U.S. Geological Survey
National Water Quality Laboratory

Quantitative Fixed-Count Method for Processing Benthic Macroinvertebrate Sample

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Quantitative Fixed-Count Method for Processing Benthic Macroinvertebrate Samples

1. Scope, application and summary

1.1. The goal of the quantitative fixed-count processing method is to sort, identify, enumerate, and estimate the total relative abundance of benthic macroinvertebrate (BMI) taxa from a sample. The fixed-count method can be applied to BMI samples collected in the field using quantitative, semi-quantitative, or qualitative sampling methods. The quantitative fixed-count method is based on a minimum number of specimens sorted from the sample, and is defined by a project's data-quality objectives (for example, 100-, or 300-specimen fixed-count minimum). Samples with more specimens than the fixed-count minimum may be subsampled using a subsampling frame partitioned into 5.1 cm x 5.1 cm grids (Moulton and others, 2000). Multiple, randomly selected portions of the original sample (stage-1 grids) are selected and the average number of specimens in each stage-1 grid is estimated. Based on the estimated number of specimens in each stage-1 grid, an appropriate subsampling strategy is determined. Grids are then randomly selected and sorted. Total sorting time is limited, depending on the fixed-count minimum (for example, 8 hours for the 300 specimen fixed-count). Large-rare specimens are sorted from any remaining unsorted portion of the sample. Specimens are identified and enumerated.

1.2. The use of trade, product, or firm names in this procedure is for descriptive purposes only and does not imply endorsement by the U.S. Government.

1.3. Lab codes supported by this method—2172

1.4. Reporting units and levels—Standard Taxonomic Assessment and Rapid Taxonomic Assessment (see NWQL SOP No. BIOB0335.x)

1.5. Detection limits—not applicable

1.6. Interferences

1.6.1. Sorting effectiveness (SE) varies with the type and amount of sample detritus. Excessive organic detritus makes it difficult to distinguish specimens (especially small, cryptic specimens) from the sample matrix.

1.6.2. Substantial amounts of inorganic detritus (for example, sand or gravel) or large organic detritus (for example, sticks and leaves) inhibit the uniform distribution of a sample in a subsampling frame. Such detritus should be removed before proceeding with the method (see NWQL SOP No. BIOP0331.x).

1.6.3. Substantial amounts of filamentous algae should be uniformly distributed in the subsampling frame as best as possible; the algae must be carefully separated or delicate organisms (for example, mayfly larvae) may be damaged, losing taxonomically valuable body parts such as gills or legs.

1.6.4. Large specimens (for example, unionid clams or crayfish) may also inhibit the uniform distribution of a sample in a subsampling frame. Such organisms may be removed before proceeding with the method (see Section 7.6.6).

1.6.5. Sample volumes in excess of 750 mL are difficult to process with this method because it is difficult to achieve a thin even distribution of sample in the subsampling frame; the volume must be reduced to less than 750 mL before using this fixed-count method.

1.6.6. If the original estimate is not accurate enough, resulting in an excessive number of specimens being sorted from the first grid, deviation from the protocol may be needed (see Section 7.11.11).

1.6.7. Estimates indicating that the original sample contains in excess of 70,560 specimens will need to be processed differently (Moulton and others, 2000).

2. Reasons for and summary of changes

2.1. This SOP is being updated to the new format as per NWQL SOP No. QUAX0001.x.

2.2. Links to NWQL Technical/Policy Memoranda are being deleted

2.3. References to documents that are not utilized are being deleted

2.4. An incorrect cross reference to another section in the SOP was corrected in section 7.4.6

2.5. Additional detail was added to section 7.12.

2.6. A statement was added to the 'Deviations' section of this SOP to allow for deviations from this SOP at the request of a customer.

2.7. The analyst is not required to submit a new demonstration of capability based upon the revised SOP.The analyst is not required to submit a document training form for the revised SOP.

3. Health, safety, and waste disposal information

3.1. Applicable Health and Safety Issues

3.1.1. Wear long pants and closed-toed shoes at all times when working in the laboratory.

3.1.2. Wear an apron, nitrile gloves, and protective eyewear during sample processing in the hood.

3.1.3. Nitrile gloves must be changed after no more than 50 minutes exposure to ethanol to avoid breakthrough.

3.1.4. Know the location of the nearest eyewash and shower stations.

3.1.5. Do not eat or drink in the laboratory.

3.1.6. Only work in the laboratory when the room ventilation system and fume hoods are working properly. Leave the laboratory and contact the BG supervisor or appropriate Support Services section representative if the ventilation systems are not working properly.

3.2. Waste Disposal and Environmental Compliance

3.2.1. Use the vacuum waste disposal system to transfer preservative waste from the fume hood to the storage drum. Contact the BG supervisor or appropriate Support Services section representative if the system is not functioning properly. Contact a representative from the National Water Quality Laboratory (NWQL) Safety, Health, and Environmental Compliance (SHE) section when the waste drum is full and needs to be replaced.

