the watershed. Sampling will not occur directly following a flood in wadeable streams (defined as >3x median discharge; Clausen and Biggs 1997). Should such a flood event occur on or prior to a target collection date, sampling should be delayed at least 5 days to allow for invertebrates to recolonize the substratum (c.f. Brooks and Boulton 1991, Matthaei et al. 1996).

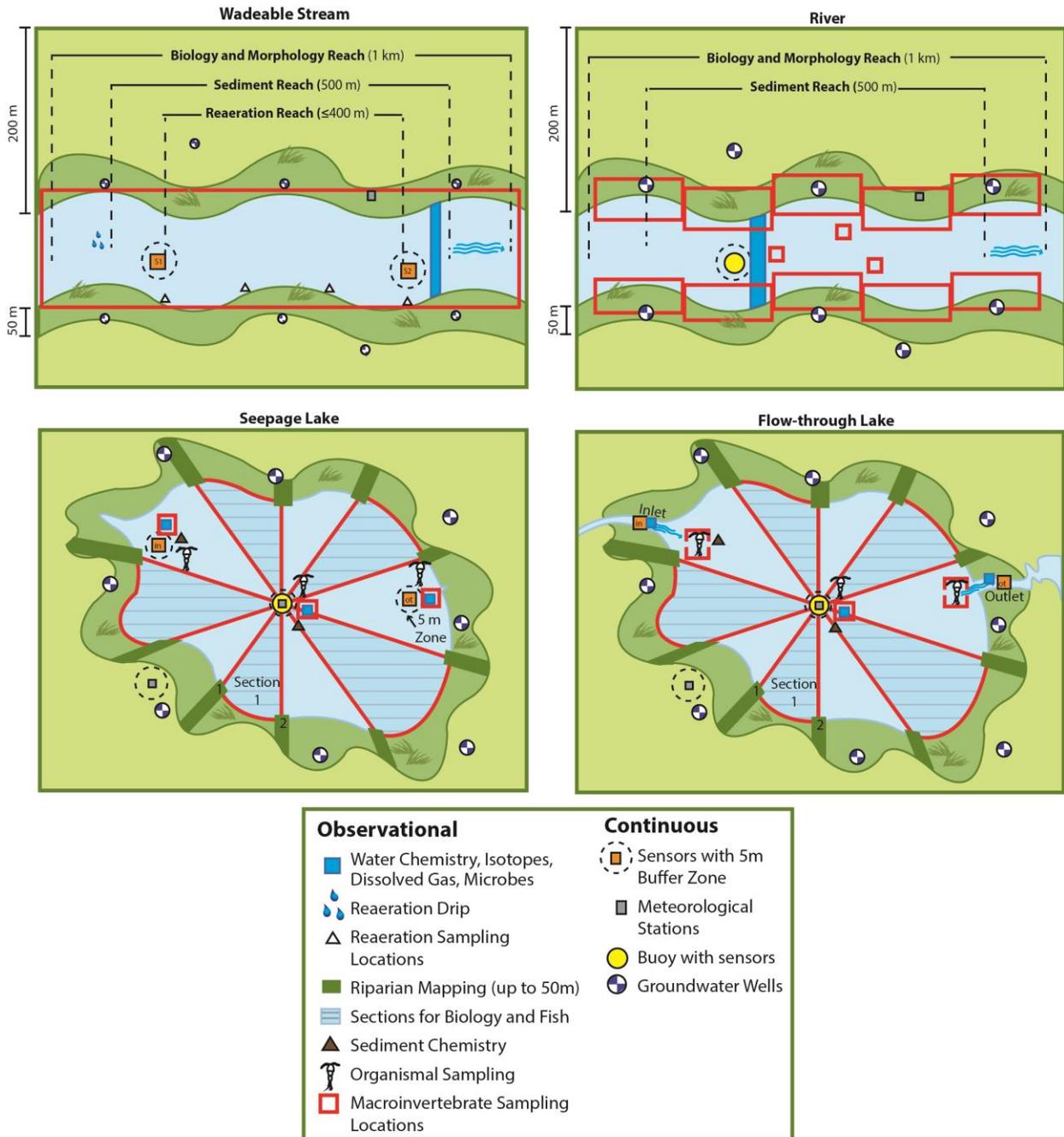


Figure 10. Generic site layouts with macroinvertebrate sampling locations in red. Seepage lakes have no true inlet or outlet stream. In flow-through streams, inlet and outlet infrastructure are located in the inlet or outlet stream channel.

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 ecologists **must** follow the protocol and



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

associated SOPs. Use NEON’s problem reporting system to resolve any field issues associated with implementing this protocol.

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

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



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

4 SAMPLING SCHEDULE

4.1 Sampling Frequency and Timing

Benthic invertebrate sampling in lakes, rivers and wadeable streams occurs three times per year at each site, roughly spring, summer, and autumn (Appendix C). Sampling must be initially scheduled within the first 21 days of the 1 month window specified in Appendix C with a minimum of two weeks between sampling dates. Accommodations for local weather conditions (e.g., late ice-off) may be made that cause the sample date to fall later in the bio bout or outside of the pre-determined window. See the appropriate Aquatic Site Sampling Design 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.

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

SOP	Site Type	Location	Bout Duration	Bouts Per Year	Bout Interval	Yearly Interval	Remarks
SOP B.1	all aquatic	any	1 day	NA	NA	opportunistic	May collect large or rare specimens that are missed with regular sampling
SOP B.2	lake, river	buoy, inlet, outlet, riparian sections, reach	1 day	3	Minimum of 2 weeks between bouts	annual	DNA samples: Bout 2 send for analysis, Bouts 1 and 3 sent to archive
SOP B.3	stream	reach	1 day	3	Minimum of 2 weeks between bouts	annual	DNA samples: Bout 2 send for analysis, Bouts 1 and 3 sent to archive

Scheduling Considerations

1. All samples for a bout must be collected during the same day.
2. **Field Work and Laboratory Processing:** After macroinvertebrate samples are collected, the following points are critical with respect to timing:
 - a. Preserve samples in the field within 60 minutes of collection.
 - b. Keep DNA samples cool (4 °C) during transportation and storage.
 - c. Replace ETOH in DNA samples within 24 hours of collection.
 - d. Replace ETOH in taxonomy samples within 72 hours of collection.
 - e. Store DNA samples at 4 °C until shipping.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

4.2 Criteria for Determining Onset and Cessation of Sampling

A range of dates for each site were determined *a priori*, based on historical data including ice on/ice off, streamflow, the accumulation of degree days, weather, and riparian phenology (Appendix C).

4.3 Timing for Laboratory Processing and Analysis

Macroinvertebrate samples must have their preservative changed after field collection, and before sending to the external labs. Preservative must be changed within 12-72 hours of field sampling for taxonomy and within 24 hours for DNA samples. Samples should ideally be shipped to the external lab within 30 days of collection, though preserved (and refrigerated DNA) samples may be held longer at the domain support facility if necessary.

4.4 Sample Timing Contingencies

All samples from this protocol in one sampling bout must be collected within one day (i.e., all 8 samples per stream or all samples per lake/rivers as detailed in this protocol) because of the fluctuating nature of aquatic habitats. Spreading sample collection over multiple days increases variability among samples. Sampling may be rescheduled due to weather or environmental conditions within the 10 days of the scheduled date provided that date is within the bout window (+ 3 days) provided in Appendix C. An incident ticket must be issued if sampling is rescheduled >10 days past the original scheduled date, or >3 days past the end of the bout window in Appendix C. A minimum of 2 weeks between sample periods shall be observed.

Table 2. Contingent decisions.

Delay/ Situation	Action	Outcome for Data Products
Hours	If weather conditions deteriorate and conditions become unsafe (e.g. approaching thunderstorm, rapid increase of water level in the wadeable stream), or the lake/river becomes too windy (>35 km hr ⁻¹) and has unsafe wave heights (>1 m) so that the boat cannot be held stationary over a sampling point while at anchor, return to shore and wait in a safe location for 30 minutes. If conditions improve, resume sampling, if not, 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.
5 Days	If flooding occurs on or prior to the targeted sampling date in a wadeable stream (>3x median discharge for the preceding year; Clausen and Biggs 1997) or unsafe wading conditions occur (Lane and Fay 1997), wait a minimum of 5 days after water level drops below 3x median discharge and is safely	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.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

	wadeable to allow the invertebrate community to recolonize.	
	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 5 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.
6 Months	Preserved macroinvertebrate samples may be held for up to 6 months at room temperature (taxonomy) or 4 °C (DNA) in the domain lab if circumstances do not allow shipping to the external lab. If held for longer than 6 months, preservative must be checked.	Holding samples >30 days affects external lab schedules, staffing, and budgets and delays data release on the NEON portal. However, sample integrity is not affected.

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 sampling locations in a given bout. For example:

- Environment – e.g., ice cover, inclement weather, or
- Management activities – e.g., controlled burns, herbicide application

Instances such as those listed above must be documented for scheduling, tracking long-term site 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 Event Reporting Protocol for more detail (RD[18]).

Missed or Incomplete Sampling Terms

Terms that inform Missed or Incomplete Sampling include:

- **Protocol Sampling Dates:** Bout-specific sampling dates (Appendix C).
- **Scheduled Sampling Dates:** Bout-specific sampling dates scheduled by Field Science and approved by Science. These dates coincide with or are a subset of the Protocol Sampling Dates.
- **Missed Sampling:** Incidence of *scheduled sampling* that did not occur. Missed 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.
- **Rescheduled:** Missed Sampling is rescheduled for another time within the *protocol sampling dates*, resulting in no change to the total number of sampling events per year.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

The documentation that must accompany missed sampling depends on the timing, subsequent action, and the audience appropriate for numerous scenarios (**Figure 11**).

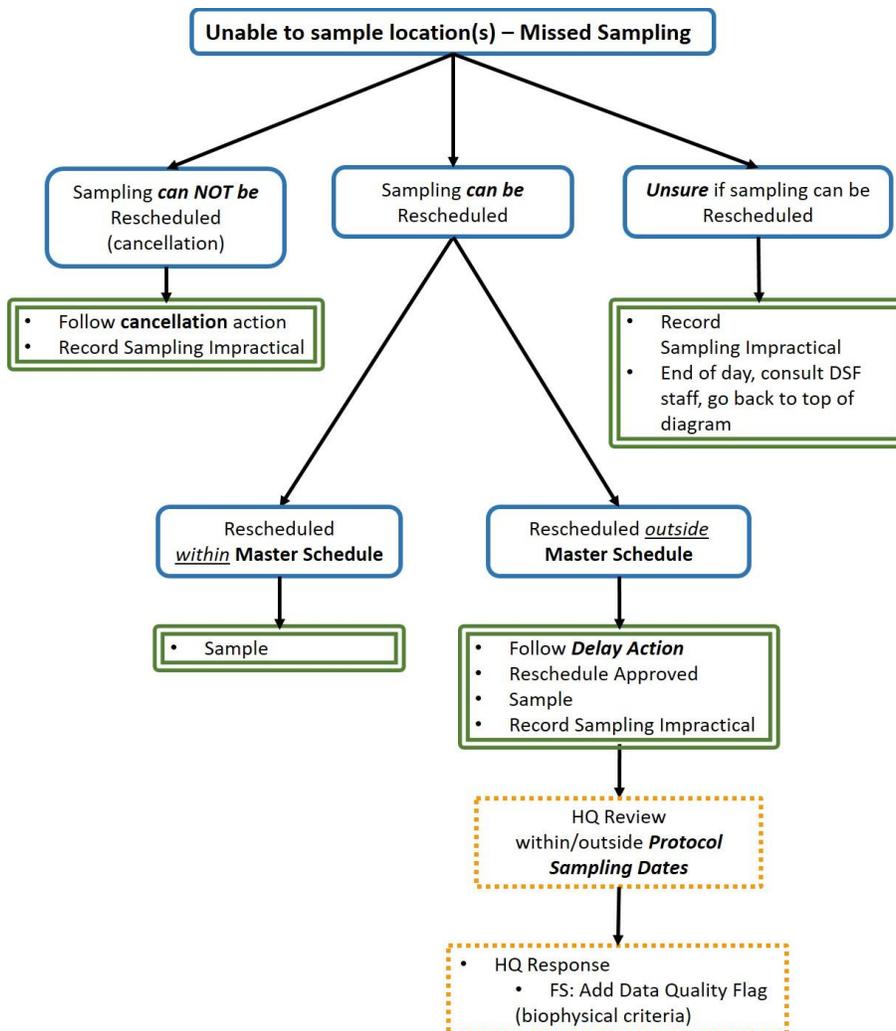


Figure 11. The documentation to account for a Missed Sampling event depends on the situation for each plot of each bout that is not sampled. Blue rounded boxes represent contingencies, green double line boxes describe the required actions, Orange dotted boxes indicate HQ actions. Required delay and cancellation actions are outlined for each protocol in the ‘Scheduled Field Activities – Delays and Cancellations’ spreadsheet available on the SSL. Missed Sampling events may also require a Data Quality flag and/or creation of a Site Management record.

To Report Missed or Incomplete Sampling:

1. Missed or Incomplete Sampling must be communicated to Science by a Service Now Incident.
 - a. For **Missed Sampling** that is **Rescheduled**, there are some cases that require approval by Science and Operations (**Figure 11**).
 - b. The lead Field Ecologist should consult the [Delayed or Cancelled Activities table](#) to best determine when reporting is required.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

2. Create a field record for each **Missed Sampling** event in the field. That is, if data are recorded in the field at the site level, a record must be made.
 - a. Example 1: Lake is ice covered and sampling cannot be attempted.
 - b. Example 2: Stream is dry and cannot be sampled.
3. For each **Missed Sampling** record, the **Sampling Impractical** field must be populated in the mobile collection device (**Table 3**).
 - a. Three macroinvertebrate taxonomy samples should be collected during each sampling event, else sampling should be re-attempted.
4. For **Rescheduled** sampling events that occur outside of the defined Protocol Sampling Dates, a protocol-specific Data Quality Flag must also be recorded (**Figure 11**).
 - a. biophysicalCriteria – An indicator of whether sampling coincided with the intended biophysical conditions
 - b. windAffected – An indicator of whether winds were >35 km hr⁻¹ at the time of sampling

Table 3. 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.

Sampling Impractical reason	Description
Location dry	Location dry
Location frozen	Location frozen
Location snow covered	Location snow covered
Other	Sampling location inaccessible due to other ecological reason described in the remarks

Table 4. Contingent decisions for sampling in a wadeable stream.

Situation	Action	Outcome for Data Products	Considerations
Wadeable stream site with <500 m aboveground stream length due to stream size or permitting restrictions	Habitat available may be insufficient to accommodate all 8 (or 11) samples without causing harm to the stream. Reduce sampling by collecting 3 taxonomy and 3 DNA samples in the dominant habitat type and 3 samples in the sub-dominant habitat.	Lower resolution for diversity metrics.	If the decision is made to decrease the number of samples collected for this protocol, it should also be reflected in the other wadeable stream biology protocols (RD[12], RD[13], RD[14]).
Wadeable stream site with seasonal drying	If the stream experiences seasonal drying such that the chosen habitat types have disappeared, select the next dominant habitat type, or use the sub-dominant habitat if still present. Reduce sampling by collecting samples only in the dominant habitat type that is present at the time of sampling (5 samples if ~200-500 m water	Less standardization of the dataset.	Habitat types sampled should be present throughout the year. The decision to switch habitat types should only occur during periods of extreme drying such that typical habitat types are



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

	<p>available, 3 samples if ~100-200 m water available). Enter “sampling impractical” for the missing habitat type.</p> <p>3 DNA samples should still be collected during in Bout 2. If enough water is not available during Bouts 1 and 3, prioritize taxonomy samples over DNA samples.</p>		no longer present at the site.
Wadeable stream site with seasonal drying	<p>If the stream dries such that 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.</p>	Missing data points	Markas “sampling impractical” in the field data.

