# Standard Operating Procedure for Preparation and Homogenization of Fish Tissue Samples

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

Effective Date: July 1, 2014

Revision Date: March 1, 2019

Revision No: 3.0

Document Control No: DOWSOP0300032

## Revision History

| Date of Revision | Page(s) Revised | Revision Explanation |
|---|---|---|
| Nov. 1, 2018 | All pages | General editing and clarification. Addition of electronic log, storage of homogenized sample in glass jar, addition of large fish subsample, add example photographs and clarification of whole body processing. |
| May 1, 2017 | All pages | Sections added include Lyophilization homogenization, dissection of whole bodies and dry weight to wet weight calculations. Revised Fish Tissue Data Sheet and created Lyophilization Data Sheet and Scale Check Log. |
| July 1, 2014 | All pages | Laboratory Procedures for Resection of Fish Fillets and Homogenization of Tissue Samples was separated from preceding document and revised/updated for general content regarding laboratory methods. |
| March 13, 2008 | All pages | **Standard Methods for Assessing Biological Integrity of Surface Waters in Kentucky** General Content-Document was re-formatted for maintaining headers, section titles, etc. in a consistent style. All references to detailed water chemistry sampling were removed, and a reference inserted directing the reader to the 'Standard Operating Procedures for Sampling and Monitoring Surface Waters for Kentucky', in draft. |
| July, 2002 | All pages | Methods for Assessing Biological Integrity of Surface Waters in Kentucky original document. |

**Suggested Citation:** Kentucky Division of Water (DOW). 2019. Standard Operating Procedure for Preparation and Homogenization of Fish Tissue Samples, Version 3.0. Kentucky Department for Environmental Protection, Division of Water, Frankfort, Kentucky.

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## Procedures

### Scope and Applicability

This manual has been developed by the Division as guidance for uniform and accurate procedures used in preparation and homogenization of tissue samples. The procedures defined herein are required for the preparation and homogenization of tissue samples and QA/QC activities resulting in information used for issuing fish consumption advisories and the biennial Integrated Report to Congress on Water Quality in Kentucky (305[b] and 303[d] Reports).

Fish consumption advisories are jointly issued by representatives from the Department for Environmental Protection, Department of Fish and Wildlife Resources and Department for Public Health when contaminants in fish tissue exceed the level considered safe for unlimited human consumption. On December 6, 2004 each agency signed an Interagency Agreement to work together on the issuance of fish consumption advisories. The Interagency Agreement was last updated June 24, 2015. The Interagency Agreement outlines the roles of each agency, but does not detail the standard operating procedures concerning how fish consumption advisories should or will be issued. Human health risk-based methodologies, based on previously developed protocols by the Great Lakes Sport Fish Advisory Task Force (GLSFATF 1993) and the U.S. Environmental Protection Agency (USEPA 2000a), are used to determine if fish consumption advisories should be issued and what restriction level the advisories recommend. The protocols provide information in greater detail to target populations such as women of childbearing age and children, and recommends the number of fish meals a person may consume to minimize health risks.

### Definitions

Division – Kentucky Division of Water
KWADE – Kentucky Water Assessment Data for Environmental Monitoring
SDS –Safety Data Sheet
PPE – Personal Protective Equipment
PTFE – Polytetrafluoroethylene (Teflon)

## Health & Safety Policy/Section

Proper PPE shall be worn by all personnel while processing samples and handling chemicals. Refer to the appropriate SDS for the correct PPE while handling chemicals. It is recommended that lab coats also be worn to protect clothing from spillage. Protective eyewear must be worn when the potential exists for particulate, vapor, liquid or foreign objects to become lodged in the eye. When working with chemicals that cause harmful fumes, personnel shall use a fume hood to reduce the threat of inhalation exposure to them and their fellow coworkers.

Toxic or caustic materials must be stored in a chemical storage cabinet. When a chemical spill (e.g. a broken mercury thermometer, broken large containers of acids or preservatives) 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. Extreme care shall be taken when processing tissue samples. When cutting frozen fish samples with a band saw or knife, fingers are to be kept away from the blade at all times. Care shall be taken when handling and cleaning blenders to reduce the chance of cutting fingers by the blades. Ear protection shall be used when loud machinery will be in use (i.e. processing fish tissue samples using saws or other electronic machinery).

If injury or exposure occurs within the laboratory facilities, 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 SDS for first aid treatment. Safety Data Sheets shall be maintained in a readily accessible location in the laboratory 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 according to the Energy and Environment Cabinet's official protocols within 24 hours of exposure or injury.

## Cautions

Several cautions exist with regard to activities and negligence that could possibly cause equipment damage, degradation of the sample and possible invalidation of the results. Potential sources of contamination include dust, instruments, utensils, work surfaces and containers that may contact the samples. All sample processing (i.e., filleting, removal of other tissue, homogenizing, compositing) will be processed in a clean environment set away from sources of potential contamination. All instruments, work surfaces, and containers used to process samples must be of materials that can be cleaned easily and that are not themselves potential sources of contamination. The predominant metal contaminants from stainless steel are chromium and nickel. If these metals are a concern, then use of stainless steel in processing should be limited and/or appropriate equipment and rinsate blanks procured. If chromium and nickel are not a concern, the use of high-quality, corrosion resistant stainless steel for sample

processing equipment is acceptable. If aluminum is of concern, the tissue samples should be placed on glass cutting boards and in glass or plastic containers. Stainless steel instruments and heavy duty aluminum foil are in use by the Division; therefore, if the above parameters are of concern, additional equipment may need to be obtained before processing of tissue samples. Equipment rinsate blanks may be used to evaluate the possibility of contamination (USEPA 2000).