3.2.2. Know the location of and be familiar with the Material Safety Data Sheets (MSDS) for each chemical used by the BG.

3.2.3. Know how to report and handle chemical and sample spills using procedures described in the NWQL Chemical Hygiene Plan (available from the NWQL SHE section).

3.3. All NWQL personnel will follow the guidance outlined in the following policy memoranda, accessible on the NWQL Home page (internal USGS access only) > Technical > Policy Memoranda. Supervisors will ensure that employees submit completed training forms, documenting that they have read and understood the information contained in these policy memoranda.

3.3.1. Safety, Health and Environmental (SHE) Compliance Section's Policy Memorandum 2006.01, *General laboratory health, safety, and waste disposal requirements*

3.3.2. NWQL Policy Memorandum 2008.01, *Conducting chemical and other potentially hazardous operations at the NWQL*

3.3.3. NWQL Policy Memorandum 04.01, *Pollution prevention and waste minimization policy*

3.3.4. NWQL Policy Memorandum 2010.02, *NWQL hours of operation*: High, Moderate, and Low Risks, pp. 3–4

3.4. All NWQL personnel will follow the guidance outlined in the NWQL SOP SHEX0355.x, *Waste disposal at the National Water Quality Laboratory*.

4. Sample preservation, handling, containers, analytical processing/holding times, and Disposal

4.1. Record all dates on jar/vial labels in a 'dd-mm-yyyy' format, using Roman numerals for the month. For example, July 12, 2004 is recorded as 12-VII-2004.

4.2. Place each portion of the sample in a ventilated metal cabinet designated by the BG Production Coordinator.

4.2.1. Unprocessed Samples

4.2.1.1. Each sample received is washed and re-preserved in 70-percent ethanol or processed within approximately 2 weeks of receipt at the NWQL (See NWQL SOP No. BIOP0331.x). Samples preserved in ethanol can be stored indefinitely.

4.2.1.2. Re-preserved samples are stored in their original container. If the original container is damaged, a suitable alternate may be used.

4.2.2. Unsorted Sample Remnant (minus large-rare specimens)

4.2.2.1. Label the outside of the sample container indicating that it contains unsorted sample detritus. Also include the first initials and last name of the individual who sorted the sample, and the date the sample was sorted.

4.2.2.2. Return the remnant to its original sample container. If the original container cannot be used, ensure that the proper sample identification code is on the replacement container.

4.2.2.3. Add enough 70-percent ethanol to cover the remnant sufficiently and secure the container lid.

4.2.2.4. Store the remnant in the designated cabinet.

4.2.3. Sorted Sample Remnant

4.2.3.1. Label the outside of the sample container indicating that it contains sorted sample detritus. Also include the sample identification code; the first initial and last name of the individual who sorted the sample, and the date the sample was sorted.

4.2.3.2. Place the remnant in an appropriately sized container so that the detritus occupies no more than about 2/3 of its volume.

4.2.3.3. Fill container with 70-percent ethanol; secure the lid.

4.2.3.4. Place the remnant with the corresponding unidentified BMIs in the designated cabinet. The sorted sample remnant is ready for the sorting effectiveness check (see Section 9.4).

4.2.3.5. Following the sorting effectiveness check, store the sorted sample remnant with its corresponding unsorted sample remnant in the designated cabinet.

4.2.4. Unidentified BMIs

4.2.4.1. Place a label appropriate to the taxonomic group (see Section 7.11.1) in each container along with the sample identification code and portion of sample from which it was obtained (for example, grids or lab large-rare).

4.2.4.2. Place BMIs in the appropriate container, preserve (for example, fill with 70-percent ethanol), and seal.

4.2.4.3. Place all sorted 'lab large-rare' specimens, regardless of taxonomic grouping, in a separate container. Label the container on the inside <u>and</u> outside with the sample identification code and "lab large-rare" (see Section 7.12.5).

4.2.4.4. Place all the vials in a rack labeled with the sample identification code.

4.2.4.5. Place the rack of vials, any associated containers, and the sorted sample remnant in the designated cabinet.

4.2.5. Identified BMIs

4.2.5.1. Place a label in each container with the determination, first initial and last name of the taxonomist, date, sample identification code, and portion of sample from which it was obtained.

4.2.5.2. Place BMIs in the appropriate container, preserve (for example, fill with 70-percent ethanol), and seal.

4.2.5.3. After non-chironomid BMIs have been identified from a sample, put the labeled vials in a rack(s) and place it on a shelf in the designated cabinet with other samples completed that week.