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, use NEON’s problem reporting system to notify Science. Please note that if sampling at particular locations requires significantly more time than expected, Science may propose to move these sampling locations.

Field sampling requires two field ecologists for four hours per site, plus travel to and from the site. Lab processing requires one ecologist for one to two hours within 12-72 hours of field sampling.

Table 5. Estimated staff and labor hours required for implementation of Macroinvertebrate Collection.

SOP	Estimated time	Suggested staff	Total person hours
SOP A.1 and A.2: Preparing for data collection and sampling	1 h	1	1 h
SOP B: Determining sampling location and sampler type	1 h first year	2	2 h first year
SOP C: Field sampling methods for each sampler type – taxonomy	4 h	2	8 h
SOP D: Macroinvertebrate DNA	1 h	2	2 h (combined with SOP C)
SOP E: Laboratory preservative swap	1 h	1	1 h
SOP F: Data Entry and Verification	1 h	1	1 h
SOP G: Shipping	1 h	1	1 h



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	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 Ecologist 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.

Safety Data Sheets (SDS) shall be made available for all chemicals used in this work (ethanol). Whenever chemicals are used, follow requirements of the site-specific Chemical Hygiene and Biosafety Plan (AD[03]) for laboratory safety and NEON EHS Safety Policy and Program Manual (AD[01]), Section HC-03, Hazard Communication.

See Section 9 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 m^2/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. All personnel must be wearing a personal flotation device (PFD) prior to entering a boat.
4. All personnel shall have access to a form of communication with other team members such as a two-way radio.
5. Be aware of any site-specific hazards and to the waters of that particular location (i.e. current status, tidal charts, water release from dams, etc.).



6 PERSONNEL

6.1 Training Requirements

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

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

Personnel will be trained in field protocols associated with this document, and trained in safe working practices for aquatic field work. Personnel must also be trained in safe handling of ethanol as required 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.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

7 STANDARD OPERATING PROCEDURES

SOP Overview

- SOP A: Preparing for Sampling
- SOP B: Determining Sampling Locations and Sampler Type
- SOP C: Field Sampling
- SOP D: Macroinvertebrate DNA Metabarcoding Field Collection
- SOP E: Post-Field Sampling Tasks
- SOP F: Laboratory Sampling and Analysis
- SOP G: Data Entry and Verification
- SOP H: Sample Shipment

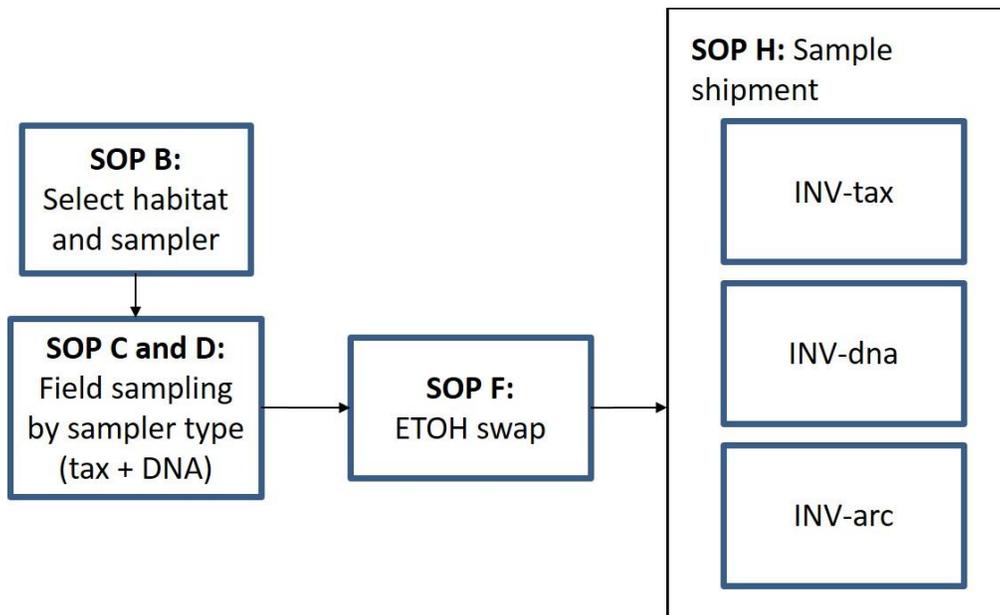


Figure 12. A high level workflow diagram that visually shows how the separate SOPs are sequentially connected.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

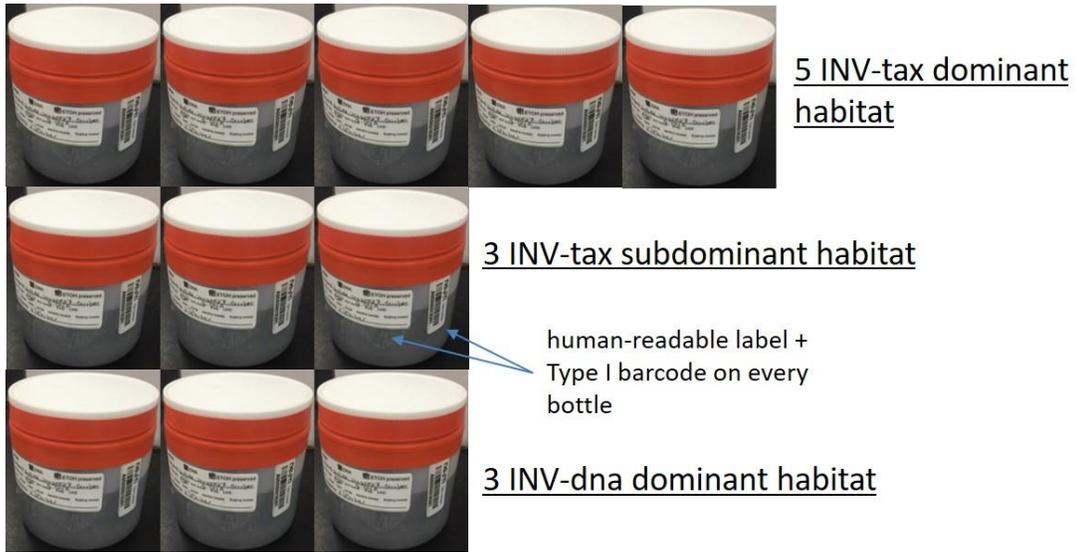


Figure 13. Macroinvertebrate sample suite, including 5 dominant INV-tax, 3 subdominant INV-tax, and 3 dominant INV-dna bottles with human readable and barcode labels. A total of 11 samples are collected per site per bout.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

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, whenever possible.

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 Labels and Identifiers

All barcodes need to be applied to dry containers for 30 mins before use. Macroinvertebrate collection uses Type I barcodes (prefix A, plus 11 numbers). Type I barcodes are for all field samples and any non-cryo applications; they have a tolerance from 4C to 105C and still scan.

1. All macroinvertebrate samples will have a weather-resistant, adhesive, human readable label on the outside of the jar (**Figure 14**).

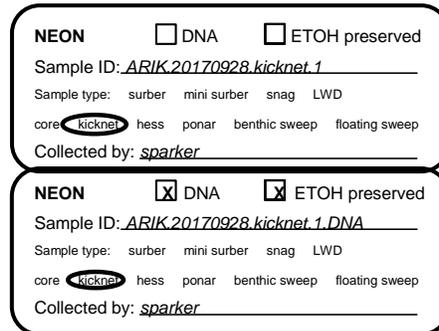


Figure 14. Example of adhesive jar labels for macroinvertebrate sampling.

2. INV-tax samples will also have a waterproof human-readable write in the rain label placed inside the jar, printed using a laser printer or written in pencil to withstand ETOH (**Figure 15**).



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

NEON DNA ETOH preserved

Sample ID: ARIK.20170928.kicknet.1

Sample type: surber mini surber snag LWD

core: kicknet hess ponar benthic sweep floating sweep

Collected by: sparker

NEON DNA ETOH preserved

Sample ID: ARIK.20170928.kicknet.1.DNA

Sample type: surber mini surber snag LWD

core: kicknet hess ponar benthic sweep floating sweep

Collected by: sparker

Figure 15. Example of write in the rain paper internal labels for macroinvertebrate taxonomy samples.

3. Adhesive barcode labels should be applied to dry, room temperature macroinvertebrate jars in advance of their use in the field, at least 30 minutes prior (**Figure 16**).
 - a. Barcode labels should be aligned lengthwise along the bottle as the scanner will not work on a curved surface (i.e. horizontally wrapped around the bottle; **Figure 13**).
 - b. Barcode labels must be associated with a unique sample and each barcode must be mapped to one sample in the database. Barcodes are unique, but are not initially associated with a particular sample, so you are encouraged to adhere barcode labels to needed containers in advance.



Figure 16. Example of adhesive barcode labels, Type I. These large-size, field-tolerant barcodes have a prefix of 'A' followed by 11 numbers.

4. Sample numbering points below are suggestions. It is **required** that the sample numbers and IDs between the mobile app and the physical label match.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

- a. Sample numbers are used to make the sample IDs unique. Numbers are selected by the field technician in the mobile app, and automatically populate into the sample ID (e.g., ARIK.20190827.SURBER.1, with “1” being the sample number)
- b. Lakes/rivers
 - i. Ponar samples should be numbered 1-3
 - ii. Littoral samples should be numbered using the riparian section number (1,3,5,7,9 or 2,4,6,8,10)
- c. Streams
 - i. Dominant habitat type (1st sampler) samples are typically numbered 1-5
 - ii. Subdominant habitat type (2nd sampler) samples are typically numbered 1-3
 - iii. If all samples at the site are collected using the same sampler type (for example, riffles and runs both use a surber), number samples 1-8.

Table 6. Sample types and barcodes used.

Sample Type	Description	Example Identifier	Fulcrum App	Container Type	Barcode Used	Barcode Required ?	Barcode Qty
INV-tax	Preserved macroinvertebrate sample	MAYF.20161027.surber.1 MAYF.20161027.core.1 MAYF.20161027.kicknet.1, etc.	(AOS) Macroinvertebrate Field Data [PROD]	HDPE jar, various sizes	Type I	Yes	1 per jar, 8 per bout
INV-dna	Preserved macroinvertebrate DNA sample	MAYF.20161027.surber.1.dna MAYF.20161027.core.1.dna MAYF.20161027.kicknet.1.dna, etc.	(AOS) Macroinvertebrate Field Data [PROD]	HDPE jar, various sizes	Type I	Yes	1 per jar, 3 per bout

A.3 Macroinvertebrate Preparation and Field Sampling

1. Collect and prepare all equipment, including sample jars and labels: paper (inside jar, **Figure 15**) and adhesive/barcode (outside jar) (RD[05], **Figure 14**).
2. Once you know the approximate size of samples at your site, consider the size of jar you are using to collect samples. Use the smallest jar you can where you can still have a ~1:1 ratio of sample:ETOH. This saves space and cost in shipping.
3. Check nets and sieves for holes, repair if necessary. Ensure that both are clean and free of debris and organic matter.
4. If collecting DNA samples, decontaminate all equipment used in DNA sample collection with 10% bleach following normal decontamination procedures (RD[08]).
5. If using a hand corer in **wadeable streams**, mark 5 and 10 cm from the bottom of the barrel with permanent marker. The contents of the nosepiece are not contained in the final sample.
6. Fill 1 L HDPE bottles (or 4 L HDPE jug) with 95% ethanol (Stein et al. 2013). Cap tightly. Clearly label bottle (suggest using Globally Harmonized System (GHS) labels) and transport to the field following EHS guidelines (AD[03]).



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

7. Fill out general aquatic field metadata mobile app upon every field sampling visit. If other protocols are done in the same day, one record for field metadata for the day is sufficient.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

SOP B Determining Sampling Locations and Sampler Type

Data for field sampling are entered in the (AOS) Macroinvertebrate Field [PROD] mobile application. Instructions for the use of this application can be found in the Sampling Support Library in the document “Manual for Fulcrum Application: (AOS) Macroinvertebrate [PROD]”.

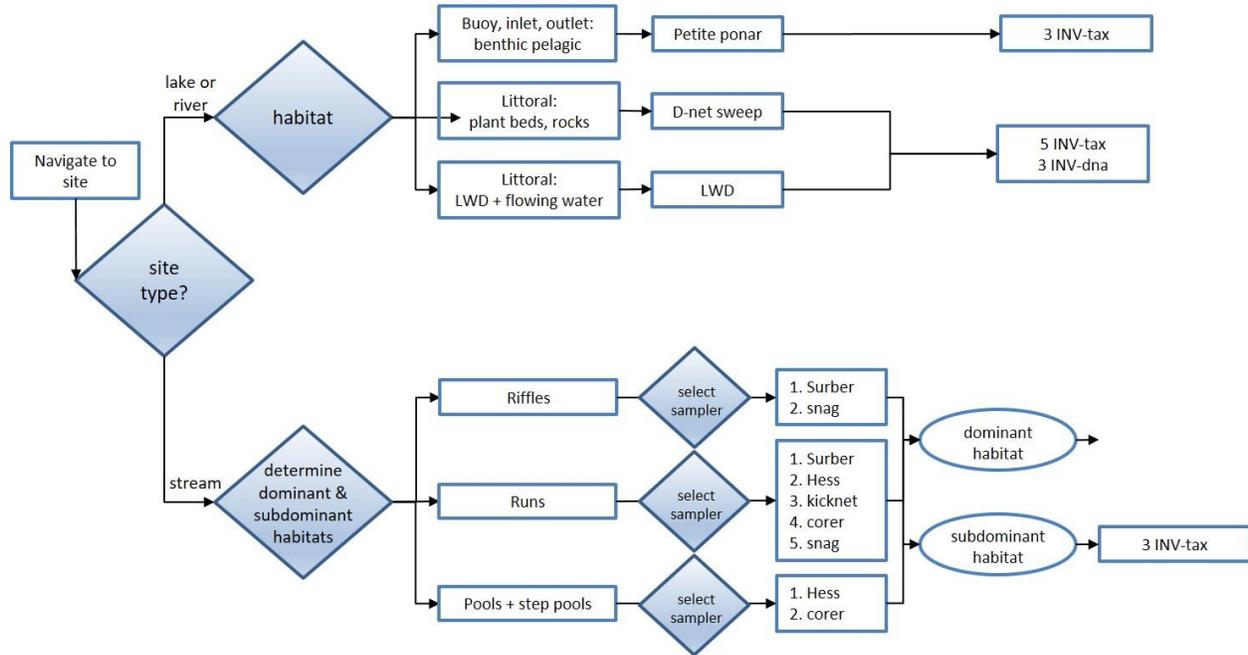


Figure 17. Workflow for habitat and sampler selection (SOP B) for macroinvertebrate collection.

B.1 Spatially and Temporally Linked Protocols

Field Metadata and Gauge Height

- Data are entered into the (AOS) Field Metadata and Gauge Height [PROD] app at the start and end of every aquatic field day.