**Personnel Qualifications / Responsibilities**

All biologists will meet at least the minimum qualifications for their job classification. In addition, fisheries biologists will be trained in the collection and identification of fish by formal academic instruction. Fisheries biologists that have not had formal academic instruction in collection and identification of fish will be deemed technically competent based on their knowledge, skills and abilities by Division management. Taxonomic education will continue with on-the-job training, interaction with experienced taxonomists and continued outside training when education opportunities become available. All laboratory personnel performing sample processing procedures should be trained or supervised by an experienced fisheries biologist in the laboratory procedures for resection of fish fillets and homogenization of tissue samples.

**Equipment and Supplies**

The following is a list of common equipment and supplies typically employed:

- Heavy duty aluminum foil
- Cutting board
- Stainless steel filet knife
- Knife sharpening stone
- High speed stainless steel blender (various sizes)
- Freezer (≤-20$^{o}$C)
- Disposable gloves (powder free)
- 60 & 250 mL Certified Widemouth Glass Jar w/PTFE lined cap #3200060 & 3200250 ThermoScientific
- Acetone – OPTIMA grade
- Deionized water
- Squirt bottles
- Whirl-pack® (various sizes)
- Ziptop bags (various sizes)
- Fish Tissue Data Sheets/Log
- Lyophilization Data Sheets/Log
- Taxonomic literature (Appendix A)
- Laboratory detergent (Liquinox®)

- Cleaning brushes
- Stainless steel trays
- Freeze dryer
- Precision balance (<=0.01 g)
- Measuring Board
- Food Processor
- Stainless steel counter tops
- Fume hood

**Methods**

The following sections describe the laboratory procedures for the preparation and homogenization of fish tissue samples. Samples are collected at designated sites for fish contaminant studies and put on ice in clean coolers for sample transport. Individual samples can be laid directly on ice in contact with other samples from the same sample site as long as they are rinsed in deionized water before resection and there is no risk of puncturing the skin or samples may be placed in zip top bags before placing on ice. Taxonomic references are listed in Appendix A.

Fish fillets and/or whole body samples will be the tissue types covered in these procedures. To assess mercury for water quality standards and consumption limits, skin-off fillets will be used. To assess the selenium water quality standard, whole body samples will be used.

**NOTE**: Spines from fish (i.e. catfish species) may be cut off prior to placing in coolers if they will puncture other fish or zip top bags

*Initial Sample Processing*

Fish will be identified, weighed (g), measured (mm) and recorded on the electronic Fish Tissue Sample log or Fish Tissue Field Datasheet (see example in Appendix B). The total body length will be determined by measuring from the anterior most part of the fish to the tip of the longest caudal fin ray (when the lobes of the caudle fin are compressed). All samples must be recorded into the electronic Fish Tissue Sample log or Fish Tissue Field Datasheet (see example in Appendix B). Data entered on the Field Datasheet must also be entered into the Sample Log after returning from the field. Information included in the Fish Tissue Data Sheet or Sample Log include:

- **SampleID**: assigned by letters “FT” + current year + consecutive order (i.e. FT18-001)
- **StationID**: KWADE station id
- **Waterbody**: Waterbody sample collected from
- **Location**: location sample collected on waterbody
- **Species**: Species of the sample collected.

- **Collection Method**: Method used to collect fish samples
- **Sample Date**: Date sample collected
- **Sample Start and End Time**: sample collection time (EST),
- **Collectors**: biologist(s) that collected sample
- **Resection Date**: Date sample processed
- **Resection Start and End Time**: sample resection time (EST),
- **Resection Analyst**: laboratory personnel who performed task
- **Sample Method:** Individual or a composite sample.
- **Tissue Type:** Whole body, Right, left or both fillets
- **Length:** Length of the sample collected in millimeters.
- **Weight:** Weight of the sample collected in grams.
- **Sex:** Sex of sample collected.
- **Aging Method: O**=Otolith; **S**=Scale; **F**=Fin; **S**=Spine
- **Age:** Age of fish collected.

*Cleaning of Work Utensils*

Equipment will be cleaned following USEPA (2000) for both organic and metals analysis between the processing of each sample. Equipment should be cleaned thoroughly with a detergent solution (i.e. Liquinox), rinsed with tap water, soaked in 50 percent $HNO_3$, for 12 to 24 hours at room temperature and rinsed in deionized water. Acids used should be at least reagent grade. Stainless steel parts and any material that may be damaged by acid may be cleaned using this recommended procedure with the acid soaking step method omitted. After cleaning, equipment is placed in drying oven (~100 $^{o}$C) to dry or allowed to air dry if not needed immediately. If equipment is not immediately used after drying, cover blender cups with aluminum foil and place spoons/knives in stainless steel pan with aluminum foil cover to prevent contamination.

