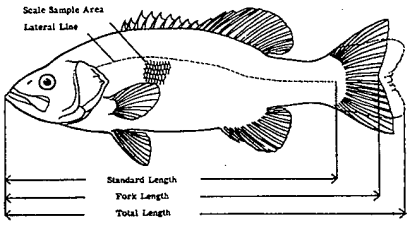
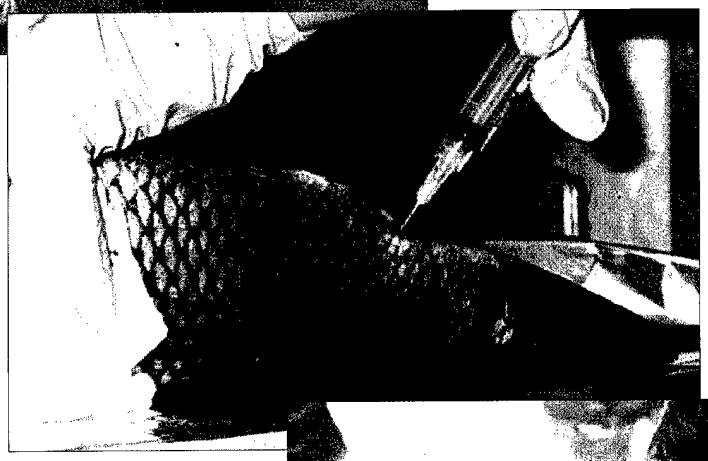
Biomonitoring of Environmental Status and Trends (BEST) Program: Field Procedures for Assessing the Exposure of Fish to Environmental Contaminants





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U.S. Department of the Interior
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Biomonitoring of Environmental Status and Trends (BEST) Program: Field Procedures for Assessing the Exposure of Fish to Environmental Contaminants

by

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Conversion Factors

Multiply	By	To Obtain
	Length	
micron (μm)	0.00003937	inch
millimeter (mm)	0.03937	inch
centimeter (cm)	0.3937	inch
meter (m)	3.281	foot
	Area	
square centimeter (cm^2)	0.001076	square foot
	Volume	
liter (L)	0.264	gallon
milliliter (mL)	0.000264	gallon
	Mass	
gram (g)	0.03527	ounce, avoirdupois
kilogram (kg)	0.453	pound

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Abstract. This document describes procedures used to collect information, tissues, and fluids for documenting the exposure of fish to environmental contaminants. For the procedures described here, fish are captured (preferably by electrofishing) and held alive until processing (generally <1 h). Fish are weighed, measured, and examined for grossly visible external lesions and pathologies. A blood sample is collected by caudal veinipuncture using a needle and syringe. The fish is subdued, and its abdominal cavity is opened. The internal organs are dissected from the fish for examination. The sex of the fish is determined by direct observation of its gonads. The liver is weighed (most species) and cut into small cubes and flash-frozen in cryogenic vials, which are stored and shipped in dry ice or liquid nitrogen. Additional liver cubes plus all grossly visible anomalies are preserved for histopathology. The gonads and spleen are weighed, and samples are preserved for histopathology. The kidneys are examined, and histopathology samples collected. A gill sample is also collected and preserved. All remaining tissues are returned to the carcass, which is wrapped in foil, labeled for chemical analysis, and chilled. Individual fish carcasses are composited by station, species, and gender; frozen; and shipped to the analytical laboratory. Procedures are also described for record keeping; processing blood to obtain serum and plasma; flash-freezing samples; cleaning equipment; and preventing the transport of living organisms among waterways. A list of necessary equipment and supplies is also provided.

Keywords: contaminants, fish health, biomarkers, chemical analysis, histopathology

1.0 Introduction

This guide describes field procedures to be used for investigations designed to monitor and assess the exposure of fish to environmental contaminants. We describe how to examine and handle freshly captured fish, and how to collect, store, and ship the tissues and fluids necessary for a suite of monitoring methods being evaluated by the U.S. Geological Survey (USGS) Biomonitoring of Environmental Status and Trends (BEST) Program. Although the guide was developed for specific studies being conducted by the BEST Program (Schmitt et al. 1995; Bartish et al. 1997), it and the specific chemical and biological methods to which it refers are potentially adaptable to a wide variety of situations and habitats. It is not a guidance document for selecting methods, nor does it contain information for interpreting study results based on the use of methods in the guide. The rationale for selecting this particular suite of methods, as well as information on the interpretation of findings, is presented elsewhere (BEST 1996; Schmitt and Dethloff in press).

The procedures we describe are primarily post-fish collection; although information on preparing for and organizing a study and suggested methods for documenting the date, time, and location of collection are included, most of the actual procedures begin after fish have been collected. Procedures are specified for making observations and collecting information, tissues, and fluids; preserving, wrapping and packaging samples; numbering samples and recording data; and storing and shipping samples and records. We also provide instructions for documenting the type of equipment used to collect the fish, the duration of the fishing effort, the exact location of the collection site, and the identity and qualifications of the personnel involved in the collection and processing of specimens and samples. A list of suggested equipment and supplies is also provided. To the extent possible, the procedures conform to Good Laboratory Practices (U.S. Environmental Protection Agency 1989); however, quality assurance and quality control issues are beyond the scope of this document. Depending on the purpose of the study, it may be necessary to develop additional project-specific protocols and standard operating procedures (SOPs) conforming to this guide, as well as a quality assurance plan. This guide and its sample data sheets may be photocopied or otherwise reproduced on waterproof paper for field use.

1.1 Background

Field portions of BEST projects using the methods described in this guide were conducted in 1995 (Schmitt et al. 1995) and 1997 (Bartish et al. 1997). In these projects, fish were collected at National Contaminant Biomonitoring Program (NCBP) stations (Schmitt et al. 1999), from selected USGS National Water-Quality Assessment (NAWQA) Program (Hirsch et al. 1988) sites, and from selected National Stream Quantity Accounting Network (NASQAN) (Hooper et al. 1996) sites in the Mississippi, Columbia, and Rio Grande basins.

The objectives of the 1995 and 1997 projects were (1) to test the feasibility of implementing biological monitoring methods recommended for use in the BEST program (Schmitt and Dethloff in press); and (2) to update information on the distribution and concentrations of organochlorine and elemental contaminants in selected U.S. rivers. Composite samples of whole fish were analyzed for elemental and organic chemical residues following NCBP protocol. The following additional methods were incorporated into the projects (Table 1): the H4IIE rat hepatoma cell bioassay, a sensitive *in vitro* method for documenting the exposure of organisms to planar polyhalogenated hydrocarbons; hepatic ethoxyresorufin-O-deethylase (EROD) activity, an indicator of exposure to polyhalogenated and polycyclic aromatic hydrocarbons; condition indices and other indicators of general fish health and well-being, including grossly observable changes and a quantitative field health assessment; and histopathological examination of selected tissues and structures. The composite fish carcass samples were also analyzed for stable isotopes of nitrogen ($\delta^{15}N$), a potential indicator of trophic position and nitrogen inputs. Because of continuing interest in the effects of chemicals on reproduction (Colborn 1991; Colborn et al. 1993; Guillette et al. 1994), biomarkers diagnostic of reproductive health and endocrine modulation were also evaluated. These included plasma vitellogenin and steroid hormone concentrations, and gonadal histopathology. Contaminants can also suppress immune system function (e.g., Matthews et al. 1990; Hutchinson and Simmonds 1994). Consequently, two immune system indicators—splenic macrophage aggregates and plasma lysozyme activity—were included.

Table 1. Methods incorporated into the large rivers projects.

Method	Description	Tissue(s) examined	Sensitivity	Primary reference(s)
Histopathology	Microscopic examination for the presence of lesions; can provide early indication of chemical exposure	Liver, gill, gonads, spleen, and kidney	Overall organism health and contaminants	Hinton et al. (1992); Hinton (1993); Goodbred et al. (1997)
Ethoxyresorufin-O-deethylase (EROD) activity	Enzyme induction by planar hydrocarbons	Liver	PCBs, PAHs, dioxins, and furans	Pohl and Fouts (1980); Kennedy and Jones (1994)
Lysozyme activity	A disease resistance factor that can be suppressed in the presence of contaminants	Blood plasma	Overall organism health	Blazer et al. (1994)
Macrophage aggregate analysis	Macrophages are important in the immune system, serving as a first line of defense for the organism and as an antigen processing cell	Spleen, hemopoetic kidney, and liver	Multiple contaminants including PAHs and metals	Blazer et al. (1994); Blazer (1997)
H4IIE bioassay	A screening tool to determine the presence of certain classes of planar halogenated compounds	Whole fish (composites)	PCBs, dioxins and furans; and PAHs	Tillitt et al. (1991)
Vitellogenin	A precursor of egg yolk, normally synthesized in the liver of female fish	Blood plasma	Endocrine modulating substances	Folmar et al. (1996)
Sex steroids (estradiol and testosterone)	Determine reproductive health and status	Blood plasma	Endocrine modulating substantces	Guillette et al. (1994); Goodbred et al. (1997)
Chemical analyses	Organochlorine chemical residues and elemental contaminants	Whole fish (composites)	Specific analytes	Schmitt et al. (1999)
Somatic indices	The relative mass of some organs is often indicative of chemical exposure	Gonads, spleen, liver	Overall organism health	Grady et al. (1992)
Stable N isotopes (^{14}N and ^{15}N)	The ratio of ^{15}N to ^{14}N ($\delta^{15}N$) increases with trophic position and sewage pollution	Whole fish (composites)	Trophic position, nitrogen sources	Cabana and Rassmussen (1996)
Necropsy-based fish health Assessment	Visual assessment of external/internal anomalies (e.g., lesions, parasites, tumors)	All	Overall organism health	Goede (1988, 1996); Adams et al. (1993); Adams (1990)

1.2 Development of this Document

This guide originated as a Standard Operating Procedure (SOP) developed by the USGS Columbia Environmental Research Center (CERC) for the 1995 and 1997 BEST Program studies of environmental contaminants and their effects on fish in the Mississippi, Columbia, and Rio Grande basins (Schmitt et al. 1995; Bartish et al. 1997). Following field portions of each study, the BEST program sponsored workshops at which participants (program staff, investigators, cooperators, and partners) identified specific procedures, documentation, and materials that could be improved to further expedite the processing of specimens and samples. These recommendations were incorporated into subsequent revisions of the SOP. This guide also incorporates information and procedures from numerous other sources, including other CERC SOPs; study plans and reports from NAWQA investigations (Goodbred et al. 1997); and books, manuals, and reports from other studies (Lagler 1956; Lagler et al. 1962; Smith 1973; Hunn 1988; Meyer and Barclay 1990; Baker et al. 1997). The health assessments based on field observations were developed from the procedures of Goede (1989, 1996) as described by Goede and Barton (1990), and parts of this guide are cross-referenced to an atlas of photographs (Goede 1988). The guide has been made more generally applicable (i.e., for use in a wider geographic area and in other programs and studies) than the SOP by eliminating references to specific collection locations and cooperators; by allowing for the collection and examination of more fishes; and by eliminating references to specific brands and trade names of supplies and equipment. Collection locations and times, preferred taxa, exact numbers of samples, modifications or additions to standard procedures, and field and laboratory cooperators should be identified in the study plan or protocol under which the procedures described in this guide are used.

Although the field procedures described here can be used to obtain tissues and fluids with which to perform many assays, they were designed specifically for conducting the quantitative health assessment and for collecting the biological materials needed for the methods being evaluated in the 1995 and 1997 BEST projects (Table 1). Consequently, the methods described here have been used by the authors to conduct only these and similar assays, and the procedures may need to be altered by investigators using the guide to collect tissue and fluid types and quantities that meet the needs of other study plans or protocols. Corresponding changes may also need to be made in the lists of required equipment and supplies.

1.3 Typographic Conventions

Within this document, the following typographic conventions are used:

- Important actions, notes, and reminders are indicated in **bold** or *italics*.
- Information to be recorded is also indicated in **bold.**
- The cross symbol (✚) indicates a health- or safety-related item.

1.4 Animal Welfare

The procedures described in this guide conform to the American Society of Ichthyologists and Herpetologists, American Fisheries Society, and American Institute of Fishery Research Biologists, "Guidelines for Use of Fishes in Field Research" (ASIH et al. 1988); and with all known SOPs and guidelines for the humane treatment and disposal of test organisms during culture and experimentation.

2.0 Roles and Responsibilities

Generally, studies based on this guide will have both field and laboratory components; we describe only the field portions. Conducting or otherwise arranging for necessary laboratory analyses is usually the responsibility of the principal investigator, project coordinator, or study director. Activities and responsibilities of field cooperators and personnel include: coordinating and conducting sampling operations; ensuring that personnel are trained in the operation of electrofishing, global positioning system (GPS), and photographic equipment (which are all beyond the scope of this guide); conforming to all pertinent health, safety, certification (watercraft operation, electrofishing, etc.), and collection permit requirements (both state and federal collection permits and their required reporting and pre-notification); securing prior written permission to enter private lands; ensuring that boats, motors, trailers, and other equipment are properly cleaned after sampling (to thwart the transport of living organisms between waterways); and shipping samples and records to cooperators and laboratories. Consult the study plan to determine the species and number of specimens required; this information is necessary under the terms of many collection permits. Walsh and Meador (1998) present additional information that may be useful in the collection of fishes.

Note: The health and safety items presented here conformed to all regulations at the time this guide was prepared. However, regulations governing the handing and shipment of chemicals and samples and the use of certain types of equipment are subject to change. It is the responsibility of the parties using this guide to ascertain and conform to these regulations.

Note: At least one representative of each field team should have documented firsthand experience in the conduct of the procedures described in this guide.

2.1 Landowner Permission

If travel across or sampling on private property is necessary, obtain permission from the landowner or a representative of the landowner, and have them sign and date a sheet prepared for that station (referred to as a Station Identification Sheet; see Appendix A for an example) to indicate landowner consent in advance of each entry.

Note: If prior written permission cannot be obtained, do not enter!

2.2 Shipment of Samples and Records

Frozen samples must be shipped to cooperating laboratories by overnight express in dry ice or in a liquid nitrogen dry-shipper. Because of the limited holding time of dry ice, it is best to sample early in the week and to ship no later in the week than Thursday morning to ensure that the samples arrive before the end of the work week. Liquid nitrogen dry-shippers, if well-charged, can be shipped on Fridays. Restrictions on shipping histopathology samples will depend on the fixative used. Check with shipping companies about samples in fixative—shipping by ground transport in properly labeled boxes will likely be required.

Project participants should consider using chain-of-custody (COC) forms when shipping or mailing samples and data, even if it is not required. An example of a COC form is enclosed within this document (Appendix B). All COC forms must be signed and dated in ink. See Section 9.0 for further details.

2.3 Shipment of Equipment and Supplies

When sampling multiple sites with multiple teams, the use of non-expendable equipment (i.e., dry ice and/or liquid nitrogen shippers, balances, centrifuges) should be coordinated among cooperators. To expedite the flow of non-expendable equipment, it is important to plan schedules as far in advance as possible. This will help to ensure that the equipment reaches each team when they need it!

Note: Centrifuges and balances are delicate and relatively expensive. They should be packed and shipped appropriately.

2.4 Determining the Age of Fish

Although this document does not specify procedures for determining the age of specimens collected, information on the age of each fish is important for interpreting the results of certain biomarkers. Because the particulars of aging fish vary among taxa and locale, field biologists are encouraged to consult local experts who specialize in aging the species to be collected before sampling begins. Techniques for aging fish are described by Tesch (1971), Jearld (1983), and in most fishery biology texts (e.g., Lagler 1956).

2.5 Photographs and Videotape

Photographs or color slides of sampling sites and operations are desirable, both for presentations and as a record. It is also advantageous to photographically record any lesions or other anomalies identified during the fish health assessment, as noted in the instructions.

Note: It is generally better (in terms of quality and expense) to take multiple photos than to duplicate slides. Videotape is an acceptable alternative to film.

2.6 Communication

Field biologists should have access to project coordinators/study directors in the event they need assistance. Pertinent telephone (including cellular telephone) and FAX numbers and email addresses should be provided.