4.2.5.4. Chironomidae larvae and pupae identified to family that are not to be mounted should have the added designation "retained" recorded on the label and be placed in the rack with the other identified non-chironomid BMIs.

4.2.5.5. Chironomidae identified to family and designated for mounting should have the added designation "TO MOUNT" recorded on the vial label and be placed in the rack designated for chironomids to be mounted from samples completed that week.

4.2.5.6. Slide mounted chironomids should be stored in slide boxes (see NWQL SOP No. BIOP0334.x) and placed on the shelf following the vial racks for samples completed during the corresponding week.

4.2.5.7. Following QC, selected vials and/or slides of identified BMI taxa will be added to the reference collection by a BG representative. All non-referenced vials/slides are stored in designated cabinets.

4.3. Sample remnants and/or BMIs can be disposed of according to NWQL Policy Memorandum 00.03.

5. Preparation of Reagents, standards, and solvents

5.1. Tap water

5.2. 70-percent ethanol (see NWQL SOP No. BIOP0331.x)

6. Apparatus

6.1. Labware

6.1.1. 2-inch wide clear tape

6.1.2. Forceps

6.1.3. Labels for vials, racks, and remnants

6.1.4. Large plastic spoon

6.1.5. Plastic washbasins

6.1.6. Scissors

6.1.7. Scoopula® or other small scoop

6.1.8. Screw-cap vials (4–6 dram preferred) with polyseal caps

6.1.9. Scrub brush

6.1.10. Small fine-bristle brush

6.1.11. Squirt bottle(s) for 70-percent ethanol and/or water

6.1.12. Various sizes/types of containers as needed

6.1.13. Vial racks

6.1.14. Watch glasses or other similar glass dishes

6.1.15. White sorting trays of various sizes (for example, 15 x 20 cm and 20 x 30 cm)

6.2. Equipment

6.2.1. Tally counter

6.2.2. Binocular microscope

6.2.3. Light source (fiber-optic illuminator or portable incandescent lamp)

6.2.4. Estimation trays (49- and 81-grid frames, each with 1.3-cm x 1.3-cm grids)

6.2.5. Subsampling frames (12-, 24-, and 42-grid frames, each with 5.1-cm x 5.1-cm grids)

6.2.6. Post-sort sieve (210 µm mesh)

6.2.7. Standard metal sieve(s) with mesh size equal to or slightly smaller than the field-collection mesh size (see Section 9.2.1)

7. Sample preparation, analysis, and instrument operation and shutdown

7.1. Work order/worksheet handling—not applicable

7.2. Obtain the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x), the BMI Identification and Enumeration Bench Data Sheet (see Attachments 7 or 9 in NWQL SOP No. BIOB0335.x), the Slide Preparations – Identification and Enumeration Worksheet (see Attachment 10 in NWQL SOP No. BIOB0335.x), the preprinted vial labels and original sample container(s) for the sample from the BG Production Coordinator or from its designated location(s).

7.3. Prepare the sample according to NWQL SOP No. BIOP0331.x.

7.3.1. Ensure that any substantial amount of inorganic detritus has been removed (see NWQL SOP No. BIOP0331.x).

7.4. If the sample volume is substantially less than 250 ml so that the sample can be easily distributed in an 81-grid estimation tray <u>and</u> the density of specimens appears to be low, proceed with a rapid estimation of specimen density.

7.4.1. Distribute the entire sample in an 81-grid estimation tray.

7.4.2. Randomly select eight (8) estimation tray grids (see Section 7.8.3) and record their coordinates on the back of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.4.3. Determine the number of specimens in each grid and record the value next to the grid's coordinates on the back of the worksheet.

7.4.4. Determine the total number of specimens in all eight (8) grids and multiply by ten (10) to obtain a rapid estimate of the total number of specimens in the sample. For example, if a total of 32 specimens were found in the eight (8) grids, the rapid estimate would be 320 specimens for the entire sample.

7.4.5. If the rapid estimate does not exceed the minimum number requested by greater than 50 percent, sort the entire sample (see Section 7.11). For example, if the requested minimum is 300, the rapid estimate should not exceed 450 specimens.

7.4.6. If the rapid estimate exceeds the requested minimum by greater than 50 percent, but is less than four (4) times the minimum, then select a 12-grid subsampling frame and proceed to Section 7.11; otherwise, proceed to Section 7.5. For example, if the requested minimum number of specimens is 300, use a 12-grid subsampling frame if the estimate is between 450 and 1200; otherwise, a larger frame is needed.

7.5. Select a stage-1 subsampling frame in which the sample can be distributed.

7.5.1. The approximated sample volume can aid in determining which frame is selected, but BMI density should also be considered.

Sample volume (mL)	Suggested subsampling frame
< 250	12 grid
250 – 500	24 grid
500 – 750	42 grid

7.5.2. Inspect the frame for detritus and/or BMIs that may have been left in the frame from the previous sample; remove and discard any that are found.

