Laboratory Procedures for Macroinvertebrate Sample Processing and Identification

Commonwealth of Kentucky
Energy and Environment Cabinet
Department for Environmental Protection
Division of Water

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Document Revision History

Date of Revision	Page(s) Revised	Revision Explanation
February, 2015	All	The macroinvertebrate processing procedures formerly found in "Laboratory Procedures for Macroinvertebrate Processing, Taxonomic Identification and Reporting" (KDOW 2011) have been updated and revised. This document only includes processing and identification procedures. A separate SOP is under development for data entry and analysis procedures.
February, 2018	All	The entire document was edited for content including details on how to enter data into the electronic data entry application (MDEA).

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Table of Contents

1. Scope and Applicability 5
2. Executive Summary 5
3. Acronyms 5
4. Health & Safety Issues 6
5. Cautions and Interferences 6
5A. Cautions 6
5B. Interferences 6
6. Personnel Qualifications/Responsibilities 6
7. Equipment and Supplies 7
8. Sample Processing Procedures 7
8A. Macroinvertebrate Data Entry Application (MDEA) 8
8B. Labeling 8
8C. Preservation 8
8D. Sample Preparation 9
8E. Processing Procedures for Semi-Quantitative Samples 9
8E.1. Full Pick Processing Method 10
8E.2. Fixed-count Subsampling (300 pick) Method 10
8F. Processing Procedures for Qualitative Samples 12
9. Taxonomic Identification Procedures 12
9A. Recording Identifications 12
9B. Subsampling Excessively Abundant Chironmidae for Identification 13
9C. Identifying Chironomids 13
9D. Archiving Specimens 14
10. Data Finalization and Upload 15
10A. Initial Data Review 15
10B. Data upload to K-WADE Database 15
11. Data and Records Management 16
12. Quality Assurance and Quality Control 16
12A. Primary QA of Macroinvertebrate Identifications 16
12B. Macroinvertebrate Sorting and Subsampling QA/QC 16
12B1. Inexperienced Sorters 16
12B2. Experienced Sorters 17
12C. Macroinvertebrate Taxonomy QA/QC 17
12C1. Sample Re-identification 17
12C2. Reference Collections 18
12D. Certification for New Macroinvertebrate Taxonomists 18
13. References 20
Appendix A. Benthic Macroinvertebrate Laboratory Bench Sheet 21
Appendix A. Benthic Macroinvertebrate Laboratory Bench Sheet, cont'd 22
Appendix A. Benthic Macroinvertebrate Laboratory Bench Sheet, cont'd 23
Appendix B. Slide Bench Sheet 24
Appendix C. Taxonomic References 25
Appendix D. KDOW Benthic Macroinvertebrate Taxonomic Level of Effort 30

1. Scope and Applicability

Macroinvertebrates are utilized extensively as indicators of water quality and are integral in the decision making process regarding the aquatic life use support of individual stream segments throughout Kentucky. This document outlines laboratory methods used by KY Division of Water (DOW) for the processing and identification of macroinvertebrate samples.

2. Executive Summary

This document includes the procedures that should be followed by the DOW staff for the uniform and accurate processing and identification of benthic macroinvertebrate samples collected from the surface waters of Kentucky. The recommended quality assurance/quality control (QA/QC) measures that should be taken when conducting these activities are also outlined.

Macroinvertebrate data are used by KDOW to:

1. Assess aquatic life use support as defined by Kentucky Water Quality Standards 401 KAR 10:026 and 10:031
2. Fulfill the requirements of sections 303(d) and 305(b) of the Clean Water Act
3. Determine effects of point and/or nonpoint source pollution on aquatic biota
4. Determine background conditions in particular watersheds or ecological regions
5. Maintain the accuracy & precision of Kentucky's Macroinvertebrate Bioassessment Index (MBI)

This manual is considered a dynamic document that is reviewed and updated as new procedures and methods are adopted.

3. Acronyms

CMC- Slide mounting media
COC- Chain of Custody
DOW- Division of Water
EtOH- Ethyl Alcohol
EPA- Environmental Protection Agency
KN- Kicknet
MACS- Mid-Atlantic Coastal Plain Streams
MBI- Macroinvertebrate Bioassessment Index
MDEA - Macroinvertebrate Data Entry Application
MH- Multihabitat
QA- Quality Assurance
QAQC – Qaulity Assurance / Quality Control
QC- Quality Control
RBP – Rapid Bioassessment Protocols
PTD - Percent Taxonomic Disagreement
SDS- Safety Data Sheet
SOP – Standard Operating Procedure
WQB – Water Quality Branch

4. Health & Safety Issues

When working with chemicals producing harmful fumes, staff should use a fume hood to reduce inhalation exposure to themselves and coworkers. When any chemical spill occurs, the first line supervisor will be notified. The first line supervisor will notify the second line supervisor and the division safety officer. The division safety officer will then notify the department safety officer. Do not attempt to clean-up a chemical spill if inhalation exposure or skin, throat, or eye irritation is a threat.

If injury or exposure occurs within the laboratory, then proper first aid attention will be administered by other lab personnel as soon as possible. If the condition is serious, the victim should be transported to a medical facility as soon as possible. For chemical exposures refer to the appropriate Safety Data Sheet (SDS) for first aid treatment. SDS sheets shall be maintained in a readily accessible location in the lab for each chemical stored or used in the lab. If any exposure occurs while in the laboratory, a 1A1 exposure or injury form needs to be submitted to the Division of Workman's Compensation within 24 hours of exposure or injury. Accidents which occur in the laboratory will be immediately reported to the acting supervisor or manager.

5. Cautions and Interferences

5A. Cautions

- Samples should be processed according to the method specified in the project study plan for which the sample was collected.
- Care and maintenance of microscopes should be performed on a regular basis by a professional service contractor.

