**Oak Ridge National Laboratory/Environmental Sciences Division**
**Benthic Macroinvertebrate Sample Processing Statement of Work**
**March 2, 2023**

## I. INTRODUCTION

Staff of the Oak Ridge National Laboratory's (ORNL's) Environmental Sciences Division (ESD) monitors the benthic macroinvertebrate communities in streams on the Department of Energy's (DOE's) Oak Ridge Reservation (ORR) in Oak Ridge, Tennessee, as well as, several nearby reference streams. Some streams have been monitored since the mid-1980's to help meet requirements mandated by National Pollutant Discharge Elimination System (NPDES) permits issued to DOE's Oak Ridge facilities or specific DOE orders, thus, continuity of sample processing procedures is of utmost importance to ensure comparability with historical data. Some samples come from locations that are either from sites known to be contaminated with low levels of isotopes of uranium and radionuclides, and sites that are suspected to be contaminated. Specifically, samples are collected from some locations that may be contaminated with isotopes of cobalt, cesium, and strontium (e.g., maximum concentrations: Co-60, <1,050 pCi/g sediment; Cs-137, <3,600 pCi/g sediment; and Sr-90, <120 pCi/g sediment), or low levels of uranium isotopes (e.g., maximum concentrations: total uranium <200 pCi/g sediment, <1% U-234, ~60 pCi/g sediment U-238 and ~4 pCi/g sediment U-235).

Unprocessed samples consist of a mixture of sediment, twigs, detritus, benthic macroinvertebrates, etc. that is preserved in ~95% denatured ethanol. On average, each sample contains ~250 grams of solids (i.e., invertebrates, sediment, and other incidental sample debris) and ~200 mL of ethanol. This Statement of Work specifically covers processing of samples collected ESD/ORNL staff.

## II. RESPONSIBILITIES/REPORTING

All benthic macroinvertebrate samples are collected, controlled, and maintained by ESD/ORNL staff until delivered for processing. The Seller will process these samples at their laboratory. Processing shall include (1) separation and removal of organisms from the sediment and detritus, and (2) identification and enumeration of the organisms per ESD/ORNL or the most recent Tennessee Department of Environment and Conservation (TDEC) protocols (TDEC 2021). A tentative estimate of samples to be processed in the first year of this contract, and the specific sample processing protocols that must be followed are provided in Table 1. It is anticipated that the number of samples expected in the remaining years of this subcontract will be similar, but because the actual number will fluctuate annually due to sponsor needs, a new list of processing needs will be provided at the beginning of each new fiscal year (i.e., October through September) covered under this subcontract. The Seller is responsible for providing all incidental supplies and equipment necessary to complete the obligations covered under this subcontract, such as vials, alcohol, taxonomic keys, sorting trays, illuminated magnifying lamps, microscopes, etc.

The Seller is responsible for obtaining and maintaining a Tennessee Radioactive Material License to handle nuclides of elements 1-96 and special nuclear materials at their off-site laboratory. The Seller shall comply with all applicable State and Federal guidelines and procedures for handling contaminated samples and hazardous materials (i.e., ethanol), and disposal of any resulting wastes (i.e., contaminated and uncontaminated liquid or solid wastes). The Seller shall be responsible for all matters pertaining to safety and health protection of their employees and the environment, including but not limited to providing all personal protective equipment and any training that is necessary for ensuring safe handling and disposal of hazardous and potentially radioactive materials (e.g., Radiological Worker training).

The Seller is responsible for creating and maintaining project-specific reference collections (see further details below in **Section VI** and project break-down in Table 1).

The Seller must submit a brief progress report within 10 days of the end of each month. The report should primarily focus on two tabular summaries: 1) an updated listing (Microsoft Excel) of the fiscal year-to-date samples received (identified by site, sample collection date, sample replicate number), sorted, identified and enumerated, and the results of quality assurance/quality control checks on sorting efficiency and taxonomic identification (**see Section VI**); 2) a summary of invoice tracking (Microsoft Excel) including fiscal year-to-date invoice numbers and corresponding breakdown of contaminated or non-contaminated samples according to different projects (i.e., watersheds). A brief description of any pertinent related activities and issues should also be included as needed.

Monthly invoices should be submitted that summarize work completed including samples sorted, identified and enumerated, and associated quality assurance/quality control checks. The Seller and ORNL will hold a business meeting every quarter to communicate progress, milestones, delivery of products, and ensure appropriate invoicing and tracking.

The DOE requires that UT-Battelle accrue monthly estimates of subcontract costs and requests that the Seller's input be the basis for the accrual. The Seller shall prepare a time-phased monthly cost estimate plan for the period of performance and include it with the subcontract proposal; any revisions must be completed within fifteen (15) days of the subcontract award. The plan is to be updated fifteen (15) days after receipt of any modification increasing or decreasing the estimated cost of the subcontract or as deemed appropriate. It is preferred that the plan be provided via electronic mail to the Project Manager (PM) at the following address: griffithsna@ornl.gov; however, it is acceptable for the plan to be provided by mail to the following address: Natalie A. Griffiths, ORNL, 1 Bethel Valley Road, Bldg. 1504, MS 6351, Oak Ridge, Tennessee 37831-6351.

## III. CHAIN-OF-CUSTODY

Chain-of-custody (COC) is maintained on all samples from their collection through final disposal. The following steps shall be taken by the Seller to ensure that COC is maintained and documented:

A. Whenever custody of samples is transferred, ESD/ORNL and the Seller's staff involved in the transfer will sign and date COC forms accompanying the samples.
B. Original COC forms shall be maintained with the samples, and a copy of signed and dated forms provided to the ESD/ORNL PM after delivery. When processed samples are returned to ESD/ORNL, the original signed and dated forms are returned as well.

## IV. SAMPLE DELIVERY

A tentative list of samples (broken down by project and deliverable number) to be processed during the first year of this subcontract is given in Table 1; this list is subject to change due to changes in funding and the actual start-date of the subcontract. Samples will be packaged and delivered by ESD/ORNL. The Buyer will make sure that each individual sample container is clearly marked with appropriate sample background information including site name, sample replicate number, collection date, COC number, and required and appropriate hazard warning labels. Each shipment will also include a radiological clearance

certification form as required for shipments from ORNL.