B.2 Unique and Rare Specimen Collection

- If you discover taxa during sampling for this or other protocols that you believe are not captured during regular macroinvertebrate sampling (e.g., bryozoan, sponges), you may collect a grab sample for identification by the external lab.
- Do not collect mussels at sites where there is a permitting restriction. Never collect endangered species.
- Preserve sample in ETOH as outlined below for taxonomy samples.
- Contact Science with a sample ID, sampling date, location, and remarks about the sample so data can be manually entered into the data collection app. Once the metadata are entered, you will be able to add this to a shipping manifest and ship on the regular schedule.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

B.3 Lakes and Rivers

1. Do not sample within a 5 m radius of the aquatic instrumentation.
2. Benthic sampling
 - a. **Lakes:** Benthic petite ponar samples (Section C.6) will be collected near aquatic chemistry sampling locations in lakes: at the buoy, inlet, and outlet. If conditions near the infrastructure are not conducive to ponar sampling (e.g., heavy plant cover), move to a location where the sampler will work properly and note in the data remarks.
 - b. **Rivers:** Benthic petite ponar samples (Section C.6) will be collected downstream of the buoy (≥ 5 m), two other deep-water locations within the 1 km reach.
 - i. If sampling a **river** with rocky substrata, use the modified kicknet method in Section C.3.
3. Littoral sampling
 - a. Samples will be collected in 5 of 10 pre-defined riparian sections (RD[09]) based on the dominant substratum (e.g., submerged plants) in each section.
 - i. A section is the littoral area between the riparian sampling coordinates.
 - ii. Choose either even or odd sections for the sampling day.
 - iii. Move to the next riparian section if the appropriate substratum type is not present in the chosen section. This may mean that section numbers are not all even or all odd.
 - b. The substratum type chosen should be present during all sampling bouts and account for at least 20% of the littoral area, ensuring that there is enough of the same substrata in which to collect all samples.
 - c. All 5 taxonomy samples (+ 3 DNA samples) must be collected from the same substratum type on each sampling bout, unless a major event (i.e., a flood) causes significant changes to the substrata.
 - i. Document changes in sampler and substratum availability through NEON's problem tracking system.
 - d. Choose the appropriate sampler based on the field conditions and substrata being sampled.
 - e. The order of preference for sampling substrata is as follows, habitat suggestions for each NEON site can be found in Appendix D:
 - i. D-frame sweep (SOP C.8): For littoral areas with plant growth and/or no flow (e.g., lakes).
 - ii. Large woody debris (SOP C.5): For submerged large woody debris (>0.3 m diameter and 5 m length) substrata in littoral areas with flowing water (rivers only) where the petite ponar is not appropriate. Water can be over the top of the net during sampling.
 - iii. Hand corer (SOP C.2): For littoral areas with sand or silt substrate and little or no plant growth. Can be submerged completely during sampling.
4. Do not sample anywhere you or other people/animals have recently disturbed (via sampling, walking, driving a boat, etc.) or locations that appear recently disturbed (e.g., overturned rocks, footprints, dislodged plants, other evidence of wildlife, cattle, humans, etc.).



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

B.4 Wadeable Streams

1. Do not sample within a 5 m radius of the aquatic instrumentation.
2. Determine the dominant habitat and sub-dominant habitat based on the Stream Morphology Map (RD[09]) or rapid habitat assessment (RD[09], SOP F) for the site. Habitat suggestions for each NEON sites are provided in Appendix D.
 - a. The habitat types chosen should be present during all sampling bouts, and should account for at least 20% of the habitat area in the permitted reach as determined by the Rapid Habitat Assessment (RD[09]). The 20% cutoff ensures that there is enough habitat in which to collect all samples.
 - b. All 5 dominant samples (and 3 DNA samples) must be collected from the same habitat type and substratum using the same sampler on each sampling bout and all 3 subdominant samples must be collected from the sample 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 (see Contingent Decisions, **Table 2**).
 - c. 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 kicknet samples and 3 core samples).
 - d. Targeted habitat types and samplers in order of sampling preference (see Definitions, Section 2.4):
 - i. Riffles
 1. Surber (SOP C.1): Requires water level lower than top of frame and gravel substrate or larger
 2. Snag (modified D-frame, SOP C.5): For riffles with significant woody debris.
 - ii. Runs
 1. Surber (SOP C.1): Requires water level lower than top of frame and gravel substrate or larger.
 2. Hess (SOP C.4): Requires water level lower than top of frame, gravel substrate or larger, best used where flow is fairly slow
 3. Modified kicknet (SOPC.3): For deeper water, water can be over top of frame. Least ideal option because it is semi-quantitative, but commonly used in runs.
 4. Hand corer (SOP C.2): For runs with sand or silt substrate.
 5. Snag (modified D-frame, SOP C.5): For runs with significant woody debris.
 - iii. Pools and step pools (if the step pool has significant flow, treat as the appropriate riffle or run category above)
 1. Hess (SOP C.4): Requires water level lower than top of frame, gravel substrate or larger.
 2. Hand corer (SOP C.2): For pools with sand or silt substrate. Most common sampler used in NEON pools.
3. 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 and cause invertebrates to drift.



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

4. Spread samples out along the reach. For example, do not collect all samples for the same substratum in the same riffle, collect samples from 5 different riffles along the reach.
5. Do not sample anywhere you or other field people/animals have walked in the reach, or locations that appear recently disturbed (e.g., overturned rocks, footprints, dislodged plants, other evidence of wildlife, cattle, humans, etc.).



SOP C Field Sampling

NEON aquatic macroinvertebrate sampling is intended to be quantitative. Although different sampler types may be used (**Figure 18**) depending on the habitat and substrate at a site, the data produced are quantitative and thus will be comparable after ingest and processing.

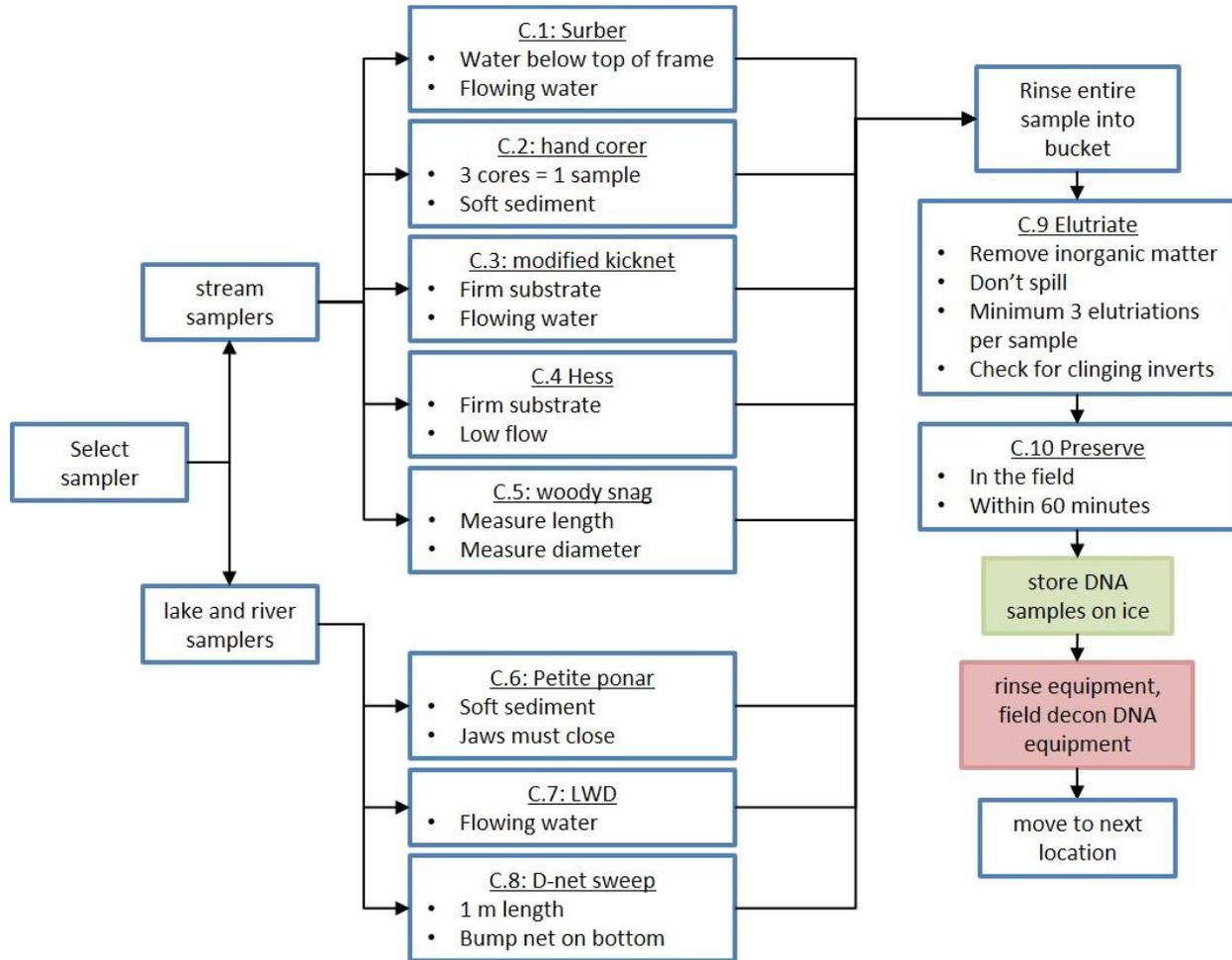


Figure 18. Workflow for macroinvertebrate collection with key points for each sampler type (SOP C).

STREAMS

C.1 Surber Sampler



In **wadeable streams**, the Surber sampler is used in shallow riffle and run habitats.

1. Ensure that all samples are collected from areas of similar depth and flow (within approximately 20%) in the **wadeable stream**. Ensure that water level is below the top of the Surber frame (best practice for sampling using a Surber).



2. Choose a location in the riffle or run that has flowing water and appears to be underwater most of the time (i.e., not underwater only at high flow). Avoid locations that are on top of, or just downstream of, large boulders (e.g., boulders that affect the flow of water at your location). Ensure that all substrata within the Surber frame are underwater.
3. Set up the Surber sampler by extending the base and clipping the arms into the screws on the base of the Surber frame (**Figure 19**).
4. Carry the 3 gallon bucket and Surber sampler with you to the sample location in the stream.
5. Approach sample location from downstream so as not to step on the cobbles that you want to sample.
6. Fill 3 gallon bucket $\frac{1}{4}$ - $\frac{1}{2}$ full with stream water so that it is weighted when resting on the stream bottom or manually hold bucket in place. Place the bucket on the stream bottom in a shallow part of the riffle (or on the shore) **that you can still reach** from the Surber location.
7. Orient the Surber so the opening of the net is facing into the stream flow (**Figure 19**).

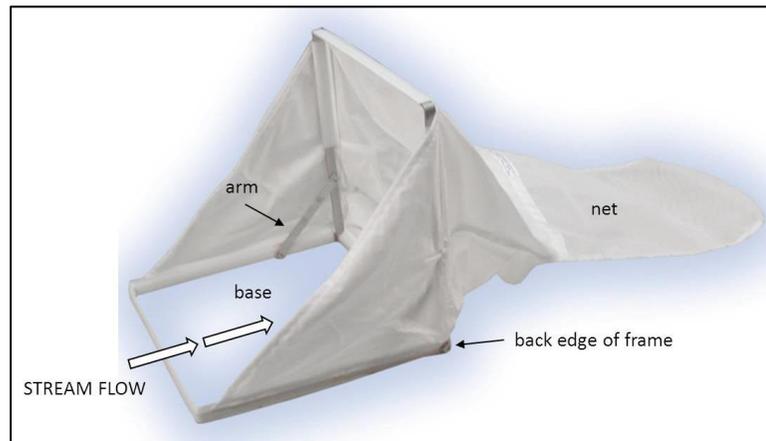


Figure 19. Diagram of Surber sampler set-up. The inside of the base is the sample area of the stream bottom (0.09 m²).

8. Place the back edge of the frame on the stream bottom first so that any material dislodged will flow into the net. Gently lower the rest of the base onto the substratum. Once base is placed on the stream bottom, do not move the sampler.
 - a. Note the dominant substratum size class at the sampling location in the mobile app.
 - b. Always stand downstream of the frame so you do not kick additional material into the sample.
 - c. Hold Surber in place using a hand or brace the frame against your leg or knee. Avoid stepping on the bottom frame as that can affect the flow of water into the net.
 - d. Ensure that nothing obstructs the flow of water into and out of the net (i.e., the net should not be kinked or partially out of the water).
9. Remove top layer of cobbles one at a time from the area inside the base of the Surber frame (**Figure 19**).
 - a. Hold each cobble near the net opening and lightly brush debris, insects, etc. from all surfaces of the cobble with your hands. The stream flow will rinse this material into the Surber net.



- b. Place cobbles in 3 gallon bucket (**Figure 20**).
- c. Continue until the top layer of cobbles has been removed from the net.
- d. For cobbles that are partially inside and outside of the frame: for every 1 cobble that you select that is partially outside of the frame, leave one cobble that is partially outside of the frame in the substratum (**Figure 21**).



Figure 20. Hold the Surber net on the stream bottom, and keeps the bucket nearby for depositing rocks from the base of the Surber.

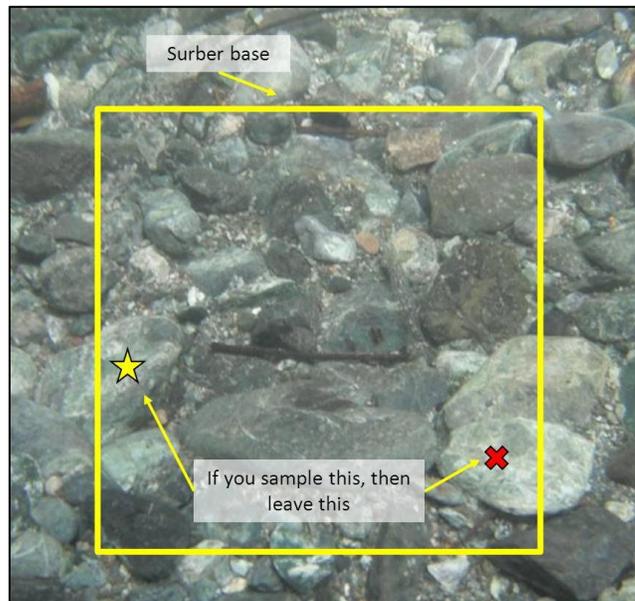


Figure 21. Cobble selection at Surber edges. The yellow square represents the bottom of the Surber sampler.



10. After the top layer of cobbles has been removed and placed in the bucket, disturb the remaining sediments (~10 cm deep in the remaining sediments; Cuffney et al. 1993) with your hand or kitchen brush so that all detritus flows into the Surber net for 1 minute.
11. After all suspended material has been washed into the net, lift the Surber sampler up off the substratum.
12. Dip the net into the stream a few times to rinse the sides of the net, always directing flow towards the back of the net. This allows material caught in the net to concentrate at the bottom of the net. Be sure to keep the net opening above the water surface so as not to accidentally lose or collect additional material in the sample.
13. Return to the processing location on the stream bank with the bucket of cobbles and the Surber sampler.
14. Set the Surber at the side of the stream so that the frame is on the shore, and the end of the net (and all material collected) is sitting in water (to keep insects alive).
15. Scrub each cobble in the bucket using the kitchen brush. Scrape off all aquatic plants using the pot scrubber side of the nylon kitchen brush or a putty knife. Brush all invertebrates into the bucket (**Figure 22**).
 - a. Scrub gently but firmly to remove insects, but not so vigorously as to damage insects. They are often identified by fragile anatomical structures such as external gills.
 - b. Rinse the cobble in the bucket. Visually inspect the cobble for organic material (e.g., moss) and clinging invertebrates (e.g., caddisflies and snails) before discarding.
 - c. Discard cleaned cobble onto the stream bank.
 - d. Repeat until all cobbles in the bucket have been scrubbed and discarded.



Figure 22. Scrub rocks from the Surber sample in the 3 gallon bucket.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

16. Invert Surber net (turn it inside-out) into bucket of water. Swirl net around in bucket water to remove all material from the net. Use your hands to brush material from the net into the bucket.