7.5.3. Record the stage-1 subsampling frame information (for example, '3 x 4' and '12') on the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.6. Transfer the sample into the stage-1 subsampling frame

7.6.1. Place the selected stage-1 subsampling frame in a large, flat-bottomed tray.

7.6.2. Transfer the sample into the stage-1 subsampling frame

7.6.3. Fill the subsampling frame slowly with just enough water to cover the sample. Too much water will cause some specimens and detritus to float and adversely affect the distribution of the sample.

7.6.4. Remove any air bubbles trapped beneath the mesh on the subsampling frame.

7.6.5. Distribute the sample uniformly over the entire subsampling frame.

7.6.6. Remove additional large detritus such as leaves and sticks if they are inhibiting uniform sample distribution (see NWQL SOP No. BIOP0331.x). Large specimens may also inhibit uniform distribution of detritus in the frame. These specimens should be rinsed, removed, and set aside for inclusion in the lab large-rare (see Section 7.12.5).

7.6.7. If necessary, re-distribute the sample as uniformly as possible over the subsampling frame.

7.6.8. Allow the sample to settle for a few seconds.

7.6.9. Keeping the subsampling frame level, carefully lift it out of the water and drain.

7.6.10. Set the subsampling frame aside, and discard the water in the large flat-bottomed tray.

7.6.11. Return the subsampling frame to the flat-bottomed tray, and cover it until the grids are removed.

7.6.12. If necessary, mist the sample with water periodically to prevent drying.

7.7. Randomly select five stage-1 grids from the stage-1 subsampling frame.

7.7.1. Select five pairs of random numbers to represent row/column coordinates on the stage-1 subsampling frame. Record the coordinates in the estimation section of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.7.1.1. Random number tables are available in ranges of 1 to 3, 1 to 4, 1 to 6, or 1 to 7.

7.7.1.2. Use two random number tables with ranges that match the respective number of rows and columns in the selected subsampling frame. For example, if a 12-grid (3 x 4) stage-1 frame is selected use a table with a range of 1 to 3 to select the rows and a table with a range of 1 to 4 to select the columns.

7.7.1.3. When a duplicate pair is selected, replace the column coordinate until the pair is unique.

7.7.1.4. Cross off the numbers on the tables as they are used.

7.7.1.5. Generate new tables if all the numbers have been crossed off.

7.7.2. Use the coordinates to locate the five grids in the stage-1 subsampling frame. For example, a 24 grid stage-1 frame has 4 rows and 6 columns; the random number pair (2,4) corresponds to the grid in row 2 of column 4.

7.7.3. Separately remove the sample detritus from each of the selected stage-1 grids and place it in a jar numbered to correspond to the row with its respective coordinate on the worksheet. Use a Scoopula® to remove most of the detritus; forceps or a fine-bristle brush may be helpful in removing small specimens and residual fine detritus.

7.8. Estimate the mean number of specimens in each of the five stage-1 grids.

7.8.1. Select either a 49-grid or 81-grid estimation tray on the basis of the average stage-1 grid volume; use the smallest tray that allows for uniform distribution of detritus that is not so dense as to obscure a clear view of the organisms. The same size estimation tray should be used for all stage-1 grids.

7.8.2. Record the estimation tray size (for example, 49 or 81) on the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.8.3. Select three pairs of random numbers for each stage-1 grid to represent row/column coordinates in the estimation tray. Record the coordinates on the worksheet.

7.8.3.1. Random number tables are available in ranges of 1 to 7 or 1 to 9.

7.8.3.2. Use a random number table with a range that matches the respective number of rows and columns in the selected estimation tray. For example, if a 49-grid tray is selected use a table with a range of 1 to 7 to select the row/column coordinates.

7.8.3.3. When a duplicate pair is selected, replace the column coordinate until the pair is unique.

7.8.3.4. Cross off the numbers on the table as they are used.

7.8.3.5. Generate a new table if all the numbers have been crossed off.

7.8.4. Distribute the sample detritus from each stage-1 grid as thinly and evenly as possible in the estimation tray.

7.8.5. Locate the estimation-tray grid corresponding to each pair of coordinates and count all specimens in the grid. For example, a 49-grid estimation frame has 7 rows and 7 columns; the random number pair (4,5) corresponds to the grid in row 4 of column 5.

7.8.5.1. If a specimen lies across two or more grids, count it if its head lies within the selected grid.

7.8.5.2. If a specimen lies across two or more grids and its head does not lie within the selected grid, then only enumerate the specimen if its head does not lie in another selected grid.

7.8.6. Record the number of specimens in each selected estimation tray grid on the worksheet.

7.8.7. Return the sample detritus from each stage-1 grid (sample and tallied specimens) to its corresponding numbered jar.