3.0 Equipment and Supplies

The equipment and supplies needed to conduct the procedures outlined in this guide are listed in Table 2. The number needed per station or per fish is indicated unless that number must be determined from the study plan. The size of sample containers and of syringes may also need to be determined from the study plan. Meador et al. (1993) and Walsh and Meador (1998) present additional useful information on the collection of fish; the latter also provide a list of suppliers and vendors of equipment and supplies.

✚ **Bring the Material Safety Data Sheets**

(MSDS) for all chemicals (solvents, preservatives, refrigerants, etc.) with you to the field. All personnel should read and become familiar with the MSDS's.

Table 2. Equipment and supplies.

Safety and Other Apparel	
Gloves	
Cryogenic (for handling frozen samples)	1 pr.
Disposable latex or vinyl (for fish processors)	40 pr. assorted sizes
Long-sleeved or rubber (for fish collectors)	3 pr.
Work (for general use)	3 pr.
Raingear, boots, etc.	enough for all
Safety glasses	3 pr.
Data Collection and Recording Supplies	
Camera and film, or cam-corder and tape	1
Clipboards	2
Forms	
Chain-of-custody	total no. varies with study
Composite Sample Worksheet	1
Fish Health Examination Sheets	1 per fish
Station Identification Sheet	1
Pens	
Ball-point	3
Cryogenic	2
Waterproof (fine-tipped)	3
Sampling guide, SOP, study plan, field notebook	1 each
Waterproof paper for labels	2 sheets
Cleaning Supplies	
Acetone (preferably reagent grade, not crude)	0.5 L, for rinsing instruments
Acetone squirt bottle w/extra 28 mm lid for shipping and transporting	1
Alcohol wipes	50, for hand cleaning
Beaker	1, 100-mL, for acetone rinse
Bleach	optional, for disinfecting
De-ionized water	4 L, for rinsing instruments
Garden sprayer or pump spray bottle	optional, for dispensing bleach solution
Plastic trash bags	no. varies, for waste disposal
Fish Processing Supplies	
Aluminum foil	300 ft, heavy duty
Bottles, 125-mL polyethylene w/ leak-proof tops	1 per fish, for histopathology samples
Ethanol, 100%	1.5 L; 95% may be used if necessary
Fixative	85 mL per fish, for histopathology samples
Heparin solution	sodium salt, 10 USP units/mL; 2 x 5 mL. Use is optional depending on study plan—Refrigerate/chill until used!
Needles (hypodermic)	1 per fish; size may vary depending on fish species and size
Rigid-walled container w/lid (i.e. coffee can or milk jug) or commercially procured sharps waste container	1
Scale envelopes	1 per fish, for scales or spines
Small towels or cloth diapers	6
Stainless steel pan for weighing fish < 2000g	optional
Syringes	5.0-mL (1 per fish); 3.0-mL (1 per fish)
Transfer pipettes	1 per fish, for plasma or serum
Vacuum containers	1 per fish, for blood; heparinized or unheparinized, depending on study plan
Weigh boats	1 of each per fish, small and large

Table 2. continued

Collecting and Processing Equipment and Related Materials	
120V AC power source (>1 amp)	1 inverter, generator, or outlet (for centrifuge)
Blunt instrument for subduing fish	1
Calibration weights	1 of appropriate mass for each balance
Centrifuge	1, only if plasma is to be collected
Centrifuge tube rack	1, plastic or styrofoam
Dissecting tools	1 kit
Electrofishing boat, backpack shocker, or electric seine and related equipment	
Ground-Fault Interrupting (GFI) extension cord	1
GPS unit	1
Hand lens	1
Hanging balance, for large fish	1
Measuring board, mm	1
Portable electric balances	3, capacities vary with study plan
Slotted serving spoon	1
Tubs or live boxes	total no. varies with study plan and when boat has no livewell
Fish and Tissue Packaging	
Coolers	small (1, for quick freezing)
	large (total number varies with study plan, for cooling fish composite samples etc.)
Cloth tags	1 per fish and 2 per composite sample, for fish carcasses
Cryogenic vials (preferably with color-coded caps or inserts)	2.0-mL (2 per fish, for plasma or serum); 1.2-mL (2 per fish, for liver tissue)
Cryogenic vial storage boxes	total no. varies with study plan
Dry ice	4-5 lb for freezing & 10-15 lb, for shipping (0.5 lb of dry ice per lb of fish)
Dry ice shipper	1
Ice (wet, cubes or block)	50 lb
Liquid nitrogen	about 15 L, for freezing/shipping samples
Liquid nitrogen dry-shipper	1
Mailing labels	1 sheet
Plastic bags (for carcass samples)	total no. varies with study plan, enough for composite samples
Shipping labels for samples	total no. varies with study plan
Miscellaneous Equipment and Supplies	
Cell phone	optional but recommended
Duct tape	1 roll, for securing sharps containers, etc.
Field guide(s) or other reference(s) for identifying fish	
Fire extinguisher (dry chemical, type A, B, and C)	1
First-aid kit	1
Flashlights and other lights	optional
Kitchen (poultry) shears or scissors	1 pr.
Material Safety Data Sheets for all chemicals (fixative, acetone, ethanol, etc.)	1 each
Paper towels	1-2 rolls
Pliers	1 pr.
Portable table and chairs	optional
Shade canopy	optional
Thermometer or other instrument for measuring water temp.	1

4.0 Record Keeping and Housekeeping Items

Housekeeping activities that should be performed throughout the sampling day are outlined in Box 1.

Quality assurance measures, including activities to ensure proper record keeping, are outlined in Box 2.

Box 1. Suggested Housekeeping Activities, to be Performed as Needed.

- Place used and disabled needles, syringes, and scalpel blades in the sharps container; place used expendables in a trash bag.
- Cap the acetone squirt bottle and the marking pens when they are not in use.
- If collecting plasma, centrifuge blood samples; remove and freeze plasma.
- If collecting serum, allow blood to clot [on (wet) ice]; centrifuge, remove and freeze serum.
- Check dry ice and slush; refresh as necessary.
- Transfer frozen liver samples from the slush to the shipping container (either storage box(es) in the dry ice cooler or aluminum canes in the liquid nitrogen dry shipper).
- After each fish is processed, thoroughly wash all contact instruments and dissecting tools with de-ionized water and rinse them with acetone.

Box 2. Suggested Quality Assurance and Record Keeping Activities.

- Prepare and retain a short-form resume (see Appendix C for an example) for all personnel involved in sampling.
- Keep a logbook of the team's activities (when the team left, who was present and what each person did, etc.).
- Make all entries in ink. Use a ball-point pen which will write under all conditions on data-entry and other forms. Forms should be printed on waterproof paper.
- PRINT legibly.
- Do not use "ditto" marks (") for observations that repeat on a series of lines. Instead, write the entry on the first and last applicable lines, and connect the observations with a single vertical line.
- If you do not obtain a measurement (equipment broken, etc.), write an explanation in the comment field on the data sheet.
- If you make a mistake, draw a SINGLE line through the incorrect entry (e.g. ~~CARP~~), and initial and date the correction. Then, indicate the nature of the error in the appropriate comment field on the data sheet.
- Number sampling sites in accordance with the protocol or study plan under which an investigation is conducted. Each sampling site should have a unique identifier. Initiate a new Station Identification Sheet for each day that you sample at a site.
- Number fish collected at a sampling site as instructed by the protocol or study plan. If no numbering system is detailed in the protocol, fish should be numbered with a unique identifier (referred to as the fish identification number) that indicates the ID of the individual specimen, the sampling site from which it was collected, and the date of collection.
- Initiate a new Fish Health Examination Sheet (see Appendix A for an example) for each specimen. Record the species, sex, length, weight, results of the field health assessment procedures, numbers of tissue and fluid samples collected, and any further remarks or comments pertaining to individual fish. To eliminate possible bias in subsequent analyses, samples bear the fish identification numbers, but no other markings.
- If you photograph (or videotape) any external or internal lesions or anomalies, use a previously prepared tag or scale envelope as a specimen identifier in each frame. There are spaces for recording the number of photos taken or the video footage shot in each section of the Fish Health Examination Sheet; filling them in will make it easier to annotate slides or tape later. It is easier for the recorder if you follow the order of the Fish Health Examination Sheet, which follows the order of this document.
- If carcass samples are composited for analysis, use the numbering system designated in the study plan or protocol. The system should be designed so that it does not conflict with fish identification or sampling site numbering schemes. Codes that include numbers and letters have been used successfully in the past. Group the samples as outlined in the study plan. The composition of composite samples should be recorded on a Composite Sample Worksheet (see Appendix A for an example).

  Note: If specimens that are designated as juvenile or indeterminate are collected, and gender identification will be made histologically at a later date, group and bag these specimens separately.
- Label cryogenic vials only with cryogenic markers, which are both water- and alcohol-resistant! Do not use a pen that is only waterproof! Label all other containers (except the cryogenic vials) with a permanent, waterproof, felt-tipped pen. If in doubt, smell the marker; waterproof markers that are not alcohol-fast smell like solvent.

  Note: Cryogenic vials with white label fields are preferred over those without label fields. Write in the label field if there is one. Stick-on labels or label tape cannot be used if the dry-ice/ethanol freezing procedure described in this guide is used; the ethanol will dissolve the adhesive.
- Check the Fish Health Examination Sheets carefully to be certain that all observations have been made and recorded before proceeding to the next fish. Do not leave blank fields on the data sheets unless specified by the study plan.

5.0 General Field Procedures

5.1 Before Leaving on a Collecting Trip

a) If time permits, pre-label enough tags, labels, envelopes, and containers (Figure 1) for the number of specimens you will be collecting given the specifications of the study plan. **Bring extra, unlabeled supplies.**

Note: It is also advantageous to label, group, and package the expendables needed for each fish prior to arrival on site. Using the list of items needed for each fish (Table 2) as a guide, put the following in a zippered plastic bag: needles and syringes (one each, 3.0- and 5.0-mL); one vacuum container, labeled; 1.2- and 2.0-mL cryogenic vials (two of each); one scale envelope; and one labeled cloth tag. These numbers may vary with the study plan; consult it before packaging.

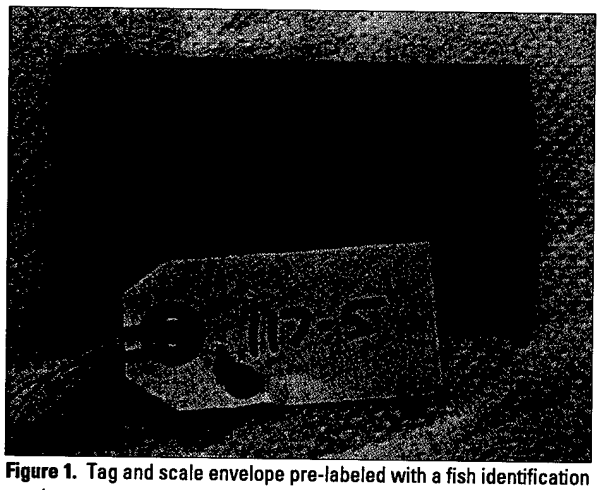


Figure 1. Tag and scale envelope pre-labeled with a fish identification number.

b) Pre-fill the 125-mL bottles with about 85 mL of fixative.

Note: The volumes suggested here are for 10% neutral buffered formalin. For other preservatives, different tissue:preservative ratios may be necessary.

✚ **10% neutral-buffered formalin is among the preferred fixatives because it can be shipped via ground transport. The fixative may vary with the project; therefore, consult the study plan. Regardless of the fixative used, it should be handled safely (i.e., in accordance with the MSDS). Fill containers under a hood or outdoors, and wear protective clothing (lab coat or long sleeves and pants; latex or vinyl gloves; and safety glasses, goggles, or face shield) to prevent eye and skin contact.**

✚ **Some preservatives and fixatives are also flammable, as are acetone and ethanol. Consult the MSDS for these materials, and avoid extreme heat and ignition sources (i.e., sparks and flames).**

c) Check the equipment and supplies list (Table 2) to be certain all materials and equipment are present, and check the operation of all equipment to ensure that it is functioning properly.

d) All equipment should be clean when it is taken out into the field — clean any equipment that needs it. Any material that will come in contact with specimens should be cleaned with cleaning agents that will not affect later analyses.

e) Fill the dry ice shipper with dry ice.

Note: Blocks are better than cubes. Also, if you will be shipping samples on dry ice, bring extra in additional coolers.

f) If a liquid nitrogen dry shipper is to be used, get it filled.

✚ **Safety notes concerning cryogenic refrigerants (i.e., liquid nitrogen and dry ice): Liquid nitrogen and dry-ice are dangerously cold. Dry ice should be handled with tools (preferably), cryogenic gloves, or both. Personnel handling liquid nitrogen-filled dewars and dry shippers should wear long sleeves and pants, face shield or eye protection, and gloves. In addition to the obvious cold hazard, these cryogenic refrigerant gases also represent an insidious hazard; because of their low temperature, they are denser than ambient air and, as they change from the solid (dry-ice) or liquid (liquid nitrogen) to the gas phase, the gasses tend to sink. Although neither carbon dioxide nor nitrogen are toxic, they can and will displace air containing oxygen. Moreover, containers for these refrigerants are designed to vent to the atmosphere. Consequently, these materials and containers should be stored in well-ventilated areas when possible. If liquid nitrogen dry shippers are stored in walk-in freezers when charged, personnel should be notified that the composition of**

the air in the freezer may be affected by the presence of the refrigerant gasses. A notice to this effect should be posted on the freezer door.

g) Check the equipment list again.

5.2 Procedures to be Followed Upon Arrival at a Station

The preferred minimum size of a sampling team is six individuals. Once at the station, do the following before you start sampling:

Divide the team into two groups (of at least three), if possible. One group (Group 1 — fish processors) should set up the sampling equipment, label containers (if not already done), and process fish while the second group (Group 2 — fish collectors) collects and ferries fish to the processing station.

Note: It is suggested that one person in the fish processing group be designated as the recorder for the duration of the sampling at any site.

Group 1, which includes the trained diagnostician and the recorder (Figure 2), should:

a) Initiate the Station Identification Sheet for the day's sampling by **recording the station number, date, station description, start time, freezing method being used, and the names of the team members**. Measure and record on the Station Identification Sheet the water temperature (C) in the general area from which fish were collected.

Figure 2. Diagnostician and recorder conducting a fish health assessment.

b) On the first day of sampling at a station, initiate a Composite Sample Worksheet by **recording the station number and date**.

c) Find the most level, shady, wind-sheltered place possible to set up the balance and centrifuge. Provide 120-V AC, 60-cycle power for the centrifuge [i.e., inverter and 12-V DC boat, vehicle, or trolling motor (1 amp minimum) battery; portable generator; electrofishing boat; or a grounded 120-V AC outlet] (Figures 3A – 3D).

A portable work surface (e.g. folding table, tailgate, cooler top) is also helpful (Figure 3B).

Figure 3A. An outdoor work area and equipment setup.

- **Important! The use of 120-V AC electrical power outdoors, especially around water, is potentially hazardous. The grounding requirements of portable power devices (i.e., generators and inverters) vary; read and follow the instructions supplied with whatever device you use. Many portable generators are now equipped with ground-fault interrupting (GFI) outlets; if yours is so equipped, use them. If it is not, use an extension cord equipped with a GFI. In addition, use this extension cord whenever working with electrical power in the field.**

- **The use of portable, engine-powered generators presents additional safety hazards—fire, burns, and fumes. Carry fuel only in approved containers, and allow the generator to cool before re-fueling to minimize fire hazards. In addition, an approved spark-arresting muffler may be required in some locales. To avoid fume hazards, do not use portable generators in buildings, vehicles, or other enclosed places.**

Figure 3B. A portable work surface. The biologist is transferring plasma into cryogenic vials.

d) Set up and level the balances (Figure 3C).

Note: Setting the balances up inside an empty cooler on a level spot works well. If it is windy or raining, close the lid between operations.