- i. Immediately release any fish or other vertebrates captured in the net. You may write a remark in the mobile app if you know what species was released.
- b. Depending on site-specific permits, mussels collected in the sampler may also need to be released. Make a remark in the data collection app if this occurs, and the species or common name if you know the type(s) of mussels collected.
- c. Use 500 mL wash bottle filled with stream water to rinse any residual organic matter or insects from the net (**Figure 23**). Check for clinging insects.



Figure 23. Removing the tip of the 500 mL wash bottle helps makes rinsing easier and increases water pressure. This tip pulls easily out of the main body of the wash bottle.

- 17. Once the Surber net is empty, all of the sample material will be suspended in water in the 3 gallon bucket. Proceed to Section C.9: Sample Elutriation and Preservation.
- 18. After elutriation and preservation, move to next location and repeat the above steps until you have the desired number of samples.
 - a. **Wadeable stream taxonomy:** 5 samples for the dominant habitat type, 3 samples for the secondary habitat.
 - b. **Wadeable stream DNA:** 3 samples in the dominant habitat type

C.2 Hand Corer



The hand corer is used in **wadeable streams** in pools or runs with sand or silt substrata. The hand corer may also be used in **lake** or **river** littoral zones with sand or silt substrata. The hand corer may be fully submerged during sampling. Each hand corer sample represents a composite of 3 core samples.

- 1. Sample in areas of similar flow and depth along the **wadeable stream** or **river** sample reach or similar depth in a **lake**. Three core samples will be combined to create a composite sample at each location.
 - a. Sample only soft sediments (sand, silt, clay). Larger sediment (i.e. gravel) will not maintain suction in the corer barrel during sampling.



2. Rinse the corer assembly to be sure that all screw threads are clean of silt and sediment.
3. Assemble the hand corer (**Figure 24**).
 - a. Screw the corer head onto the top of the stainless steel core tube (Figure 24). **NOTE:** Petroleum jelly may be applied to the threads to aid sealing.
 - b. Cores are usually more stable without the PVC liner tube. The corer may be used with or without the liner tube and core catcher, this decision may be made by the field ecologist at the time of sampling.
 - c. Screw the stainless steel nosepiece onto the bottom of the core tube (**Figure 24**).
 - d. Petroleum jelly may be applied to the flutter valve to help create suction in the corer.

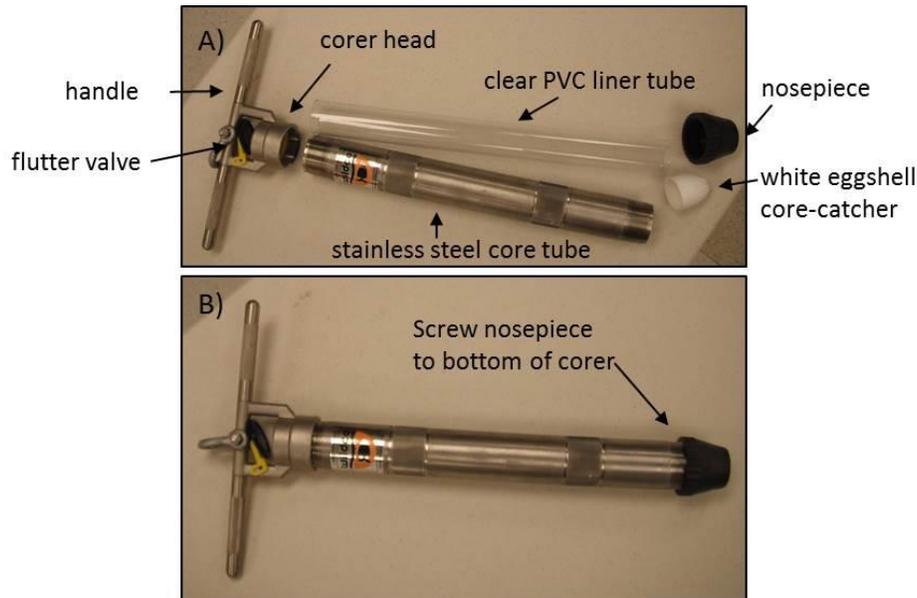


Figure 24. Hand-corer assembly: A) Disassembled corer. Use of the liner tube and core catcher are optional. B) Assembled corer. Note: the nosepiece may be stainless steel (not black Lexan as in photo) in some domains

4. Approach sample location from downstream so as not to step in the location where you want to sample.
5. Hold the core sampler firmly by the handle (**Figure 24**).
6. Push the core sampler through the water and into the sediments in one smooth, continuous movement (**Figure 25**). Allow the flutter valve to remain open.
 - a. Depending on the sediment type, it may take a lot of force to push the corer into the sediments.
 - b. Do not hammer or pound the corer into the sediments.
 - c. Keep barrel of corer at a 90° angle with the sediments.
 - d. Note the dominant substratum size class at the sampling location in the mobile app.



Figure 25. Push the hand corer through the water and into the sediments at a 90° angle

7. Push corer approximately 5-10 cm into the substratum while keeping the blue flutter valve open.
 - a. Pre-measure 5 cm and 10 cm on the outside of the corer before sampling.
8. If the corer is not completely submerged, wet the blue flutter valve (or apply petroleum jelly) on the top of the corer head and close by hand (**Figure 24**).
 - a. The valve must be kept wet to maintain a good seal and prevent loss of sample when the corer is extracted. Petroleum jelly may help keep the flutter valve sealed.
 - b. If the corer is submerged, allow air to escape from the flutter valve. When the corer is pulled up, the flutter valve should close by itself.
9. Holding the flutter valve closed, **slowly** pull the corer straight up and out of the sediments.
 - a. If the corer is pulled up too fast, you may lose the contents.
 - b. You may also use a spatula or your hand to cap the bottom of the core before pulling it out of the sediments if maintaining suction is a problem at the site
10. Continue to hold the flutter valve closed and lift the entire core sampler clear of the water, keeping the corer vertical so as not to spill the sample.
 - a. If the sample spills before reaching the bucket, you may rinse the corer and start over at Step 3 approximately 0.5 m away from the original location, as long as it has not been disturbed.
 - b. If some, but not all, of the sample spills and is not deposited in the bucket, discard the entire sample and start over.
 - i. Use a secondary container to deposit each core so that if one core fails, the entire composite sample is not lost.
11. Quickly hold the core sampler over the 3 gallon bucket and release the flutter valve, allowing the sample to drop into the bucket.
 - a. Depending on suction in the corer, the sample may drop into the bucket soon after it is removed from the water.
12. Fill the 500 mL wash bottle with stream water and rinse any residual organic matter or insects from inside the corer into the bucket.
 - a. Check for clinging insects.





13. Once the corer is empty, all of your sample material will be suspended in water in the 3 gallon bucket.
14. Repeat Steps 3-13 two more times, until there is a composite of 3 cores in the 3 gallon bucket. Proceed to Section C.9 Sample Elutriation and Preservation.
 - a. 3 cores = 1 composite sample
15. After elutriation and preservation, clean corer in stream water and move to next location and repeat the steps above until you have the desired number of composite samples.
 - a. **Wadeable stream taxonomy**: 5 samples for the dominant habitat type, 3 samples for the secondary habitat
 - b. **Wadeable stream DNA**: 3 samples in the dominant habitat type
 - c. **Lake or river taxonomy**: 5 samples in the littoral zone
 - d. **Lake or river DNA**: 3 samples in the littoral habitat

C.3 Modified Kicknet Sampler (Semi-quantitative)



The modified kicknet may be used for deep runs in **wadeable streams**, or benthic sampling in **rivers**. Water may flow over the top of the net as this is a semi-quantitative sampling method.

1. Choose runs (or deep riffles) that are similar in flow and depth.
2. Make sure net is firmly attached to handle.

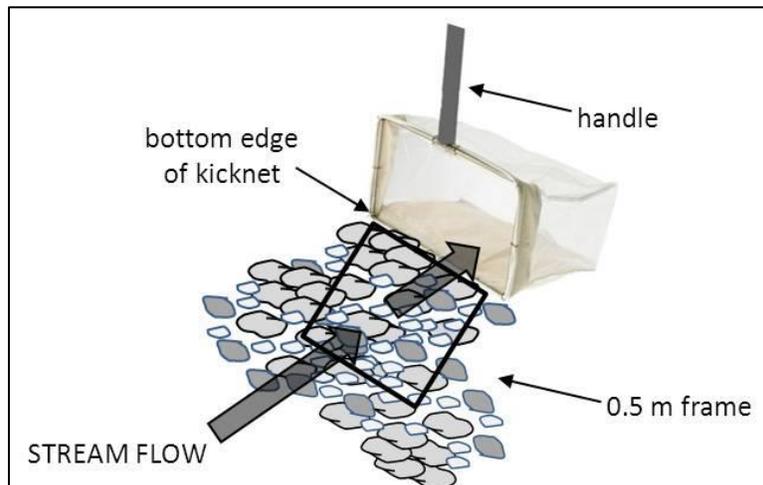


Figure 26. Example of modified kicknet setup.

3. Approach sample location from downstream so as not to disturb the location where you want to sample.
4. Orient the net so the opening of the net is facing into the stream flow.
5. Place the bottom edge of the frame on the stream bottom first, hold in place. Once the net edge is placed on the stream bottom, do not move the net (**Figure 26**).
6. Disturb the substrata within the ~0.25 m² quadrat upstream of the kicknet.
 - a. **Wadeable stream**: Place 0.25 m² quadrat just upstream (1-5 cm) of the kicknet edge.



- i. If you can reach the stream bottom inside the quadrat with your hands:
 - 1) Pick up each cobble and brush the surface with a kitchen brush so that the stream current carries all insects/organic matter into the net.
 - 2) For cobbles that are partially inside and outside of the frame: for every 1 cobble that you select that is partially outside of the frame, leave one cobble that is partially outside of the frame in the substratum (**Figure 21**).
 - 3) Once a cobble is cleaned, discard to the side or downstream of the sample area. Do not resample cobbles.
 - 4) Follow by using feet to disturb the sediments by kicking back and forth for 60 seconds inside the quadrat, so all organic matter flows into the net.
 - ii. If you cannot reach the stream bottom, step into the quadrat. Use feet to disturb the sediments by kicking back and forth for 60 seconds inside the quadrat, so all organic matter flows into the net.
 - b. **Rivers:** If water is too deep for wading, disturb substrata using a long-handled brush so all organic matter flows into the net and estimate the area of the 0.25 m² quadrat as you will not be able to place and hold the quadrat.
7. Sweep the kicknet toward the water surface, taking care to keep the opening facing upstream so no contents will spill.
 8. Dip the net into the stream a few times to rinse the sides of the net, always directing flow towards the back of the net. This allows material caught in the net to concentrate at the bottom of the net. Be sure to keep the net opening above the water surface so as not to accidentally lose or collect additional material in the sample.
 9. Take the net to a processing location on the stream bank.
 10. Note the dominant substratum size class at the sampling location in the mobile app.
 11. Half-fill 3 or 5 gallon bucket with native water.
 12. Invert net (turn it inside-out) into bucket of water. Swirl net around in bucket water to remove all material from the net. Use your hands to brush material from the net into the bucket.
 -  13. Use 500 mL wash bottle to rinse any residual organic matter or insects from the net.
 - a. Check for clinging insects.
 14. Once the net is empty, all of the sample material will be suspended in water in the 3-5 gallon bucket. Proceed to Section C.9 Sample Elutriation and Preservation.
 15. After elutriation and preservation, clean net in stream water and move to next location and repeat the steps above until you have the desired number of samples.
 - a. **Wadeable stream taxonomy:** 5 samples for the dominant habitat type, 3 samples for the secondary habitat
 - b. **Wadeable stream DNA:** 3 samples in the dominant habitat type
 - c. **River taxonomy:** 3 samples if replacing petite ponar samples, 5 samples if littoral sampling
 - d. **River DNA:** 3 samples in the littoral habitat



C.4 Hess Sampler



In **wadeable streams**, the Hess sampler should be used in shallow riffles, runs, or pools where the other samplers above will not work due substratum type or stream flow. The Hess is best used in areas with firm substrate (gravel or larger) with very little flow.

1. Ensure that all samples are collected from areas of similar depth and flow (within a approximately 20%). Water level must be below the top of the Hess frame.
2. Choose a location that appears to be underwater most of the time (i.e., not underwater only at high flow). Avoid locations that are on top of, or just downstream of, large boulders (e.g., boulders that affect the flow of water at your location).
3. Carry the 3 or 5 gallon bucket and Hess sampler with you to the sample location.
4. Fill 3-5 gallon bucket $\frac{1}{4}$ - $\frac{1}{2}$ full with stream water so that it is weighted when resting on the stream bottom or manually hold bucket in place. Place the bucket on the stream bottom in a shallow part of the riffle (or on the shore) **that you can still reach** from the sampling location.
5. Position the Hess frame securely on the stream bottom, with the screened opening facing upstream and the net facing downstream (**Figure 27**)
 - a. Hold the sampler in position by applying pressure with your knees to the back of the sampler, or have a second person hold the sampler.
 - b. Note the dominant substratum size class at the sampling location in the mobile app.



Figure 27. Hess sampler with collection net and dolphin bucket.

6. Reach into the sampler and remove any large substrata (i.e., cobbles) and place in the 3 or 5 gallon bucket.
7. After the top layer of cobbles has been removed and placed in the bucket, disturb the remaining sediments (~10 cm deep in the remaining sediments; Cuffney et al. 1993) with your hand or kitchen brush so that all detritus flows into the Hess net (typically ~30-60 seconds).
 - a. If there is insufficient stream flow to wash sample and detritus into the net, create flow with your hands inside the sampler until the water inside the Hess sampler appears clear.
8. Dip the net into the stream a few times to rinse the sides of the net, always directing flow towards the back of the net and dolphin bucket. This allows material caught in the net to concentrate at the bottom of the net. Be sure to keep the net opening above the water surface so as not to accidentally lose or collect additional material in the sample.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

9. Return to the processing location on the stream bank with the bucket of rocks and the Hess sampler.
10. Set the Hess at the side of the stream so that the frame is on shore, and the end of the net (and all material collected) is sitting in water (to keep insects alive).
11. Scrub each cobble in the bucket using the kitchen brush. Scrape off all aquatic plants using the pot scrubber side of the brush. Brush all invertebrates into the bucket.
 - a. Scrub gently but firmly to remove insects, but not so vigorously as to damage insects. They are often identified by fragile anatomical structures such as external gills.
 -  b. Rinse the cobble in the bucket. Visually inspect the cobble for organic material (e.g., moss) and clinging insects (e.g., caddisflies) before discarding.
 - c. Discard cleaned cobbles on the stream bank.
 - d. Repeat until all cobbles in the bucket have been scrubbed and discarded.
12. Rinse all material in the net into the dolphin bucket.
13. Holding the end of the net and dolphin bucket over the 3 gallon bucket, unscrew the dolphin bucket and rinse its contents into the 3 gallon bucket.
 - a. Invert net into 3 gallon bucket and rinse any remaining insects to the sample.
14. Once the Hess net is empty, all of the sample material will be suspended in water in the 3 gallon bucket. Proceed to Section C.9: Sample Elutriation and Preservation.
15. After elutriation and preservation, clean net in stream water and move to the next location and repeat the steps above until you have the desired number of samples
 - a. **Wadeable stream taxonomy**: 5 samples for the dominant habitat type, 3 samples for the secondary habitat
 - b. **Wadeable stream DNA**: 3 samples in the dominant habitat type

C.5 Woody Snag Sampling (small woody debris, semi-quantitative)



Woody snag sampling is used in **wadeable streams**, primarily in riffles and runs with a silty or sandy bottom.