7.8.8. Enter the information from the estimation process into the appropriate Microsoft® Excel version of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x) to obtain a recommended subsampling strategy. Available fixed-count worksheets include:

300 MINIMUM COUNT.xls

100 MINIMUM COUNT.xls

7.8.8.1. Enter the sample identification code, the size of the stage-1 subsampling frame (for example, 12, 24, or 42), and the size of the estimation tray used (for example, 49 or 81).

7.8.8.2. Enter the three estimation tray grid counts for each stage-1 grid into the corresponding fields of the Microsoft® Excel version of the worksheet. (Note: the spreadsheet will perform all calculations to obtain the average number of specimens in each stage-1 grid.)

7.8.8.3. After the calculations are made, processing guidance is displayed in the spreadsheet. Transcribe the processing guidance onto the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x). For example, "Sort 4 grids from a 24 grid stage-1 frame."

7.8.8.4. Save the estimation results with the file name "*sampleID*.xls" (for example, WILL0904IRM0003A.xls) in a directory designated by the BG Production Coordinator.

7.9. Using the information obtained from the estimate, either sort the sample (see Section 7.11) or perform additional subsampling (see Section 7.10) as recommended by the Microsoft® Excel version of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.9.1. If the Microsoft® Excel version of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x) is not available, select an appropriate subsampling strategy using the guidance presented in Moulton and others (2000).

7.9.2. Consult the BG Production Coordinator if in doubt how to proceed.

7.10. Procedures when 2-stage subsampling is recommended.

7.10.1. Record the stage-2 subsampling frame size on the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.10.2. Transfer the recommended number of stage-1 grids to the stage-2 subsampling frame and evenly distribute the detritus (see Section 7.6).

7.10.2.1. If the recommendation is to transfer fewer than five (5) stage-1 grids to the stage-2 subsampling frame, select and transfer the first three (3) or four (4) stage-1 grids as they are listed in the estimation worksheet.

7.10.2.2. Fewer than three (3) stage-1 grids should not be transferred to subsequent subsampling frames.

7.10.3. Randomly select the appropriate number of stage-2 grids from the stage-2 subsampling frame in the same fashion they were selected from the stage-1 subsampling frame (see Section 7.7). Record the grid coordinates, in order, in the sorting section of the worksheet.

7.10.4. Separately remove the sample detritus from each selected stage-2 grid and place in a jar numbered to correspond to the row with its respective coordinates in the sorting section of the worksheet.

7.11. Sort specimens from the randomly selected grids.

7.11.1. Prepare a labeled rack of vials filled with 70-percent ethanol corresponding to the following taxonomic groupings as needed: Gastropoda, Bivalvia, Oligochaeta, Hirudinea, Acari, Decapoda, Amphipoda/Isopoda, Ephemeroptera, Odonata, Plecoptera, Heteroptera, Megaloptera, Trichoptera, Lepidoptera, Coleoptera, Diptera (excluding Chironomidae), Chironomidae, Other, or Miscellaneous.

7.11.2. Sort selected grids in order.

7.11.2.1. If sorting stage-1 grids, transcribe their coordinates, in order, from the estimation section to the sorting section of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x), and sort them in the order they are recorded.

7.11.2.2. If sorting stage-2 grids, sort them in the order they are selected and recorded on Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.11.3. Record the time (hh:mm) when sorting begins on the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x).

7.11.4. Place each grid or a portion of the grid in an estimation tray. Grid marks on the estimation tray can be used as a guide while sorting so that portions of the sample are not missed.

7.11.5. Begin sorting specimens from the estimation tray under a dissecting microscope at 10X magnification.

7.11.5.1. Do not sort the following specimens or life stages: vertebrates, arthropod exuviae, empty mollusk shells, branchiobdellids (worm-like crayfish parasites), eggs, microcrustaceans, or terrestrial specimens. If in doubt, sort the specimen but do not count it.

7.11.5.2. Do not pry open detritus to search for burrowing specimens.

7.11.6. Place specimens into vials by taxonomic grouping (see Section 7.11.1).

7.11.7. Use a counter to keep track of the number of specimens sorted from each grid.

7.11.8. Always sort a grid to completion.

7.11.9. Composite the sorted detritus to form the sorted sample remnant.

7.11.10. Upon completion of a grid, record the stop time (hh:mm), number of specimens sorted and the total sort time in the appropriate space in the sorting section of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x). Total sort time should be rounded to the nearest 0.1 hours (6 minutes) and recorded as decimal hours (for example, 19 minutes should be recorded as 0.3 hours).

7.11.11. After completely sorting either the first or second grid, if it is estimated that the minimum number of specimens will be exceeded by more than 50 percent (for example >150 specimens sorted from the first grid, when using a 300 specimen minimum), consult the BG Production Coordinator for guidance or calculate a revised density estimate and consult Mouton and others (2000) to determine a more appropriate subsampling strategy.