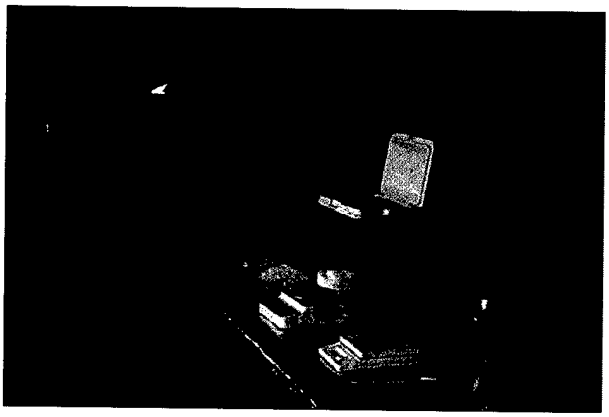


Figure 3C. An indoor work area and equipment setup.

e) Power up the balance(s) and ascertain that it (they) is (are) operating properly by weighing the appropriate calibration mass. **Record the make, model, and ID or serial number of the balance(s) and the "Before" calibration weight(s)** on the Station Identification Sheet.

All weights should be recorded in grams and the recorder should confirm that the balance is in the correct mode at all times. Also, personnel working with the balances should ensure that any weigh boats or other equipment being used to hold fish or organs are resting completely and solely on the weighing platform of the balance. Check the zero of the hanging balance, and adjust if necessary using the thumb screw at the top of the cylinder.

Figure 3D. An outdoor work area and equipment setup, with biologists processing fish.

f) Unpack the expendables (cryogenic vials, centrifuge tubes, bags, tags, labels, etc.) (Figure 4), and label the containers (if not done already).

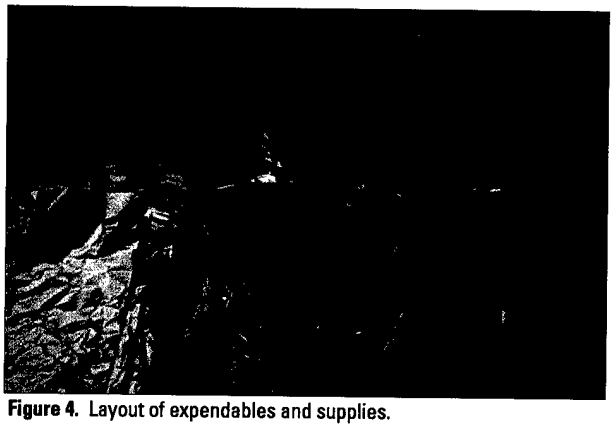


Figure 4. Layout of expendables and supplies.

g) Fill a container (tub, bucket, cooler) with ambient water and pre-soak the diapers or towels to be used to handle the fish (to remove any detergents or other chemicals).

h) Keep heparin, syringes, and vacuum containers chilled on ice until needed.

✚ **For protection against pathogens and parasites associated with either the fish or water, disposable latex or vinyl gloves**

water, disposable latex or vinyl gloves should be worn by team members handling the fish and collecting tissues and fluids. The gloves should be replaced as necessary if they become torn or punctured, and disposed of properly. Hands should be washed with disposable alcohol wipes. In waters known or suspected to harbor *Pfisteria* spp. or other pathogens, fish collectors and handlers should take extra precautions by wearing long gloves or sleeve protectors.

Group 2 (fish collectors) should:

a) Obtain the Station Identification Sheet and **record settings for the electrofishing equipment (in the General Comments) and location coordinates**. Then, initiate sampling (Figure 5). **Record the range of latitude and longitude sampled in degrees (000°), minutes (00'), and seconds (00") and the stop time** when finished.

5.3 Collecting and Holding Fish

The target taxa and desired number of specimens should be found in the study plan or protocol for the specific project. The collection of larger, adult fish is encouraged, for three main reasons: first, obtaining the necessary amounts of blood and tissues for the procedures outlined here may be difficult with smaller, juvenile fish; second, many biomarkers are neither valid nor well-documented in juvenile fish; and third, the observations called for may be difficult to perform with small fish. The latter is especially true for determining fish gender and weighing organs in the field. For studies that require identification of fish gender, specimens large enough to be mature but which are of indeterminate gender may be used if gender can and will be determined histologically (using preserved tissues).

The preferred collection methods are DC electrofishing (Figure 5), seining, or DC electric seining (the methods of capture that are least injurious to the fish); however, be certain that nets have not been chemically treated or used previously in heavily contaminated waters. Hook-and-line capture is also permissible. Trap-, gill-, or trammel-netting, and AC electrofishing should not be used because these methods are more injurious to the fish and can bias the quantitative health assessments and biomarker analyses. To complete the procedures described here, **fish must be held alive.**

Note: Holding fish for prolonged periods can affect biomarkers and health assessments; you should begin processing specimens before finishing the collection. Although predatory fish may be more available for capture at night, holding fish overnight for next-day processing is discouraged because it may bias physiological parameters and biomarkers to an unknown degree. In addition, holding fish for prolonged periods in tanks or live wells with abrasive surfaces or in net pens can cause external lesions.

Note: Depending on the species and the time of year, it may not be possible to ascertain the sex of a fish by external observation. Therefore, if a given number of each sex is desired, more than the target number of fish will have to be collected to meet the objectives of the study, and extra supplies will be needed.

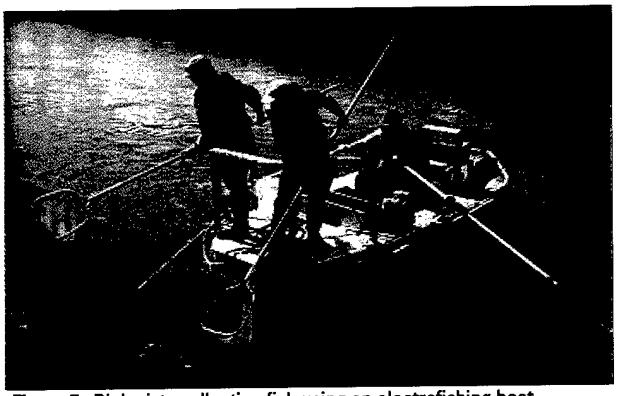


Figure 5. Biologists collecting fish using an electrofishing boat.

5.4 Preparing Slush for Quick-Freezing

When the first fish is ready to be processed, prepare the dry ice slush as follows:

Place a brick-sized block of dry ice in a small cooler. Gradually add about 1/3 the cooler volume (ca. 400 mL) of 100% ethanol and stir (with the slotted spoon) to make slush (i.e., snow-cone consistency). If the dry ice available is too solid for slush, use chips of dry ice to super-cool the ethanol solution. Ethanol can be re-used from one station to the next.

✚ **As noted earlier, liquid nitrogen, dry ice, and the dry ice-ethanol slush are dangerously cold; avoid skin and eye contact. Personnel handling samples and containers with the cryogenic coolants should wear long pants and sleeves, eye protection, and cryogenic gloves.**

- **Avoid splashing or touching the slush!**

- **Reminder: Ethanol is flammable.**

Close the cooler lid (loosely) and place the small cooler in a large cooler filled with (wet) ice. Open the coolers only when necessary. Check the wet and dry ice regularly and replace as needed. You should be able to stand the cryogenic vials in the slush; if you cannot, add more dry ice.

Note: Samples in cryogenic vials should be quick-frozen in either slush or dry ice. Liquid nitrogen may be used in lieu of theses procedures; however, the operation of liquid nitrogen dewars is not addressed in this guide.

Note: In the event that the dry ice supply becomes low, abandon the slush, especially if you are going to ship or transport samples in dry ice. If you suspect that you will have to use dry ice from the shipper to maintain the slush, insert all cryogenic vials directly into the dry ice. Record the change in procedure on the Station Identification Sheet under "Freezing Method Used". It is best to know in advance where you can obtain more dry ice.

6.0 Processing Fish

The actual processing of the fish begins here. The order in which the procedures are performed is important to ensure that activities conducted in one step do not compromise observations, tissues, or fluids obtained in subsequent steps, and that tissues and fluids with short holding times are collected as rapidly as possible. Consequently, the procedures that are described in the following sections and diagrammed in the accompanying flow charts should be performed in the order in which they are presented, which also conforms to the organization of the fish data sheets upon which the observations are recorded.

6.1 Blood Collection and Initial Fish Processing (Flow Charts 1 and 2)

Note: Some of the biomarkers used in studies supported by this guide can be performed on either serum or plasma. Serum is the liquid remaining after blood has been allowed to clot. Plasma is the liquid obtained after centrifugation, without clotting. To obtain plasma, an anti-coagulant (such as heparin) is used to prevent clotting. The terms "plasma" and "serum" are often used (incorrectly) interchangeably. Consult the study plan or protocol to determine which fluid to collect. Note: This guide only describes drawing blood from the posterior caudal artery and vein. Another blood sampling location and method (e.g. cardiac puncture) may be specified in the study plan. Within a study, be consistent in the location and method of blood collection.

- **This is a reminder that personnel handling and dissecting the fish should wear gloves.**

a) Prepare the work surface by covering it with a piece of foil (dull side up/shiny side down) large enough to wrap the fish you will be processing.

b) Secure a towel or diaper (from the soaking container). Wring it dry, then tare it on the large balance.

c) Assemble the needle and syringe (if necessary). Generally, use 3.0-mL syringes for fish < 300 mm TL, and 5.0-mL syringes for fish > 300 mm TL.

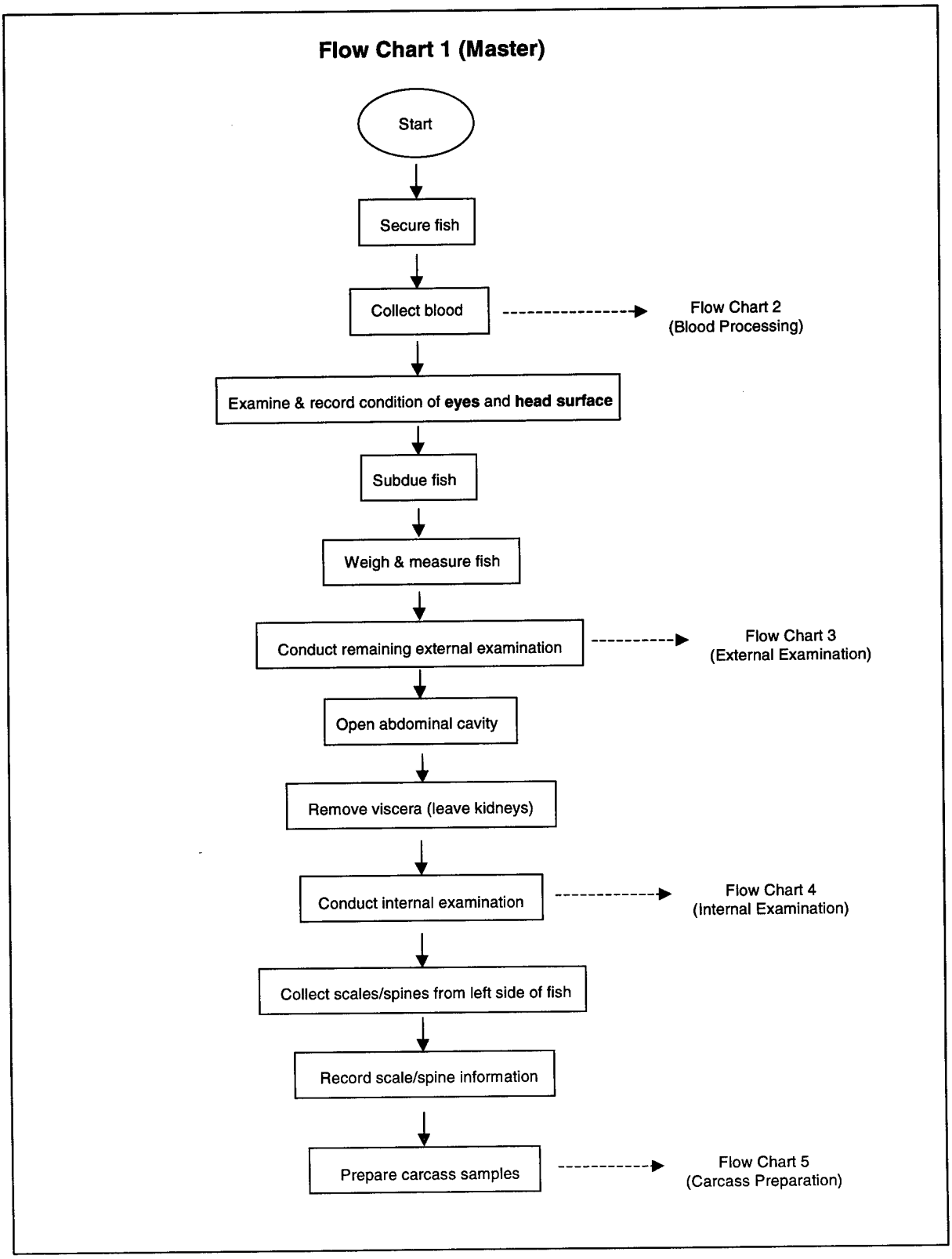
Note: If serum is to be obtained rather than plasma, no anticoagulant will be used. Skip to step (e).

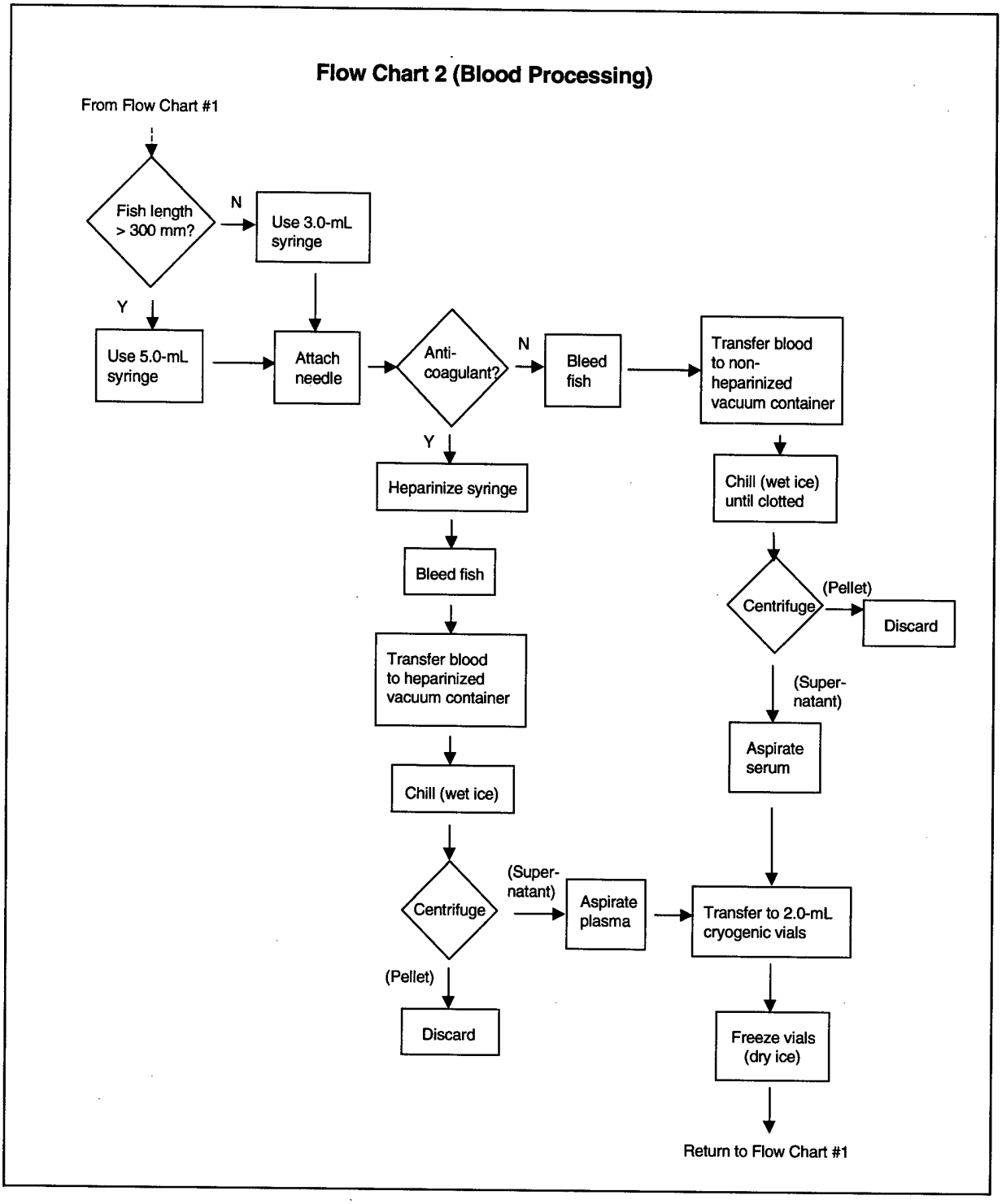
d) Heparinize the needle and syringe by uncapping the needle and drawing a few milliliters of heparin solution into the syringe, inverting it (i.e., needle up), and withdrawing the plunger to its full extent. Dispense the heparin back into its container. The amount remaining on the walls of the syringe and in the hub of the needle will prevent clotting for most fishes. The exception is the black basses and other sunfishes (Centrarchidae), the blood of which clots readily. For these fishes, leave a visible meniscus (ca. 0.2 mL) in the syringe.