**NOTE**: If glass sample containers have a certificate for Certified Environmental Sample Containers, the cleaning process for those containers is omitted.

**NOTE**: most equipment in the Division's fish tissue laboratory is composed of stainless steel or other material that would be damaged by the use of $HNO_3$. Caution should be exercised when using $HNO_3$ to clean equipment.

**NOTE**: If cutting boards are covered in heavy duty aluminum foil they may be cleaned with detergent and rinsed in tap water. Aluminum foil must be changed after each sample is processed, but the cutting board will not need to be cleaned between samples (USEPA 1993).

*Tissue Preparation*

All samples will remain on ice until tissue preparation can begin. Tissue preparation is to occur within 48 hours of collection (USEPA 2000). If tissue preparation cannot be performed within 48 hours of collection in the laboratory, tissue preparation will be performed in the field or samples will be frozen. Frozen samples need to be partially thawed once returned to the laboratory for preparation (USEPA 2000). If tissue preparations are performed in the field, a clean area will be set up away from sources of exhaust and areas where gasoline or grease are used to help reduce the potential for surface and airborne contamination of the samples. A notation will be made on the Fish Tissue Data Sheet of the location of tissue preparation. Powder-free gloves are to be worn when handling the samples. A protective glove may be worn under a powder-free disposable glove to help prevent cutting injuries while resecting fillets. Knives with stainless steel blades will be used in the resection of fillets. Specimens will be prepared on cutting boards or table surface covered with heavy duty aluminum foil that is changed after each sample (USEPA 1993 and 2000). Only parts of the specimen that will not be sent to the analytical laboratory for analysis should come in contact with aluminum foil if possible.

**NOTE:** Changing heavy duty aluminum foil after each use on cutting boards does not require the cutting board to be cleaned between each sample. However, if the cutting board has body fluid or tissue on it from a previous resection, the board must be cleaned.

*Resection of Fish Fillets*

Target fillet (or composite) weight is >50 g wet weight. Fillets from the right side of the fish will be used as part of the sample. However, if the target weight is not met, the left fillet should be removed, added to the sample and noted. Qualifying composite samples are described below and will only be composed of right fillets or both fillets from each sample. Fillets will be processed as boneless skin-off and will include all flesh and fatty deposits from the nape to the caudal fin and from the dorsal fin of the back down to venter including the belly flap area of the fish (Figure 1). If skin-on fillet is required by a particular project, each fish will be scaled prior to the resection of the fillet and rinsed in de-ionized water after scaling. Any bones should be removed from the fillet if present after resection.

**NOTE**: Rinse fillet in deionized water if blood, scales or other material are on the fillet after resection.

**NOTE**: The Division has observed that a skin-on fillet often is difficult to homogenize and skin-off fillets are preferred.

**NOTE**: Care must be taken to avoid contaminating tissues with material released from inadvertent puncture of internal organs. If the tissue is contaminated by materials released from the inadvertent puncture of the internal organs during resection, the tissue will be rinsed in deionized water.

Figure 1. Resection of fillet from a largemouth bass (*Micropterus salmonids*).

After the fillet is removed from the skin of the fish, it should be placed on a clean glass cutting board. In order to facilitate lyophilization and homogenization, fillet(s) will be cut into small pieces (≤ 1 in). The small pieces are then placed in a zip top bag or whirlpak® that is labeled with sample information and stored at ≤-20 $^{o}$C until lyophilization/homogenization (USEPA 2000) or placed in a freeze dryer for lyophilization.

In order to determine percent moisture, the container must be weighed to the nearest 0.01 g before storing the fillet inside. Then the container and fillet is weighed (nearest 0.01 g wet weight) together. If the sample volume is too large to be placed in one container (~300 g), the sample can be divided and placed in multiple containers creating a subsample. These data (subsample number and container weights) will be recorded on the Lyophilization Data Log (Appendix C). If aging structures or organism sex is required for a project, collect this information after resection of fillets has occurred.

**NOTE:** Small pieces of tissue should be arranged in the container so that no pieces of tissue touch if possible (*Figure 2*). This allows greater surface area contact during the lyophilization process.

Figure 2. Photo showing tissue arrangement in whirlpak®

**NOTE**: The Division has observed that making horizontal and vertical cuts in the fillet prior to removing fillet (Figure 3) from the skin allows small pieces to be removed from the skin without placing fillet on a separate cutting board. This can eliminate the use of a second cutting board.

Figure 3. Horizontal and vertical cuts in fillet.

**NOTE**: Large fish (fish that weigh >1 kg) may be sub sampled by taking equal portions from the anterior, posterior, mesial and belly flap sections of the resected fillet (Figure 4). The subsample must weigh >50 g.