Receipt and transfer of samples will be documented by signature and transfer date on the accompanying COC forms: Any discrepancies or problems concerning samples must be resolved as soon as possible after the exchanges. The Seller shall keep each original COC form, and a copy shall be provided to the ESD/ORNL PM or representative.

## V. SAMPLE ANALYSIS PROCEDURES

Two types of benthic macroinvertebrate samples are routinely collected: quantitative collected following ESD/ORNL protocols and semi-quantitative collected following the most current TDEC (e.g., 2021) protocols. Each sample type shall be processed following different and specific procedures. Quantitative samples collected with ESD/ORNL protocols shall be processed following Standard Operating Procedures (SOPs) developed by ORNL staff specifically for these samples (MCS-SOP-11 and MCS-SOP-12 in Appendix A). Semi-quantitative samples collected with TDEC protocols shall be processed per the most current TDEC sample processing protocols (e.g., TDEC 2021 at the time of this Statement of Work): there shall be no deviations from the prescribed TDEC procedures.

Samples processed via ESD/ORNL protocols shall include (1) removal of **all** macroinvertebrates from the entire sample (i.e., no subsampling) and (2) identification and enumeration of all the removed organisms Most organisms shall be identified to the lowest practical taxon (i.e., genus); exceptions to this are included in MCS-SOP-12 (Appendix A). Requests for deviations from these guidelines must be submitted in writing and approved by the ESD/ORNL PM before implementation.

TDEC protocols use a random sorting procedure to obtain a subsample of 160 – 240 organisms for enumeration and identification. The required level of identification for TDEC samples is detailed in their most current protocols (TDEC 2021).

Identifications of organisms for both procedures should be made with the most up to date standard taxonomic keys available (e.g., Merritt et al. 2019, Thorp and Rogers 2016), but available region- or taxon-specific taxonomic keys may also be useful for identifying some taxa.

For samples processed following TDEC protocols, all sorted specimens shall be stored in one vial unless the presence of large specimens requires use of additional vials; slide mounts must be stored vertically in an appropriate slide box. Details of final storage for specimens from sample processed via ESD/ORNL protocols are provided in MCS-SOP-12 (Appendix A). For all samples, vials and slides shall be appropriately identified on a label with site name, date of collection, sample replicate number, and COC number (this information is normally on the original label). A final preservative consisting of 80% ethanol and 5% glycerin shall be used. For vials with screw-top lids, a piece of Parafilm should be placed between the cap and the vial after processing is complete. The unprocessed portion of samples processed following TDEC protocols shall be preserved with 95% ethanol before they are returned to the ESD/ORNL PM in their original containers with the original labels.

## VI. QUALITY ASSURANCE/QUALITY CONTROL

The integrity of every sample must be maintained from collection to final disposal. Samples are accompanied with COC records, and every exchange of a sample must be documented on these records. Regular quality control checks of sorting efficiency and accuracy of taxonomic identification must be

performed by the Seller; suitable methods are described in the attached ESD/ORNL protocols (Appendix A) and in the most current TDEC (2021) protocols. Any requested deviations from QA/QC procedures by the Seller must be submitted in writing to, and approved by, the ESD/ORNL PM before implementation.

The Seller is responsible for creating and updating project-specific reference collections. These reference collections shall consist of representatives from each distinct taxon for which the identification of has been verified by an appropriate taxonomic expert as needed. The source of each specimen in the reference collection must be adequately documented on the label inside each specimen vial (i.e., all information on original sample label and the taxonomic name). A list of the taxa and specimens in the reference collection (along with pertinent sample identification) shall be maintained in a Microsoft Excel spreadsheet. Identifications of organisms in the reference collection from contaminated sites will not require verification.

## VII. DELIVERABLES

Sample results must be submitted electronically to the ESD/ORNL PM in a Microsoft Excel or comma-delimited file either via electronic mail or compact disk by agreed upon deadlines. For data entry of samples processed with ESD/ORNL protocols, each distinct taxon in each replicate sample represents an observation, and the following information shall be included in separate fields of each observation in the following order: site name, collection date, sample replicate number, "genus" (i.e., taxon name for lowest level of identification), species (only if there's an entry for specimens identified to species, otherwise leave the field blank), and number of specimens in sample. An example of the format for entering data for samples processed with TDEC protocols will be provided after the first such samples are delivered for processing. All character entries must be uppercase. Copies of laboratory bench sheets completed by the Seller for each sample must also be provided with submittal of electronic data. A tentative list of projected delivery dates for samples processed in the first year of this subcontract is provided in Table 1.

All processed (invertebrates only) and partially processed (i.e., unprocessed portion of samples processed via TDEC protocols) samples shall be returned to ESD/ORNL. To avoid a backlog in the delivery of these samples, they should be returned on a regular basis. This exchange must include documentation on the original chain of custody form that accompanied the samples with their delivery to the laboratory. The completed reference collections must be submitted to the ESD/ORNL PM at the end of the subcontract period.

## VIII. REFERENCES

Merritt, R.W., K.W. Cummins, and M.B Berg (eds). 2019. An Introduction to the Aquatic Insects of North America. 5h ed. Kendall Hunt, Dubuque, IA.

TDEC (Tennessee Department of Environment and Conservation). 2021. Quality system standard operating procedure for macroinvertebrate stream surveys. Tennessee Department of Environment and Conservation, Division of Water Resources, Nashville, TN. https://www.tn.gov/content/dam/tn/environment/water/policy-and-guidance/DWR-PAS-P-01-Quality_System_SOP_for_Macroinvertebrate_Stream_Surveys-122821.pdf

Thorp, J. H., and D.C. Rogers (eds). 2016. Thorp and Covich's Freshwater Invertebrates: Ecology and General Biology. Volume 1. 4th ed. Academic Press, Inc., New York, NY.