1. Woody snag sampling should be used where snag/woody debris fits inside a D-frame net for sampling. This method is considered semi-quantitative because although all of the sample material fits inside the net, we are estimating surface area of the snag. Sample in areas of similar flow and depth for each sample.
 - a. Snags must be submerged and appear to be underwater most of the time (i.e., not underwater only at high flow).
 - b. Snags must account for >20% of habitat throughout the sampling area.
 - c. Snags may include leaf litter packs, overhanging branches, submerged wood (< 0.3 m (6 inches) in diameter and < 5 m long. If using leaf litter packs/debris must be present throughout the year (not just seasonally).
 - d. Note the dominant substratum size class below the snag at the sampling location in the mobile app.



Figure 28. Sites such as D08 Mayfield Creek are well-suited to snag sampling with their sandy/silty substrate and abundance submerged large woody debris.

2. Two people are needed to collect the sample. Place the 243 μ m mesh bag (D-frame net) around the end of the snag with the net opening facing upstream so invertebrates will not escape.
 - a. If there is slow- or no flow at the sampling location, try to angle the net toward the water surface to prevent macroinvertebrates from swimming away.
3. The second person should quickly saw or lop the snag near the end of the sampling net (near the mouth of the D-frame net).
4. Remove the snag section covered by the net by sawing with the bow saw or breaking the snag at the end of the net.
 - a. If you are unable to remove the snag or need to leave it in the stream for other sampling, you may scrub into the net without breaking off the piece as long as the stream flows into the net to collect any organisms that are brushed off the snag. This step requires flow to be present at the sampling location so organisms are not lost from the net.
5. Fill 3 or 5 gallon bucket $\frac{1}{4}$ - $\frac{1}{2}$ full with native water.
6. Remove snag from net and place in the bucket of stream water.
7. Invert net (turn it inside-out) into the bucket of water. Swirl net around in bucket water to remove all material from the net. Use your hands to brush material from the net into the bucket.
8. Use the 500 mL wash bottle filled with native water to rinse any residual organic matter or insects from the net. Check for organisms clinging to the sides of the net or in the seams.
9. Brush insects off snag surface using the nylon kitchen brush.
 - a. Rinse snag surface into bucket using the 500 mL wash bottle.
 - b. Measure length and width of snag and record in the mobile app.
 - c. Visually inspect snag and remove any remaining insects using flexible forceps or disposable pipet.
10. All sample material should now be in the 3 or 5 gallon bucket.
11. Fill out the mobile app with location, date, and type of samples.





12. Proceed to Section C.9 Sample Elutriation and Preservation.
13. After elutriation and preservation, rinse net in stream, move to next sampling location and repeat the steps above until you have the desired number of samples
 - a. **Wadeable stream taxonomy**: 5 samples for the dominant habitat type, 3 samples for the secondary habitat
 - b. **Wadeable stream DNA**: 3 samples in the dominant habitat type

LAKES AND RIVERS

C.6 Petite Ponar



The petite ponar is primarily used for benthic sampling in lakes and rivers.

1. Navigate to sampling location:
 - a. **Lakes** (3 samples):
 - i. Deepest point in the lake, determined by bathymetric site map and GPS coordinates (location = c0)
 - ii. Near the lake inlet (location = in)
 - iii. Near the lake outlet (location = ot)
 - iv. Please note that you can move away from the inlet or outlet sensor locations if conditions are inappropriate (e.g., too much plant cover, hard substrata) for sampling due to changes in water level or plant growth. If this occurs, please note approximate distance from the infrastructure in the date remarks.
 - b. **Rivers** (3 samples, near water chemistry sampling location and 2 other haphazardly chosen locations):
 - i. Sensor/buoy location (location = c0)
 - ii. Choose two other locations representative of the sensor location. Locations should be ± 10 m of the river depth at the buoy and a minimum of 50 m apart from each other.
2. In lakes and rivers, gently lower an anchor at the bow and allow boat to float back with wind or current to sampling location. Drop a second anchor at the stern to hold boat in place.
 - a. Allow ~5 minutes for sediments to settle after lowering the anchor, you can use this time to prepare the sampling equipment.
 - b. Using a bow anchor rope 2 times the water depth will minimize disturbance of the sediment at the sampling location.
 - c. Sample on the side of the boat, away from the motor and anchor, so as not to interfere with the ponar or disturb the sediments
3. Release the safety pin on the ponar sampler and open the bottom of the ponar (**Figure 29**). Place the pinch-pin (with spring) in the ponar and hold the ponar by the rope at the top. The sampler should stay in the open position as long as there is tension on the rope. The ponar will close automatically when the tension is released (i.e., the sampler hits sediment).



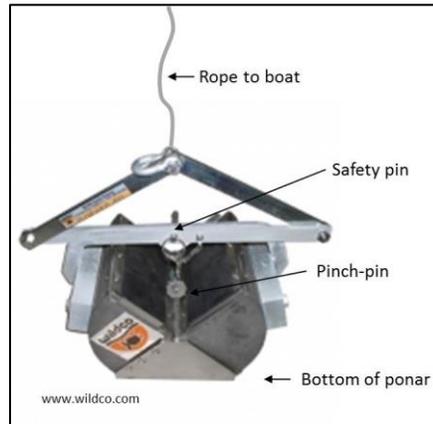


Figure 29. Example of a petite ponar setup.

4. Hold the open ponar above the water surface above the point where you want to sample.
5. Slowly lower the ponar in the water and maintain rope tension.
6. When the ponar hits the sediments and the lines goes slack, pull up on the rope to close the jaws.
7. If the jaws do not close when the sampler hits the lake/river bottom, attach a messenger to the line and drop onto the ponar to trigger the jaws to close.
8. Pull the ponar up to the surface using the rope.
9. Check to see if the ponar properly closed. If it did not close or there is an object (such as a stick) holding the jaws open, dump the sample away from your next sampling location and return to Step 4 in a different location (at least 2 m away from original location in lakes and rivers).
10. If the ponar is properly closed, place the sampler over the bucket and open the jaws to release the sample.
 - a. NOTE: Ponar sampling in areas of heavy macrophyte growth may cause the ponar to close improperly. Sample in areas of lighter or no plant growth if possible.
11. Rinse the inside of the ponar into the bucket using the 500 mL wash bottle.
 - a. If in a lake, rinse the inside of the ponar into the bucket using the 500 mL wash bottle filled with filtered (through the 250 μ m sieve) lake water to exclude zooplankton.
 - b. If in a river and zooplankton is apparent in rinsewater to the naked eye, use 250 μ m filtered water as detailed in the previous step.
12. Once ponar is empty and clean, all of your sample material will be in the 3 gallon bucket.
13. Carefully add water to the bucket to until bucket is about $\frac{1}{2}$ full.
 - a. In a lake, use >250 μ m filtered water to remove zooplankton. You may either use the 250 μ m sieve or 243 μ m Nitex mesh (used for preservative change in lab) to filter water.
14. Proceed to Section C.9 Sample Elutriation and Preservation.
15. After elutriation and preservation, rinse sampler with native water, move to the next sampling location and repeat the steps above until you have the desired number of samples.
 - a. Lakes and rivers: 3 samples



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

C.7 Large Woody Debris Sampling (Semi-quantitative)

Large woody debris (LWD) sampling is intended for logs in **rivers** that are too large to cut off and sample via the snag method (SOP C.5). This sampling method is considered semi-quantitative because the entire substrate that is sampled does not fit inside the net.

1. Sample in areas of similar flow for each sample.
 - a. LWD must be submerged and appear to be underwater most of the time (i.e., not underwater only at high flow).
 - b. LWD must account for >20% of habitat throughout the sampling area.
 - c. LWD is typically characterized by trees that have fallen into the river from the bank, or have been carried downstream by the current and become lodged against the river bottom.
 - d. In a **river**, choose either 5-even or 5-odd riparian sections for sampling
 - i. Choose sections by looking at the time: if the minute is even, sample even sections. If the minute is odd, sample odd sections.
 - ii. If there is no LWD in the section chosen, proceed to the next section. You may sample 2 snags from the same section if necessary.
 - iii. The sample number on the sample labels should correspond to the Riparian Habitat Section numbers. The mobile app will generate the sample ID using the riparian section number you have entered.
 - iv. Corresponding taxonomy and DNA samples should be collected from the same riparian section. If you are unable to collect both from the same section, notify Science through the problem reporting system.
 - e. Note the dominant substratum size class below the snag at the sampling location in the mobile app.
2. Ensure that the LWD is submerged to at least 0.6 m (2 ft) deep, in flowing water (**rivers** only), and has a diameter of ≥ 0.15 m (6 inches). The snag should either break the water surface or come within 0.3 m (1 ft) of the water surface for ease of sampling (Angradi et al. 2006).
 - a. Navigate boat to first LWD, approaching slowly from downstream.
 - b. Carefully drop and anchor the boat or have the boat driver hold the boat in place in the current using the boat motor.
 - c. Place the modified kick-net (13" x 20" opening) on top of the LWD with the opening facing up-current, just below where the snag breaks the water surface. Make sure the frame of the net is touching the LWD surface
 - d. Using the long-handled deck brush, scrub ~1 m of snag so that organisms and debris wash into the sampling net (**Figure 30**). Scrub the top and sides of the snag.
 - i. As this is a semi-quantitative method, not all organisms will be washed into the net.
 - e. The length is assumed to be 1 m and the width is assumed to be the width of the modified kicknet, you do not need to record length or width.
 - f. At the end of the sweep, turn the net opening toward the water surface and lift out of the water to prevent organisms from escaping.



- g. Dip net into the water a few times to rinse the sides of the net, always directing flow towards the back of the net. This allows material to concentrate at the bottom of the net. Be sure to keep the net opening above the water surface so as not to accidentally lose or collect additional material in the sample.
- h. Pull the net into the boat.



Figure 30. From Angradi et al. 2006, example of snag sampling in a large river. While the boat driver holds the boat in position, one person holds the modified kicknet against the snag, while a second scrubs the snag with a long-handled deck brush.

3. Fill 3 or 5 gallon bucket $\frac{1}{4}$ - $\frac{1}{2}$ full with native water.
4. Invert net (turn it inside-out) into the bucket of water. Swirl net around in bucket water to remove all material from the net. Use your hands to brush material from the net into the bucket.
5. Use the 500 mL wash bottle filled with native water to rinse any residual organic matter or insects from the net. Check for organisms clinging to the sides of the net or in the seams.
6. All sample material should now be in the 3 or 5 gallon bucket.
7. Fill out the mobile app with location, date, and type of samples.
8. Proceed to Section C.9 Sample Elutriation and Preservation.
9. After elutriation and preservation, rinse net in stream, move to next sampling location and repeat the steps above until you have the desired number of samples
 - a. **River taxonomy:** 5 samples in the littoral habitat
 - b. **River DNA:** 3 samples in the littoral habitat

C.8 D-net Measured Sweep Samples (Semi-quantitative)



In **lakes** and **rivers**, the D-net sweep samples are the preferred sampling method for littoral samples.

1. Sampling locations will correspond to the 10 habitat stations set forth in the Riparian Habitat Assessment Protocol (RD[15]). This divides the **lake/river** margins into 10 sections (**Figure 31**).

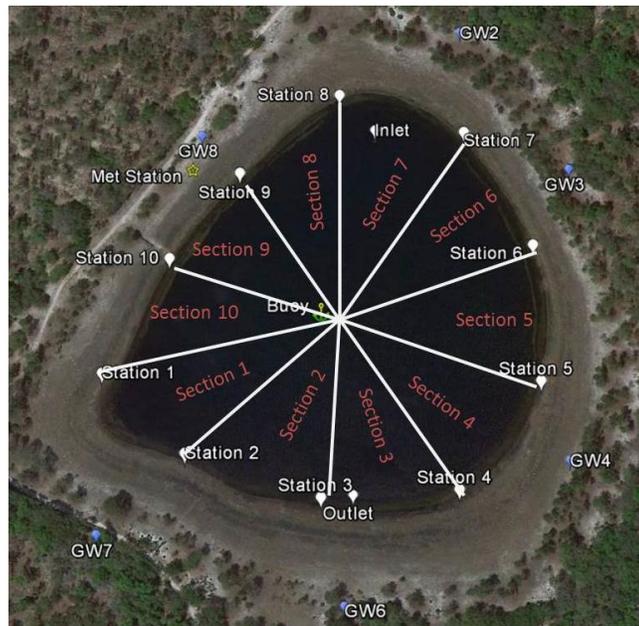


Figure 31. Example of Riparian Stations splitting the lake into sections at D03 Lake Barco.

2. Choose either the 5-even or 5-odd habitat sections for sampling.
 - a. Choose sections by looking at the time. If the minute is even, sample even sections. If the minute is odd, sample odd sections.
 - b. The sample number on the sample labels should correspond to the Riparian Habitat Section numbers. The mobile app will generate the sample ID using the riparian section number you have entered.
 - c. Sample in rooted plant habitat <1m deep if possible. Choose similar depth and habitat to sample in each section.
 - i. If the lake has no rooted plant habitat, sample floating vegetation mats.
 - ii. Senescent or dead plant material is acceptable for sampling.
 - d. You may sample anywhere along the shoreline of the section in order to find the preferred sampling habitat. If no preferred habitat is available, skip to the next section, then continue with the previously selected sections.
3. Navigate boat to an aquatic plant bed in the chosen sampling section. Ensure that you are able to reach the lake/river bottom with the sampling net.
4. If permits allow and the area of the lake or river is shallow enough to wade, step out of the boat and sample by wading (permits will be provided by NEON Permitting). If permits are not available, sample near the bow of the boat, away from the motor so the motor will not interfere with the sweep net or disturb the sediments.
5. Attach handle to D-frame net (**Figure 32**).



Figure 32. D-frame net, used for sweep samples in vegetation and silty habitats.

6. Sweep the D-frame net through the vegetation one time across a 1 m distance in one direction (DiFranco 2006, USEPA 2011).
 - a. To determine the sweep length, measure 1 m on the net handle (typically 1 m = the wooden part of the handle). Using this as a reference, place feet 1 m apart on the **lake/river bottom** and sweep from foot to foot. Distance is estimated if sampling from the boat.
 - b. The net should remain submerged during the entire sweep, and the bottom of the net should brush the sediments during the entire sweep (**Figure 33**).
 - c. For rooted aquatic plants: bump or jab the bottom of net frame against the sediment several times (> 3 times) to collect benthic organisms while keeping the net frame close to, but not scraping, the bottom.
 - d. For floating vegetation mats: scoop all vegetation into the net.
 - e. Keep net in motion to prevent organisms from swimming out of the net. Conduct sweep quickly, entire sweep should last ~3 seconds.
 - f. At the end of the sweep, turn the net opening toward the water surface and lift out of the water to prevent organisms from escaping.
 - g. Retain all plants that are completely in the net in the sample. If plants are hanging on the net frame, break the plants and include the portions that are in the net with the sample, discard those that are hanging outside the net.
 - h. Rinse net by dipping in the water, keeping the mouth of the net above the water line so as not to introduce additional organisms to the sample.



Figure 33. D-frame sweep technique in a littoral area; the D-frame net must remain submerged and close to the substrate throughout the entire sweep.