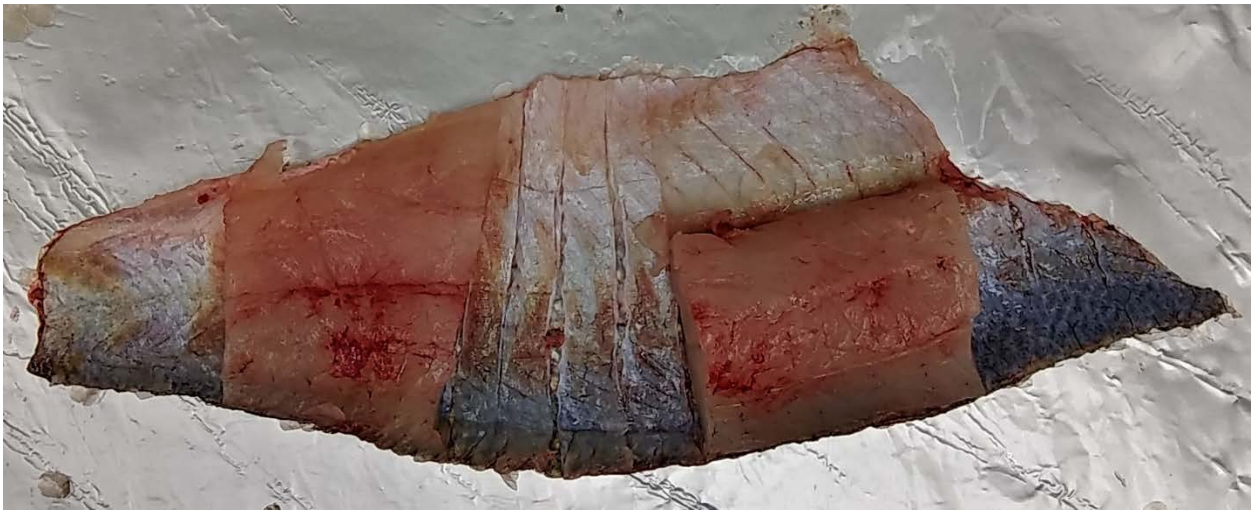

Figure 4. Large fish fillet subsample.

*Processing Whole Body Samples*

Processing of whole body samples will be processed using all body parts, bones, body liquids and scales. Stomach contents are to be included in the sample as well. Target whole body sample weight is >50 g wet weight unless other amounts required by analysis (i.e. Se analysis may only require 5 g).

**NOTE:** ageing structures should not be removed in whole body samples.

*Processing Small Fish*

Small fish (i.e. *Semotilus*) in a zip top bag or whirlpak® may be placed directly into the freeze dryer without any processing. In order to facilitate lyophilization, these fish may need to be cut into two pieces or slits made into the abdominal cavity after the first lyophilization cycle.

*Processing Large Fish*

Large fish may be processed in one of two ways: 1) cutting the whole fish into small pieces and freeze drying the whole fish or 2) cutting the fish into pieces and processing the pieces in either a commercial food processor or meat grinder (USEPA 1993).

*Cutting the Whole Fish* – Large fish whole body samples will be diced into small pieces (≤ 1 in or as small as it can be safely dissected) with a stainless steel saw blade and/or knife on stainless steel table tops/tray or cutting boards covered with heavy duty aluminum foil or glass cutting

boards. Care must be taken when dissecting large specimens because it can be difficult to slice through bones and scales. Small pieces of fish are then place inside the sample container(s).

*Commercial Food Processor or Meat grinder* – Large fish will be cut into sections small enough to fit into processor or grinder. The tissue should be processed until no discernable parts are identifiable. The whole fish may not be able to be processed in one batch. In these circumstances the fish should be processed in batches. Once a batch is processed, the tissue should be placed into a stainless steel pan and the next batch processed. Once all batches have been processed, all tissue should be mixed in the stainless steel pan, divide into quarters, opposite quarters mixed together and the two halves mixed back together. This process must be repeated a minimum of two times and the mixture is placed back into the processor/grinder one more time. After final processing, an aliquot should be obtained sufficient for laboratory analysis and placed into the sample container. Excess sample can be discarded unless needed for other purposes defined in QAPP.

**NOTE**: Skin, large scales and large bones may not homogenize with some species of fish. However, every effort should be made to homogenize skin, scales and bones. The skin, scales and bones that are not homogenized should not be included in any sample sent to the analytical laboratory.

*Sample Container for Whole Body Samples*

The recommended sample container is a sterile whirlpak®, but can be any certified clean container that can be lyophilized without damage or weight loss. The container should be weighed to the nearest 0.01 g before storing the whole body sample inside. Next the container and the whole body sample should be weighed (nearest 0.01 g wet weight) together. If the samples volume is too large to be placed in one container and the project requires retention of all sample material, the sample can be divided and placed in multiple containers creating a subsample. These data (subsample number and container weights) will be recorded on the Lyophilization Data Sheet (Appendix C). Sample information will be written on the outside of the container. The sample is then stored at ≤-20 $^{o}$C until lyophilization/homogenization (USEPA 2000) or placed directly into the freeze dryer for lyophilization.

**NOTE**: Whole body aliquot samples should be arranged in the sample container in a thin layer (≤~1 in). This thin layer will facilitate the lyophilization process and can be broken into smaller pieces after the first cycle in order to increase surface area.