**Table 1. Benthic macroinvertebrate samples to be processed in Q3-4 FY2023 and Q1-2 FY2024.**

| Project (deliverable no.) | Collection date | Tentative delivery date[a] | Number of contaminated samples | Number of uncontaminated samples | Processing protocols[b] | Tentative processing deadline[a] |
|---|---|---|---|---|---|---|
| **A. WRRP (Bear Creek)** | | | | | | |
| Bear Creek | | | | | | |
| (1) | Apr-23 | 04/30/23 | 12 | 9 | ESD/ORNL | 08/30/23 |
| (2) | Aug-23 | 08/30/23 | 4 | 0 | TDEC | 11/30/23 |
| (3) | Oct-23 | 10/30/23 | 12 | 9 | ESD/ORNL | 01/30/24 |
| **Sub-total** | | | **28** | **18** | | |
| McCoy Branch | | | | | | |
| (4) | Apr-23 | 04/30/23 | 6 | 0 | ESD/ORNL | 08/30/23 |
| (5) | Aug-23 | 08/30/23 | 2 | 0 | TDEC | 11/30/23 |
| (6) | Oct-23 | 10/30/23 | 6 | 0 | ESD/ORNL | 01/30/24 |
| **Sub-total** | | | **14** | **0** | | |
| **B. WRRP (Melton Branch)** | | | | | | |
| Melton Branch (MEK 0.6) | | | | | | |
| (7) | Apr-23 | 04/30/23 | 3 | 0 | ESD/ORNL | 08/30/23 |
| (8) | Aug-23 | 08/30/23 | 1 | 0 | TDEC | 11/30/23 |
| **Sub-total** | | | **4** | **0** | | |
| **C. ORNL BMAP** | | | | | | |
| (9) | Apr-23 | 04/30/23 | 12 | 12 | ESD/ORNL | 08/30/23 |
| (10) | Aug-23 | 08/30/23 | 4 | 1 | TDEC | 11/30/23 |
| **Sub-total** | | | **16** | **13** | | |
| **D. ETTP BMAP** | | | | | | |
| (11) | Apr-23 | 04/30/23 | 9 | 3 | ESD/ORNL | 08/30/23 |
| (12) | Aug-23 | 08/30/23 | 3 | 1 | TDEC | 11/30/23 |
| **Sub-total** | | | **12** | **4** | | |
| **E. Y-12 BMAP** | | | | | | |
| (13) | Apr-23 | 04/30/23 | 12 | 8 | ESD/ORNL | 08/30/23 |
| (14) | Aug-23 | 08/30/23 | 3 | 2 | TDEC | 11/30/23 |
| (15) | Oct-23 | 10/30/23 | 12 | 8 | ESD/ORNL | 01/30/24 |
| **Sub-total** | | | **27** | **18** | | |
| **Totals** | | | **101** | **53** | | |

[a]Delivery and deadline dates may be adjusted to reflect the actual contract start date and to adjust for possible changes in schedule priority.

[b]ESD/ORNL = ESD/ORNL processing protocols MCS-SOP-11 and MCS-SOP-12 (See Appendix A of Statement of Work); TDEC = Tennessee Department of Environment and Conservation (TDEC) 2021 protocols (https://www.tn.gov/content/dam/tn/environment/water/policy-and-guidance/DWR-PAS-P-01-Quality_System_SOP_for_Macroinvertebrate_Stream_Surveys-122821.pdf).

**APPENDIX A**

**ENVIRONMENTAL SCIENCES DIVISION/OAK RIDGE NATIONAL LABORATORY**
**BIOLOGICAL MONITORING AND ABATEMENT PROGRAM**
**STANDARD OPERATING PROCEDURES:**
**MCS-SOP-11 AND MCS-SOP-12**

# *Aquatic Ecology Group (AEG) Standard Operating Procedures*

**Benthic Macroinvertebrate Community Studies**
**QAP-ESD-03**

**MCS-SOP-11**

**Revision 4 - Date: 01/16/17**

**Subject: Laboratory Procedures for Sorting Quantitative Benthic Macroinvertebrate Samples**

**This document is available on the ESD Internal Web as a controlled document. If a copy is made for use offline, the user should check the revision number on the web site to verify this SOP is the most current revision.**

## 11.1 Purpose/Scope

There are many acceptable procedures for sorting benthic macroinvertebrate samples (e.g., Barbour et al. 1999, Klemm et al. 1990); the procedure selected usually depends upon project requirements, objectives, and goals. The sorting procedures covered in this SOP include only those for samples that have been collected using a quantitative technique such as those described in SOP-5. If a project requires the use of procedures not covered in this SOP, this SOP must either be modified to include the new steps, the sponsor's or regulatory agency's (e.g., TDEC 2011) procedures be adopted copies of their current procedures maintained, or new a procedure must be prepared.

Sorting macroinvertebrate samples must be performed by trained personnel. Therefore, this procedure is not intended for use without proper training, as determined by the Principal Investigator.

## 11.2 Equipment

5-gal Aluminum can with lid
Laboratory fume hood
2-gal Plastic bucket
8-in Brass U.S. Standard Sieves, No. 50 (297 Φm mesh) and No. 60 (250 Φm mesh)
Metal tripod large enough to fit inside a 2-gal bucket and support an 8-in diameter brass sieve
8" X 10" White plastic trays, e.g., photo trays (2 or more)
Blotter paper or other absorbent paper for wicking water
Disposable nitrile laboratory gloves per RSS 661
Denatured ethanol (95%)
Tap water
Fine-tipped forceps (surgical grade)
Small, metal spatula, scoop, or spoon (.1 teaspoon capacity)
Illuminated desk magnifier (~1.75X magnification)
Glass screw cap vials or patent lip vials with neoprene stoppers (∃20 ml or ≥2 dram)
Inner sample labels made of 100% rag paper or other water resistant paper
No. 2 lead pencil, ink pen with indelible ink, or ink jet printer for making labels
Fine-tipped permanent marking pen (black or blue)
Vial holder or rack
Alpha and/or beta-gamma meter (if processing samples from sites that require setting up a radiological area)

Benthic Macroinvertebrate Laboratory Chain-of-Custody and Log Sheet (Exhibit 12-1, SOP-12)
Small, plastic container with 12 slips of paper (2 cm x 2 cm), 6 labeled "yes" and 6 labeled "no", or 10 slips of paper numbered consecutively from 1 to 10