- **Heparin is a powerful anti-coagulant—be careful! Also, carefully assemble and disassemble needles and syringes.**

e) Secure a fish from the holding tank. Ascertain its condition (i.e., that it is alive) and identify it to species. **Make a reasonably positive identification at this time**, using a guide to species names and abbreviations. A subset of species names and codes for fish that are likely to be used with these procedures can be found in Appendix D. Initiate a Fish Health Examination Sheet for the fish by assigning the fish an ID number. **Record the Station ID number, date, fish ID number, and species** on the Fish Health Examination Sheet. If you are not sure of the fish species identification, note on the data sheet that you will confirm it at a later time.

Note: For fish nomenclature see Robins et al. (1991).




Flow Chart 2 (Blood Processing)
From Flow Chart #1
Fish length > 300 mm?
N
Use 3.0-mL syringe
Y
Use 5.0-mL syringe
Attach needle
Anti-coagulant?
N
Bleed fish
Transfer blood to non-heparinized vacuum container
Y
Heparinize syringe
Bleed fish
Transfer blood to heparinized vacuum container
Chill (wet ice)
Centrifuge
(Super-natant)
Aspirate plasma
(Pellet)
Discard
Chill (wet ice) until clotted
Centrifuge
(Pellet)
Discard
(Super-natant)
Aspirate serum
Transfer to 2.0-mL cryogenic vials
Freeze vials (dry ice)
Return to Flow Chart #1

f) To collect blood, wrap the fish securely in the towel or diaper, leaving the caudal peduncle exposed. The posterior caudal artery and vein lie together just ventral to the vertebral column. These vessels can be reached with a needle and syringe inserted between the hemal arches and spines from either of two directions:

Lateral approach (Figure 6): Lay the fish on its (right) side, head elevated. Remove a few scales from the lateral line near the anal fin. Insert the needle from the side, just below the lateral line, to the approximate mid-sagittal depth.

Figure 6. Drawing blood from the caudal artery and vein using a lateral approach. Note that the biologist handling the fish is wearing gloves.

Ventral approach (Figure 7): Hold the fish on its back, head elevated, with the ventral surface facing you. Remove a few scales from the narrowest part of the caudal peduncle, and insert the needle in the mid-sagittal plane. Push the needle toward the vertebrae (dorsally), angled anteriorly.

Note: This method does not work for all species.

✚ **Be careful! Using a needle and syringe with an un-anesthetized, live fish can be dangerous. Two people are generally required—one to hold the fish, the other to obtain the sample. Do not stab yourself or a co-worker.**

Exert **slight** suction. When the needle contacts the base of the vertebrae (if drawing ventrally), withdraw the needle slightly. Too much suction will collapse vessels and tissues around the needle, restricting blood flow. It

Figure 7. Drawing blood from the caudal artery and vein using a ventral approach. Note that the biologist handling the fish is wearing gloves.

may be necessary to try more than once; if so, move anteriorly with each new attempt. However, repeatedly withdrawing the needle increases the likelihood of a clot forming. If a clot does form, withdraw the needle from the fish and expel the clot with slight pressure on the plunger of the syringe.

Fill the syringe with blood (if possible). **Record the volume of blood obtained**, as well as any problems encountered, on the Fish Health Examination Sheet.

Note: The minimum quantity of blood that yields a useable volume of plasma or serum should be specified in the study plan or protocol. Blood should be collected from a single location on all fish in a study to avoid introducing variation into the results.

g) Using pliers, remove the needle from the syringe, crush or bend it, and put it in the sharps container for later disposal.

h) Remove the cap from a labeled, chilled, vacuum container. If the study requires serum, use a non-heparinized vacuum container. If plasma is required, use a heparinized vacuum container. To avoid bubbles and hemolysis, **gently** dispense the blood from the needle-less syringe into the vacuum container—tilt the vacuum container slightly and run the blood down the side of the tube. Gently rock the vacuum container from side-to-side about 10 times to mix the heparin (if used) into the blood. Replace the cap and place the vacuum container in the tube rack cradled in ice (wet) and chill until processed.

i) Crush the syringe and place it in the trash bag for later disposal.

j) Conduct the external examination of the head surface and the eyes at this time (per instructions under Section

6.2.1 and 6.2.2). **DO NOT remove anomalies** at this time —the fish has not been subdued.

k) Expose the anterior dorsal surface of the specimen and, with a blunt instrument, subdue the fish with a sharp blow to the rear of its head.

l) Place the fish, in its towel, on the previously tared electronic balance. If the fish is too heavy for the balance, unwrap it and weigh it on the hanging balance. A tared holding container (e.g. a stainless steel pan) can be used to weigh fish < 2000 g on the larger electronic balance.

m) **Record the weight of the fish (± 1 g)** on the Fish Health Examination Sheet.

Note: Check the zero and the operational mode of the balances between each fish, and re-adjust or tare as necessary.

n) Unwrap the fish and measure it on the measuring board (total length, in millimeters; squeeze the caudal fins together – Figures 8A and 8B).

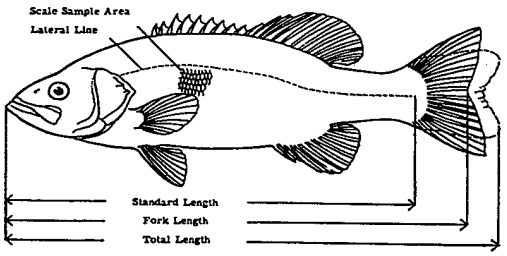


Figure 8A. Length measurements and scale sampling areas for spiny-rayed fish. From Lagler, *Freshwater Fishery Biology*, (1956) Wm. C. Brown Publishers, used with permission from the McGraw-Hill Companies.

o) **Record the total length (mm)** on the Fish Health Examination Sheet.

p) If you have not already done so, **label a tag and a scale envelope (fish identification number)** (Figure 1); use a permanent, waterproof felt-tipped pen and write in large, block letters. Use one or the other as a label in any photographs or video footage.

6.2 External Examination (Flow Chart 3)

Work quickly; although working times may vary with temperature, species, and endpoint, the activities of many enzymes decline rapidly after the death of the fish,

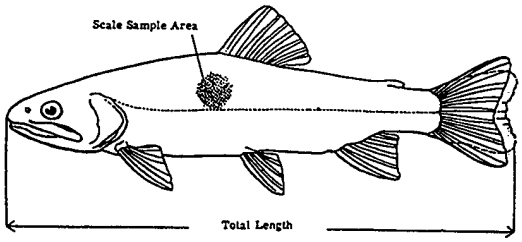


Figure 8B. Length measurements and scale sampling areas for soft-rayed fish. From Lagler (1956).

and tissues become necrotic. As a general rule, not more than 10 minutes should elapse from the time the fish is subdued until the cryogenic vials containing liver tissue are transferred to the slush for freezing. If more than 10 minutes elapses, note the approximate length of time elapsed in the "General Comments" field on p. 2 of the Fish Health Examination Sheet.

Place the subdued fish on the measuring board to make general observations (Figure 9). **Record general remarks about fins, skin, and other external features** on the Fish Health Examination Sheet before you begin the specific observations of particular organs, tissues, and structures. Important conditions to note are deformities, scale loss, external parasites, etc.

Note: Aesthetic observations are unnecessary.

Begin the external observations as outlined on the Fish Health Examination Sheet; use Appendix E as a guide. Within each section, be sure to **record all observations by marking (X) in all boxes on the data sheet that apply**. Recorded examinations of eyes, opercles, pseudobranchs, and fins may include marking both "normal" and deteriorated conditions since more than one structure is involved.

Make a clear, precise mark for data entry for the external and internal examinations. If the observation does not seem to fit any of the listed categories, **check "Other" and describe your observations** in the indicated areas. **Record further remarks about listed categories** in the General Comments section.

Note: The general procedure during the external and internal examination is to record all observations that apply, and to collect and preserve a sample of anything that appears abnormal. If an abnormal sample is collected, it is helpful to the histopathologist to also collect and preserve some normal tissue of the same type. To ensure proper preservation, no piece of

Figure 9. Biologists examining the external features of the fish.

preserved tissue should be larger than 1-cm^3; cut larger pieces to this size if necessary.

Note: This line is 1 cm long. ——

6.2.1 Body Surface. Examine all surfaces for any **Tumors, Lesions, or Parasites.** Remove them and place them in the labeled bottle of fixative. **Record anomaly type(s)** found by checking the appropriate blocks. **Describe any anomalies noted as "Other". Note the general location of all anomaly type(s) in the "Other" field and record the number of pieces in fixative.**

6.2.2 Eyes (Goede 1988, Pictures 1 - 6). The left and right eyes should be examined and scored separately, as follows (check all that apply):

Normal — No aberrations evident; eyes "clear", not protruding, milky, opaque, or bloody.

Exophthalmic —Swollen, protruding eye. More commonly referred to as "pop-eye" or "goggle-eye".

Hemorrhagic — Bleeding in the eye.

Opaque —This is a very graphic category and you need not know whether the eye is functionally blind. It generally refers to opaque or cloudy eyes, or the appearance of cataracts. The nature of the opacity is not important here.

Missing — An eye appears to be missing from the fish. *(Note: it may actually be scarred over).*

Emboli —Gas bubbles visible.

Other — Any manifestations that do not "fit" the above (i.e., parasites, spots, cuts, abrasions); describe.

Record the condition of each eye and the number of pieces in fixative for each eye on the Fish Health Examination Sheet.

6.2.3 Opercles (Goede 1988, Picture 60). It is necessary only to observe the degree of shortening of the opercular flaps. Score the opercles according to the following criteria (check all that apply):

Normal — No shortening; gills completely covered.

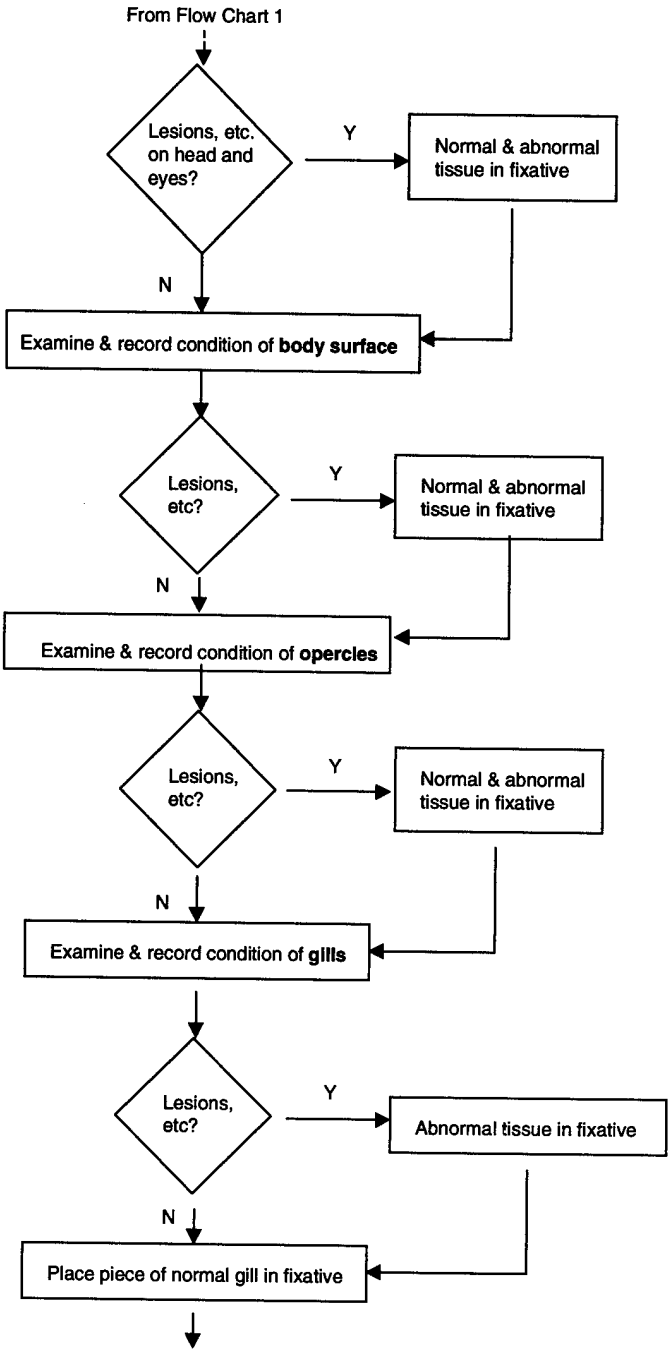
Slight shortening — Slight shortening of the opercle with a very small portion of the gill exposed.

Severe shortening — Severe shortening of the opercles with a considerable portion of the gill exposed.

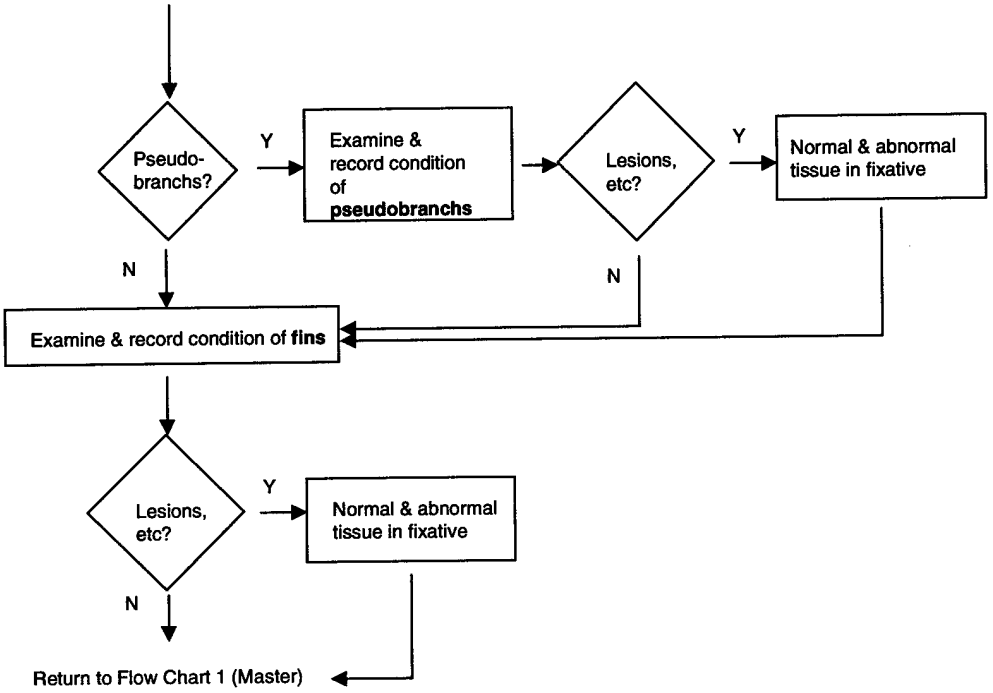
Other — Any observation that does not fit above; describe.

Record the condition of the opercles and the number of pieces in fixative.

Flow Chart 3 (External Examination)



Flow Chart 3 cont'd (External Examination)



6.2.4 Gills (Goede 1988, Pictures 7 - 15). The gills are examined and evaluated separately. Be very careful when observing the gills. Gill structure and tissue can easily be affected by the manner in which the fish is handled during and after collection. Evaluate the gills as follows (check all that apply):

Normal — No apparent aberration.