7. For floating aquatic plants only, hold the net in the water with the mouth above the water line. Agitate the plant material with your hands, in water in the net for 1 minute. Rinse plant material with 500 mL wash bottle if necessary. Inspect vegetation to ensure that you have removed invertebrates. Remove and discard plant material while keeping macroinvertebrate sample in the net (FDEP 2011).
 - a. Keep in mind that this is a quantitative sampling approach, so do not discard material that contains macroinvertebrates.
8. With the macroinvertebrate sample in the net, dip D-frame net into the water a few times to rinse the sides of the net, always directing flow towards the back of the net. This allows material to concentrate at the bottom of the net. **Be sure to keep the net opening above the water surface** so as not to accidentally lose or collect additional material in the sample.
9. Pull the net into the boat.
10. Fill the 3 gallon bucket $\frac{1}{4}$ – $\frac{1}{2}$ full with 250 μm filtered lake water to exclude most zooplankton.
11. Invert net (turn it inside-out) into bucket of filtered rinsewater. Swirl net around in rinsewater to remove all material from the net. Use your hands to brush material from the net into the bucket.
12. Use 500 mL wash bottle filled with 250 μm filtered lake water to rinse any residual organic matter, plants, or organisms from the net into the bucket. Check for organisms clinging to the sides of the net or in the seams.
 - a. All sample material should now be in the 3 or 5 gallon bucket.
13. Fill out field mobile application and label with location, date, and type of sample.
14. Complete Sample Elutriation and Preservation (SOP C.9).
15. After elutriation and preservation, rinse net in native water and move to the next location, repeating the steps above until you have the desired number of samples.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

- a. **Lake or river taxonomy**: 5 samples in the littoral habitat
- b. **Lake or river DNA**: 3 samples in the littoral habitat

ALL SAMPLES

C.9 Sample Elutriation and Preservation

1. Samples only need to be elutriated if they have inorganic material that you do not want to include with the sample. If the sample is all organic material, include all material in the sample and proceed to preservation (Step 15).
2. Gently swirl contents in bucket to create a “whirlpool” and suspend organic material. This process is called elutriation (**Figure 34**).



Figure 34. Process of elutriation (Dates and Byrne 1997).

3. Carefully pour contents of bucket into 250 μm sieve or sieve bucket (**Figure 35**). If you are in a boat, hold the sieve over a 5 gallon bucket to capture the waste water. Some material will be retained in the original bucket.
 - a. Be careful not to slosh sample water over the edge of the sieve. All material must be contained within the sieve to maintain a quantitative sample.
 - b. The sieve bucket should be used for petite ponar samples for thick, fine sediments.
 - c. If the sieve fills up with organic matter, you may pick clumps out of the sieve and place in sample jar.
 - d. You may use your fingers or shake the sieve or sieve bucket to stir the sample and move fine material through the sieve.
 - e. You may use low-pressure water to wash sediment through sieve mesh. High pressure will damage organisms.



- f. Sieving the sample in small portions may help prevent clogging.
- g. Continue rinsing to reduce the sediment in the sample, especially for petite ponar samples. Reduce samples to ≤ 750 mL if possible.



Figure 35. Carefully pours water from the 3 gallon bucket into the sieve.

- 4. Partially refill bucket with water (bucket opening should be facing upstream) without losing any of the organic matter contents of the bucket.
 - a. Use 250 μm filtered water in lakes to exclude zooplankton. You may use the 250 μm sieve or 243 μm Nitex mesh (used to change preservative in the lab) for filtering rinsewater.
- 5. Repeat elutriation (Steps 2-4) until the water appears relatively clean. There may be some sand or silt in the bottom of the bucket. Elutriate a **minimum** of 3 times per sample.
 - a. Elutriating ~ 3 -5 times (until you no longer see insects in the swirled water in the bucket) is typically sufficient to remove insects from the inorganic matter.
 - b. At sites with fine sediments (i.e., petite ponar samples in lakes), elutriation may be particularly difficult. If using the sieve bucket, dunk the bottom of the sieve bucket directly into the lake water, without adding new water to the top of the bucket, to facilitate sediment rinsing.
- 6. Check sides of bucket for clinging invertebrates. Check sand/inorganic material at the bottom of the bucket for caddisflies (rock cases) or other heavy invertebrates (e.g., snails), place in sample container using forceps or fingers. Retain any plant material in the sample jar.
 - a. Place any invertebrates from this inspection in the sieve with the rest of the sample.
 - b. Mussels may need to be returned to the stream depending on permitting requirements.
- 7. Discard inorganic material remaining in the bucket, and rinse bucket with native water. Set bucket aside.





8. The entire sample should now be in the sieve or sieve bucket.
9. Add a small adhesive label and barcode label to the outside of the sample jar with the sample ID for use by the external taxonomy lab. Write in pencil as ethanol will erase permanent marker. Make sure the label sample ID matches the sample ID generated by the data ingest. For taxonomy samples, also add a waterproof paper label (write in pencil) inside the sample jar.
 - a. Sample ID format: *SITE.DATE.sampleType.sampleNumber* (**Table 6**)
 - b. Example: *ARIK.20140620.kicknet.3*
 - c. Paper labels are added inside the jar because ETOH can degrade the external labels during shipping.
10. Fill the 500 mL wash bottle with native water. Use 250 μ m filtered water in lakes to exclude additional floating invertebrates.
11. Rinse sieve screen and edges with wash bottle and tap sieve until organic material is collected at one edge of the sieve (**Figure 36**).



Figure 36. Rinse the sample from the sieve into the open container with the 500 mL wash bottle.

12. Open the sample jar and carefully rinse (using the 500 mL wash bottle) sample from the sieve into the sample jar. You can also use your fingers to move the sample from the sieve into the sample jar.
 - a. Use as little water as possible. The more water used, the more ETOH is needed. Be sure to rinse sieve and fingers into the sample jar to thoroughly remove all material.
 - b. If there is too much rinse-water, you may re-sieve sample by dumping contents of sample jar back into the sieve, and repeating Steps 11-12.
13. If sample is too large for one sample jar, you may use multiple jars. Each jar must be properly labeled with site and date information, as well as “Sample 1 of 2” or “Sample 2 of 2”.





Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

- a. Use a Type I barcode label on sample jar 1 and band or tape the two jars together, or place both containers in one zip-top bag. The external lab will re-combine both into one sample.
14. Check sieve for clinging insects that may not have been washed into the sample jar.
15. **Carefully** add preservative. Preserving immediately prevents predation and damage to insects in the samples.
 - a. Wear latex gloves when preserving samples if your domain's chemical hygiene plans calls for it. Always wear eye protection (e.g., sunglasses).
 - b. Preserve samples in a well-ventilated location (e.g., outdoors).
 - c. Add enough ethanol preservative to the sample to preserve in a 1:1 sample material: preservative ratio (sample material includes water in the jar). Ideal preservation is 70% ethanol, but using 1:1 as an eyeball estimate is usually a good start for the field preservation.
16. Record data in the mobile app.
 - a. Scan the barcode label with the tablet or hand-held scanner (**Figure 37**).
 - b. Record all relevant metadata for each sample in the mobile app.
 - c. **Ensure that the human-readable sample ID matches the sample ID generated by the mobile app.**

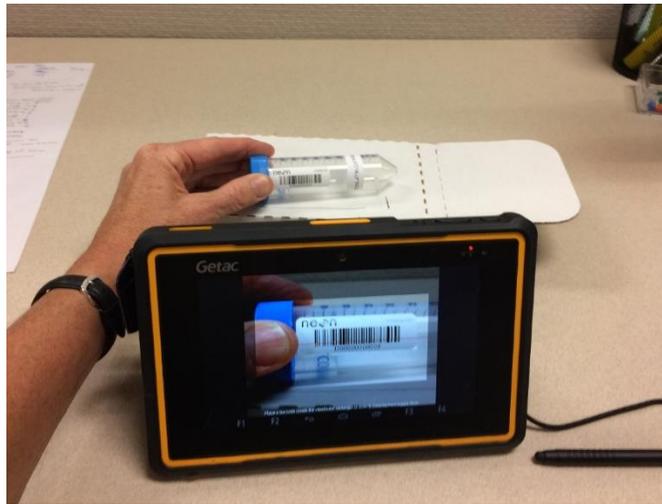


Figure 37. Barcode label scanning.

17. Close the sample jar tightly.

C.10 Sample Preservation

Preserve samples within 60 minutes using 95% ethanol streamside/lakeside or at the field vehicle (see details above in each sampling section) to prevent predation within the samples to a final concentration of ~70% ethanol.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

SOP D Macroinvertebrate DNA Metabarcoding Field Collection

Macroinvertebrate DNA samples are collected during each sampling bout, three times per year. Samples collected specifically for DNA metabarcoding will be analogs to three of the samples already being collected in the dominant (or littoral) habitat type for morphological taxonomy. This sampling approach uses the community metabarcoding approach, where replicate samples from the site are preserved in high-concentration ethanol for sequencing analysis.

D.1 Sterilize equipment

All equipment must be cleaned and sterilized prior to sample collection at the site to prevent contamination of DNA from the person collecting the sample, DNA from another site, or DNA from the environment outside of the site (Laramie et al. 2015). Equipment is difficult to sterilize in the field, so thorough rinsing of all equipment between samples is necessary.

1. Cleaning equipment:

- a. Wearing nitrile gloves, clean all nets, waders, and other equipment that comes in contact with samples during macroinvertebrate sampling using a 10% bleach solution. Follow with a DI or tap water rinse to remove the residual bleach (Jane et al. 2014). Note that this is a higher concentration what is usually used in the Decontamination Protocol (RD[08]).
 - i. Field equipment to decontaminate with stronger solution:
 1. Sampling net(s)
 2. Sieve
 3. Bucket
 4. Brush
 5. Forceps
 6. Boots/waders
 7. Sample jars if they are being reused from other sampling, or come uncapped from the manufacturer
 - ii. Lab equipment to decontaminate
 1. Filter cup
 2. Filter mesh
 3. Sieve (if using)
 4. Pipette (if using)

2. Wearing clean nitrile gloves, place equipment and consumables in a clean bag so as not to contaminate it on the way to the field site.

D.2 Field collection

1. Fill out and place an adhesive human-readable label and barcode label on the sample jar. Circle "DNA" on label. **Do not** use a paper label inside the sample jar for this protocol because this could introduce contamination.



2. Collect an additional sample at 3 of the 5 dominant habitat sampling locations chosen above (SOP B). Use the same sampler already chosen to use for taxonomy samples at each of those locations. Be sure to thoroughly rinse all equipment that comes in contact with the DNA samples with stream water between samples.
 - a. Choose 3 riparian sections or habitat units that 1) have enough habitat to support two samples and 2) are spread out along the stream/river reach or lakeshore (ideally samples 1, 3, and 5).
 - b. If following contingent decisions, still collect 3 samples. Three is the minimum number of samples you can collect for the data to be statistically relevant to users
3. Wear nitrile gloves while sampling for DNA. You can re-use and clean gloves by wiping with an ethanol wipe or spritzing with ETOH.
4. Prior to sampling, prime the sampler by rinsing well with local source water to remove any residual debris or organisms.
 - a. You do not need to decontaminate between samples with bleach or ETOH within one day at a site because it is difficult and largely ineffective to sterilize nets in the field. Rinse well with local water, tap water, or DI.
 - b. Clean nitrile gloves with ETOH spray and or wipes between samples, or use new gloves for each sample.
5. Collect samples according to SOP B for the appropriate habitat type and sampler.
 - a. If it appears that a sample has no macroinvertebrates in it (e.g., D-frame sweep samples, snag, or LWD samples), recollect the sample at an adjacent location.
 - b. It is particularly important to pay attention to the elutriation step. Samples will be homogenized in a blender at the external facility, and inorganic matter (pebbles, sand) will interfere with homogenization.
6. Choose “DNA” in the mobile app, and the appropriate habitat and sampler metadata.
7. Sample ID = *SITE.YYYYMMDD.sampleType.sampleNumber.DNA*
8. After sample collection, use forceps or clean-gloved hands to pick out large organic matter, leaving the macroinvertebrates in the sample.
 - a. Pick large plant material out of the sample only if you are sure that it is free of macroinvertebrates. The external lab will homogenize these samples in a blender, so minimizing extra material in the sample helps keep lab costs down.
 - b. Samples will be homogenized in a blender at the external facility. Make sure there are no large sticks or other material in the sample that could interfere with homogenization.
9. After the entire sample is in the sample jar, carefully drain the some of the extra water off the top. You may want to use the sieve to help drain without losing macroinvertebrates from the sample.
10. Add 95% ETOH to sample jar to completely cover sample (Stein et al. 2012). ETOH concentration should be as close to 95% as you can get (as little extra water as possible. You do not need to fill the jar to the top with ETOH.
11. Return sample to domain lab and store in the dark at 4 °C and proceed to Section F.3.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

SOP E Post-Field Sampling Tasks

1. Refresh the sampling kit
 - a. Replace sample jars and resealable bags.
 - b. Print new field labels and field data sheets. Make sure barcode labels are available.
 - c. Refill/restock preservative containers.
2. Equipment maintenance, cleaning and storage
 - a. Decontaminate all equipment that has come in contact with site water according to the Decontamination Protocol (RD[08]).
 - b. Dry all equipment thoroughly between sites and before storage.
 - c. Check all nets for holes and patch if necessary.
3. 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 jars.
 - ii. 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.
 - b. Nice to check
 - i. Site ID, collect date, sampling protocol version

E.1 Document Incomplete Sampling Within a Site

Macroinvertebrate sampling is scheduled to occur at all prescribed sampling locations according to the frequency and timing described in Section 4.1. Ideally, sampling will occur at these sampling locations for the lifetime of the Observatory (core sites) or the duration of the site's affiliation with the NEON project (gradient sites). However, sampling may be shifted from one location to another when sampling is compromised. In general, a sampling location is compromised when sampling becomes so limited that data quality is significantly reduced.

There are two main pathways by which sampling can be compromised. First, sampling locations can become inappropriately suited to answer meaningful biological questions (e.g., a terrestrial sampling plot is compromised after road-building activities, or a stream moves after a flood and the location is no longer within the stream channel). Second, sampling locations may be located in areas that are logistically impossible to sample on a schedule that is biologically meaningful.

Macroinvertebrate sampling locations may become compromised if a stream dries or is snow or ice covered, or if the water level of the lake drops too low to samples near the inlet and outlet infrastructure. If sampling at a given location or habitat type is not possible during a given bout a problem ticket should be submitted by Field Science staff.

Please note that 3 macroinvertebrate samples of either type (INV-tax or INV-dna) is the minimum that can be collected during a bout to ensure scientifically useful data. Therefore, it is preferable to collect a sample in a different location rather than not collect a sample at all.



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

To document locations not sampled during the current bout:

1. Review Fulcrum records to determine which locations were scheduled for sampling but were not sampled.
2. Create an incident with the following naming convention to document the missed sampling: 'AOS Sampling Incomplete: INV – [Root Cause Description]'
 - a. Example: 'AOS Sampling Incomplete: INV – Could not access inlet and outlet due to cows'
3. Staff scientists review incident tickets periodically to determine whether a sampling location is compromised.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

SOP F Laboratory Sampling and Analysis

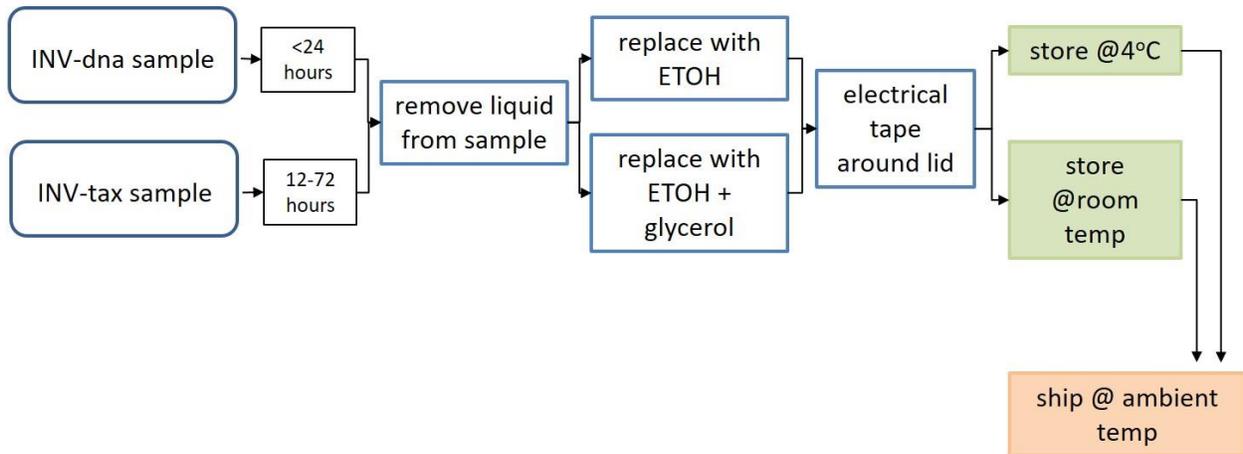


Figure 38. An expanded diagram of the lab workflow for the macroinvertebrate lab SOP.