**11.3 References**

Barbour MT, Gerritsen J, Snyder BD, Stribling JB (1999) Rapid bioassessment protocols for use in wadeable streams and rivers. EPA 841-B-99-002. U. S. Environmental Protection Agency, Office of Water, Washington, DC. http://www.epa.gov/OWOW/monitoring/rbp/

Klemm, D. J., P. A. Lewis, F. Fulk, and J. M. Lazorchak. 1990. Macroinvertebrate field and laboratory methods for evaluating the biological integrity of surface waters. EPA/600/4-90/030. U.S. Environmental Protection Agency, Washington, DC.
http://www.epa.gov/bioindicators/html/benthos_methods.html

TDEC (Tennessee Department of Environment and Conservation). 2011. Quality system standard operating procedures for macroinvertebrate stream surveys. July 2011 Revision. Division of Water Pollution Control, Nashville, TN.
https://www.tn.gov/assets/entities/environment/attachments/bugsop11.pdf

**11.4Procedures**

**11.4.1** Don required personal protective equipment (PPE) as defined by the project's Research Safety Summary (RSS). If samples are from sites known or suspected of being contaminated by radionuclides, a Radiological Work Permit (RWP) must be obtained from Radiation Protection personnel. Follow the requirements in the RWP regarding PPE, boundary demarcation for a radiological area, and accumulation of waste.

**11.4.2** Select a sample and document its selection on a Benthic Macroinvertebrate Laboratory Chain-of Custody and Log Sheet or similar record (see Exhibit 12-1, SOP-12).

**11.4.3** Rinse the ethanol from the sample material, and separate the lighter materials in the sample (e.g., organisms, leaves, twigs, algae, etc.) from the heavier materials (e.g., gravels) following steps **11.4.3.1** - **11.4.3.3**. When samples are suspected or known to be contaminated, these steps should be completed in a laboratory fume hood. If the sample is not contaminated, these steps may be completed on a laboratory bench top.

**11.4.3.1** Place an 8-in, No. 60 sieve on a tripod in a 2-gal bucket. Grab and firmly hold the jar with the sample and swirl the jar vigorously to suspend the lighter materials. Before the suspended material settles, remove the lid from the jar and carefully pour the supernatant through the sieve into the bucket.

**11.4.3.2** Refill the sample jar with ~250 mL of tap water, replace the lid securely, and swirl the jar vigorously to suspend any remaining lighter materials. Before the suspended material settles, remove the lid and pour the supernatant through the sieve into the bucket.

**11.4.3.3** Repeat step **11.4.3.2** a minimum of four times. With guidance from the Waste Management representative, determine if the ethanol concentration of resulting rinsate is low enough (and radionuclides if from a contaminated location) to allow disposal down a process drain. If acceptable, pour the rinsate down the drain, but if not, accumulate the waste per current Waste

Management guidelines.

**11.4.4** Most of the organisms and plant materials (e.g., leaves, twigs, algae) from the sample will end up in the sieve, while most of the heavier materials (primarily gravels) will remain in the sample jar. The material in the jar can remain there until processed. Place the sieve containing the unprocessed sample material into a separate 8" x 11" tray and partially fill it with tap water to keep the material damp. Add enough tap water to the material remaining in the jar to keep it damp as well, and replace the lid.

**11.4.5** Quality assurance checks of sorting efficiency must be performed on an ongoing basis. Several methods exist for incorporating quality control into the sorting process. BMAP samples may be checked with one of the two following methods: (1) 50% pan-check and (2) the 10% sample-check. The 10% sample-check method is preferred, but if samples for a project have been checked historically with the 50% pan-check method, then that is the preferred method.

**11.4.6** If using the 10% sample check method, fill a least two vials approximately half full of 95% ethanol, and if using the 50% pan-check method, at least three vials of ethanol are needed. Midges (i.e., Chironomidae) should be placed in a separate vial from the other taxa for both methods. Insert a water resistant label into each vial that includes the site name, date, replicate number, and the taxonomic group contained in the vial (e.g., "midges", "other taxa"). If using the 50% pan-check method a third vial labeled "check vial" also will be needed. If more than one vial is required for one of the groups, make sure that sequential numbering is also marked on the label and lid of the vial. For example, if two vials are required for the "other taxa", the first would be marked "1 of 2" and the second would be marked "2 of 2". Labeling information can be applied to the label paper either with a #2 pencil, an ink pen with black India ink, or an ink jet printer; make sure the ink from a pen or printer is dry before placing labels in ethanol.

**11.4.7** To aid in keep track with scanning location and progress while sorting a sample, mark the bottom of an 8.5" x 11" white photographic tray (i.e., sorting tray) with three or more equally spaced horizontal or vertical lines with a permanent marking pen (blue or black ink).

**11.4.8** Using a rigid tool such as a spatula, small spoon, or small scoop, remove a small aliquot of sample material (. 1 teaspoon) from the sieve and place it into a sorting tray. Depending on the type of debris in the sample, more material (and sometimes less) may be added, but the quantity scanned at any one time should not exceed one tablespoon. Pour enough tap water into the tray to cover the sample material with ~ ¼ inch of water, and then gently rock the tray to evenly disperse the material.

**11.4.9** Systematically scan the material in the entire sorting tray at least twice while using an illuminated magnifying lamp; samples with a lot of organisms may require more than two scans. Between each scan, gently rock the tray to redistribute the material. Carefully remove all invertebrates with fine-tipped forceps (surgical grade forceps are less likely to damage specimens), and place them in appropriately labeled vials (e.g., "midges", "other taxa"). When uncertain if a piece of debris is an invertebrate, place it in a vial for later determination under a microscope. This is especially important for less experienced sorters. Place vials in a rack or similar holder to keep from spilling their contents while processing a sample.

**11.4.10** If using the 50% pan-check method to assess sorting efficiency, follow steps **11.4.11 – 11.4.18**; if using the 10% sample-check method, follow steps **11.4.19 – 11.4.22**.

**11.4.11** If sorting following the 50% pan-check method, large pieces of sample debris from

uncontaminated samples may be placed in the trash. If there is a large quantity of debris, it is best to first place it on some type of absorbent material (e.g., blotter paper or paper towel) to allow some of the moisture to be wicked away before disposal in a sanitary trash container. Debris from contaminated samples should be accumulated in a plastic storage bag and placed partially open under a fume hood to allow the material to air dry.