Frayed — This generally refers to actual erosion of tips of gill lamellae resulting in "ragged" appearing gills. Mere separation of gill lamellae can be construed as "frayed" but the condition may have been caused by something as simple as the manner in which the gill was exposed by the investigator.

Clubbed — This refers to swelling of the tips of the gill lamellae. They can often appear bulbous or "club" - like.

Marginate — A graphic description of a gill with a light discolored margin along the distal ends or tips of the lamellae or filaments. Margination can be and often is associated with "clubbing". If both seem to apply, check both.

Pale — This refers to gills that are very light in color. Extreme anemia can discolor the gills to the point of being white. Severe bleeding induced during sampling of blood can also result in somewhat pale gills. Gills also begin to pale somewhat after death, which is common in fish taken from nets. All of this should be considered in making the observation.

Other — Any observation that does not fit the above; describe.

Record the results of the examination of each gill on the Fish Health Examination Sheet.

Cut one small section of normal-appearing gill as well as any grossly visible anomalies and place them in the fixative.

Record the number of pieces in fixative for each gill on the Fish Health Examination Sheet.

6.2.5 Pseudobranchs. For all fishes without pseudobranchs, **check the box marked "Other", write "NA"** in the pseudobranchs comments, and proceed to the examination of the Fins (Section 6.2.6).

Pseudobranchs (Goede 1988, Pictures 16 - 23). Lift each opercular flap and locate the pseudobranchs, which lie dorsal and anterior to the gills. Score the pseudobranchs as follows (check all that apply):

Normal — Concave or flat, with no visible aberrations.

Swollen — Convex (not flat or concave).

Lithic — Mineral deposits producing a granular appearance or white spots.

Hemorrhagic — A general category that includes all forms of redness beyond the normal red color, which can be caused by hemorrhage, infection, etc.

Other — Any observation that does not fit the above; describe.

Record the condition of the pseudobranchs and the number of pieces in fixative on the Fish Health Examination Sheet.

6.2.6 Fins (Goede 1988, Pictures 61 - 63). Eroded or "ragged" fins are a departure from normal condition and health. Previously eroded fins that are healed over and show no evidence of active erosion are considered normal in this assessment. The evaluation of fins is relative to the degree of active erosion in evidence. If only one fin is actively eroding or has other abnormalities, **record the observation and fin location**. If several fins are eroding with unequal severity, **check all that apply and note in the fin comments** which fins are abnormal.

Note: Erosion usually involves both the fin membrane and rays, whereas fraying involves only the membrane.

Normal — No active erosion, fraying, or hemorrhage. This includes previously eroded fins that are completely healed over.

Mild erosion — Active erosion but no evidence of hemorrhage or secondary infection.

Severe erosion — Active erosion with hemorrhage and/or evidence of secondary infection.

Frayed — Margins of fins ragged or torn.

Hemorrhagic — Reddened (i.e., bloody) areas visible within the intact fin.

Emboli — Gas bubbles visible within the fin.

Other — Any observation that does not fit above; describe.

Record the condition or conditions that apply if one or more fins is affected. **Note the location of any lesions** in the General Comments **and the number of pieces in fixative.**

6.3 Internal Examination and Sample Collection (Flow Chart 4)

Lay the subdued fish on the foil and open the abdominal cavity with a cut from the vent forward to the pectoral girdle; cut through or closely to one side of the pelvic girdle. Do **NOT** insert the scissors so far that the internal organs are damaged.

Note: You will be asked to weigh the liver (for some fishes), gonads, and spleen. Use the balance that will yield the greatest number of significant digits based on the size of the fish and its internal organs.

Reach into the anterior end of the abdominal cavity with one gloved finger and find the esophagus. Cut the esophagus with the scissors, and remove the entire viscera except for the kidneys, which will remain in the carcass (Figure 10). Use only gloved fingers and a blunt probe to free the internal organs from the carcass. Put the carcass aside (on the foil) while working with the excised internal organs.

Begin the internal examination with the liver, which must be sampled and flash-frozen rapidly. Use Appendix E as a guide.

6.3.1 Liver, Gall Bladder, and Bile (Flow Chart 4a).

Although treated separately here and on the Fish Health Examination Sheet, these three components are examined and sampled together.

Figure 10. Viscera removed from the fish for the purpose of conducting the internal examination.

6.3.1.1 Liver Weight

Note: The following apply only for fishes with a discrete liver (salmonids, largemouth bass, and others). For fish without a discrete liver (i.e. carp), write "NA" after Weight, and proceed to the Liver Observation (Section 6.3.1.2).

Tare a weigh boat or small piece of foil (dull side up/shiny side down) on the balance. Tease the liver and gall bladder free of mesenteric membranes with gloved fingers, and remove them together (Figure 11).

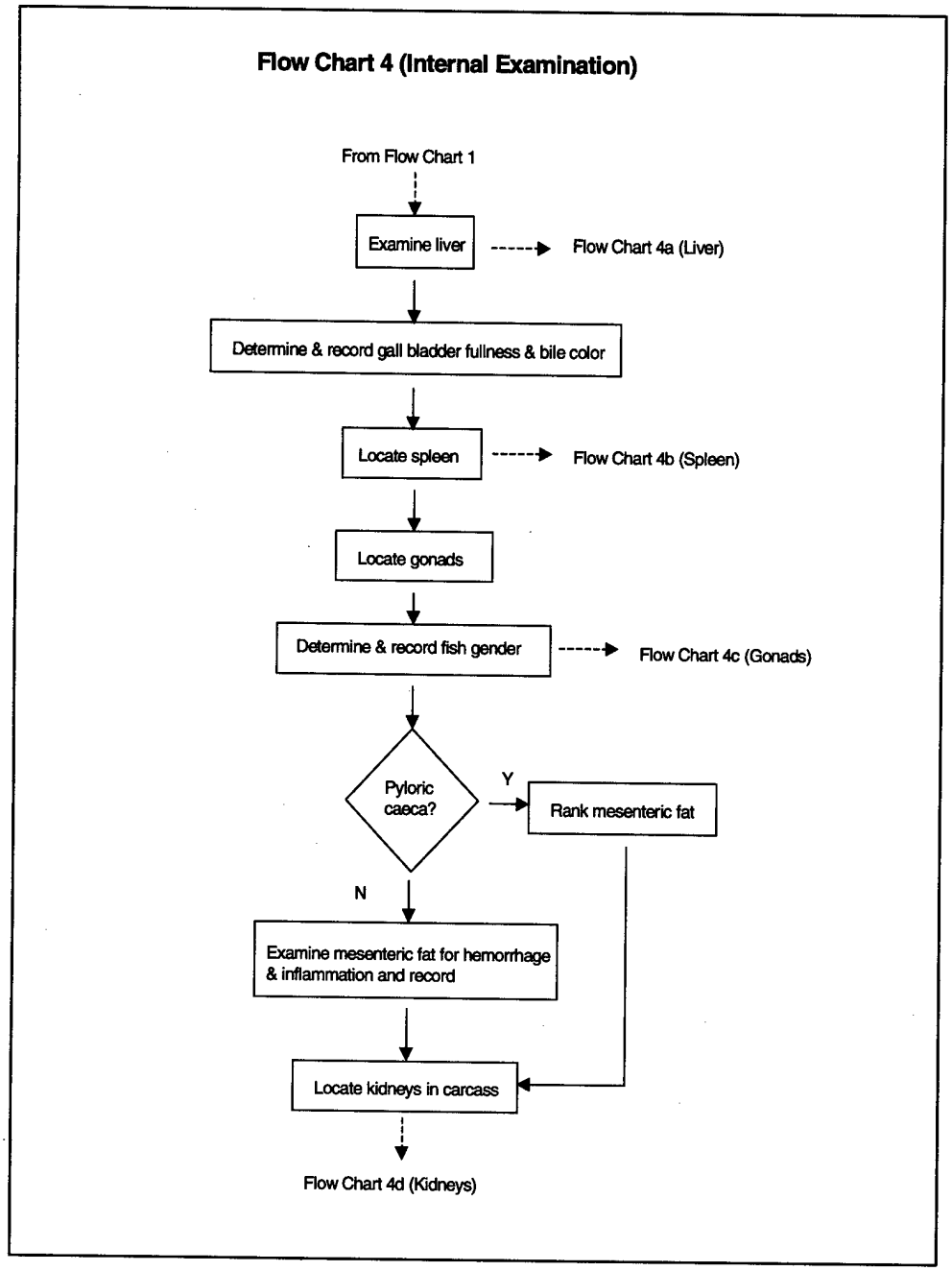
Figure 11. Removal of the liver and gall bladder.

Leave the intact gall bladder attached, and place the liver on the tared balance.

Reminder: Use the smallest capacity balance possible, based on the size of the fish and the liver.

Note: Should you inadvertently puncture the gall bladder, wash any contaminated liver,

spleen, or kidney pieces with de-ionized water before putting them into the fixative or a cryogenic vial. If the contaminated parts of the organs are to be left in the fish or returned to the carcass later, they need not be washed.



Record the liver weight (± 0.1 g or less) on the Fish Health Examination Sheet.

6.3.1.2 Liver Observation (Goede 1988, Pictures 44 - 52). The appearance of the liver may be an artifact of the sampling; the observer should take that into consideration. Note that the appearance of the intact liver and gall bladder may, for example, vary with the length of time from collection to observation. It also depends on the extent of blood loss during sampling. Check all that apply.

Dark- to light-red color — Dark red is the normal color. However, the liver is a blood storage organ, and it may be a lighter red color after bleeding, but not so pale as to be classified as general discoloration or as tan. Color may also vary among species and taxa.

Tan or "coffee with cream color" – "Fatty" liver (i.e., more or less uniformly light tan color).

General discoloration — Uniform color other than the above (gray is common); describe.

Focal discoloration — Color change in part of the liver, giving it a mottled appearance; describe.

Nodules in liver — Nodules (i.e., white parasitic cysts or bacterial granulomas) and incipient nodules, such as those in hepatoma (dark blotches) or cholangioma ("popcorn" look); swollen areas.

Other — Aberration or deviation in the liver that does not fit into above scheme; describe.

Record the liver condition on the Fish Health Examination Sheet.

6.3.1.3 Liver Sample Collection. For fish **with** a discrete liver, use **acetone-rinsed scissors** to cut any grossly observable foci or lesions from the liver and put them into the fixative. **Try not to puncture the gall bladder.** Cut the remainder of the liver into 1-cm cubes in the weigh boat or on the foil.

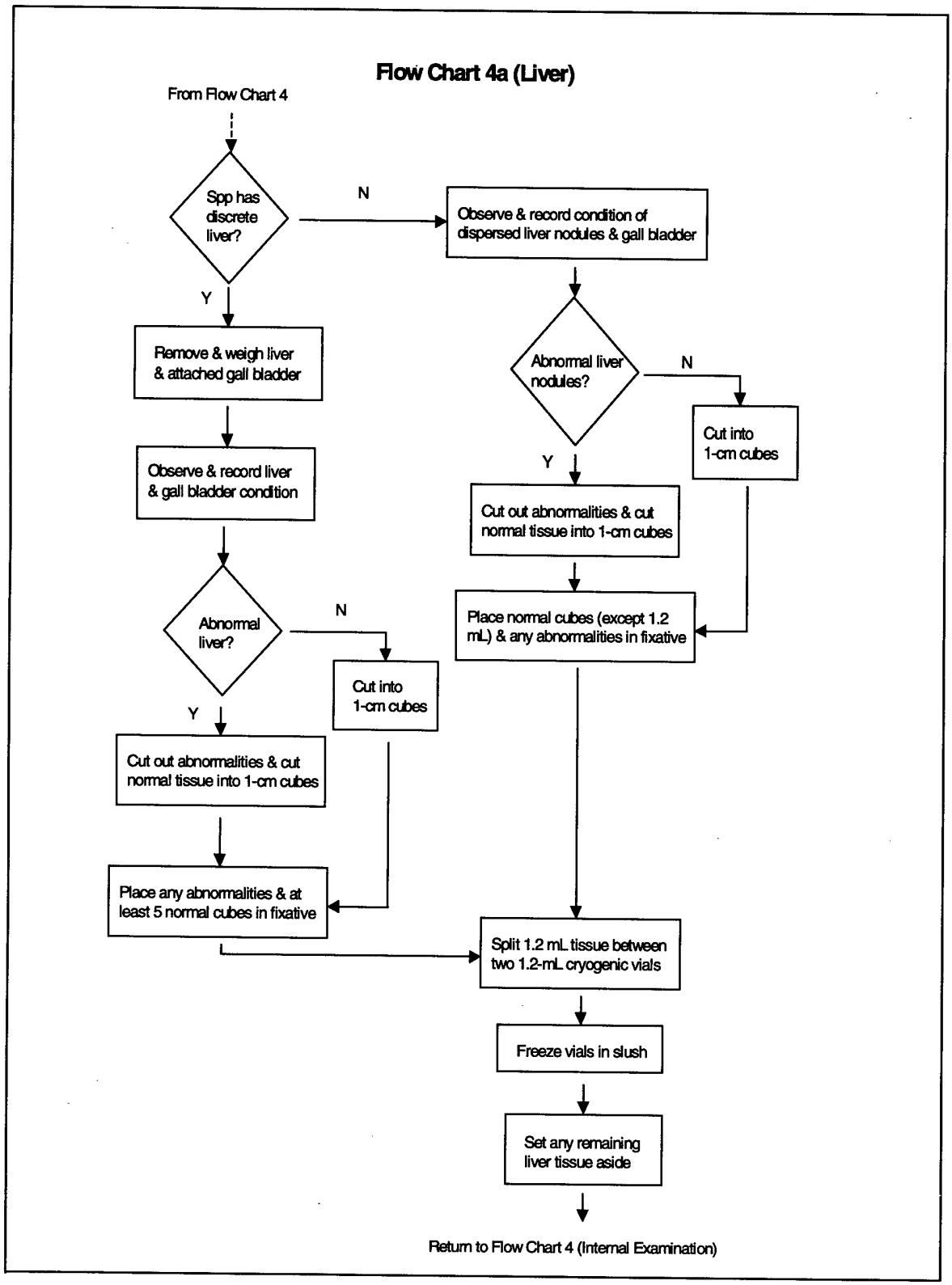
Using **acetone-rinsed forceps**, fill labeled, 1.2-mL cryogenic vials (number specified in the study plan) about half-full with liver tissue (Figure 12), and quick-freeze them in the dry-ice slush (you may use the slotted spoon). In addition to the grossly observable lesions (if any), place a minimum of five 1-cm cubes in the fixative for histopathological analysis. **Record the number of liver samples in fixative and the number of liver sample cryogenic vials** on the Fish Health Examination Sheet. Set the weigh boat or foil containing the remainder of the liver aside.

For fish **without** a discrete liver, such as common carp and goldfish, the procedure is analogous, but the liver cannot be removed and weighed. Upon completion of the visual observation, locate and inspect as many hepatic nodules as possible.

Note: Here, nodule refers to the dispersed liver organ, not to a pathological condition.

Using acetone-rinsed scissors and forceps, collect a minimum of five nodules that appear normal, cut them into 0.2-cm pieces, and fill the labeled, 1.2-mL cryogenic vials (number specified in the study plan) about half-full (Figure 12). Quick-freeze the cryogenic vials in the dry-ice slush. Collect any additional normal-appearing nodules, as well as all that appear abnormal, cut them into 1-cm pieces, and put them in the fixative. **Record the number of nodules (liver pieces) in fixative and the number of liver sample cryogenic vials** on the Fish Health Examination Sheet.

Figure 12. Placing liver tissue in a 1.2-mL cryogenic vial for cold preservation.