*Qualifying Composite Samples*

Individual samples are preferred over composite samples. Individual samples provide a direct measure of the range and variability of contaminants in the target fish population. Composite samples can be utilized when both the right and left fillets of an individual sample weighs <50 g wet weight and multiple individuals are required to reach the target weight, whole body

samples or when it is cost-prohibitive to analyze individual samples. Qualifying composite samples must adhere to these guidelines:

1. All tissue in the composite must be the same species.
2. Right fillets or both fillets should only be used unless it is a whole body sample.
3. All tissue in the composite must be of similar size so that the smallest individual in a composite is no less than 75% (with a target of 90%) of the total length of the largest individual (USEPA 2000).

Notation shall be made on the Fish Tissue Data Sheet as to which individuals make up the composite sample. Sample details such as the length and weight can be averaged to describe the composite sample.

*Preservation*

Once samples are received from the field and resection of fillets or processing of whole body samples has occurred, samples are placed in the laboratory freezer and stored at ≤-20 $^{o}$C until samples can be lyophilized.

*Lyophilization*

Since the Division of Environmental Program Support Sampling Guidelines have established a six-month holding time for metals and no defined holding times have been established for percent lipids or PCB analysis, lyophilization is to occur as soon as possible but within six months of collection to allow for analytical analysis. A detailed illustration of lyophilization procedures are shown in Appendix D. Personnel are to use powder-free gloves when handling samples. Samples are placed inside the freeze dryer for lyophilization. The sample container will be left slightly open for the evacuation of moisture. Care must be taken when opening the sample container as to not allow any contamination or sample loss, including frozen moisture to occur. The freeze dry cycle will consist of a nine-hour freeze and seven-hour drying time. The process may be changed based on the amount of moisture in each sample cycle. The freeze cycle may be shortened (i.e. 4 hours) if samples are completely frozen before placing in freeze dryer. If reporting in wet weight, the percent moisture will be recorded before removing the sample from the lyophilized container. See the "Dry Weight to Wet Weight Conversion" section for the percent moisture calculation. After the percent moisture is calculated and recorded, the sample should be transferred to a stainless steel blender for homogenization. A fillet sample with ≥75% moisture loss and a whole body sample with ≥70% moisture loss is normally sufficient for homogenization. However, some tissue may not reach these targets (i.e. tissue with a high lipid content). If percent moisture targets are consistent (≤5% change) after three cycles, note in log and proceed to homogenization.

**NOTE**: When reporting in dry weight (i.e. selenium analysis), samples need to be dried to a constant weight. It may take several lyophilization cycles. When there is no weight loss and the sample weights remain constant (≤5% change), the sample is completely dry.

*Homogenization*

Depending on the samples volume, the appropriate blender cup size will be used. Most fillets can be homogenized in the small blender cups (32 ounces) while whole body samples may require the large blender cups (1 gallon). If subsamples were created, they are to be combined in the blender cup for homogenization. The sample is homogenized until no obvious masses are visible, stirring with a stainless steel spoon for verification. If any samples will not fit into a blender cup, samples should be homogenized in batches and then homogenized samples should be mixed according to USEPA (2000). Mix all samples together in a stainless steel pan of sufficient size, divide into quarters, mix opposite quarters together, and then mix the two halves back together. This process must be repeated a minimum of two times. After homogenization, the sample will be placed into a receiving vessel. If the receiving vessel has been filled with homogenized sample, excess sample tissue can be discarded unless needed for other purposes defined in QAPP.

**NOTE**: Homogenization should take place under a fume hood in order to reduce exposure to small particles produced during the homogenization process.

To ensure that a record of the sample weights for the calculation of moisture loss is recorded, a Lyophilization Data Log or Sheet (see example in Appendix C) will be used. The Lyophilization Data Log or Sheet field definitions are described below.

- **Sample ID:** assigned by letters "FT" + current year + consecutive order (i.e. FT18-001).
- **Subsample Number:** Detailed as # of specified subsample of # of containers used for the sample.
- **Container Weight:** Weight of empty sample container.
- **Sample Wet Weight:** Wet weight of the sample subtracting the container weight in grams.
- **Reporting Type Goal:** Wet weight (i.e. mercury) or Dry weight (i.e. selenium) or both.
- **Cycle Weights:** Sample weight + container weight after lyophilization cycle.
- **% Moisture:** The results of the calculation: [(Wet Weight–Dry Weight)/ Wet Weight].

*Transferring Homogenized Sample to Receiving Vessel and Storage*

After homogenization, the sample is placed in a certified level 1 glass container with a PTFE lid (USEPA 2000) (Figure 5) liner using a stainless steel spoon. After the sample is placed in a certified level 1 glass container with a PTFE lid liner, the sample is placed in a freezer at ≤-20 $^{o}$C until processed for analysis in the analytical laboratory. The receiving vessel should be labeled with the Sample ID.

Figure 5. Homogenized sample.