**11.4.12** If after the rinsing steps (steps **11.4.3** - **11.4.4**) there appears to be more than two aliquots of sample material in the sieve to sort, then approximately 50% of the trays must be resorted by another individual. Proceed to step **11.4.14** to complete processing of a sample with > 2 aliquots to sort**.**

**11.4.13** If two or fewer trays are likely, all trays must be resorted by another individual following step **11.4.9**. Place any observed organisms into the "check vial." After completing the resort, pour the contents of the pan into a No. 50 sieve (295 µm) resting in the sink if the sample is not contaminated. If the sample is contaminated, and it has been determined that it cannot be disposed of in a process drain, the material should be poured into a sieve resting on a tripod placed in a bucket in the fume hood.

**11.4.14** The 50% pan-check method incorporates a simple technique to randomly select 50% of the sample aliquots for resorting. Place six labels marked "yes" and six marked "no" into a small plastic container and thoroughly shake it. Without looking, remove one slip of paper. If the slip of paper is marked with a "yes", have another individual resort the tray of material following step **11.4.9** above. If the slip of paper is marked with a "no", the tray of material is not resorted and its contents are poured into a process drain through a No. 50 sieve. Return the slip of paper to the cup.

**11.4.15** While resorting, remove all organisms encountered and place them into the vial labeled "check vial". After an aliquot has been resorted, pour the material into the sieve in the sink and place the lid on the "check vial". Keep a record of the number of aliquots resorted and not resorted on a Benthic Macroinvertebrate Laboratory Chain-of-Custody and Log Sheet after completing a sample (see Exhibit 12–1 in SOP-12).

**11.4.16** Repeat steps **11.4.8** - **11.4.16** until all sample material in the sieve has been processed.

**11.4.17** After all of the sample material in the sieve has been processed, sort small aliquots (.1 to 2 teaspoons) of the material remaining in the sample jar following step **11.4.9**. Trays from this portion of the sample are not included in the checks of sorting efficiency, therefore, after sorting each aliquot, pour the water and debris into the sieve over the process drain.

**11.4.18** To complete a check of sorting efficiency following the 50% pan-check method, the organisms placed in the "check vial" from resorted aliquots must be identified and enumerated following procedures in SOP-12. If the number of organisms in the "check vial" exceeds the combined total number of organisms in the vials containing "other taxa" and "midges" by >10%, then 100% of the trays sorted by the deficient sorter must be checked until their sorting efficiency consistently exceeds 90% (i.e., the number of organisms in the "check vial" must be ≤ 10% of the number initially sorted).

**11.4.19** If following the 10% sample-check method, sort invertebrates from the entire sample (i.e., from the sieve and jar) following steps **11.4.8** and **11.4.9,** but retain the initial ethanol preservative poured from the sample during step **11.4.3.1** and collect all extraneous debris associated with the sample in a **250 µm sieve** (No. 60). Before rinsing the sample following steps **11.4.3.2** and

**11.4.3.3**, pour the ethanol from the bucket back into the original sampling jar. Collect all extraneous sample debris in the sieve in the sink (or a sieve on a tripod in a bucket if the sample is contaminated), and then return the debris from the sieve to the jar with the original ethanol. Make sure that the original sample label is also replaced in the jar.

**11.4.20** Repeat step **11.4.8** and **11.4.9** until the entire sample has been processed.

**11.4.21** After an individual has sorted 10 samples and the invertebrates have been identified and enumerated (SOP-12), number the jars with the samples that have been sorted consecutively from 1 to 10, with a pencil or marking pen on the jar's lid. Place the slips of paper labeled consecutively 1 through 10 into a small plastic cup, shake vigorously, and withdraw one slip of paper without looking. The sample having the same number as the slip of paper should then be resorted in its entirety following steps **11.4.3 – 11.4.4** and **11.4.6-11.4.9**, except all of the liquid and sample debris may now be disposed of.

**11.4.22** The total number of organisms removed from the resorted sample must not exceed 10% of the total number from the first sort. If ≤10%, then it and the remaining nine samples that were processed may be disposed of. If the number found in the resorted samples is >10% of the number found in the first sort, then another of the sorted samples must be randomly selected and resorted. If the number organisms found during resort of the second sample is ≤10%, then it and the remaining eight processed samples may be disposed of. If the second resorted sample exceeds 10%, then all eight of the remaining samples must be resorted.

**11.4.23** After a sample has been completely sorted, fill each vial at least 3/4 full of 95% denatured ethanol, tightly close the vials, and place them in a secure flammable liquid storage cabinet. The 95% ethanol is needed to compensate for any excess dilution from water added incidentally while transferring organisms from the tray to the vial. If the sample was from a contaminated site, scan the specimen vials for external contamination using an alpha and/or beta-gamma meter before placing them in the cabinet. If contamination is found, contact RADCON. Enter the number of vials for each sample in the appropriate column of the Benthic Macroinvertebrate Laboratory Chain-of-Custody and Log Sheet (Exhibit 12-1).

**11.4.24** Allow the waste debris from the sample (i.e., gravel, sand, and detritus) to air dry. If the material is from an uncontaminated location, it can dry on top of a nearby laboratory bench, and then it can be disposed of in a sanitary waste container. If the debris is from site know or suspected of being contaminated with radionuclides, allow it to air dry in a fume hood by leaving the plastic storage bag open for 24 or more hours. To facilitate descriptions of waste, keep contaminated materials separated by project. Once a bag is full or another project begins, seal the used bag and start filling a new bag; the presence of the original sample labels with the waste sediment will allow the bags to be identified at a later date by project or stream if necessary. After the contents in a bag are dry, place the bag into a covered, 5-gal aluminum storage can that is lined with a heavy plastic bag. See Radiation Protection for the correct labeling of the storage container.

**11.4.25** Contact the Waste Management representative for proper disposal of all liquid and solid wastes that could not be disposed of in the process drain or sanitary waste container.