7.11.12. After completely sorting at least three (3) grids:

7.11.12.1. If it is estimated that the final number of specimens sorted from the <u>entire</u> subsampling frame will be more than 25-percent <u>below</u> the minimum (for example, an estimate of less than 225, when using a 300 specimen minimum) <u>and</u> that the time limit (see Section 7.11.13.2) will not be exceeded by sorting the entire frame, the remaining detritus should be sorted completely. Systematic random grid removal is not necessary. If the minimum is exceeded by more than 50% while sorting in this fashion, the sample will need to be resorted

7.11.12.2. If the total number of organisms sorted from the sample is more than 50-percent above the minimum (for example, greater than 450 when using a 300 specimen minimum), the sample should be recombined and processed appropriately based on a revised estimated density that can be calculated from the total number of organisms sorted. Consult the BG Production Coordinator for guidance.

7.11.13. If at least three (3) grids have been sorted, sorting can stop when any of the following conditions are met:

7.11.13.1. The minimum number of specimens is exceeded by at least 5-percent at the completion of a grid. For example, at least 315 specimens have been sorted, when using a minimum of 300.

7.11.13.2. The time limit for a given minimum is reached at the completion of a grid.

7.11.13.2.1. 8 hours for a 300-specimen minimum

7.11.13.2.2. 3 hours for a 100-specimen minimum

7.11.13.3. Sorting one more grid will cause the time limit to be exceeded, even if the minimum has not been reached. For example, if the total sorting time is 7.8 hours and the average time per grid has been 0.4 hours, sorting can stop.

7.12. When sorting is complete (see Section 7.11.13),

7.12.1. Determine and record the total count for specimens sorted and total sort time, rounded to the nearest 1/10th hour (6 minutes) on the worksheet. For example, 3 hours 11 minutes is recorded as 3.2 hours.

7.12.2. Calculate and fill in the 'Correction factor' section of the worksheet. The correction factor entered here is different than that referred to in Section 8.1, as it is used to facilitate ease of data entry.

7.12.2.1. Example 1: If four (4) stage-1 grids are sorted from a 24-grid, stage-1 subsampling frame, enter *4:24*.

7.12.2.2. Example 2: If five (5) stage-1 grids are combined and transferred from a 24-grid, stage-1 subsampling frame into a 12-grid, stage-2 subsampling frame, and four (4) stage-2 grids are sorted, enter *20:288*.

7.12.3. Ensure that the size(s) of the subsampling frames used and the number of grids either sorted or transferred to subsequent trays is recorded accurately on the worksheet.

7.12.4. Obtain the initials of an individual designated by the BG Production Coordinator, indicating that the actual subsampling strategy corresponds to the information recorded on the worksheet.

7.12.5. Perform a 15-minute visual-sort for large-rare specimens occurring in any unsorted sample detritus. Regardless of relative abundance or size, specimens may be included if they are likely to represent new taxa.

7.12.6. Enter the final subsampling information into the Microsoft® Excel version of the worksheet.

7.12.7. Save the file and print a hardcopy of the Microsoft® Excel version of the completed worksheet.

7.12.8. Update the sample status is sorted with the sorter's last name and date (mm/dd/yyyy) that the sorting was completed.

7.13. Clean-Up

7.13.1. Preserve and label all sample components as indicated in section 4.

7.13.2. Inspect all subsampling frames, estimation trays, sieves, sorting trays, and washbasins for retained specimens. Return any specimens found to the sample.

7.13.3. Rinse and clean all estimation trays, sieves, sorting trays, subsampling frames, and washbasins used to process the sample.

7.13.4. Scrub sieves with a brush and rinse from both sides to remove entrained sample detritus.

7.13.5. Wipe up water and clean workstation.

7.13.6. Put away all supplies and equipment.

7.14. Identify the Sample

7.14.1. Identify all unmounted taxa referring to NWQL SOP No. BIOB0335.x

7.14.2. Mount taxa as per NWQL SOP No. BIOP0334.x.

7.14.3. Identify all mounted taxa as per NWQL SOP No. BIOB0335.x

8. Data acquisition, processing, and evaluation

8.1. Calculation of the laboratory subsampling correction factor [W = total grids in the stage-1 subsampling frame, X = total grids sorted from the stage-1 subsampling frame, Y = total grids in the stage-2 subsampling frame, Z = total number of grids sorted from the stage-2 subsampling frame]

	Subsampling strategy	
	1-Stage subsampling	**2-Stage subsampling**
Laboratory subsampling correction factor (*L*)	$L = \frac{W}{X}$	$L = \frac{W}{X_1} \times \frac{Y}{Z}$

[1]In 2-stage subsampling, X will typically be 3, 4, or 5, depending on the actual strategy.