5B. Interferences

- Samples should be stored in a secure location to ensure sample integrity.
- All samples should be entered in an appropriate logbook or tracking system.
- Samples stored in containers using ethyl alcohol (EtOH) are not always airtight. Therefore, EtOH may evaporate leading to sample desiccation. EtOH levels should be checked periodically to ensure adequacy.
- Any equipment/supplies should be free and clear of debris and organisms. Always wash and inspect sorting pans, mesh sieves and storage containers before and after use.
- Sample alcohol must be decanted and refreshed within 12 hours of collection.

6. Personnel Qualifications/Responsibilities

Processing procedures are performed by personnel trained in this SOP. Macroinvertebrate identifications are performed by personnel with special expertise. Personnel must have a basic understanding of laboratory practices and safety. Personnel performing these procedures are responsible for reading and fully understanding the methods and QAQC procedures presented in this document.

7. Equipment and Supplies

An itemized list of common equipment and supplies typically used to process macroinvertebrates can be found below.

Microscopes

- Stereomicroscope (dissecting scope) with 10X ocular and appropriate zoom magnification
- Fiber optic light source
- Compound microscope with 10x, 20x, 40x, 100x objectives and phased contrast

Sorting and Identifying

- Approved Subsampling Pans
    - 25-square Caton trays (5x5 grid)
    - 36-square Petri-dish for midge subsampling
- Sorting Pans of Various Sizes
- Glass Petri dishes of various sizes
- Watch glasses of various sizes
- Random numbers table/generator

Record Keeping

- Sample log book (Appendix A)
- Macroinvertebrate bench sheet (Appendix B)
- Chironomid slide bench sheet (Appendix C)

Tools

- Dumont #5 Fine-tip forceps
- Probes
- Counter

Preservation & Storage

- 75% EtOH
- Glass specimen jars
- Glass shell vials
- CMC mounting media
- Glass slides and cover slips
- Cotton

Taxonomic Literature

- See Section 8.0 (Appendix D)

8. Sample Processing Procedures

KDOW uses separate field collection methods for low- and high-gradient streams. These methods are outlined in "Methods for Sampling Benthic Macroinvertebrate Communities in Wadeable Waters" (KDOW 2016), which should be consulted to address questions in determining low- vs high- gradient streams. In summary, in high-gradient streams, collections produce two types of samples; a semi-quantitative (1m^2 kicknet or KN) sample composed of four composited 0.25 m^2 quadrat KN samples and a qualitative (multihabitat or MH) sample consisting of collections from various habitats present in the reach. Low-gradient streams are sampled using a proportional sampling technique which follows the

Mid-Atlantic Coastal Plain Streams Workgroup (MACS) protocol (EPA 1997) also detailed in Barbour et al. (1999). Using this methodology, a semi-quantitative collection consists of 20 D- or A- frame net jabs composited into a single container. The following laboratory processing methods approved for these field collection procedures were developed from guidance provided by Barbour et al. (1999).

8A. Macroinvertebrate Data Entry Application (MDEA)

The bench sheet for recording all macroinvertebrate sample collection, processing and identification data is in the form of a Microsoft Excel spreadsheet application. The MDEA will also complete all macroinvertebrate metric calculations automatically. When a macroinvertebrate sample is returned to the lab, it must be entered into K-WADE as a field activity during data entry from the field station visit (i.e. the collection method must be entered on the "Macroinvertebrate Sample Information" tab.

When laboratory processing of the sample begins, a copy of the master MDEA (located in the project-year data folder) is placed in the appropriate project folder on the network drive. This copy should be renamed as the Station ID for the sample site. If more than one macroinvertebrate sample is to be collected from the same stream reach in the same year, then sequential replicate numbers (starting with 1) will be appended to the filename (i.e. station ID) after a dash (-) (e.g. -rep1, -rep2, -rep3, etc.). If the sample is from a station visit duplicate, then the suffix "-dup" will be appended to the filename. If the sample is being completed for QA or TRAINING purposes then the suffixes "-QA" and "-Training" must be appended to the filename. For new taxonomist training, the sample completed by the new taxonomist is labeled as training, while the re-identification by the experienced taxonomist is the original sample. These samples will then be added to the macroinvertebrate sample tracking system so that subsequent activity on each sample is tracked throughout the entire process of completing the sample. The MDEA will be used to store all raw data, including sample processing, identifications, and QA/QC, until the data is uploaded to K-WADE.

8B. Labeling

Each sample container must have two labels: one on the inside of the container and one on the outside of the container, but not on the lid. The label must include the station ID, stream name, county, date sampled, collection method, and the collectors' initials. After processing, jars containing residual unpicked material should be labeled "Residual".

Vial and slide-mounted invertebrates must also be properly labeled after identification. These labels include the station ID, stream name, county, sample date, collector, the vial or slide number, and the macroinvertebrate lab analyst's name. The MDEA produces labels that meet vial and slide labelling specifications and must be printed on cotton fiber paper for long-term (> 1 month) curation in alcohol. Printable labels for slides and vials can be found on the labels tab of the MDEA.

8C. Preservation

Upon returning to the laboratory, the EtOH in each sample should be replaced with fresh 75% EtOH for storage until processing occurs. This must be done by draining the contents over a U.S. Standard No. 30 sieve (600 µm wire mesh) to prevent organism loss. Organisms captured in the sieve must be returned to the sample container.