**Review**

This procedure has been approved by the QA Coordinator prior to revision/or issuance.
Reviewed by: M. K. McCracken, BMAP Quality Assurance Coordinator
Signature date: 01/16/17

**Approval**

The revision and/or issuance of this standard operating procedure must receive the signed approval of the Principal Investigator.
Approved by: Ryan A. McManamay, Principal Investigator
Approval date: 01/16/17

Approval signature on file.

Questions, comments or suggestions concerning this procedure should be directed to the Principal Investigated listed above.

# *Aquatic Ecology Group (AEG)*
# *Standard Operating Procedures*

**Benthic Macroinvertebrate Community Studies**
**QAP-ESD-03**

**MCS-SOP-12**

**Revision 3 - Date: 01/16/17**

**Subject: Identifying and Enumerating Macroinvertebrates**

**This document is available on the ESD Internal Web as a controlled document. If a copy is made for use offline, the user should check the revision number on the web site to verify this SOP is the most current revision.**

## 12.1 Purpose/Scope

The following procedure covers the steps for identifying and enumerating benthic macroinvertebrates collected with quantitative sampling techniques such as those described in MCS-SOP-5 and sorted with the procedures in MCS-SOP-11. These procedures may easily be adapted to most other sampling and processing techniques by simply changing the level of taxonomic identification. However, since this SOP requires sorting a sample in its entirety, it may be necessary to modify these procedures if techniques are used that affect enumeration (e.g., subsampling). If a project requires specific procedures not fully covered in this SOP, then those procedures shall be followed (e.g., TDEC 2011), and the Principal Investigator must obtain and retain the most up-to-date version of those procedures.

The identification of macroinvertebrates is a highly specialized skill that must be performed by properly trained and qualified personnel. Therefore, this procedure is not intended for use without such proper training and qualifications, as determined by the Principal Investigator.

## 12.2 Equipment

Dissecting microscope with magnification of at least 80X
Compound microscope with magnification of at least 400X
Microscope slides (3" X 1")
Microscope cover slides (12-mm, round, no. 1 thickness)
Small glass stirring rod
CMC-10 mounting media
Denatured ethanol solution (80%)
Solution of 80%/5% denatured ethanol/glycerin
Fine-tipped forceps (surgical grade)
Probe with flexible, fine point (e.g., insect mounting pin inserted point out in a small round stick)
Plastic or glass petri dishes (sizes 60 mm X 15 mm to 100 mm X 15 mm)
Plastic, multi-cell trays
Assortment of up-to-date macroinvertebrate identification keys
Multiple-tally denominator
Benthic Macroinvertebrate Laboratory Chain-of-Custody and Log Sheet (Exhibit 12-1)
Macroinvertebrate Identification and Sorting Guidelines (Exhibit 12-2)
Benthic Macroinvertebrate Laboratory Bench Sheet (Exhibit 12-3)
Inner sample labels made of 100% rag paper or other water-resistant paper

No. 2 Lead pencil, pen with indelible ink (black or blue), or ink jet printer (for making inner sample labels)
Parafilm cut in squares of .2" X 2"

### 12.3 References

TDEC (Tennessee Department of Environment and Conservation). 2011. Quality system standard operating procedures for macroinvertebrate stream surveys. July 2011 Revision. Division of Water Pollution Control, Nashville, TN.
https://www.tn.gov/assets/entities/environment/attachments/bugsop11.pdf

There are many books and other publications that provide updated and current keys that are taxon-specific that should be used when possible to ensure that the most current names are used for all taxa. Two excellent books for identifying aquatic insects and other aquatic invertebrates should also be used including the following:

Merritt, R.W., K.W. Cummins, and M.B Berg (eds). 2008. An Introduction to the Aquatic Insects of North America. 4th ed. Kendall Hunt, Dubuque, IA.

Thorp, J. H., and D. C. Rogers (eds). 2016. Thorp and Covich's Freshwater Invertebrates: Ecology and General Biology. *Volume 1 of Thorp and Covich's Freshwater Invertebrates*. Fourth Edition. Academic Press, Inc., New York.

For keys to the Chironomidae, the following may be useful:

Epler, J. H. 2001. Identification manual for the larval Chironomidae (Diptera) of North and South Carolina. A guide to the taxonomy of the midges of the southeastern United States, including Florida. St. Johns Water Management District Special Publication SJ2001-SP13. (consult the internet for the most recent version and updates).

### 12.4 Procedures

**12.4.1** Select a sorted sample for identification and document its selection by entering the current date in the sample's entry on a Benthic Macroinvertebrate Laboratory Chain-of-Custody and Log Sheet or similar record (see Exhibit 12-1). If the sample was sorted with the 50% pan-check method (MCS-SOP-11), it will have at least three vials: one each labeled "other taxa", "midges", and "check-vial". If a sample is sorted with the 10% sample-check method (MCS-SOP-11) it will have at least two vials: one each labeled "other taxa" and "midges." For both sorting procedures there may be additional vials for samples with large numbers of organisms or large numbers of snails or clams/mussels.

**12.4.2** Identify specimens with the most up-to-date taxonomic keys. Most macroinvertebrates can be identified with generalized keys, such as Merritt et al. (2008) and Thorp and Rogers (2016), but several other excellent taxon-specific or regional faunal keys are also available.

**12.4.3** Follow the general guidelines given in Exhibit 12-2, Tables 12-2a and 12-2b for the level of identification. In most cases identification will be to genus, but several groups are identified to higher taxonomic levels. Table 12-2a also lists taxonomic groups that should not be included in identification and enumeration because they are either not benthic or they are normally classified as microinvertebrates, thus, estimates of their presence/absence and/or abundance are less likely to be reliable if collected and sorted following protocols in MCS-SOP-5 and MCS-SOP-11.

Deviation from these guidelines must be approved and a record of such modifications maintained by the Principal Investigator.

**12.4.4** Pour the contents of the vial containing "other taxa" into a petri dish and place the dish on the base of the dissecting microscope. Handling of organisms is facilitated with the use of fine-tipped forceps (surgical grade) and a probe with a flexible, fine tip. Count the organisms by taxon as they are identified; devices such as a multiple-tally denominator will facilitate this process. Enter taxa names and their final numbers in the appropriate spaces on a Benthic Macroinvertebrate Laboratory Bench Sheet such as that shown in Exhibit 12-3. If the organisms will not be weighed (MCS-SOP-13), return them to a vial 3/4 full of a solution of 80%/5% denatured ethanol/glycerin; make sure the sample label is also in the vial.