9. Quality control (QC) and quality assurance (QA)

9.1. Individuals sorting quantitative samples need to have been trained by the BG and been approved to sort samples under the routine sorting effectiveness protocols (see Section 9.4).

9.2. Before starting method

9.2.1. If the field-collection mesh size is unknown, stop work immediately and contact the BG Production Coordinator.

9.2.2. Inspect all sieves, washbasins, and sorting trays, and subsampling frames to make sure there are no specimens or sample detritus remaining from a previously processed sample.

9.2.3. If applicable, compare the information on the internal and external sample labels; report any discrepancies to the BG Production Coordinator.

9.2.4. Contact the BG Production Coordinator immediately with any problems related to the processing of the sample.

9.3. Verify that information recorded on the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x) is correct prior to the large-rare sort. An individual designated by the BG Production Coordinator performs this verification.

9.4. Determine Sorting Effectiveness

9.4.1. The sorting effectiveness check detects gross errors. Typical errors include incompletely sorting one or more grids or routinely missing certain taxa (for example, tiny case-building caddisflies).

9.4.2. An individual designated by the BG Production Coordinator checks sorting effectiveness on each sample prior to taxonomic identification.

9.4.3. Samples sorted by personnel inexperienced in the processing methodology are re-sorted one grid at a time while the sample is being processed. In this manner, a designated individual reviews at least the first five samples processed. If the BG Production Coordinator deems their performance satisfactory, routine sorting effectiveness checks are performed on future samples.

9.4.4. Routine Sorting Effectiveness

9.4.4.1. Each sorted sample remnant is re-sorted at 10X magnification.

9.4.4.2. Re-sort time is limited to 10-percent of the original sort time. For example, if a sample was originally sorted in 3.0 hours, then the re-sort is limited to 0.3 hours (18 minutes).

9.4.4.3. Specimens obtained during a re-sort are counted and added to the appropriate original sorting vials.

9.4.4.4. The sorting effectiveness statistic (E_s) is calculated as:

$$E_S(\%) = 100\% \cdot \frac{S}{R+S}$$

where, R = the total specimens obtained during the re-sort of the sorted sample remnant

S = the total specimens originally obtained from the sorted sample remnant

9.4.4.5. E_s should be ≥ 90 percent for the sample to pass.

9.4.4.6. The total number of specimens recovered, initials of the individual performing the sorting effectiveness check, and the date (mm/dd/yyyy) are recorded on the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x). 'PASS' or 'FAIL' should also be circled.

9.4.5. Corrective Actions

9.4.5.1. Regardless of whether or not the sample passes or fails, recommendations made by the individual that performed the sorting effectiveness check are implemented in the sorting of future samples.

9.4.5.2. The intent of the sorting effectiveness check is to discover gross errors that might have resulted in different processing decisions during the sorting phase (for example, fewer grids sorted). This is a minimum count method, so unless it is determined that significant savings in time can be gained during the identification phase, gross errors have been made, or the errors persist, the recommendations will be limited to future modifications of the technician's procedures.

9.4.5.3. If a sample fails the SE check, 1-stage subsampling was performed, and the following is true, consult with an individual designated by the BG production coordinator to determine if the sample should be reprocessed. See Table 2 for general guidance.

$$T < R + S - \left[\frac{R+S}{X} \right]$$

where, R = the total specimens obtained during the re-sort of the sorted sample remnant as in section 9.4.4.4

S = the total specimens originally obtained from the sorted sample remnant as in section 9.4.4.4

T = the minimum sorting goal as defined in section 7.11.13.1

X = total number of grids sorted from the stage-1 subsampling frame as in section 8.1

9.4.5.4. If the sample fails the SE check, 2-stage subsampling was performed, and the following is true, consult with an individual designated by the BG production coordinator to determine if the sample should be reprocessed, See Table 2 for general guidance.

$$T < R + S - \left[\frac{R+S}{Z} \right]$$

where, R = the total specimens obtained during the re-sort of the sorted sample remnant as in section 9.4.4.4

S = the total specimens originally obtained from the sorted sample remnant as in section 9.4.4.4

T = the minimum sorting goal as defined in section 7.11.13.1

Z = total number of grids sorted from the stage-2 subsampling frame as in section 8.1

Table 2. Minimum total number of specimens indicating action might be warranted for common final grid counts.
[1]action level differs from formula due to the fact that it is assumed that either 150 or 300 organisms would have been sorted from either the 1st or 2nd grid.

Final Grids Sorted	Action Level
3	450[1]
4	420
5	394
6	378
7	368

9.5. Taxonomic QC—as described in NWQL SOP No. BIOB0335.x.

10. Data management and record

10.1. Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x):

10.1.1. The individual that sorted the sample should initial in the appropriate space.

10.1.2. The individual that confirms that the subsampling and processing is accurately reflected on the worksheet should initial in the appropriate space and record the date (mm/dd/yyyy) the check is performed.