8D. Sample Preparation

Remove the semi-quantitative sample from the container using the following steps:

1. Remove the lid and place a No. 30 (600 µm) mesh sieve over the container opening.
2. Holding firmly, invert and drain any remaining liquid from the container into the sink, keeping the sample in the container.
3. Flip the sieve and container right side up after draining and examine the sieve for organisms that may have escaped during draining. Return any organisms in the sieve to the container.
4. Alternatively, the entire sample may be gently rinsed with water in a No. 30 sieve to remove preservative, fine sediment, large organic material, rocks, twigs, whole leaves, *etc*. Rinse any material removed from the sample, visually inspect for organisms, and discard.
5. Transfer the sample into an appropriately sized Caton tray that is placed in a larger sorting pan.
    a) A larger Caton tray should be used with samples that contain a lot of material
    b) A smaller Caton tray should be selected for samples with sparse material
    c) Regardless of site, a Caton tray containing 25 squares in a 5 x 5 grid must be used for processing. Using this tray, each square represents 4% of the sample.
6. Use a small amount of water to rinse the container of any remaining sample, dumping it into the pan, until the entire sample and all organisms are transferred to the pan.
7. If the semi-quantitative sample was stored in more than one container, the contents of all containers for a given semi-quantitative sample should be combined in the pan following steps 1 - 6
8. Evenly distribute the sample in the Caton tray by filling the white sorting pan in which the Caton tray sits with water to aid in dispersion of the sample. If large clumps of filamentous algae are present, cut them into small pieces with scissors to allow for easier sample spreading and distribution.
9. Once the sample is evenly distributed in Caton tray, water from the sorting pan can be drained. This is done by gently removing the Caton tray from the sorting pan and pouring out the water. The Caton tray should then be placed back in the larger sorting pan.
10. If the sample is dehydrated, it may be rehydrated using the following method:
    a) Place a No. 30 sieve over the container opening.
    b) Holding firmly, invert and drain any remaining liquid from the container into the sink, keeping the sample in the container.
    c) Flip the sieve and container right side up and examine the sieve for organisms that may have escaped during draining. Return any organisms in the sieve to the container.
    d) Refill the container with water and allow the sample to sit for several minutes to rehydrate organisms.
    e) Dump the sample into an appropriate caton tray and ensure all organisms and sample material are removed from the container, as in steps 4 and 5 above.

8E. Processing Procedures for Semi-Quantitative Samples

KDOW utilizes two processing methods: 1) full pick and 2) fixed-count subsampling. Both methods may be applied to the semi-quantitative sample, regardless of collection method (KN or 20 Jab) . To determine the appropriate method, first visually estimate how many organisms are in the sample. If there obviously appears to be 300 or fewer organisms, apply the full pick method. If there appears to be greater than 300 organisms, apply the 300 pick method. The following rules apply to picking organisms, regardless of method:

1. Samples should be picked at 10× magnification using a dissecting microscope.
2. Pick all organisms encountered regardless of condition or maturity.

Young individuals directly attached to adults – common in leeches and many crustaceans - should not be counted in the sample unless they are detached completely from the adult.

8E.1. Full Pick Processing Method

1. Remove the contents of one square from the Caton tray and place in a small sorting pan or Petri dish (Table 1).
2. Add enough water to disperse and thin out the sample.
3. Using a dissecting microscope, carefully scan the sample and pick encountered organisms.
4. Picked organisms are placed in glass shell vials containing 75% EtOH.
5. Continue picking until sample is devoid of organisms.
6. Debris from the sample can be discarded as the sample is picked if sample processing QA (i.e. pick check) will not be completed.
7. When processing is complete, store organisms in a sample container filled with 75% EtOH. Ensure that the container is labeled with all pertinent sample information (section 8B Labeling).
8. Enter Pick Processing Information in the "Laboratory Processing Information" section of the "Pro-An" tab of the MDEA. All fields must contain data.

8E.2. Fixed-count Subsampling (300 pick) Method

The fixed-count subsampling method was adapted from "Rapid Bioassessment Protocols for Use in Wadeable Streams and Rivers" (Barbour et al. 1999). The semi-quantitative sample (KN or 20 Jab) should be randomly subsampled to a fixed-count of 300 individual organisms ± 20%, with an effort made to count a *minimum* of 300 organisms.

Subsampling Procedures

The following rules must be applied during macroinvertebrate subsampling.

1. An organism lying on the line between two squares is considered to be in the square in which its head lies. In those instances where it may not be possible to determine the location of the head (i.e. worms), the organism is considered to be in the square containing most of its body.
2. Picking doesn't stop once the target number is achieved, but continues until the current square is completely picked.

Level 1 Subsampling

A minimum of four randomly chosen squares (i.e. 16% of the entire sample) must be picked from the sample, but it may take > 4 squares to achieve the target number (300 ± 20%), with an effort made to count at least 300 organisms.

Note: If it is apparent that there are > 80 organisms in one square (i.e. the number of organisms that may result in > 360 organisms being picked from four squares), the processor can proceed straight to Level 2 subsampling.

1. Generate a random number and pick the corresponding square in the Caton tray. Place the contents of the square in a Petri dish (**Hint:** A credit card-sized piece of plastic is excellent for removing square contents).
2. Add enough water to disperse and thin out the sample.
3. Pick all organisms from this square and place in vials filled with 75% EtOH.
4. Using a counter, count the number of organisms as they are picked.
5. If > 80 organisms are picked from the first square, then stop picking and proceed to Level 2 Subsampling.
6. If < 80 organisms are picked in the first square, continue using randomly generated numbers to pick squares until 300 ± 20% of individuals are picked remembering a minimum of 4 squares must be picked.

7. Debris from the sample can be discarded as the sample is picked if sample processing QA (i.e. pick check) will not be completed.
8. When processing is complete, store organisms in a sample container filled with 75% EtOH. Ensure that the container is labeled with all pertinent sample information (section 8B Labeling).
9. Enter Level 1 Pick Processing Information in the “Laboratory Processing Information" section of the “Pro-An” tab of the MDEA. All fields must contain data.