F.1 Preparation

1. Fill 500 mL wash bottle with 475 mL 95% undenatured ethanol and 25 mL 99% glycerol (final concentration: 5% glycerol; Stein et al. 2013) for taxonomy preservation. Clearly label bottle.

F.2 Processing Samples – Taxonomy

1. Replace preservative between 12 and 72 hours of field collection in the domain lab.
2. Wearing safety glasses and nitrile gloves, open field-preserved sample under fume hood.
3. Carefully decant liquid over a clean 250 μ m sieve or filter cup similar to that used in the TOS Beetle Protocol (RD[10]) over an ETOH waste container. Take care not to lose any sample material.
 - a. Rinse down sides of sample jar with ETOH/glycerol wash bottle so no material remains near the top of the sample jar.
 - b. If using sieve, check for any macroinvertebrates or other organic material that may have come from the sample. Using featherweight forceps, place this material back in the sample jar.
 - c. If using the TOS beetle filter cup, rinse down the sides of the cup with ETOH. You may either include the mesh and all samples material on it in the sample jar, or rinse sample material from the mesh into the jar and discard the mesh. Use 250 μ m mesh, not the TOS mesh as it is a different mesh size.
4. Check that sample label is still readable and inside the sample jar. Check that the external adhesive label/barcode label is on the outside of the sample jar.
5. Re-preserve sample with 95% ethanol/5% glycerol solution. Fill jar with liquid to prevent sample damage during shipping.
6. Close the sample jar tightly.
7. Carefully clean sieve or filter cup before decanting the next sample.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

8. Continue until preservative in all samples has been replaced and proceed to Sample Shipping (SOP H).

F.3 Processing Samples – DNA

Preservative in DNA samples must be changed within 24 hours to preserve the integrity of DNA. This process replaces the water in the organisms with ethanol.

1. Change ethanol within 24 hours in the domain lab. This helps to ensure that DNA is preserved quickly and will be in good condition for analysis.
2. **Do not add glycerol** to DNA samples.
3. Decontaminate the filter apparatus, nylon mesh, and any equipment that comes in contact with the DNA sample during processing, or keep a separate, decontaminated filter apparatus for DNA.
4. Wearing safety glasses and nitrile gloves, open field-preserved sample under fume hood.
5. Follow Section F.2 to change out the ethanol in the sample, with the exception of adding glycerol.
 - a. Clean and decontaminate any equipment or consumables that come in contact with the sample material with 10% bleach prior to the sampling day, and rinse with 10% bleach followed by DI or tap water between samples.
6. Replace ethanol, preserving as close to 95% as possible.
 - a. Container does not need to be filled to the top with liquid since these specimens do not need to remain intact for processing.
7. Close the sample jar tightly, place in 4 °C and dark until shipping.
8. ETOH wipe and sterilize filter apparatus or any equipment that comes in contact with the DNA sample material between samples.
9. Carefully clean and dry all equipment prior to storage.

F.4 Sample Storage

Taxonomy samples may be stored at the domain support facility at room temperature or 4 °C until shipping. For shipping instructions see SOP H.

DNA samples from may be stored at 4 °C or frozen at -20 °C until shipping



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

SOP G 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 error and improve data quality. Adhesive barcode labels will be used and scanned into the mobile application. 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. For detailed instructions on protocol specific data entry into mobile devices, see the NEON Internal Sampling Support Library (SSL).

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.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

SOP H Sample Shipment

Information included in this SOP conveys science-based handling and packaging instructions for the samples types included in this protocol. For shipping instructions, see RD[20].

1. Keep morphological taxonomy samples and DNA samples separate.
 - a. Bout 2 DNA samples will be shipped to an external lab for analysis.
 - b. Bout 1 and Bout 3 DNA samples will be shipped directly to the archive facility.
2. Seal jar lids with electrical tape prior to shipping. This helps prevent leaks and prevent ethanol fumes from turning labels black during shipment.
 - a. Tip: Assigning a different color of electrical tape to each site in the domain helps keep samples organized.
3. Bag samples
 - a. INV-tax: Place sealed sample jars into one or several gallon-sized resealable zip-top bags, grouped by site. Sample jars are acceptable “inner containers” required for shipping.
 - b. INV-dna: Individually bag each DNA sample in a zip-top bag to prevent contamination of DNA from sample to sample during shipment.
4. See RD[20] for further shipping instructions.

H.1 Handling Hazardous Material

Follow procedures for shipping ethanol in limited quantity ground shipments in AD[03] and RD[20].

H.2 Supplies/Containers

See section SOP H Steps 1-3 and Appendix E specific shipping materials.

H.3 Timelines and Conditions

Taxonomy samples: Shipping should occur within one week of sampling if possible, however samples may be held for up to 30 days at the domain support facility if necessary. Samples may be stored at the domain support facility room temperature or 4 °C until shipping. Samples are shipped ground at ambient temperature.

DNA samples: Samples from Bout 2 must be held at 4 °C and will be shipped to the external facility for analysis on a CLA-defined schedule. Samples from Bouts 1 and 3 will be shipped directly to the archive facility on a CLA-defined schedule. Samples may be held for a maximum of 6 months if batch shipping is requested by the external lab. Samples are shipped ground at ambient temperature.

H.4 Grouping/Splitting Samples

Group samples by site per bout in plastic bags. Samples from multiple sites may be sent in the same shipment. Macroinvertebrate samples may be shipped with zooplankton samples (RD[17]) if going to the same external lab.



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

H.5 Return of Materials or Containers

Include return shipping label if any shipping materials need to be returned to the domain support facility (e.g., cooler). Sample jars will not be returned.

H.6 Shipping and Chain of Custody

The mobile shipping applications are used to create chain of custody documents. Shipments are to include a hardcopy of the shipping manifest (RD[11]) in each box as well as an electronic shipping manifest that is emailed to the receiving laboratory and NEON Collections and Laboratory Analysis at the time of shipment (created by the shipping application). The shipping manifest must accurately document the physical samples inside the shipping container.

H.7 Laboratory Contact Information and Shipping/Receipt Days

See the Shipping Information for External Facilities on NEON's CLA intranet site.



8 REFERENCES

- Allan, J. D. 1995. *Stream Ecology: Structure and Function of Running Waters*. Kluwer Academic Publishers, Boston, MA. 388 pages.
- Angradi, T.R. (editor). 2006. *Environmental Monitoring and Assessment Program: Great River Ecosystems, Field Operations Manual*. EPA/620/R-06/002. U.S. Environmental Protection Agency, Washington, D.C.
- Baker, John R., David V. Peck, and Donna W. Sutton (editors). 1997. *Environmental Monitoring and Assessment Program Surface Waters: Field Operations Manual for Lakes*. EPA/620/R-97/001. U.S. Environmental Protection Agency, Washington, D.C.
- Barbour, M. T., Gerritsen, B. D. Snyder, and B. Stribling. 1999. Benthic Macroinvertebrate Protocols. Pages 7.1-7.34 *in* *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 Protection Agency, Office of Water, Washington, D.C.
- Brooks, S. S. and A. J. Boulton. 1991. Recolonization dynamics of benthic macroinvertebrates after artificial and natural disturbances in an Australian temporary stream. *Australian Journal of Marine and Freshwater Research* 42:295-308.
- Clausen, B. and B. J. F. Biggs. 1997. Relationships between benthic biota and hydrological indices in New Zealand streams. *Freshwater Biology*. 38: 327-342.
- Cuffney, T. F., M. E. Gurtz, and M. R. Meador. 1993. *Methods for collecting benthic invertebrate samples as part of the National Water-Quality Assessment Program*. U.S. Geological Survey Open-File Report 93-406. Raleigh, NC.
- Dates, G. and J. Byrne. 1997. *Living Waters: Using Benthic Macroinvertebrates and Habitat to Assess Your River's Health*. River Watch Network, Montpelier, VT.
- DiFranco, J. 2006. *Protocols for Sampling Aquatic Macroinvertebrates in Freshwater Wetlands*. DEPLW0640. Maine Department of Environmental Protection, Bureau of Land and Water Quality, Portland, Maine.
- FDEP. 2011. *Sampling and use of Stream Condition Index (SCI) for assessing flowing waters: A primer*. DEP-SAS-001/11. Florida Department of Environmental Protection Bureau of Assessment and Restoration Support, Tallahassee, FL.
- Hauer, F. R. and V. H. Resh. 2006. Macroinvertebrates. Pages 435-463 *in* F. R. Hauer and G. A. Lamberti, editors. *Methods in Stream Ecology*, Second Edition. Academic Press, Boston, MA.
- Hynes, H. B. N. 2001. *The Ecology of Running Waters*. The Blackburn Press, Caldwell, NJ. 555 pages.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

- Jane, S. F., T. M. Wilcox, K. S. McKelvey, M. K. Young, M. K. Schwartz, W. L. Lowe, B. H. Letchers, and A. R. Whitley. Distance, flow, and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*. 15: 216-227.
- Lane, S. L. and R. G. Fay. 1997. Safety in Field Activities: U.S. Geological Survey Techniques of Water-Resources Investigations, Book 9, Chapter A9. Accessed 11 April 2013, at <http://pubs.water.usgs.gov/twri9A9/>.
- Laramie, M. B., D. S. Pilliod, C. S. Goldberg, and K. M. Strickler. 2015. Environmental DNA sampling protocol: Filtering water to capture DNA from aquatic organisms. *USGS Survey Techniques and Methods, Book 2, Chapter 113*. <http://dx.doi.org/10.3133/tm2A13>.
- Matthaei, C. D., U. Uhlinger, E. I. Meyer, and A. Frutiger. 1996. Recolonization by benthic invertebrates after experimental disturbance in a Swiss prealpine river. *Freshwater Biology* 35: 233-248.
- Merritt, R. W. and K. W. Cummins. 2006. Trophic Relationships of Macroinvertebrates. Pages 585-609 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.
- 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.
- Stein, E. D., B. P. White, R. D. Mazor, P. E. Miller, and E. M. Pilgrim. 2013. Evaluating ethanol-based sample preservation to facilitate use of DNA barcoding in routine freshwater biomonitoring programs using benthic macroinvertebrates. *PLoS ONE* 8: e51273.
- USEPA. 2002. Standard operating procedure for benthic invertebrate field sampling. EPA LG406. U.S. Environmental Protection Agency, Washington, D.C.
- USEPA. 2009. National Lakes Assessment: A Collaborative Survey of the Nation's Lakes. EPA 841-R-09-001. U.S. Environmental Protection Agency, Office of Water and Office of Research and Development, Washington, D.C.
- USEPA. 2011. National Lakes Assessment. Field Operations Manual. EPA 841-B-11-003. U.S. Environmental Protection Agency, Washington, D.C.
- Wetzel, R. G. 2001. *Limnology: Lake and River Ecosystems*, 3rd Edition. Academic Press, San Diego, CA. 1006 pages.



APPENDIX A QUICK REFERENCES

A.1 Steps for Macroinvertebrate Sampling

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

Step 2 – Prepare internal (below), external sample labels (2" x 4"), and barcode labels.

Step 3 – Ensure the General AQU Field Metadata and Gauge Height (RD[06]) app is completed per field site visit (mobile app or datasheet).

Step 4 – Determine habitat sampling locations from the Rapid Habitat Assessment (RD[09]).

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

Wadeable streams

1. Riffles (Surber, Hess, or snag)
2. Runs (Surber, Hess, modified kicknet, hand corer, or snag)
3. Pools and step pools (Hess or hand corer)

Lakes/Rivers – Benthic

1. Petite ponar
2. Modified kicknet

Lakes/Rivers – Benthic

1. Aquatic plant beds or floating mats (D-frame net), dead vegetation is acceptable
2. Snags/large woody debris (Modified kicknet snag sampler – rivers only)
3. Littoral – firm substrate (modified kicknet)
4. Littoral – soft substrate (hand corer)

Step 6 – Collect taxonomy samples (Wadeable Streams: 5 per dominant habitat type, 3 per subdominant dominant habitat type; Lakes/Rivers: 3 in benthic sediments, 5 in the dominant littoral habitat type with the appropriate sampler). Collect DNA samples (Wadeable Streams: 3 per dominant habitat type; Lakes/Rivers: 3 per dominant littoral habitat type (with same sampler as taxonomy)).

Step 7 – Elutriate and pour sample over sieve or sieve bucket.

Step 8 – Transfer sample from sieve to sample jars

Step 9 – Preserve with 95% ethanol to a final concentration of ~70% ethanol in the field.

Step 10 – Taxonomy: Change preservative and add glycerol at the Domain Support Facility within 12-72 hours of sample collection. DNA: Change preservative (no glycerol) at the Domain Support Facility within 24 hours of sample collection.



<i>Title:</i> AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		<i>Date:</i> 03/16/2022
<i>NEON Doc. #:</i> NEON.DOC.003046	<i>Author:</i> S. Parker	<i>Revision:</i> F

Step 11 – Store appropriately and ship samples to external facility.



APPENDIX B REMINDERS

Before heading into the field:

- Collect and prepare all equipment, including sample jars and labels.
- Pre-print waterproof labels, small adhesive labels, and barcode labels.
- Check for holes in nets and sieve, assure that both are clean and free of debris.
- Bleach clean all equipment between sites for DNA sampling.
- Fill 1 L HDPE bottles with 95% undenatured ethanol. Cap tightly and clearly label bottle. Transport to the field following EHS guidelines.

Sample collection:

- When making contingent decisions (
- **Table 4**), a suite of 3 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 collect fewer than 3 samples.
- Determine the habitats to sample based on the Stream Morphology or rapid habitat assessment, Bathymetry and Riparian Maps.
- Choose the appropriate sampler and follow depth and substrate rules for sampler.
- Start sampling at the bottom of the reach in flowing water, working upstream so as not to decrease visibility and cause invertebrates to drift (**wadeable streams**).
- Spread samples out along the reach or among riparian sections.
- Do not sample anywhere you or other field people/animals have walked in the reach, recently sampled, or locations that appear recently disturbed.
- **Lakes/Rivers**: Collect 3 samples from benthic sediments and 5 samples from the dominant littoral habitat for taxonomy. Collect 3 samples from the dominant littoral habitat for DNA.
- **Wadeable Streams**: Collect 5 samples from the dominant habitat type and 3 samples from the sub-dominant habitat for taxonomy. Collect 3 samples from the dominant habitat for DNA.
- Macroinvertebrates often become trapped in the folds of the nets (near the stitching). Check net seams between each sample to ensure that specimens are added to the correct samples, and do not remain in the net.
- Macroinvertebrates are often lodged in the edges of the sieve. Check the sieve between samples to avoid sample-to-sample specimen contamination.
- Samples must be preserved in the field within 1 hour of sampling to prevent predation within the sample, as predatory insects tend to eat smaller insects when put into sample jars.