10.1.3. The individual performing the sorting effectiveness check should record the number of specimens obtained during the sorting effectiveness check, their initials, the date of the check (mm/dd/yyyy), and circle either 'PASS' or 'FAIL' as appropriate.

10.1.3.1. When checking the sorting effectiveness during the training period (see Section 9.4.3), instead of recording the number of specimens obtained, enter "training", the date of sorting completion, and circle 'PASS'. Make notes on the back of the estimation worksheet as to your overall impression of the sorting effectiveness of the trainee.

10.1.4. All spaces not highlighted in gray should be completed, with the exception of sorting data in the sorting section beyond the number of sorted grids (see Section 7).

10.1.5. Problems or errors associated with the sample (for example, sample was spilled or wrong sieve was used). Attach additional sheets as needed.

10.1.6. File the completed hand-written worksheet with the remaining sample paperwork.

10.2. Save the Microsoft® Excel version of the Subsampling and Preliminary Enumeration Worksheet (see Attachment 6 in NWQL SOP No. BIOB0335.x) in the directory designated by the BG Production Coordinator and back it up regularly. A printed hardcopy is filed with the remaining sample paperwork.

10.3. BMI Identification and Enumeration Bench Data Sheet (see Attachment 7 in NWQL SOP No. BIOB0335.x):

10.3.1. Circle either '100' or '300' as appropriate depending on the minimum number of specimens requested or enter the minimum number of specimens requested in the space provided.

10.3.2. The individual that sorted the sample should record their first initial, full last name, and the date (mm/dd/yyyy) that the sorting was completed.

11. Definitions, abbreviations, and acronyms

11.1. Detritus—organic and/or inorganic matter of any size present in a BMI sample, excluding the specimens.

11.2. Estimation tray—a small gridded tray (either 49- or 81-grids) used to estimate the number of specimens in a stage-1 grid.

11.3. Large-rare—large and generally rare specimens (occurring at low frequencies) present in a sample that may or may not be represented by those previously sorted.

11.4. Visual sort—Sorting specimens from a sample without the aid of magnification.

11.5. 1-stage subsampling—A procedure to obtain randomly selected square-grid subsamples from the original sample.

11.6. Stage-1 subsampling frame—gridded subsampling frame (either 12-, 24-, or 42-grids) used to obtain square-grid subsamples from the original sample.

11.7. Stage-1 grid—a randomly selected square-grid from a stage-1 subsampling frame.

11.8. Stage-1 subsample—the resulting composite of all sorted stage-1 grids.

11.9. 2-stage subsampling—A procedure to obtain randomly selected square-grid subsamples from a stage-1 subsample.

11.10. Stage-2 subsampling frame—gridded subsampling frame (either 12-, 24-, or 42-grids) used to obtain square-grid subsamples from a stage-1 subsample.

11.11. Stage-2 grid—a randomly selected square- grid from a stage-2 subsampling frame.

11.12. Stage-2 subsample—the resulting composite of all sorted stage-2 grids.

12. Deviations

12.1. This SOP allows for the removal of large organisms prior to subsampling if it is deemed that they are inhibiting the uniform distribution of the detritus in the subsampling frame.

12.2. NAWQA is asking the Biological Group (BG) to evaluate modifications to this procedure to gain efficiencies that may reduce the amount of time and/or supplies required to process samples. Certain sections of OFR 00-212 (http://nwql.usgs.gov/Public/pubs/OFR00-212.pdf) require that specific procedures, equipment, and/or supplies be used. Since the BG does not have access to samples that can be used for evaluation, actual field samples must be used to evaluate process and/or equipment modifications. As a result, the BG may deviate from the requirements specified in this SOP to evaluate potential procedural modifications. Deviations that are isolated to a single sample will be documented on the appropriate sample paperwork. Documentation of deviations that are more global in nature will be kept in a laboratory notebook dedicated for that purpose. All samples to which this statement applies will have a sticker affixed to them referring to this section of the appropriate SOP. In any case, the BG staff will use its best professional judgement to ensure that deviations do not violate the intent of OFR 00-212. If there is concern regarding whether or not the deviation will violate the intent of the OFR, BG staff will consult with the customer and the supervisor of the BG.

13. References

Moulton, S.R., II, Carter, J.L., Grotheer, S.A., Cuffney, T.F., and Short, T.M., 2000, Methods of analysis by the U.S. Geological Survey National Water Quality Laboratory — processing, taxonomy, and quality control of benthic macroinvertebrate samples. U.S. Geological Survey Open-File Report 00-212.

14. Key Words

benthic macroinvertebrate, fixed-count method, quantitative sample processing,

15. Attachments

None