Level 2 Subsampling (subsampling a subsample)
Some samples may contain a very large number of organisms that may result in >360 individuals being picked from four squares. Therefore, if > 80 individuals are picked in the first square in Level 1, then the processor must proceed with Level 2 subsampling.

No minimum number of squares is required in Level 2 subsampling, so it is recommended to pick one square at a time until the desired number (300 ± 20% with an effort made to count a *minimum* on 300 organisms) is achieved.

1. Randomly choose 4 squares from the larger Caton tray used in level 1 subsampling and place them in a smaller 25-square Caton subsampling tray, which is appropriate for the amount of material.
2. Randomly select a square from the Level 2 pan and pick all organisms from this square and place in glass shell vials filled with 75% EtOH.
3. Using a counter, track the number of organisms as they are picked.
4. Continue randomly picking squares until the desired number (300 ± 20%) is achieved, with an effort made to count a *minimum* of 300 organisms. Again, no minimum number of squares is required for Level 2 subsampling.
5. Enter Level 2 Pick Processing Information in the “Laboratory Processing Information" section of the “Pro-An” tab of the MDEA. All fields must contain data.
6. In rare circumstances, if picking through the first square or any subsequent squares at level 2 will likely result in > 360 organisms, then the square that will result in >360 organisms can be subsampled further with Level 3 subsampling. For example:

    a. If grid 1 from level 2 subsampling had >360 organisms, place all material from grid 1 from level 2 subsampling and place in a smaller caton try and follow steps outlined in level 3 subsampling.
    b. If grid 1 from level 2 subsampling had 200 organisms, it is likely that picking a second square will result in > 360 organisms. Keep the 200 organisms from grid 1, but place the entire content of grid 2 in a smaller Caton tray and follow steps outlined in level 3 subsampling.
    c. If grids 1 and 2 from level 2 subsampling had ~125 organisms each (250 total), it is likely that picking a third square will result in > 360 organisms but a minimum of 300 has not yet been achieved. Keep the 250 organisms from grids 1 and 2, but place the entire content of grid 3 in a smaller Caton tray and follow steps outlined in level 3 subsampling.

Level 3 Subsampling (subsampling a subsample of a subsample)
Under Level 3 Subsampling, the following procedure should be implemented:

1. Spread contents of the last grid from Level 2 subsampling into an appropriately sized 25-square Caton subsampling tray.

2. Randomly select a square from the Level 3 pan and pick all organisms from this square.
3. Using a counter, track the number of organisms as they are picked.
4. Continue randomly picking squares until the desired number (300 ± 20%) is achieved, keeping in mind that picking will not necessarily begin at zero with level 3 subsampling.
5. Effort should be made to count a *minimum* of 300 organisms. No minimum number of squares is required at Level 3 subsampling.
6. Enter pick processing information in the "Processing" tab of the MDEA. The total number of squares for each subsampling level should be noted. All fields in the MDEA must contain data.

8F. Processing Procedures for Qualitative Samples

The qualitative (MH) sample collected in high gradient streams should be picked such that all unique taxa are removed from the sample with the exception of Chironomidae, which must all be entirely picked from the multihabitat sample. If > 50 Chironomidae individuals are present, then chironomids should be subsampled following the methods outlined in section 9B of this document. Organisms must be preserved in a labeled jar containing 75% EtOH for identification.

9. Taxonomic Identification Procedures

Identifications are made to the lowest practical level of identification following the standard taxonomic effort table (Appendix D) using a dissecting scope for most organisms. Specimen condition (damaged, early instar, poor slide mount) may result in the situation of having to leave identification at more course levels (e.g., family or higher). Individuals in the family Chironomidae are mounted on slides using CMC mounting medium and identified using a compound microscope. A list of the taxonomic references currently used by KDOW can be found in Appendix C.

9A. Recording Identifications

1. Select the sample from the Lookup List located at the top of the "Sample" tab of the MDEA.
2. Enter all processing and analysis information on the Pro-An tab of the MDEA.
3. All non-slide-mounted identifications are recorded on the "Vial" tab of the MDEA.
4. Type the final identification of the taxon into the next available combo box in the Final ID column. This box contains a dropdown and also autocompletes entries to facilitate data entry. If a name is misspelled, the adjacent cell in the Quant column will be highlighted red, and the notes column will be highlighted in red and will display "***CRITICAL SPELLING ERROR, Fix the Spelling of the taxon name***", indicating revisions are needed to the entered name.
5. Enumerate individuals from the semi-quantitative sample in the "Semi-Quant" column.
6. Mark the presence of taxa from the multihabitat (MH) sample by typing a "Y" in the "MH" column. Taxa identified in the multi-habitat sample are not enumerated.
7. In some cases, the analyst must determine if a taxon is to be included in richness metrics. This typically occurs when organisms are identified to levels above genus because they are early instars or damaged. If this decision must be made, then the cell in the "Incl." column will be highlighted yellow. If the taxon is to be included in the richness calculations, then a "Y" is placed in the "Incl." column. In contrast, if the taxon is to be excluded in the richness calculations, then an "N" is placed in the "Incl." The Incl. column contains a dropdown menu to facilitate data entry.
8. The life stage of the organism MUST be entered into the Life Stage column using the available dropdown menu. The cell will highlight as yellow indicating its status as a mandatory data entry cell. All non-insects and Hemiptera should be entered as "UNDIFFERENTIATED" and insects should be distinguished between "LARVA" and "ADULT". The MDEA defaults all non-insects and

Hemiptera to “UNDIFFERENTIATED” and other insects to “LARVA”. The analyst must switch the insect life stage to adult when necessary for non-Hemipterans.

9. Difficulties encountered during identification (e.g., missing gills) are noted in the “Notes” column.
10. If individuals are removed for QA/QC purposes or to add to reference collections, then make a note in the “Notes” column.
11. Notes should also be included detailing why any taxa were included/excluded in the “Incl.” column.
12. Archive samples according to the methods in Section 9D.