Sample preservation:

- Preserve the samples streamside/lakeside or at the vehicle to reduce predation.
- Change ethanol and add glycerol within 12-72 hours of field sampling for INV-tax, and replace ETOH within 24 hours for INV-dna.
- Fill jar with ETOH to prevent destruction of organisms during shipping.



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

Data QA/QC:

Required checks

- Check that the sample IDs generated by the mobile application(s) match the sample IDs written on the sample jars.
- 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.

Nice to check

- Site ID, collect date, sampling protocol version



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

APPENDIX C ESTIMATED DATES FOR ONSET AND CESSATION OF SAMPLING

Preliminary date ranges for biological sampling bouts. General guidelines for developing these dates are presented in the NEON Aquatic Sample Strategy Document (RD[07]). Also see the Site Specific Sampling Strategy Document for each domain (RD[19]) on NEON’s FOPS intranet site.

Domain	Site		Bout 1	Bout 2	Bout 3
D01	Hop Brook	HOPB	11Apr-9May	9Jul-6Aug	3Oct-31Oct
D02	Lewis Run	LEWI	19Mar-16Apr	5Jul-2Aug	18Oct-15Nov
D02	Posey Creek	POSE	19Mar-16Apr	5Jul-2Aug	18Oct-15Nov
D03	Flint River	FLNT	21Feb-21Mar	27Jun-25Jul	7Oct-4Nov
D03	Lake Barco	BARC	9Feb-9Mar	27Jun-25Jul	29Oct-26Nov
D03	Lake Suggs	SUGG	9Feb-9Mar	27Jun-25Jul	29Oct-26Nov
D04	Rio Guilarte	GUIL	26Jan-23Feb	21Jun-19Jul	9Nov-7Dec
D04	Rio Cupeyes	CUPE	24Jan-21Feb	21Jun-19Jul	10Nov-8Dec
D05	Crampton Lake	CRAM	20Apr-18May	5Jul-2Aug	13Sep-11Oct
D05	Little Rock Lake	LIRO	20Apr-18May	5Jul-2Aug	13Sep-11Oct
D06	Kings Creek	KING	23Mar-20Apr	3Jul-31Jul	3Oct-31Oct
D06	McDiffett Creek	MCDI	23Mar-20Apr	3Jul-31Jul	3Oct-31Oct
D07	Leconte Creek	LECO	15Mar-12Apr	30Jun-28Jul	12Oct-9Nov
D07	Walker Branch	WALK	9Mar-6Apr	1Jul-29Jul	19Oct-16Nov
D08	Mayfield Creek	MAYF	5Mar-2Apr	29Jun-27Jul	31Oct-28Nov
D08	Black Warrior River	BLWA	19Feb-19Mar	27Jun-25Jul	31Oct-28Nov
D08	Tombigbee River	TOMB	22Feb-22Mar	26Jun-24Jul	2Nov-30Nov
D09	Prairie Lake	PRLA	18Apr-16May	5Jul-2Aug	11Sep-9Oct
D09	Prairie Pothole	PRPO	20Apr-18May	5Jul-2Aug	11Sep-9Oct
D10	Arikaree River	ARIK	21Mar-18Apr	4Jul-1Aug	20Sep-18Oct
D11	Pringle Creek	PRIN	17Feb-17Mar	29Jun-27Jul	23Oct-20Nov
D11	Blue River	BLUE	7Mar-4Apr	1Jul-29Jul	12Oct-9Nov
D12	Blacktail Deer Creek	BLDE	1May-29May	13Jul-10Aug	30Aug-27Sep
D13	Como Creek	COMO	20May-17Jun	14Jul-11Aug	30Aug-27Sep
D13	West St. Louis Creek	WLOU	2May-30May	5Jul-2Aug	3Sep-1Oct
D14	Sycamore Creek	SYCA	12Jan-11Feb	24Mar-23Apr	3Jun-3Jul
D15	Red Butte Creek	REDB	29Mar-26Apr	6Jul-3Aug	29Sep-27Oct
D16	McRae Creek	MCRA	10Apr-8May	11Jul-8Aug	23Sep-21Oct
D16	Martha Creek	MART	6Apr-4May	5Jul-2Aug	22Sep-20Oct
D17	Teakettle 2 Creek	TECR	10Apr-8May	9Jul-6Aug	25Sep-23Oct
D17	Upper Big Creek	BIGC	2Apr-30Apr	9Jul-6Aug	28Sep-26Oct
D18	Oksrukuyik Creek	OKSR	21May-18Jun	29Jun-27Jul	7Aug-4Sep
D18	Toolik Lake	TOOK	19Jun-17Jul	27Jul-24Aug	3Sep-1Oct
D19	Caribou Creek	CARI	2May-30May	26Jun-24Jul	18Aug-15Sep



APPENDIX D SITE-SPECIFIC INFORMATION: HABITAT AND SAMPLER RECOMMENDATIONS

Sampler types should be consistent at a location from year to year.

Domain	Site		Habitat 1 (5 reps)	Habitat 2 (3 reps)
D01	Hop Brook	HOPB	Riffle (Surber)	Pool (corer)
D02	Lewis Run	LEWI	Run (core)	Riffle (Surber)
D02	Posey Creek	POSE	Riffle (Surber)	Pool (Hess)
D03	Flint River	FLNT	Littoral (LWD)	Benthic (petite ponar)
D03	Lake Barco	BARC	Littoral (D-net benthic sweep)	Benthic (petite ponar)
D03	Lake Suggs	SUGG	Littoral (D-net floating sweep)	Benthic (petite ponar)
D04	Rio Guilarte	GUIL	Riffle (Surber)	Pools (Hess)
D04	Rio Cupeyes	CUPE	Riffle (Surber)	Run (Surber)
D05	Crampton Lake	CRAM	Littoral (D-net benthic sweep)	Benthic (petite ponar)
D05	Little Rock Lake	LIRO	Littoral (D-net benthic sweep)	Benthic (petite ponar)
D06	Kings Creek	KING	Riffle/run (Surber)	Pools (corer)
D06	McDiffett Creek	MCDI	Riffle (Surber)	NA
D07	Leconte Creek	LECO	Riffle (Surber)	Pool (corer)
D07	Walker Branch	WALK	Riffle (Surber)	Run (Surber)
D08	Mayfield Creek	MAYF	Riffle/run (snags)	Run (core)
D08	Black Warrior River	BLWA	Littoral (LWD)	Benthic (petite ponar)
D08	Tombigbee River	TOMB	Littoral (LWD)	Benthic (petite ponar)
D09	Prairie Lake	PRLA	Littoral (D-net benthic sweep)	Benthic (petite ponar)
D09	Prairie Pothole	PRPO	Littoral (D-net benthic sweep)	Benthic (petite ponar)
D10	Arikaree River	ARIK	Run (modified kicknet)	Pool (core)
D11	Pringle Creek	PRIN	Run (core)	Riffle (Surber)
D11	Blue River	BLUE	Run (modified kicknet)	Riffle (Surber)
D12	Blacktail Deer Creek	BLDE	Riffle (Surber)	Run (Surber)
D13	Como Creek	COMO	Riffle (Surber)	Run (modified kicknet)
D13	West St. Louis Creek	WLOU	Riffle (Surber)	Pool (core)
D14	Sycamore Creek	SYCA	Run (Surber)	Pool (core)
D15	Red Butte Creek	REDB	Step pool (Hess)	Run (Hess)
D16	McRae Creek	MCRA	Step pool/Riffle (Surber)	Step pool/Pool (Surber)
D16	Martha Creek	MART	Riffle (Surber)	Pool (Hess)
D17	Teakettle 2 Creek	TECR	Riffle-cobble (Surber)	Riffle-bedrock (Surber)
D17	Upper Big Creek	BIGC	Run (Surber)	Riffle (Surber)
D18	Oksrukuyik Creek	OKSR	Run (modified kicknet)	Riffle (Surber)
D18	Toolik Lake	TOOK	Littoral (D-net benthic sweep)	Benthic (petite ponar)
D19	Caribou Creek	CARI	Run (modified kicknet)	Riffle (Surber)



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

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 7. Equipment list – General equipment.

Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
RD[09], RD[15], RD[16]	N	Site-specific Rapid Habitat Assessment or Riparian Sampling Locations	Determining sampling locations	1
CDW-G: 4452963	N	Mobile data entry tablet	Field data entry	1
	N	Cooler, 9-28 qt	Field sample storage; use size appropriate to samples being collected	1
Grainger, W.W. Forestry Suppliers, Inc. Cabela's	N	Waders (hip or chest) or knee boots	Wading	1 pair/ person
	N	Personal flotation device (PFD)	Boating safety in lakes/ rivers	1 per person
Forestry Suppliers, Inc.: 39481 Cabela's Inc.: IK-270217 Recreational Equipment Inc.: 895022	N	Handheld GPS unit (with batteries, ±4 m accuracy) or Humminbird	Navigating to sampling locations in lakes/rivers	1
West Marine Products, Inc.: 15131444	N	Depth finder (see Bathymetry Protocol, RD[17])	Determining depth at sampling location in lakes/rivers	1
Consumable items				
Forestry Suppliers, Inc.: 010510-1 49247	N	General AQU Field Metadata Sheet and field data sheets (all-weather copier paper, write in pencil)	Recording metadata in case tablet fails	1
Forestry Suppliers, Inc.: 010510-1 49247	N	Pre-printed paper labels (all-weather copier paper, write in pencil)	Labeling samples, inside sample jar	1 sheet
Amazon Capital Services Inc.: 7530-01-498-9209	N	Pre-printed 1" x 2 5/8" adhesive labels (write in permanent marker)	Labeling samples, outside sample jar	1 sheet



Item No.	Exact Brand	Description	Purpose	Quantity
	N	Adhesive barcode labels (Type I)	Labeling sample bottles with barcode-readable	1 sheet
	N	Pencils	Recording data	4

Table 8. Equipment list – Macroinvertebrate Samplers.

Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
Forestry Suppliers, Inc.: 227439	N	Surber sampler (243 um Nitex mesh), 0.093 m ²	Sample collection in riffles and runs	1
Forestry Suppliers, Inc.: 227441	N	Hess sampler (243 um Nitex mesh), 0.086 m ²	Sample collection in riffles, runs, shallow pools	1
Forestry Suppliers, Inc.: 223578	N	Petite ponar sampler with rope	Sample collection in soft sediments in lakes/streams	1
	N	Messenger	Used with petite ponar in lakes/streams	1
Forestry Suppliers, Inc.: 223583	N	Hand corer, stainless steel, 20 in	Sample collection in sandy or silty habitats (streams)	1
Forestry Suppliers, Inc.: 227442	N	Modified kicknet sampler (rectangular, 243 um Nitex mesh)	Sample collection in runs (streams) and large woody debris	1
Forestry Suppliers, Inc.: 279992	N	Collapsible quadrat (0.5 x 0.5 m ²)	Used with modified kicknet sampler in streams	1
Forestry Suppliers, Inc.: 425D21	N	D-frame net (243 um Nitex mesh)	Sample collection for woody snags and littoral (lakes/streams)	1
Forestry Suppliers, Inc.: 75080 Home Depot: 643278	N	Bow saw, 21 in or loppers	Used with D-frame snag sampler in streams	1
	N	Work Gloves (pair)	Used with petite ponar for safe handling of ponar rope	1
Forestry Suppliers, Inc.: 71112 Fisher Scientific Company, LLC: S90532C	N	Meter stick, meter tape, or metric ruler	Used with snag and LWD samplers (streams and lakes/streams)	1



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

Item No.	Exact Brand	Description	Purpose	Quantity
Grainger, W.W.: 2RWC7	N	Kitchen brush with nylon bristles	Used with Surber, Hess, snag, and LWD samplers for rock and wood scrubs	2
Grainger, W.W.: 3H382 3H384	N	Deck brush with polypropylene bristles, 60 inch handle	Used with LWD/modified kicknet sampler in rivers	1
Grainger, W.W.: 4YP29	N	Putty knife	Scrape plant material off cobbles in streams	1
Consumable items				
Amazon Capital Services Inc.: B001ECQ4L2	N	Petroleum jelly	Used with hand corer to maintain suction in streams	1
VWR: TWTX3044P	N	Sterile 70% ethanol wipes (e.g., www.soscleanroom.com item TX3044P pre-wetted wipe OR TX3215 dry wipe)	Field-sterilization for gloves for DNA samples	3
	N	ETOH spray bottle (with 95% ETOH)	Field sterilization for gloves for DNA samples	1

Table 9. Equipment list – Macroinvertebrate field elutriation and preservation.

Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
Fisher Scientific Company, LLC: 0340910E	N	Polyethylene wash bottle, unitary (500 mL; Figure 23)	Rinsing sieve, bucket	2
Forestry Suppliers, Inc.: 134576	N	Sieve, 250 um	Sample sieving; elutriation	1
Forestry Suppliers, Inc.: 217009	N	Sieve bucket, 242 um	Sample sieving; elutriation in <u>lakes/rivers</u>	1
Grainger, W.W.: 34A216	N	Plastic bucket, 3 or 5 gallon	Substrate scrubbing (streams), elutriation, collecting waste water on boat (lake/river)	1-2
BioQuip Products Inc.: 4748	N	Flexible forceps, featherweight	Collecting clinging insects	1



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

Item No.	Exact Brand	Description	Purpose	Quantity
Consumable items				
Thomas Scientific, Inc.: 1709C04 (clear) 1185B56 (amber) 1212W94 (clear jug)	N	HDPE bottles (1 L) or jug (4 L) with lids (clear or amber)	Transporting ethanol to the field	1-4
United States Plastic Corp: 66286 66027 66028	N	Sample jars, HDPE with screw-top lid, 6, 16, or 32 oz.	Sample container, field staff choose sizes appropriate for site	11
Amazon Capital Services Inc.: B0051QTSE0	N	Disposable pipets (polyethylene), 3 mL	Collecting clinging insects	2
Fisher Scientific Company: 4355601 Thomas Scientific, Inc.: C954K61	N	Ethanol, 95% non-denatured	Preservative	1-4 L
	N	Latex or Nitrile gloves (pair)	Preventing preservative contact with skin	5
Grainger, W.W.: 5LH30	N	Resealable plastic zip-top bags (gallon)	Organizing samples	5

Table 10. Equipment list – Laboratory processing: preservative replenishment.

Item No.	Exact Brand	Description	Purpose	Quantity
Durable items				
Fisher Scientific Company, LLC: 0340910E	N	Unitary wash bottle, 500 mL, ETOH + glycerol	Adding preservative to sample jars and rinsing filter cup	1
Forestry Suppliers, Inc.: 134576	N	Sieve, 250 um	Changing preservative	1
Fisher Scientific Company, LLC: 033134E Amazon Capital Services Inc.: 24-C44	N	Filter cup (500 mL HDPE bottle and 243 um Nitex mesh square, see TOS beetle protocol)	Changing preservative	1



Title: AOS Protocol and Procedure: INV - Aquatic Macroinvertebrate Sampling		Date: 03/16/2022
NEON Doc. #: NEON.DOC.003046	Author: S. Parker	Revision: F

Item No.	Exact Brand	Description	Purpose	Quantity
BioQuip Products Inc.: 4748	N	Featherweight forceps	Picking up insects	1
	N	Safety glasses	Preventing preservative contact with eyes	1 pair
Consumable items				
Fisher Scientific Company: 4355601 Thomas Scientific, Inc.: C954K61	N	Ethanol, 95%, undenatured	Preservative	5 L
Fisher Scientific Company: g331	N	Glycerol, >99%	Preservative	1 L
Amazon Capital Services Inc.: 24-C44	N	243 um Nitex mesh cloth, cut into squares for filter cup	Catching insects over the filter cup	8
	N	Nitrile gloves	Preventing preservative contact with skin	1 pair