**Dry Weight to Wet Weight Conversion**

When the Reports of Analysis are delivered from the analytical laboratory, all samples that have been lyophilized will be reported in dry weight and converted to wet weight for the Integrated Report and consumption advisories unless dry weight is recommended (i.e. selenium). The conversion from dry weight to wet weight requires obtaining the percent moisture of the sample being analyzed (USEPA 2016). To calculate percent moisture, samples must be weighed before and after lyophilization. Samples will be weighed on a scale to the nearest 0.01g. Percent moisture must be included on the chain of custody and presented with the official lab report of analysis. The conversion formulas and additional information are found in Appendix E.

**Quality Control and Quality Assurance**

*Delivery to the Analytical Laboratory*

Samples will be delivered to the analytical laboratory following KDOW (2009). A chain of custody will be assigned with the samples (see example in Appendix C).

*Balance Calibration Checks*

All samples are to be weighed on a balance that is calibrated and of adequate accuracy and precision (USEPA 2000). Balance checks must be recorded at the beginning of each weighing session using the reference weights 200 g, 100 g and 50 g and after every 20 samples. For the calculation of percent moisture, the acceptable tolerance between reference weights and the balance readings is ± 0.1 grams. Care must be taken to avoid balance interferences. Reference weight handling and standardization procedures are found in ESB (2015). Balance calibration and corrective actions for out-of-control data will follow procedures outlined in ESB (2015). If the instrument fails to meet accuracy specifications after re-calibration, the balance will be tagged "Out of Service" until repair or replacement of the balance has occurred. An example Balance Check Log is available in Appendix G.

*Laboratory Splits and Rinsate Blanks*

Laboratory split samples will be collected by submitting two independent samples of homogenized tissue from the same sample to the analytical laboratory, if required by the study plan.

Rinsate blanks are a deionized water sample collected by rinsing the equipment that typically comes in contact with the tissue during homogenization. The equipment should be cleaned prior to rinsing using the protocols described in the above section "Cleaning of Work Utensils." The sample will be collected in appropriate bottles and submitted for analysis if required by the study plan.

*Data Storage, Entry and Verification*

All field and laboratory data will be recorded on the Fish Tissue Log (Appendix B) and Lyophilization Log (Appendix F). Results from the analytical laboratory must be filed in the project's e-files and recorded into K-WADE according to KDOW (2015). The project coordinator will be responsible for reviewing the received data for accuracy and resolving any corrective actions if needed.

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## Appendix A. Suggested Taxonomic References


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**Appendix B. Fish Tissue Data Sheet**

## FISH TISSUE FIELD DATA SHEET-*example*

| | |
|---|---|
| **Waterbody:** *Cave Run Lake* | **Collection Date:** *05/29/2017*<br>**Start Time:** *1200* **End Time:** *1400* |
| **Location:** *Near Banger Ramp* | **Basin:** *Licking* |
| **Site ID:** *DOW05036025* | **County:** *Rowan* |
| **Coordinates (Latitude/Longitude):** *38.04375 -83.43882* | **Collection Method:** *Large Boat Electrofisher* |
| **Tissue Preparation Location:** ☐Field ☒ Lab | **Collectors:** *Garrett Stillings, Rodney Pierce and Robert Johnson* |
| **Notes:** *Lesions were found on Field Sample ID: 17-001* | |

| Sample ID (Year-Number; Ex. 17-001; 17-002) | Sample Method (Individual or Composite) | Tissue type (RF,LF, BF,WB) | Species | Length (mm) | Weight (g) | Sex | Aging Method * | Age |
|---|---|---|---|---|---|---|---|---|
| *17-001* | *Individual* | *RF* | *Largemouth Bass* | *507* | *1975* | *F* | *O* | *9* |
| *17-002* | *Individual* | *BF* | *Channel Catfish* | *414* | *700* | *M* | *S* | *5* |
| *17-003* | *Composite* | *RF* | *Bluegill* | *182* | *125* | *NR* | *NR* | *NR* |
| *17-003* | *Composite* | *RF* | *Bluegill* | *167* | *100* | *NR* | *NR* | *NR* |
| *17-003* | *Composite* | *RF* | *Bluegill* | *168* | *115* | *NR* | *NR* | *NR* |
| *17-003* | *Composite* | *RF* | *Bluegill* | *147* | *60* | *NR* | *NR* | *NR* |
| *17-003* | *Composite* | *RF* | *Bluegill* | *171* | *105* | *NR* | *NR* | *NR* |
| *17-004* | *Composite* | *WB* | *Creek Chub* | *50* | *40* | *NR* | *NR* | *NR* |
| *17-004* | *Composite* | *WB* | *Creek Chub* | *60* | *50* | *NR* | *NR* | *NR* |

**Resection of fillets or sample dissection by:** *Garrett Stillings and Rodney Pierce*
**Resection Date:** *05/30/2017*
**Resection Start Time:** *1015*
**Resection End Time**: *1130*

***Aging Method:** **O**=Otolith; **S**=Scale; **F**=Fin; **S**=Spine
**RF**=Right Fillet; **LF**=Right Fillet; **BF**=Both Fillets; **WB**=Whole Body; **NR**=Not Recorded