9B. Subsampling Excessively Abundant Chironmidae for Identification

If ≤ 50 Chironomidae are found in any part of the sample (e.g. semi-quantitative or qualitative), then all individuals are identified and enumerated for that part of the sample. However, excessively abundant Chironomidae may occur in some samples (i.e. > 50 individuals). If there are more than 50 individuals in any part of the sample (e.g. semi-quantitative or qualitative), then individuals must be subsampled to 50 individuals to reduce the effort and time it would require identifying all individuals in the sample. A 6 x 6 cm gridded Petri dish with 36 total squares shall be used to randomly subsample Chironomidae individuals until 50 individuals are selected using the following methods:

1. Place all Chironomidae individuals in the 6 x 6 cm gridded Petri dish and distribute the organisms across the dish.
2. Randomly select a square, pick all organisms in the square, and place them in vials of 75% EtOH.
3. Continue randomly picking entire squares until 50 Chironomidae individuals have been selected for identification.
4. If the final square that is picked will result in more than 50 individuals being selected for identification, then individuals within the square must be randomly selected until 50 individuals are attained for mounting and identification.
5. Place remaining individuals, not to be identified, in a separate vial. This vial should be stored with the original sample and should be labeled as “residual”.
6. Enter the Chironomidae subsampling information in the “Pro-An” tab of the electronic data entry sheet including whether the Chironomidae were subsampled and the total number of Chironomidae originally picked.

9C. Identifying Chironomids

1. Mount larvae on slides using techniques found in Epler (2001).
2. Every slide must be labeled by hand or using the labels generated on the “Slides” tab of the MDEA. The Semi-quantitative slides and multi-habitat slides should be numbered separately (i.e. both starting with Slide #1). Slide labels must include the following information:
   - Site number
   - Stream name
   - County
   - Sample date
   - Sample method (MH, KN or 20 jab)
   - Collector(s) initials
   - Slide number
3. Enter the total number of quantitative and multihabitat slides on the “Pro-An” tab of the MDEA.
4. Type the final identification of each taxon into the next available combo box in the Lookup Value column of the “Slide” tab. This box contains a dropdown box and also autocompletes entries to facilitate data entry. If a name is misspelled, the adjacent cell in the Quant column will be highlighted red, and the notes column will be highlighted in red and will display “***CRITICAL SPELLING ERROR, Fix the Spelling of the taxon name***”, indicating revisions are needed to the entered name.

5. In some cases, the biologist must determine if a taxon is to be included in richness metrics. This typically occurs when organisms are identified to levels above genus because they are early instars or damaged. If this decision must be made, then cells in the "Incl." column will be highlighted yellow. If the taxon is to be included in the sample, then a "Y" is placed in the "Incl." column. In contrast, if the taxon is to be excluded from richness, then an "N" is placed in the "Incl." column. The "Incl." column contains dropdown menus to facilitate data entry.
6. The life stage of the organism **MUST** be entered into the Life Stage column using the available dropdown menu. The cell will highlight as yellow indicating its status as mandatory for data entry.
7. Difficulties encountered during ID (e.g., poor mount) are noted in the "Notes" column.
8. If individuals are removed for QA/QC purposes or to add to reference collections, then add a comment in the "Notes" column.
9. Notes must be included detailing why any taxa were included/excluded in the "Incl." column.
10. For each individual in each identified taxon (listed across Row 1 after the Notes Column-Indiv. 1, Indiv. 2, etc.), record the slide number, cover slip ID and specimen number for each individual in the "Loc" column and in the "Samp" column determine whether the sample is from the semi-quantitative (Q) sample or multihabitat (M) sample. See Figure 1 for the schematic to be used to assign specimen numbers to Chironomids.
11. Archive samples according to the methods in Section 9D.