Flow Chart 4a (Liver)
From Flow Chart 4
Spp has discrete liver?
N
Observe & record condition of dispersed liver nodules & gall bladder
Y
Remove & weigh liver & attached gall bladder
Abnormal liver nodules?
N
Cut into 1-cm cubes
Observe & record liver & gall bladder condition
Y
Cut out abnormalities & cut normal tissue into 1-cm cubes
Abnormal liver?
N
Place normal cubes (except 1.2 mL) & any abnormalities in fixative
Cut into 1-cm cubes
Y
Cut out abnormalities & cut normal tissue into 1-cm cubes
Place any abnormalities & at least 5 normal cubes in fixative
Split 1.2 mL tissue between two 1.2-mL cryogenic vials
Freeze vials in slush
Set any remaining liver tissue aside
Return to Flow Chart 4 (Internal Examination)

6.3.1.4 Gall Bladder Fullness and Bile Color (Goede 1988, Pictures 53 - 57).

The bile is observed indirectly through observation of the gall bladder. The ranking scheme considers "fullness" of the bladder and degree of "green-ness", as follows:

Bile color should be classified as

Yellow or straw color;
Light green to grass green; or
Dark green or dark blue-green.

Gall bladder fullness may be either

Empty or nearly so;
Partly full; or
Full or distended.

Record the color of the bile and the fullness of the gall bladder on the Fish Health Examination Sheet.

Note: If bile color can be determined, bile is present and the gall bladder should be described as either "partly full" or "full". If the gall bladder is empty, do not check a color.

6.3.2 Spleen (Flow Chart 4b) (Goede 1988, Pictures 31 - 33).

Locate the spleen and dissect it whole from the viscera. Tare a weigh boat or small piece of foil. Weigh the spleen and **record the spleen weight (± 0.002 g)** on the Fish Health Examination Sheet.

Reminder: Use the smallest capacity balance possible based on the weight of the fish and the spleen.

Observe the condition of the spleen as follows (check all that apply):

Red to Black – "Black" is actually a very dark red color; both conditions are considered normal.

Granular — Granular or "rough" appearance of the spleen.

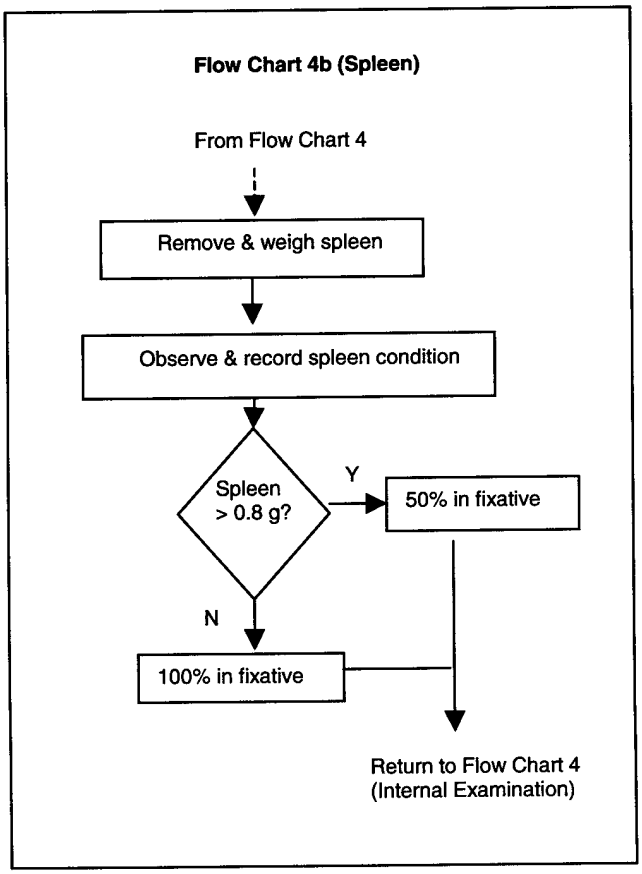
Nodular — The spleen contains or manifests fistulas or nodules of varying sizes. These are often cysts, such as those caused by parasites or chronic mycobacterial infections.

Enlarged — The spleen can be significantly and noticeably enlarged.

Other — Occasionally there are grossly visible aberrations that do not fit any of the classes above. The spleen may be mottled gray, and some fishes may have very small spleens. These should be classified as "Other" and described.

Record the spleen condition on the Fish Health Examination Sheet.

If the spleen is large (> 0.8 g), cut it in half, and place one half in the fixative, and retain the other with the carcass. Otherwise, place the entire spleen in fixative. **Record that the spleen in fixative was collected** on the Fish Health Examination Sheet.



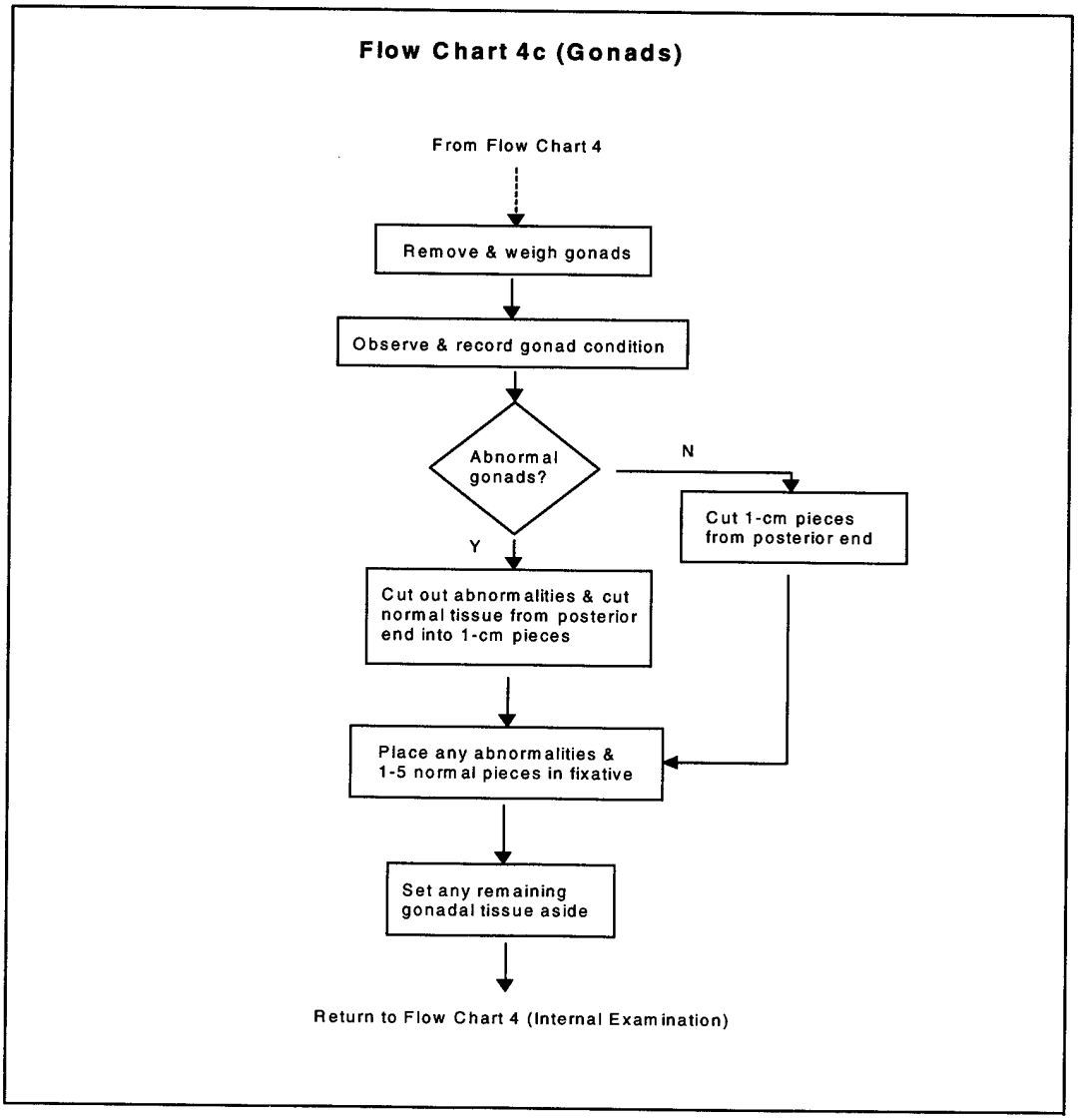
6.3.2 Gonads (Flow Chart 4c). Locate the gonads and determine the gender of the fish, if possible.

Note: The gross visible appearance of the gonads varies greatly among taxa and over the life of the fish. Generally, ovaries have a granular appearance and are yellow, orange, or pink; in contrast, testes are lighter in color (i.e., whitish to yellow) and have a less granular appearance. Some local experience with the species to be collected will be required before gender and reproductive stage can be accurately determined in the field.

Record the gender on the Fish Health Examination Sheet (under the Gonads section and in the first section of page 1). Dissect the gonads from the viscera. Tare a weigh boat or piece of foil on the appropriate balance.

Reminder: Use the smallest balance possible, based on the size of the fish and the gonads.

Record the gonad weight (± 0.1 g or less) and the gonadal condition (i.e., ripe, spent, intermediate) on the Fish Health Examination Sheet. Ripe fish have distended abdomens and large, full gonads; ovaries contain many large eggs. The gonads of spent fish are small; ovaries contain at most a few small eggs, and the gonads are vestigial or nearly so. An intermediate condition is not distinctively either ripe or spent.



Note any lesions, parasites, etc. by checking "Other" (preserve a sample), and describe.

Cut 1-cm pieces from the posterior end (bottom tips) of the gonads, and place a maximum of five gonad pieces in the fixative. If the gonad is small, preserve the entire organ.

Record the number of pieces in fixative, and set the weigh boat or foil containing the remainder of the gonad aside.

6.3.4 Mesenteric Fat (Goede 1988, Pictures 27 - 30). Pyloric caeca are present in certain fish families (eg. Salmonidae, Centrarchidae). The following ranking system of mesenteric fat deposition was developed for salmonids and has been adapted for the black basses and other fishes here. For fishes without pyloric caeca, check the mesenteric tissues in the visceral cavity for hemorrhage and inflammation. If these conditions are present, **record them** in the General Comments section on the Fish Health Examination Sheet, write "**NA**" in the Mesenteric Fat section, and skip to the examination of the Kidney(s) (section 6.3.5).

No fat — No fat deposited around the pyloric caeca and no fat deposited anywhere in the visceral cavity.

Slight fat — Less than 50% of each caecum is covered with fat.

50% — about 50% of each caecum is covered with fat.

> 50% — More than 50% of each caecum is covered with fat.

Completely covered — Pyloric caeca are completely covered by a large amount of fat.

Record the mesenteric fat rating on the Fish Health Examination Sheet.

6.3.5 Kidney(s) (Flow Chart 4d). In some fishes (common carp, centrarchids), the anterior (head) and posterior (trunk) kidney are separate; in others (salmonids, ictalurids), they are continuous. We treat them as being together for examination, but separate for sample collection. Observe and sample both.

6.3.5.1 Posterior (Trunk) Kidney (Goede 1988, Pictures 36 - 43). The trunk kidney is exposed by removing the layer of mesentery at the posterior dorsal border of the abdominal cavity. Observe its condition as follows (check all that apply):

Normal — Firm, dark red color lying relatively flat dorsally in the visceral cavity along the length of the ventral surface of the vertebral column.

Swollen — Wholly or partly enlarged or swollen.

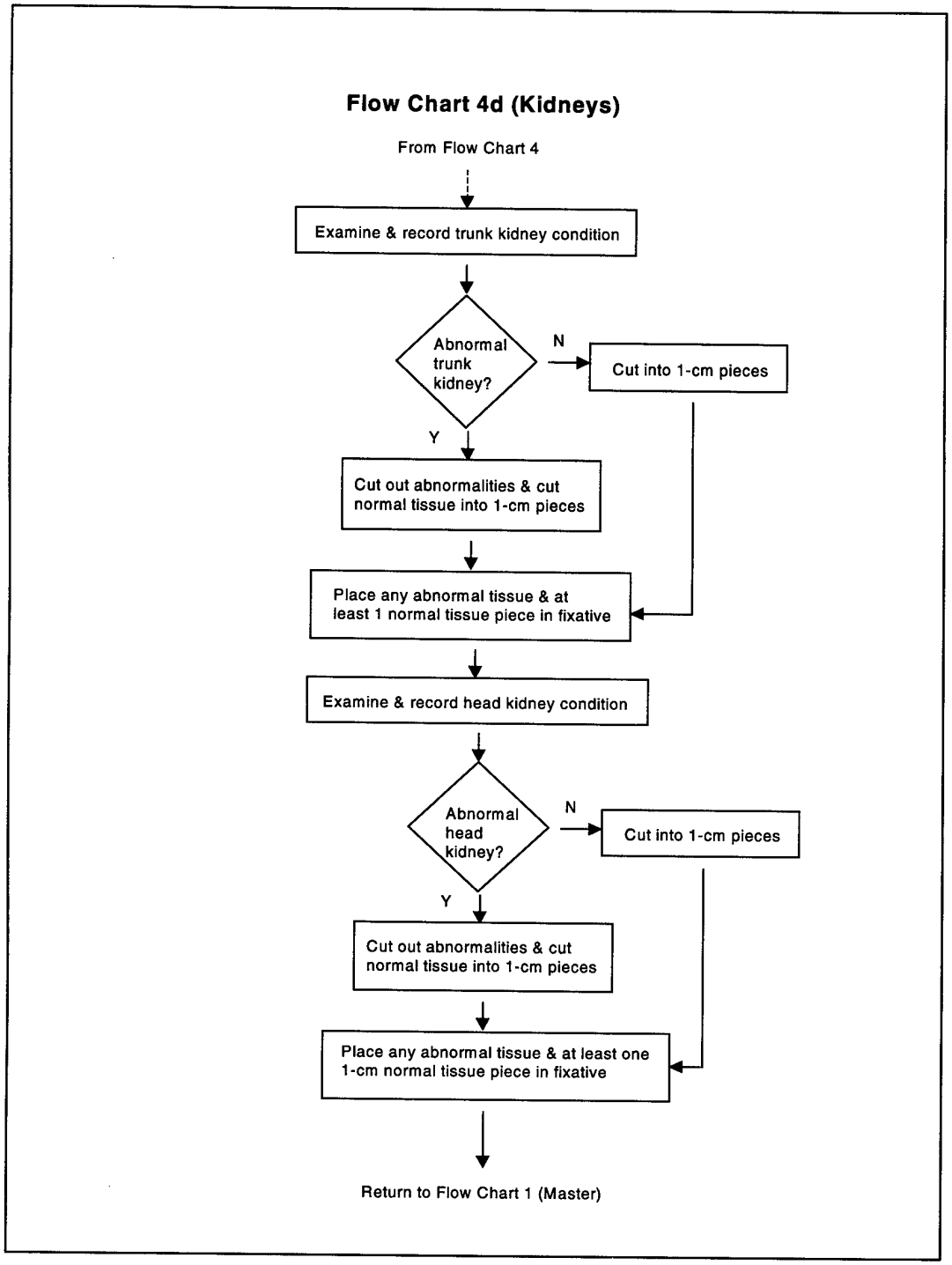
Mottled — Gray discoloration, mottled or "patchy" in appearance, ranging from scattered patches of gray to mostly or totally gray discoloration. This is not to be mistaken for the superficial gray appearance caused by the mesenteric membranes on the surface of the kidney. Move these membranes aside before examining and recording observations.

Granular — The kidney has a "granular" or irregular surface which may be due to the presence of granulomas, hemorrhages, congestion or other causes. These areas are generally not hard and "gritty".

Urolithiasis — Urolithic deposits are hard and "gritty". This condition is also known as nephrocalcinosis and involves deposition of white or "cream-colored" amorphous mineral material in the tubules of the kidney. It can range in appearance from very small white spots to severe conditions with very large "serpentine" deposits.

Note: These sites of deposition are not to be confused with the Stannius bodies (corpora of Stannius), which are the white nodular tissues present in salmonid kidneys and which have an endocrine function. The Stannius bodies are generally not associated with the tubules and usually occur at the "edges" in an area about midway along the kidney. They appear more globular than do urolithic deposits.

Other — This is used to classify any aberrations that do not fit into the above


Flow Chart 4d (Kidneys)
From Flow Chart 4
Examine & record trunk kidney condition
Abnormal trunk kidney?
N
Cut into 1-cm pieces
Y
Cut out abnormalities & cut normal tissue into 1-cm pieces
Place any abnormal tissue & at least 1 normal tissue piece in fixative
Examine & record head kidney condition
Abnormal head kidney?
N
Cut into 1-cm pieces
Y
Cut out abnormalities & cut normal tissue into 1-cm pieces
Place any abnormal tissue & at least one 1-cm normal tissue piece in fixative
Return to Flow Chart 1 (Master)

scheme; describe.