## Appendix C. Lyophilization Data Sheet

## Lyophilization Data Sheet – example

| Sample Details | | | | | | | Sample Weight + Container Weight After Lyophilization Cycle | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | A | B | C | | | | | | | |
| Sample ID | Date/Time | Subsample Number (ex. 1 of 2; 2 of 2) | Container Weight (g) | Container Weight + Sample WW (g) | Sample WW (g) (A - B) | Reporting Type Goal (WW or DW) | Cycle #1 Weight (g) Date:5/10/16 Time: 1015 | Cycle #2 Weight (g) Date:5/11/16 Time:1200 | Cycle #3 Weight (g) Date:5/12/16 Time:1030 | Cycle #4 Weight (g) Date:5/13/16 Time:1100 | Cycle #5 Weight (g) Date:5/14/16 Time:1115 | Cycle #6 Weight (g) Date: Time: |
| *16-010* | *4/15/16 1015* | *1 of 1* | *6.61* | *72.71* | *66.10* | *WW* | *65.24* ☐ | *54.87* ☐ | *24.87* ☒ | - ☐ | - ☐ | - ☐ |
| *16-011* | *4/15/16 1030* | *1 of 2* | *6.59* | *197.25* | *190.66* | *WW* | *125.65* ☐ | *98.33* ☐ | *57.33* ☐ | *57.01* ☒ | - ☐ | - ☐ |
| *16-011* | *4/15/16 1045* | *2 of 2* | *6.65* | *152.91* | *146.26* | *WW* | *100.37* ☐ | *93.35* ☐ | *82.35* ☐ | *70.26* ☐ | *69.23* ☒ | - ☐ |
| *16-012* | *4/15/16 1100* | *1 of 3* | *6.63* | *40.21* | *33.58* | *DW* | *20.53* ☐ | *15.36* ☐ | *15.30* ☒ | - ☐ | - ☐ | - ☐ |
| *16-012* | *4/15/16 1115* | *2 of 3* | *6.65* | *25.13* | *18.48* | *DW* | *16.35* ☐ | *10.89* ☐ | *8.56* ☒ | - ☐ | - ☐ | - ☐ |
| *16-012* | *4/15/16 1130* | *3 of 3* | *6.64* | *55.23* | *48.59* | *DW* | *35.45* ☐ | 20.79 ☐ | 15.47 ☒ | - ☐ | - ☐ | - ☐ |

**WW**=Wet Weight; **DW**=Dry Weight ☐=Final Cycle Weight ☐ Check if the cycle was the final lyophilization cycle and the sample was homogenized. Subsamples should be composited before homogenization.

| Use This Section Only if Reporting Type is WW. | | | | | |
|---|---|---|---|---|---|
| | W | X | Y | Z | |
| Sample ID | Sample Weight + Container Weight (g) (∑ Final Cycle weights with the same Sample ID) | Container Weight (g) (∑ **A** with the same Sample ID) | Sample WW (g) (∑ **C** with the same Sample ID) | Sample DW (g) (**W** – **X**) | % Moisture ((**Y** –**Z**)/**Y**) x 100 |
| *16-010* | *24.87* | *6.61* | *66.10* | *18.26* | ***72.38*** |
| *16-011* | *126.24* | *13.24* | *336.92* | *113.00* | ***66.46*** |
| | | | | | |

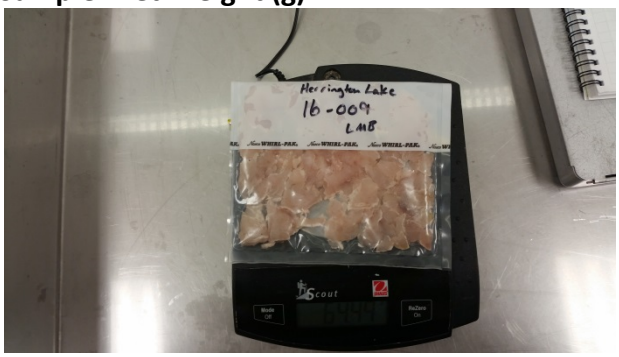
| Use This Section Only if Reporting Type is DW and Samples with the Same Sample IDs have been Composited and Homogenized. Reporting Type DW does not require % Moisture. | | | | |
|---|---|---|---|---|
| | Sample Weight + Container Weight After Lyophilization Cycle | | | |
| Sample ID | Cycle #1 Weight (g) Date:5/15/16 Time:1030 | Cycle #2 Weight (g) Date:5/16/16 Time:1045 | Cycle #3 Weight (g) Date:5/17/16 Time:1300 | Cycle #4 Weight (g) Date: Time: |
| *16-012* | *35.32* | *34.66** | *34.66** | - |
| | | | | |
| | | | | |
| | | | | |

*Samples should be lyophilized until a consistent weight is measured.

## Appendix D. Lyophilization Procedures

1. Weigh a clean opened whirlpak® to record **Container Weight (g)**.

2. Insert diced fish fillet/whole body into the opened whirlpak® and weigh to record **Container Weight + Sample Wet Weight (g)**.




3. Arrange whirlpaks® on trays and place into freeze dryer.

4. Verify if the freeze dryer settings and vacuum pump oil levels are correct for the lyophilization process. Read freeze dryer instruction guide for more information.