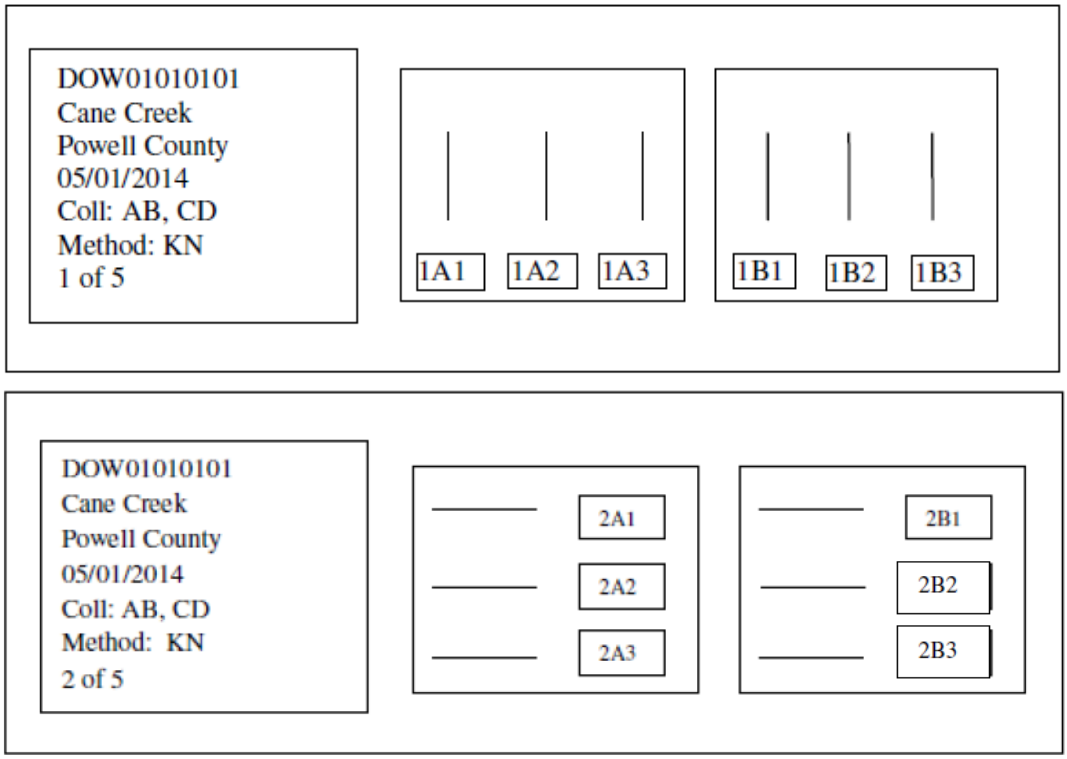

Figure 1. Schematic for Assigning Specimen Numbers to Chironomids

9D. Archiving Specimens

All organisms identified from the same sample should be placed in a jar filled with 75% EtOH. The jar should be labeled with the following information: station ID, stream name, county, date sampled, sample type (KN, 20 jab, MH), collector(s) initials, and check mark (or some other marking) indicating the sample has been identified. The EtOH levels should be examined regularly and replenished as needed.

Slides should be stored in appropriately labeled slide boxes. Samples should be stored in a secure location for a minimum of five years.

10. Data Finalization and Upload

When identifications are complete, the ID date and staff name on the "Pro-An" tab of the data entry sheet should be entered.

10A. Initial Data Review

Initial data review refers to verification checks performed immediately following completion of the macroinvertebrate field activity (i.e. processing and identification). Initial data review will be completed by the macroinvertebrate sample analyst immediately prior to data upload. Initial data review for macroinvertebrate samples includes:

- Check that the MDEA is correctly and completely filled out.
- Project/Trip/Station Visit information and date/time on bench sheet matches field activity information in K-WADE.
- All data qualifiers and comments have been recorded.

After completing these tasks, the macroinvertebrate sample analyst will fill out initial data review information on the "processing tab", which includes the analyst's name and the date of initial data review. This must be done prior to upload of the PDF bench sheet to K-WADE so that the PDF benchsheet shows that initial data review has been completed on the sample.

10B. Data upload to K-WADE Database

After initial data review is completed, macroinvertebrate data must be uploaded into the K-WADE database for permanent storage. First, PDF copies of macroinvertebrate bench sheets (i.e. the red "Inv" and "Slides" tabs of the electronic data entry sheet) must be uploaded to the corresponding station visit in K-WADE during data entry. Additionally, any other documents pertinent to completing the macroinvertebrate field activity must also be uploaded to the appropriate station visit in K-WADE (e.g. taxon voucher images, if available).

The data from the MDEA must be entered into the correct macroinvertebrate sample for the appropriate station visit in K-WADE. This is done by uploading the data using the brown upload tabs in the MDEA (i.e. the Samp, Proc, An, Res, Metric, and Index tabs). These tabs are all necessary for data upload and are autopopulated as the analyst fills out the MDEA during processing and identification. The process for this is as follows:

1. Once the MDEA is final for the sample being completed, then the analyst will run the "Upload Preparation" macro within the MDEA, which will complete final processing of the MDEA for upload.
2. The result of the "Upload Preparation" macro must be saved as its own file in *.XLSX format in the upload folder of the working project folder. The "Upload Preparation" macro prompts the analyst to complete this save.
3. The analyst can then navigate to the appropriate "Macroinvertebrate Sample Information" tab in K-WADE, and click on the "Import Sample Data Package" button to complete data upload.
4. The analyst then selects the appropriate upload file (i.e. the result of the "Upload Preparation" macro). The upload process imports all macroinvertebrate sample processing information, analysis information, taxon lists, and metrics & indices calculated for the sample.
5. After successful upload all data must be reviewed for completeness.

Altenatively, if upload errors cannot be resolved in the MDEA, then the data can be re-entered manually into the database after navigating to the appropriate trip/station visit in the database. If manual data entry is necesary, then all fields (even notes) must be transcribed from the bench sheet to the database.

11. Data and Records Management

All documents, electronic data, and slides are retained in accordance to Quality Assurance Project Plans (QAPP). As mentioned in section 10B, PDF copies of macroinvertebrate bench sheets (i.e. the red "Main" , "Taxa", and "Slides" tabs of the MDEA) must be uploaded to the corresponding station visit in K-WADE during data entry. Additionally, these pdfs should be saved in the appropriate folder on WQB servers after completing each sample. This can be done by selecting the tabs using the CTRL key and then either printing the tabs to a PDF file using a PDF printer option (e.g. Acrobat Distiller, etc.) or using an "Export PDF" option (preferred).

12. Quality Assurance and Quality Control

12A. Primary QA of Macroinvertebrate Identifications

Primary QA of macroinvertebrate samples involves checking the results for 'unusual' taxa that are not typically found in the geographic region where the sample site is located. To facilitate the completion of this QA task, functionality has been incorporated into the macroinvertebrate data entry application to identify any taxa names that are entered that have not been found in the geographic area of the sample site based on DOW historic data. Specifically, three columns have been added to the "Inv_in"tab: BioRegion, EcoRegion and County. As taxon names are entered into the data entry application, these columns will return a "Y" if the taxon has historically been collected by DOW staff in that particular BioRegion, EcoRegion or County. Otherwise, these columns will return an "N", which is also highlighted red. These columns must be checked as data is entered and any questionable identification must be confirmed by a second taxonomist. Furthermore, in instances where a taxon is identified, but not known historically from that bioregion, it must be confirmed by a second taxonomist regardless of the certainty of the identification. All confirming taxonomist names must be listed in the notes column for that taxon.