Record the trunk kidney appearance on the Fish Health Examination Sheet.

Place at least one 1-cm piece of the trunk kidney in the fixative, and **record the number of pieces in fixative** (if possible—fish kidney tends to fall apart) on the Fish Health Examination Sheet.

6.3.5.2 Anterior (Head) Kidney. In fishes with separate kidneys, the head kidney is located dorsal to the liver and may be viewed after the latter has been removed.

Record the head kidney appearance according to the criteria above; record observations in the same section as above.

Remove as much of the head kidney as you can (at least one 1-cm piece) and place it in fixative. **Record the number of pieces in fixative** (if possible) on the Fish Health Examination Sheet.

6.4 Scales or Spines (Flow Chart 1)

Collect a scale sample (most fishes) or pectoral fin spine (ictalurids) from the **left** side of the fish. If the left side is damaged (i.e., scales or pectoral fin not present), collect from the right side.

Spiny-rayed fishes (bass, walleye, etc.) — from the area of the appressed pectoral fin (Figure 8A).

Soft-rayed fishes (common carp, suckers, etc.) — beneath the anterior portion of the dorsal fin, above the lateral line (Figure 8B).

Ictalurids (i.e., catfishes) — entire **disarticulated** pectoral spine; cut away as much fin and flesh as possible. Be sure to get the base of the spine (i.e., twist off like a chicken leg).

Note: These structures are included as representative because most fishery biologists have the training, experience, and equipment necessary to read them. Other hard structures (otoliths, opercular bones, vertebrae, etc.) may be used if these structures are known to be valid indicators of age in the species you are working with and you have the capability to read them.

Place the scale or spine sample in a labeled scale envelope (Figure 1) and allow to air-dry. Store it until it can be read. **Record that the sample was collected and the side of the fish from which it was collected** on the Fish Health Examination Sheet.

6.5 Preparing Carcass Samples (Flow Chart 5)

Prepare the carcass for chemical analysis by placing unused parts of the liver and gonads back into the abdominal cavity of the fish. To avoid fins poking through the aluminum foil, cut fin spines at base. Wrap the carcass securely in the aluminum foil that was used in the examination (dull side in/shiny side out). Large fish may need to be double-wrapped. Tie the previously prepared fish ID label (Figure 1) securely to the caudal peduncle (outside of the foil). Place the carcass sample in a large plastic bag of the proper composite (see study plan) and chill on (wet) ice.

Add the carcass sample to the tally of the appropriate composite sample (i.e., write the ID number of the individual fish) in the appropriate block on the Composite Sample Worksheet.

Reminder: Keep inter-sex or indeterminate individuals separate until the gender has been confirmed histologically.

6.6 Cleaning Equipment

Thoroughly rinse all contact surfaces (measuring board, table, etc.) with tap or ambient water. Wash all dissecting equipment with D.I. water, and follow with an acetone rinse.

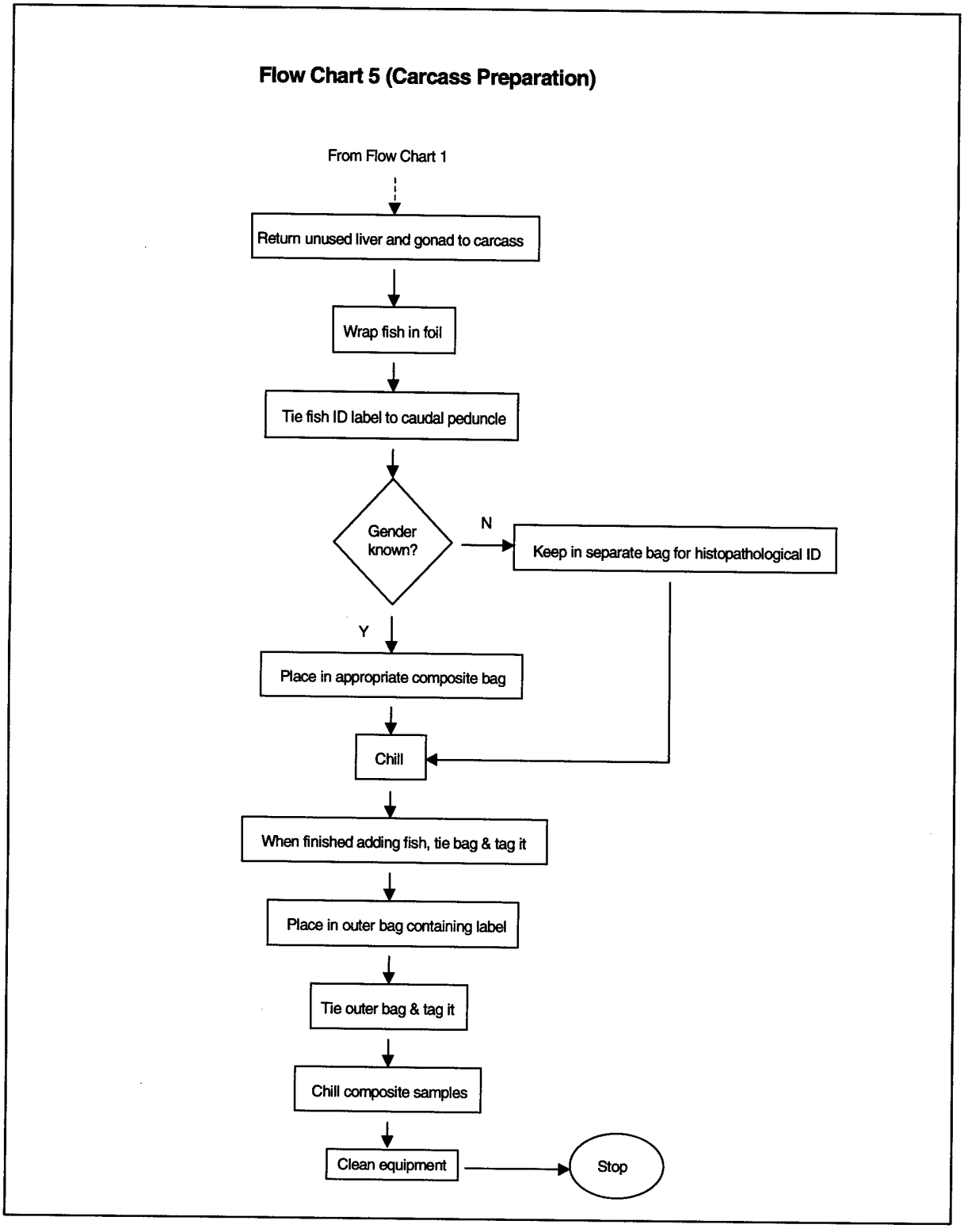
✚ **Reminder: Acetone is flammable.**

7.0 Processing Plasma or Serum Samples (Flow Chart 2)

7.1 Plasma

This procedure can be initiated at any time, but the centrifuge rotor must be balanced. Balance the rotor by spacing tubes evenly. If an even number of samples is not available, fill an empty tube with water and use it to balance the rotor. **Remove the caps** from the chilled vacuum containers to be centrifuged.

Centrifuge the tubes for 10 min @ 3500 rpm, then check the samples; the plasma should be transparent and


Flow Chart 5 (Carcass Preparation)
From Flow Chart 1
Return unused liver and gonad to carcass
Wrap fish in foil
Tie fish ID label to caudal peduncle
Gender known?
N
Keep in separate bag for histopathological ID
Y
Place in appropriate composite bag
Chill
When finished adding fish, tie bag & tag it
Place in outer bag containing label
Tie outer bag & tag it
Chill composite samples
Clean equipment
Stop

straw-colored. If it is not, spin the samples for another 10 min. To avoid warming, do not go beyond 20 min total without re-chilling the sample(s).

Note: Pink serum or plasma color that does not clear with centrifugation indicates hemolysis (breakage of red-cell membranes), which may be caused by over-aspiration during blood collection or rough handling (agitation, dispensing too vigorously) during processing. If it occurs regularly, some adjustment of technique may be indicated.

Aspirate the plasma with a transfer pipette (Figure 3B); if necessary, **divide each sample** among the labeled 2-mL cryogenic vials (number specified in the study plan). Don't try to get every drop of plasma. It is better to leave a little in the cryogenic vials than to contaminate the plasma with debris. If you disturb the red blood cell pellet in the bottom of the tube, re-centrifuge the sample for a few minutes.

Securely cap the cryogenic vials, then **freeze them in dry ice (not slush due to potential ethanol contamination)**. Discard the excess plasma and pellet. **Record the number of plasma samples collected** on the Fish Health Examination Sheet.

7.2 Serum

Allow the blood samples to clot on (wet) ice. When the samples have completely clotted and the clot has retracted, centrifuge as per Section 7.1. **Aspirate the serum** with a transfer pipette. If necessary, **divide each sample** among the labeled 2-mL cryogenic vials (number specified in the study plan). Do not disturb the clot. If you disturb the clot, allow the particulate materials to settle again before attempting to aspirate the serum.

Securely cap the cryogenic vials, then **freeze them in dry ice (not slush due to potential ethanol contamination)**. Discard the excess serum and clot. **Record the number of serum samples collected** on the Fish Health Examination Sheet.

8.0 Post-collection and Processing Procedures

8.1 Package and Store Composite Samples (Flow Chart 5)

Using a permanent, waterproof, felt-tipped pen, prepare a label [small (2-cm x 4-cm) piece of waterproof paper] and two tie-tags for each composite sample by writing the correct code on the label and both tags.

On the Composite Sample Worksheet, check the identity of the composite samples you have generated at the station. **Double-bag the composite carcass samples**. Place the composite sample label in the outer bag, and tie each bag shut with a matching labeled tag. **The team leader checks to see that the tag codes match what is recorded on the Composite Sample Worksheet**. After confirming that the actual codes match those recorded on the sheet, initial the sheet in the "Reviewer's Initials" box. Store the composite samples in coolers with (wet) ice.

8.2 Complete and Check Fish Health Examination and Station Identification Sheets

Post-calibrate the balances by re-weighing the calibration masses, and **record the "After" calibration readings** on the Station Identification Sheet. Turn off the balance(s) and re-pack them in their case(s).

The team leader checks the Fish Health Examination Sheets and the Station Identification Sheets again to be certain that all information has been recorded and is legible. Be certain that the genders specified in the "gonads" section matches what is written at the top of page 1. After each sheet has been reviewed and approved, it should be initialed in the "Reviewer's Initials" box.

8.3 Clean-up

Fasten the lid on the sharps container (tape it closed) and place it in the trash bag. Properly dispose of used expendables. Thoroughly clean all work surfaces.

Thoroughly clean and inspect all equipment used in the field to protect against the transportation of living organisms among waterways.

Note: Procedures for cleaning equipment are beyond the scope of this document and may vary among agencies, regions, states, and water bodies.

Pay particularly close attention to boats (including live wells), motors, trailers, and tubs used to hold fish. A stop at a car wash may be advantageous (be sure to rinse thoroughly with clean water). A garden sprayer or pump spray bottle containing a weak bleach solution can be used for a final rinse of all equipment.

Tap water is sufficient for cleaning smaller equipment (pans, measuring boards, coolers, live boxes), followed by the bleach solution. De-ionized water should be used to rinse dissecting equipment and the weighing pans of electronic balances (be careful!). Give dissecting equipment a final acetone rinse.

9.0 Transferring and Shipping Frozen Liver, Plasma, and Serum Samples

If you are shipping liver samples in dry ice, use the slotted spoon to transfer any frozen liver samples in the slush bath to the plastic storage box(es), and transfer the boxes to the dry ice cooler. Transfer plasma or serum samples that have been frozen in dry ice to the plastic storage box(es) and back into the dry ice cooler. Keep the samples separated by type (liver and plasma or serum) and load with the labeled surfaces facing in one direction so receiving personnel can easily read them.

If you are using a liquid nitrogen dry-shipper, transfer the frozen liver and plasma or serum samples to the dry shipper. Load the aluminum canes, and keep the samples separated by type (liver and plasma or serum). Load the cryogenic vials with the labeled surfaces facing outward so receiving personnel can read them.

When all samples have been transferred, empty the slush bath; as noted earlier, you may re-use the ethanol.

If liver, plasma, or serum samples will be shipped in dry ice, they should be shipped to cooperating laboratories or otherwise transferred to a -80C freezer within 24 hours of collection. If you are visiting more than one station, this implies that you will have to ship samples before you return to your duty station. **Find out in advance how and from where you will be shipping samples, and plan your trip accordingly!** Samples in liquid nitrogen can be kept longer, depending on ambient temperatures and the length of time since the shipper was charged. Consult the study plan or protocol to determine the recipient(s) of frozen samples.

Note: It is essential that any receiving laboratory be notified by telephone in advance of shipping frozen samples. Speak directly to the designated individuals. Do not ship if you have only left a voice-mail message or sent a FAX or E-mail message that has not been confirmed.

Complete and sign a COC form, and attach it and the addressed shipping label to the outside of the cooler (if shipping in dry ice). For liquid nitrogen dry-shippers, place the COC inside the lid of the shipping case. Be sure that the dry shipper is properly labeled ("DRY SHIPPER, NON-HAZARDOUS") on the outside of the shipping container. **Before loading samples, be sure that the dry shipper contains no LIQUID nitrogen by tipping it on its side (outdoors). Shipping companies will not accept containers that contain any LIQUID nitrogen (see Appendix F).**

Note: Store the dry ice or liquid nitrogen shippers in a freezer if possible (remember that escaping refrigerant gasses may displace the air from the freezer!). Check dry ice regularly and supplement it as necessary. Most importantly, check your dry ice or liquid nitrogen immediately before you ship! If you are shipping samples on dry ice, remember to ship them by Thursday.

10.0 Upon Return from the Field

10.1 Ship Frozen Liver, Plasma, and Serum Samples (per Section 9.0)

10.2 Process and Freeze Composite Fish Samples

Before freezing the composite fish samples, confirm the identity of any specimens you remain unsure of; solicit expert advice if necessary. Make any necessary changes on the Worksheets and re-bag samples as necessary to ensure the correct composition of each sample.

Be sure to **initial and date any corrections** you make on any Worksheet.

Freeze the composite samples, and store them frozen until they are ready to be shipped to the analytical laboratory. Ship composite samples in dry ice with a completed COC form.

10.3 Photocopy and Mail Data Sheets and Other Records

If the project coordinator/study director is stationed elsewhere, field teams should make two copies of all data sheets and field notes. One set of copies and the notebook should be retained by the team leader. The second set of copies and the original data sheets should be sent to the project coordinator/study director along with a completed COC form. If the project coordinator/study director is a member of the field team, retain both the original data sheets and field notes and at least one set of photocopies. For additional security, store the originals and copies in separate locations.

10.4 Ship Histopathology Samples

Before you ship these samples, top off the fixative and check each bottle to be certain that the tops are tightly fastened. Samples should be shipped with a completed COC form. **Check transport restrictions for your given fixative to determine the method of transportation and packaging instructions.**

10.5 Process Film

As soon as possible, **annotate your slides, photographs or videotape** - mark the station number, fish identification number (if applicable), and date.

10.6 Age Fish and Mail Age Data

Fish should be aged using the techniques developed for the structure (e.g. scale, spine, opercle) that was collected. These techniques may vary with species. If the original data must be sent to a cooperating laboratory, original data sheets should be shipped with a completed COC form. COC is optional if photocopied data sheets are being shipped.

10.7 Thoroughly Clean Non-Expendable Equipment

Equipment and supplies should be cleaned as soon as possible after returning from the field. Do not use detergents or other cleaning agents that could damage specimens or bias laboratory analyses during the next sampling trip.

Reminder: Special care should be taken when cleaning equipment to ensure that no living organisms are transported from one waterway to the next.