**5.** When cycle is finished, weigh whirlpaks® and calculate percent moisture. Multiple cycles may be needed. ≥70% of moisture loss is normally sufficient for homogenization of fish tissue. If reporting in dry weight additional lyophilization cycles are needed after homogenization until weights remain constant.

## Appendix E. Wet/Dry Weight Conversion Information

In 2016, the Kentucky Division of Water elected to homogenize fish tissue samples by lyophilization, also known as freeze drying, instead of liquid nitrogen or dry ice homogenization. It was concluded that lyophilization would simplify and accelerate the sample homogenization process and limit chances of contamination. There were specific observations where the process of homogenizing samples that were frozen with liquid nitrogen/dry ice had damaged blender blades, introducing contaminants in the sample. Lyophilized samples can be homogenized easily in a blender with no risk of damaging the blender cups or blades.

From personal communication with the Environmental Services Branch staff, lyophilization aids in the analytical analysis of samples. Dry samples make digestion easier because it pre-concentrates elemental compositions by eliminating water while digestion reagents are not diluted. In an extraction when the sample is not homogenized thoroughly the solvent cannot reach all parts of the sample, which can create inconsistent results. Additionally, there is a significant reduction in solvent usage for primary extraction since the volume/mass of the sample will effectively be reduced by > 1/5$^{th}$.

Samples homogenized by lyophilization will be reported in dry weight from the Environmental Services Branch and can be converted to wet weight by the user for fish consumption advisories and Integrated Reports. The conversion from dry weight to wet weight requires obtaining the percent moisture of the sample being analyzed (USEPA 2016). To calculate the percent moisture, samples must be weighed before and after lyophilization. Samples will be weighed on a balance at least to the nearest 0.01 grams. When converting between weights, the least number of significant figures in any number of the conversion determines the number of significant figures in the result. The conversion formulas are described below.

**Dry Weight to Wet Weight Conversion Formulas**

***Wet Weight = Total sample weight before lyophilization***

***Dry Weight = Total sample weight after lyophilization***

***% Moisture = [(Wet Weight - Dry Weight)/Wet Weight] x 100***

***Wet Weight Concentration = Dry Weight Concentration x [1 - (% Moisture/100)]***

***Dry Weight Concentration = Wet Weight Concentration / [1 - (% Moisture/100)]***

**Examples:**

***14-112** -- [(35.32-6.58)/35.32]x100=81.37=% Moisture*
*1.870 x [1– (81.37/100)]=0.348=WW Concentration*

***14-125** -- [(28.73-6.24)/28.73]x100=78.28=% Moisture*
*0.262 x [1– (78.28/100)]=0.059=WW Concentration*

| Field ID | Dry Weight (g) | Wet Weight (g) | % Moisture | DW Hg Concentration (mg/kg) | WW Hg Concentration (mg/kg) | WW Hg (mg/kg) from past runs in 2014 |
|---|---|---|---|---|---|---|
| 14-112 | 6.58 | 35.32 | 81.37 | 1.87 | 0.35 | 0.36 |
| 14-125 | 6.24 | 28.73 | 78.28 | 0.26 | 0.06 | 0.06 |

## Appendix F: Chain of Custody

**CHAIN OF CUSTODY**

***Program/Code: Fish Tissue – A20***

| Sample Type | County | Sample ID | Sample Description | % Moisture Removed | Sample: Matrix | Collection Method | Collection Date/Time | Container 1 | Container 2 | Container 3 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ☐ Fillet<br>☐ Whole Body<br>☐ Egg/Ovary | ☐ Composite<br>☐ Grab | | ☐<br>LAB Report # | ☐ | ☐ |
| | | | | | ☐ Fillet<br>☐ Whole Body<br>☐ Egg/Ovary | ☐ Composite<br>☐ Grab | | ☐<br>LAB Report # | ☐ | ☐ |
| | | | | | ☐ Fillet<br>☐ Whole Body<br>☐ Egg/Ovary | ☐ Composite<br>☐ Grab | | ☐<br>LAB Report # | ☐ | ☐ |

Sample Type: FS=Field Sample, FD= Field Duplicate, LSP=Lab Split

**Continued on Page 2** ☐ **YES**

**Shipment Temp:**

Analysis Requested:

Sample Matrix: ☒ Tissue-Fillet ☐ Tissue-Whole Body

Sample Type: ☒ Dry Weight ☐ Wet Weight

**Container 1:** Metals($3130T CALC)/Hg(3340T CALCD)/MeHG(3350T CALCD)/Pesticides($6260T CALC)/PCB($6300T CALC)/%Lipids(5460T)/%Moisture(9540)

**Container 2:**

**Container 3:**

**Samples Collected By:**

**Relinquished by:** _____ **Date:** _____ **Received by:** _____ **Date:** _____

**Representing:** _____ **Time:** _____ **Representing:** _____ **Time:** _____

## Appendix G. Scale Check Log

## Balance Check Log

Scale:
B636994848

| Weighing Session | | | Reference Weight Balance Readings | | | Check if Recalibrated |
|---|---|---|---|---|---|---|
| Date | Time | Analyst | 200 g | 100 g | 50 g | |
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