12B. Macroinvertebrate Sorting and Subsampling QA/QC

It is required that the first two samples processed by any staff be checked by another staff member to ensure that processing is completed correctly. Sorters are categorized as either inexperienced or experienced. All new staff are deemed inexperienced until they have passed the inexperienced sorter requirements for an entire year - starting at the date of first sample picked - at which time they are considered experienced sorters. The following procedures are followed based on experience category:

12B1. Inexperienced Sorters

1. As the sample is picked, the sorter saves all sorted debris residue in a separate container labeled "sorted residue". The number of organisms picked is recorded in the "Sorting Pan QA" section of the QAQC_in tab of the bench sheet.
2. A second sorter scans the debris for remaining organisms, tallies the organisms found and records it on the bench sheet in the "Sorting Pan QA" section of the QAQC_in tab of the bench sheet.
3. Sorting efficiency (%) is calculated as follows and recorded on the bench sheet:

$$= \left(\frac{\text{\# organisms picked from sample}}{\text{\# organisms picked from sample + \# organisms found during check}} \right) \text{ x } 100$$

4. If >90% sorting efficiency is achieved for both samples, then every 10th sample will be examined for efficiency. Any macroinvertebrates found during this QA/QC procedure will be discarded since the original processor was within allowable error.
5. If <90% sorting efficiency occurs, subsequent samples will be checked for efficiency until a sorting efficiency of >90% is achieved. Also, all macroinvertebrate individuals found by the staff member completing QA will be added to the sample.

12B2. Experienced Sorters

1. At the beginning of each calendar year, follow steps 1-3 for inexperienced sorters for the first two samples processed by each experienced staff member.
2. If >90% sorting efficiency is achieved on those samples, then no more samples will be checked for efficiency that year. Any macroinvertebrates found during this QA/QC procedure will be discarded since the original processor was within allowable error.
3. If <90% sorting efficiency occurs, subsequent samples will be checked for efficiency until a sorting efficiency of >90% is achieved. Also, all macroinvertebrate individuals found by the staff member completing QA will be added to the sample.

Sorting pan QA/QC activities **MUST** be entered in the "QAQC_in" tab of the MDEA.

12C. Macroinvertebrate Taxonomy QA/QC

12C1. Sample Re-identification

It is recommended that five percent (5%) of all identified samples be re-identified by a second taxonomist. Samples selected for re-identification are chosen randomly using a random numbers table, or other random selection methodology, and are identified in house, by a second KDOW macroinvertebrate taxonomist. A second electronic data entry sheet is completed. This second bench sheet must be saved as a PDF and appended to the original pdf bench sheet in the appropriate program folder.

Percent Taxonomic Disagreement (PTD) is used to determine the taxonomic precision of KDOW biologists as described in Stribling et al. (2003) where:

$$\text{PTD} = \left[1 - \left(\frac{comp_{pos}}{N}\right)\right] \text{X } 100$$

$comp_{pos}$ = number of agreements, and
N = total number of specimens in the larger of the two counts.

Table 2 (reproduced from Stribling et al. 2003) demonstrates how to determine counts of agreement, which is utilized in the PTD calculation. A PTD value ≤ 10% is the target criterion. If a sample comparison fails to meet the target criterion, taxonomic discrepancies must be reconciled by the biologists, and if necessary, a third macroinvertebrate taxonomist is consulted to settle unresolved identifications. After completing the re-identification of the sample, "ID QA" section in the "QAQC_in" tab is filled out on the original MDEA as well.

Table 2. Example comparisons of re-identification results by 2 taxonomists showing counts of agreements. Target taxonomic level is based on program specifications.

Target taxonomic level	Identification	Taxonomist 1	Taxonomist 2	No. agreements
Genus	Baetidae		1	0
	Procloeon/Centroptilum	1		
Genus	*Argia*	1	2	1
	Coenagrionidae	1		
Genus	*Bratislavia*		2	2
	Bratislavia unidentata	2		
Genus	*Ceratopsyche morosa*	12		12
	Ceratopsyche bronta		12	
Genus	*Physa*		4	0
	Physidae	4		
Genus	*Dugesia tigrina*	1	25	1
	Cura foremanii	25		
Genus	*Glyptotendipes*	58	32	32
Species	*Polypedilum halterale*		9	0
	Polypedilum obtusum	9		
Genus	*Hexatoma*	4	4	4

12C2. Reference Collections

It is required that every macroinvertebrate taxonomist maintain a verified reference collection. The concept for the reference collection is that if a taxonomist identifies macroinvertebrate 'X' correctly in the reference collection, then the same is true for that organism in all other samples. Specimens that are archived in taxonomists' collections will be sent to an outside agency for confirmation. Specimens are not considered to be verified reference taxa until a third party taxonomist confirms that the specimen was correctly identified. Reference collections should be developed using the following guidelines:

- All reference specimens are archived in screw-top vials.
- The collection is built by cataloguing taxa as they are encountered.
- Reference specimens are labeled with a unique identifier that provides the taxonomist's initials, the year of collection, and a sequential number (i.e. klm14-001, klm14-002).
- No more than five individuals of the same taxon are placed in the same vial.
- Individuals of the same taxon should originate from the same sample.
- Specimens chosen for reference should be of high quality.

12D. Certification for New Macroinvertebrate Taxonomists

New macroinvertebrate taxonomists will be required to pass internal QA/QC steps prior to beginning work as a taxonomist identifying macroinvertebrate samples to ensure that sample identifications are accurate and consistent with sample processing and identification protocols. The QA/QC procedures as outlined in this section (12D) will be followed by new macroinvertebrate taxonomists at KDOW. This is a one-time process to initially examine the accuracy and quality of taxonomic data produced by new taxonomists at KDOW.