**12.4.5** If weights are to be obtained, place all individuals by taxon into the separate cells of a plastic, multi-celled tray filled with water. If weights cannot be taken within 24 hr, replace the water within the cells with 80% ethanol. The procedures for weighing organisms are given in SOP-13; the need for obtaining weights will be determined by project requirements or the Principal Investigator.

**12.4.6** After all "other taxa" have been identified, pour the specimens from the "midges" vial into a petri dish and place the dish on the base of the dissecting microscope; identify and enumerate as done for the "other taxa". However, identifications of Chironomidae may require slide mounts of some individuals and use of a compound microscope with a magnification capability of at least 400X. Once several specimens of most taxa have been identified from slide mounts, it may be possible to identify most specimens under the dissecting microscope without mounts.

**12.4.7** Several techniques are available for making slide mounts; descriptions of some of these techniques may be found in, for example, Epler (2001). The preferred procedure is to mount small specimens whole and the head and body of large individuals separately in a mounting medium such as CMC-10 if available. If CMC-10 is available the following steps may be followed; if not, follow the instructions for the mounting medium available. With CMC-10, place a drop of the mounting media on a 3" X 1" glass slide with a blunt object such as a glass stirring rod. If midges are small, multiple specimens may be mounted under a single cover slide and additional CMC-10 made be needed. However, the ideal position of a chironomid is to have the ventral surface of the head facing up and the body lying sideways, so mounting more than one specimen under a single cover slide make this more difficult. Also, because a compound microscope inverts the image, it is best to mount specimens in the same direction (e.g., antennae pointing towards the top of the slide).

**12.4.8** Grab the specimen(s) to be mounted with the forceps (specimens may be mounted directly from water or ethanol), place it (them) in the drop of CMC-10, and twist the head and body in the proper positions (see previous step).

**12.4.9** Use the forceps to grab a cover slide (12-mm microscope cover slides, No. 1 thickness are best). Carefully place it on top of the drop of mounting media at an angle, make any needed positioning adjustments of the specimen(s), and then apply gentle but steady pressure on the head to spread the mouth parts, and the anal end to spread the claws on the hind parapods. Pressure on the cover slide may be applied with a blunt object such a pencil eraser, blunt forceps, or the base end of the fine-tipped forceps. Normally two or three mounts can be placed on each slide if desired.

**12.4.10** Place a small piece of clear adhesive tape on the unused end of the slide, and write the site name, sample number, collection date, and chain-of-custody number with an indelible ink pen or no. 2 lead pencil, and make sure room is left to also write the organism's taxonomic name.

**12.4.11** Set slides aside and keep them horizontal for at least 3 hr before turning them up vertically to, for example, place them in a slide box. After 3 hr add additional mountant if air bubbles are forming under the cover slide, and if necessary, it may still be possible to make minor adjustments in positioning of specimens.

**12.4.12** Allow slides to dry ~24 hr before trying to identify specimens to provide enough time for significant clearing to occur; clearing may take longer for larger specimens. If the mount is poor and it's not possible to determine the identification from mounts of other specimens, the slide may be placed in water to soak for several hours, and then the specimen(s) can be retrieved and remounted.

**12.4.13** Count specimens by taxon as they are identified; devices such as a multiple-tally denominator will facilitate this process. Record taxa names and their final numbers in the appropriate spaces on a Benthic Macroinvertebrate Laboratory Bench Sheet such as that shown in Exhibit 12-3.

**12.4.14** After midges have been identified, if the unmounted specimens will not be weighed, place them into a vial that is ~3/4 full of a solution of 80%/5% denatured ethanol/glycerin, and make sure the sample label is also in the vial.

**12.4.15** If weights are to be obtained, place all individuals by taxon into separate cells of a plastic, multi-celled tray filled with water. If weights cannot be taken within 24-hr, replace the water within the cells with 80% ethanol. The procedures for weighing organisms are in SOP-13; the need for obtaining weights will be determined by project requirements or the Principal Investigator. Adjust weights to account for slide-mounted individuals using the assumption that individual weights equal average weights obtained from those individuals that were weighed. If all of the individuals for a given taxon are mounted, use the average weight from a similarly size taxon to estimate the weight. After specimens are weighed, return them to their original vial that is at least 3/4 full of a solution of 80%/5% denatured ethanol/glycerin making sure the label includes word "midges" on it.

**12.4.16** If a sample was sorted following the 50% pan-check method (MCS-SOP-11), then next identify and enumerate organisms in the "check-vial". Enter the taxa names and numbers in the appropriate spaces on the Benthic Macroinvertebrate Laboratory Bench Sheet (Exhibit 12-3). Do not enter a taxon's name a second time if it is already listed; just enter the number in the column for check vial totals for that taxon. Results from the "check vial" are used to adjust final total numbers of all individuals found in this quality assurance step.

**12.4.17** After specimens from all vials have been identified, enumerated, and recorded on the Benthic Macroinvertebrate Bench Sheet, enter the data into a commercially available electronic data base (e.g., Microsoft Excel). For data entry, each taxon represents an observation, and the following information should be included in separate fields as follows: site, collection date, sample number, genus (taxon name for lowest level of identification), species [if specimen(s) are identified to species, if no species then leave blank], number of specimens in sample, and weight if measured.

**12.4.18** After completion of a sample, make sure that the final preservative is a solution of 80%/5% denatured ethanol/glycerin.

**12.4.19** Cut a square of Parafilm large enough to cover the opening of the vial with the cap on (. 2 in x 2 in), and place in on top of the vial opening. Then, tightly replace the vial cap.

**12.4.20** After completing the sample, enter the completion date on the Benthic Laboratory Chain-of-Custody and Log Sheet (Exhibit 12-1), and return the processed sample to a secure storage area.

**Exhibit 12-1**
**Exhibit 12-2**
**Exhibit 12-3**

**Review**

This procedure has been approved by the QA Coordinator prior to revision/or issuance.
Reviewed by: M. K. McCracken, BMAP Quality Assurance Coordinator
Signature date: 01/16/17

**Approval**

The revision and/or issuance of this standard operating procedure must receive the signed approval of the Principal Investigator.
Approved by: Ryan A. McManamay, Principal Investigator
Approval date: 01/16/17

Approval signature on file.