New taxonomists will be provided with samples in which they complete the entire processing and identification of the sample following the methods outlined in this SOP. Once the sample is completed, a second experienced taxonomist will re-identify and re-enumerate these samples again, following the methods described in this SOP. The PTD metric (as outlined in Section 12C1) will be used to compare the

results obtained by the new taxonomist with the identifications completed by the certified taxonomist. A PTD value ≤ 10% is the target criterion. If a sample comparison fails to meet the target criterion, taxonomic discrepancies must be reconciled by the biologists, and if necessary, a third macroinvertebrate taxonomist will be consulted to settle unresolved identifications. The new taxonomist will be certified to start working independently on new samples once they have passed certification training on a minimum of three re-identified samples and supervisor approval. Once certification has been completed, the taxonomist will follow the QA/QC procedures outlined in Section 12C1 of this document for sample re-identifications.

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Appendix A. Benthic Macroinvertebrate Laboratory Bench Sheet

KDOW Benthic Macroinvertebrate Laboratory Bench Sheet

Cover Page

Station Information and Collection Information

Program:		Project:		Trip:					
Locale Name:		Location Desc:							
Station Name:		County:		Latitude:		Long.:		Act. Date /Time:	
Bioregion:		Stream Type:		Catchment Area (mi2):		Fld Col. Meth.:		Primary Collector	

Processing		Analysis	
Pick By (Date):		ID By (Date):	
Processing Type:			
Processing Method:		Analysis:	
Replicate			
Total Picked:		Taxonomic level:	
Quant. Midges Picked:			
Quant. Midges Subsampled:		# Organisms ID'd:	
Midge % Subsample:			
# Quant Slides:		Quant Midges ID'd:	
MH Midges subsampled:			
# Multi slides:		MH Midges ID'd	
Level 1 Squares:			
Level 2 Squares:		Use Alt Taxa Traits:	
Level 3 Squares:		N	
% Sub Sample:		Taxa Traits Version	
Original TNI:		1-Jan-2017	

MBI

Metric	Raw Value	Scaled	Ref. Cond.?
Genus Taxa Richness			
Genus EPT Richness			
mHBI			
m%EPT			
%Ephem			
% Chiro & Oligo			
% Clinger			
MBI-W			

Modified MBI

Metric	Raw Value	Scaled	Ref. Cond.?
m % Clinger			
mMBI			

O / E Results

Total # of Expected Taxa ID'd:		Ref. Cond.?
O/E Index:		

Supplementary Metrics

Metric	Raw Value	Scaled	Ref. Cond.?
Genus Clinger Richness			
% Intolerant			
Genus Intolerant Richness			
% Tolerant			
% 5 Dominant			
% Hydropsychidae			
% Shredder			
% Predator			
Genus Shredder Richness			
Genus Predator Richness			
% Nut. Tolerant Taxa			
Hilsenhoff Biotic Index		N/A	N/A
% Non-insect		N/A	N/A
# Exhibiting Ref. Condition			
% Reference			

Activity Notes	Processing and ID Notes	Analyst's Assessment Notes

Data Review and Upload Information

Initial Data Review:		Upload:	

Appendix A. Benthic Macroinvertebrate Laboratory Bench Sheet, cont’d

Project:		**Station Name:**		**Taxa List Page 1**	
Trip:		**Locale Name:**			
Collection Date:		**Collector:**		**Identified By:**	

Final ID	**SQ**	**MH**	**Incl.**	**Stage**	**Notes**

Appendix A. Benthic Macroinvertebrate Laboratory Bench Sheet, cont'd

Project:		Station:		Slides List Page 1
Trip:		Locale:		
Collection Date:		Collector:	Identified By:	

Final ID	ID'd	SQ	MH	Slide Locations

Appendix B. Slide Bench Sheet

Kentucky Division of Water Bench Sheet - Slides					Page 1
Stream Name:			Station ID:		
Coll. 1:	Date:	ID By:		Date:	
Final ID	**Slide Locations**	**ID'd**	**Quant**	**MH**	**Comments / Tax. QC**

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Appendix D. KDOW Benthic Macroinvertebrate Taxonomic Level of Effort

All macroinvertebrates must be identified to the lowest determinable level based on current taxonomic references and resources available. The table below provides a general standardized level of effort that is required for mature and well preserved specimens. Generally, it is required to identify organisms to at least the genus level. Due to taxonomic limitations, some groups cannot be identified to the genus or species level and therefore should be taken to the level specified below. For all taxonomic groups, if the level can easily go lower, for example monotypic genera, or if only one genus or species is known to occur in a certain geographic area, then these specimens should be identified at the lowest possible taxonomic level.

Phylum	Class	Order	Family	Taxonomic Resolution
Annelida				
	Oligochaeta			Family
			Hirudinidae	Family
Arthropoda				
	Insecta			
		Coleoptera		Genus/Species
		Diptera		Genus/species, except the following families:
			Dolichopodidae	Family
			Phoridae	Family
			Scathophagidae	Family
			Syrphidae	Family
		Ephemeroptera		Genus/Species
		Hemiptera		Genus/Species
		Lepidoptera		Genus/Species
		Megaloptera		Genus/Species
		Odonata		Genus/Species
		Plecoptera		Genus/Species
		Trichoptera		Genus/Species-except Hydropsyche where morosa group (formerly Ceratopsyche) **MUST** be identified as Hydropsyche morosa gp.
	Crustacea			
		Amphipoda		Genus/Species
		Decapoda		Genus/Species
		Isopoda		Genus/Species
	Arachnida	Trombidiformes		Hydracarina (unranked)
Cnidaria				Genus/Species
Ectoprocta				Genus/Species
Mollusca				
	Bivalvia			Genus/Species
	Gastropoda			Genus/Species
Nematomorpha				Genus/Species
Nemertea				Genus/Species
Porifera				Genus/Species