Questions, comments or suggestions concerning this procedure should be directed to the Principal Investigator listed above.

## EXHIBIT 12-1. BENTHIC MACROINVERTEBRATE LABORATORY CHAIN-OF-CUSTODY AND LOG SHEET

| PROJECT NAME: | | | | | | | | | | | PAGE NO. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site name | Sample number | Collect date | No. of containers | Relinquished by | Record by | Trans. date | Sorter | Sort date | No. of vials | Taxonomist | ID. date | No. Of vials | Comments |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |
| | | | | | | | | | | | | | |

## EXHIBIT 12-2. MACROINVERTEBRATE IDENTIFICATION AND SORTING GUIDELINES

**Table 12-2a. General guidelines for the removal and identification of organisms from benthic macroinvertebrate samples collected with ORNL BMAP protocols (MCS-SOP-5).**

Deviations from these guidelines must be approved by the Principal Investigator.

---

A. Except where noted below and in Table 12-2b, identify specimens to genus or the lowest practical taxon if damaged or immature. If a taxonomist has the expertise to identify specimens to species without significant additional effort, then such identifications may be made.

B. In general, if uncertain of generic identification, identify to the next highest taxonomic level which is normally family, suborder, or order (e.g., *Stylogomphus*? should be entered as "Gomphidae"; if uncertain if a stonefly is a Perlidae or Perlodidae, then the entry should be "Systellognatha"). When identification is not to genus or species, the "Species" field should be left blank.

C. Coleoptera

Combine counts for larval and adult Coleoptera of the same genus. In the comment field on the data sheet indicate either how many are adults or how many are larvae.

D. Trichoptera

Combine counts for larvae and pupae of the same genus. In the comment field on the data sheet indicate how many are pupae.

E. Other limited identifications

1. Nematoda - No further identification
2. Oligochaeta - No further identification except for the distinct species, *Branchiura sowerbyi*
3. Turbellaria - No further identification
4. Hirudinea - No further identification
5. Chironomidae - Identify only to subfamily except the Chironominae should be identified to tribe (Chironomini or Tanytarsini)
6. Hydrachnidia (water mites) - No further identification
7. Nemertea – Note further identification

F. The following taxa shall neither be sorted from samples nor enumerated.

1. Collembola - all (Surface dwellers/semi-aquatic)
2. Hemiptera - all surface dwellers/"skaters" (e.g., Gerridae, Macroveliidae, Mesoveliidae, Veliidae), semiaquatic taxa (e.g., Gelastocoridae, Hebridae, Ochteridae, Hebridae), and non-benthic (e.g., Corixidae).
3. Microcrustacea - Cladocera, Copepoda, and Ostracoda (Too small/ineffective sampling and sorting)
4. Tardigrada - all (Too small/ineffective sampling and sorting)
5. Coelenterata - all (Too small/ineffective sampling and sorting)
6. Branchiobdellidae - (Commensal on crayfish, thus not truly benthic)

---

**Table 12-2b. List of the taxa that may be encountered that are monospecific or generally identifiable to species with readily available taxonomic keys. Except for possible other monospecific taxa, identification of species (and number if multiple species) not on this list should only be noted in the comment column and not entered as a separate observation.**

Additions/deletions to this list shall be approved by the Principal Investigator. This list applies only to samples processed via ORNL protocols.

*Dicranopselaphus variegatus*

*Anchytarsus bicolor*

*Ancyronyx variegata*

*Basiaeschna janata*

*Branchiura sowerbyi*

*Chauliodes pectinicornis*

*Chauliodes rastricornis*

*Clioperla clio*

*Corbicula fluminea*

*Corydalus cornutus*

*Diplectrona modesta*

*Eccoptura xanthenes*

*Habrophlebia vibrans*

*Hagenius brevistylus*

*Hylella azteca*

*Leucotrichia pictipes*

*Lype diversa*

*Macronychus glabratus*

*Microcylloepus pusillus*

*Nigronia serricornis*

*Nigronia fasciatus*

*Oulimnius latiusculus*

*Psephenus herricki*

*Pseudosuccinea columella*

*Stylogomphus albistylus*

**EXHIBIT 12-3. BENTHIC MACROINVERTEBRATE LABORATORY BENCH SHEET (FRONT)**

**Page _____ of _____**

| **Project Name:** | | | | **Sorted by:** **Date Started:** **Date Finished:** | | | |
|---|---|---|---|---|---|---|---|
| **Stream Name:** | | | | **Taxonomist:** **Date Started:** **Date Finished:** | | | |
| **Site Name:** | | | | **Chain-of-Custody Number:** | | | |
| **Taxon** | **Number** | **Ck. no.[a]** | **Total no.[b]** | **Taxon** | **Number** | **Ck.no.[a]** | **Total no.[b]** |
| **Ephemeroptera** | | | | **Odonata** | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | **Diptera** | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| **Plecoptera** | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| **Trichoptera** | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | **Other Taxa** | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| **Coleoptera** | | | | | | | |
| | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

**[a]Number of organisms in QA check vial.**
**[b]Total number of organisms corrected for number in check vial. This number is derived by multiplying the check vial number (Ck. no.) by the proportion of pans checked during sorting QA, and then adding to number (no.).**

## BENTHIC MACROINVERTEBRATE LABORATORY BENCH SHEET (BACK)

| | |
|---|---|
| **Total Number of Organisms _____** | **Total Number of Taxa _____** |
| **Proportion of Pans Checked _____** | |

**QC / Sorting:**

**Checked by: _____ Date: _____**

**Sorting efficiency**

**(Sorting efficiency = Number in check vial (Ck. No.) ) Number originally sorted (No.) H 100)**

**_____**

**(∃80% Sample passes or < 80% sample fails)**

**Action taken:**

**QC / Taxonomy:**

**Checked by: _____ Date: _____**

| **Original identification** | **Verification identification** |
|---|---|
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |

| | |
|---|---|
| **Subsampled? Yes_____ No _____** | **Explain:** |

**General comments:**