11.0 Acknowledgments

In addition to the objectives stated in Section 1.1, the basin-wide sampling conducted in 1995 and 1997 also sought to evaluate the administrative and logistical feasibility of implementing the BEST Program through partnerships with other agencies and USGS programs. Consequently, the studies were undertaken jointly by the BEST program, the Division of Environmental Contaminants of the FWS, and the USGS - NAWQA program. Field portions of the studies were coordinated and implemented by FWS Environmental Contaminants Specialists stationed at Ecological Services Field Offices and by NAWQA biologists, who were trained in the procedures described in this guide. We greatly appreciate the assistance of the FWS and USGS biologists and others who participated in the 1995 and 1997 studies, especially those who provided input into the development of this guide; the BEST program for supporting the bulk of the 1995 and 1997 studies and the preparation and publication of this guide; and the NAWQA program for supporting parts of the 1995 study and contributing to the preparation of the guide. A. Donahue, R. Jung, and D. Nicks also contributed substantially to the development of this guide. The review comments of C. Densmore and S. Goodbred were very helpful. R. Lipkin and R. Young prepared the final copy.

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APPENDIX A

Data Sheets

USGS

STATION IDENTIFICATION SHEET STATION ID # _____ DATE ____/____/____

STATION DESCRIPTION (River and Location): _____

Sampling START Time: ☐ Sampling STOP Time: ☐

LANDOWNER'S PERMISSION: _____ Date: _____

_____ Date: _____

Freezing Method Used: ☐ Liquid Nitrogen ☐ Dry Ice Slush

Field Shipper Used: ☐ Liquid Nitrogen Dry-Shipper ☐ Dry Ice

Team Leader: _____

Other Personnel: _____ _____

_____ _____

_____ _____

_____ _____

GPS COORDINATES: (Degrees - Minutes - Seconds)

	LATITUDE DD	MM	SS	LONGITUDE DD	MM	SS
MAXIMUM:	_____	_____	_____	_____	_____	_____
MINIMUM:	_____	_____	_____	_____	_____	_____

BALANCE: *(make and serial #)*	*MASS:*	**CALIBRATION:** Before	After

Water temperature _____ °C

RECORDER'S INITIALS: _____

USGS

FISH HEALTH EXAMINATION SHEET DATE: ___/___/___ STATION ID# _____ FISH ID# _____

Blood: _____(mL) Plasma/Serum: _____ Cryovial 1 _____ Cryovial 2

SPECIES: _____ Weight: _____(g) Length: _____(mm) Sex: _____

EXTERNAL EXAMINATION: *(check all that apply)*

EYES:

Left:
- ☐normal
- ☐exopthalmic
- ☐hemorrhagic
- ☐opaque
- ☐missing
- ☐emboli
- ☐OTHER *specify:* _____

in fixative ___ # of Photos ___

Right:
- ☐normal
- ☐exopthalmic
- ☐hemorrhagic
- ☐opaque
- ☐missing
- ☐emboli
- ☐OTHER *specify:* _____

in fixative ___ # of Photos ___

HEAD:
- ☐normal
- ☐tumors
- ☐lesions
- ☐parasite
- ☐OTHER

BODY SURFACE:
- ☐normal
- ☐tumors
- ☐lesions
- ☐parasite
- ☐OTHER

OTHER *specify* (include location): _____

in fixative ___ # of Photos___

OPERCLES: ☐OTHER *specify:* _____
- ☐normal
- ☐slight shortening
- ☐severe shortening

in fixative ___ # of Photos ___

GILLS:

Left:
- ☐normal
- ☐frayed
- ☐clubbed
- ☐marginate
- ☐pale
- ☐OTHER *specify:* _____

in fixative ___ # of Photos ___

Right:
- ☐normal
- ☐frayed
- ☐clubbed
- ☐marginate
- ☐pale
- ☐OTHER *specify:* _____

in fixative ___ # of Photos ___

PSEUDOBRANCHS: ☐OTHER *specify:* _____
- ☐normal
- ☐swollen
- ☐lithic
- ☐hemorrhagic

in fixative___ # of Photos___

FINS:
- ☐normal
- ☐mild erosion
- ☐severe erosion
- ☐frayed
- ☐hemorrhagic
- ☐emboli

☐OTHER (*specify; identify affected fins)* _____

in fixative___ # of Photos___

INTERNAL EXAMINATION: ***(check all that apply)*** FISH ID# _____

LIVER:
- ☐ dark to light red
- ☐ tan (coffee with cream)
- ☐ general discoloration
- ☐ focal discoloration
- ☐ nodules

☐ OTHER *specify:* _____

Weight _____(0.1g) *with gallbladder intact*

_____ (Cryovial 1)
_____ (Cryovial 2)

in fixative _____ # of Photos _____

BILE: Color:
- ☐ yellow
- ☐ light-grass green
- ☐ dark green to blue-green

Fullness:
- ☐ empty
- ☐ partly full
- ☐ full

☐ OTHER *specify:* _____

# of Photos _____

SPLEEN:
- ☐ red to black
- ☐ granular
- ☐ nodular
- ☐ enlarged

☐ OTHER *specify:* _____

Weight _____(0.002g)

in fixative _____

of Photos _____

GONADS:
- ☐ male
- ☐ female
- ☐ intermediate
- ☐ juvenile

☐ OTHER *specify:* _____

- ☐ ripe
- ☐ spent
- ☐ intermediate

Weight _____(0.1g)

in fixative _____ # of Photos _____

MESENTERIC FAT: ☐ no fat ☐ slight fat ☐ 50% ☐ >50% ☐ completely covered # of Photos _____

KIDNEY:
- ☐ normal
- ☐ swollen
- ☐ mottled
- ☐ granular
- ☐ urolithiasis

☐ OTHER *specify:* _____

HIND KIDNEY:
in fixative _____

HEAD KIDNEY:
in fixative _____

of Photos _____

GENERAL COMMENTS:

☐ SCALES:
side sampled:
- ☐ left
- ☐ right

☐ SPINES:
specify:
_____
_____

INITIALS:
RECORDER: _____
DISSECTOR: _____
REVIEWER: _____

USGS

COMPOSITE SAMPLE WORKSHEET STATION ID # _____ DATE ____/____/____

COMPOSITION OF COMPOSITE SAMPLES:						
SAMPLE	SPECIES	FISH ID #'S	M	F	Int	Juv
A						
B						
C						
D						
E						
F						
G						

STATION ID # _____

SAMPLE	SPECIES	FISH ID #'S	M	F	Int	Juv
H						
I						
J						
K						
L						
M						
N						
O						

REVIEWER'S INITIALS: _____

APPENDIX B

Chain-of-custody Form

DEPARTMENT OF THE INTERIOR
U. S. FISH AND WILDLIFE SERVICE
DIVISION OF LAW ENFORCEMENT

CHAIN OF CUSTODY RECORD

FILE NO.
INV-

DATE AND TIME OF SEIZURE:	REGION:	EVIDENCE/PROPERTY SEIZED BY:
SOURCE OF EVIDENCE/PROPERTY (person and/or location): ☐ TAKEN FROM: ☐ RECEIVED FROM: ☐ FOUND AT:		CASE TITLE AND REMARKS:

ITEM NO.	DESCRIPTION OF EVIDENCE/PROPERTY (include Seizure Tag Numbers and any serial numbers):

ITEM NO.				
	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.				
	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.				
	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	

☐ ADDITIONAL TRANSFERS ON REVERSE SIDE

FORM 3-2063 (3/90)

FILE NO.
INV.

CHAIN OF CUSTODY RECORD (continued)

ITEM NO.	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	
ITEM NO.	FROM: (PRINT NAME, AGENCY)	RELEASE SIGNATURE:	RELEASE DATE	DELIVERED VIA: ☐ U.S. MAIL ☐ IN PERSON ☐ OTHER:
	TO: (PRINT NAME, AGENCY)	RECEIPT SIGNATURE:	RECEIPT DATE	

* U.S. G.P.O.:1997 508-455

APPENDIX C

Sample Resume

RESUME

Christopher J. Schmitt, Research Fishery Biologist
U.S. Geological Survey, Biological Resources Division
Columbia Environmental Research Center (CERC)
4200 New Haven Rd., Columbia, MO 65201

Education

University of Massachusetts (Amherst), B.S. (Fisheries Biology), 1971
Michigan State University (East Lansing), M.S. (Fisheries Biology), 1974

Professional Experience (last 10 years)

Research Fisheries Biologist, Acting Division/Branch Chief, and Team Leader

1978-1993--U.S. Fish and Wildlife Service (FWS), Columbia National Fisheries Research Laboratory/National Fisheries Contaminant Research Center, Columbia, MO.

1993-1996--National Biological Survey/Service, Midwest Science Center, Columbia, MO.

1996-present--USGS/BRD/CERC, Columbia, MO.

Professional Memberships

Society of Environmental Toxicology and Chemistry, American Fisheries Society, Ecological Society of America, American Water Resources Association, Sigma Xi.

Selected Publications (from last 5 years)

Schmitt, C.J. 1999. Environmental contaminants. *in* M.J. Mac, P.A. Opler, P.D. Doran, and C.E. Puckett Haecker eds. *Status and trends of the Nation's living resources.* Volume 1. Pp. 131-166. U.S. Department of the Interior, National Biological Service. Washington, D.C.

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Ellis, G.S., J.N. Huckins, C.E. Rostad, C.J. Schmitt, J.D. Petty, and P. McCarthy. 1995. Evaluation of lipid-containing semipermeable membrane devices and gas chromatography-negative chemical ionization-mass spectrometry for monitoring organochlorine chemical residues in large rivers. *Environ. Toxicol. Chem.* 14:1875-1884.

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APPENDIX D

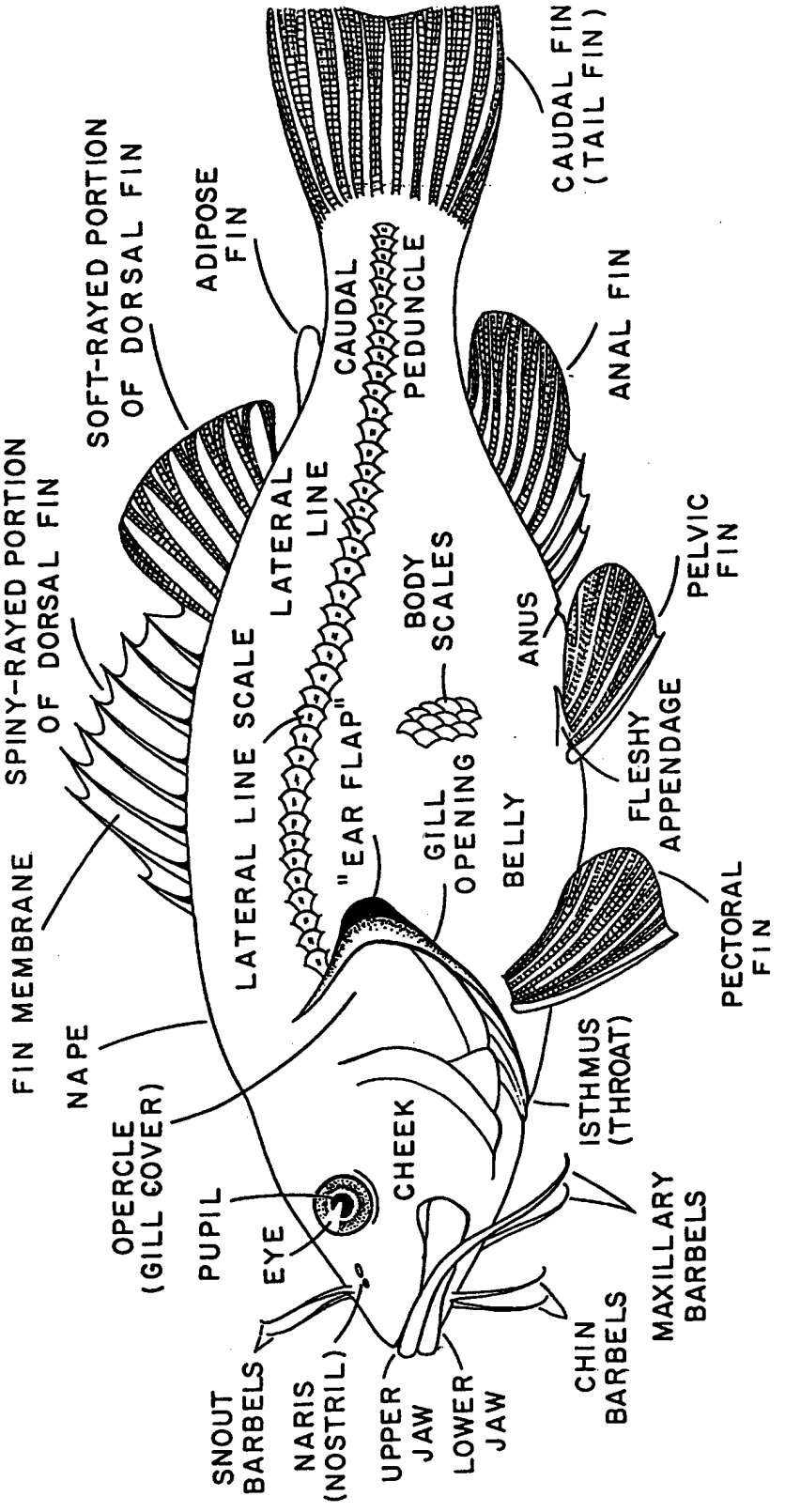
Species List

COMMON NAME	TSN	GENUS	SPECIES	SPECIES CODES
Alligator gar	201897	*Actractosteus*	*spatula*	AGAR
Arctic grayling	162016	*Thymallus*	*arcticus*	AGRY
Bigmouth buffalo	163956	*Ictiobus*	*cyprinellus*	BMBF
Black bullhead	164039	*Ameiurus*	*melas*	BKBL
Black crappie	168167	*Pomoxis*	*nigromaculatus*	BKCP
Bloater	161943	*Coregonus*	*hoyi*	BLOT
Blue catfish	163997	*Ictalurus*	*furcatus*	BLCF
Bluegill	168141	*Lepomis*	*macrochirus*	BLGL
Bowfin	161104	*Amia*	*calva*	BWFN
Bridgelip sucker	163897	*Catostomus*	*columbianus*	BSUK
Brown bullhead	164043	*Ameiurus*	*nebulosus*	BRBL
Brown trout	161997	*Salmo*	*trutta*	BNTT
Burbot	164725	*Lota*	*lota*	BRBT
Carpsucker	163916	*Carpiodes*	*Spp.*	CPSK
Chain pickerel	162143	*Esox*	*niger*	CHPL
Channel catfish	163998	*Ictalurus*	*punctatus*	CNCF
Chinese catfish	164122	*Clarias*	*fuscus*	CICF
Chiselmouth	163531	*Acrocheilus*	*alutaceus*	CHMO
Coho salmon	161977	*Oncorhynchus*	*kisutch*	COHO
Common carp	163344	*Cyprinus*	*carpio*	CARP
Cuban limia	165911	*Poecilia*	*vittata*	CULI
Dolly Varden	162000	*Salvelinus*	*malma*	DOLL
Flannelmouth sucker	163906	*Catostomus*	*latipinnis*	FSUK
Flathead catfish	164029	*Pylodictis*	*olivaris*	FHCF
Freshwater drum	169364	*Aplodinotus*	*grunniens*	FWDM
Gizzard shad	161737	*Dorosoma*	*cepedianum*	GZSH
Golden shiner	163368	*Notemigonus*	*crysoleucas*	GOSN
Goldeye	161905	*Hiodon*	*alosoides*	GDEY
Goldfish	163350	*Carassius*	*auratus*	GOLD
Green sunfish	168132	*Lepomis*	*cyanellus*	GRSF
Humpback whitefish	161937	*Coregonus*	*pidschian*	HBWF
Klamath sucker	163913	*Catostomus*	*snyderi*	KSUK
Lake chubsucker	163922	*Erimyzon*	*sucetta*	LKSK
Lake herring	161942	*Coregonus*	*artedii*	LKHR
Lake trout	162002	*Salvelinus*	*namaycush*	LKTT
Lake whitefish	161941	*Coregonus*	*clupeaformis*	LKWF
Largemouth bass	168160	*Micropterus*	*salmoides*	LMBS
Largemouth bass x spotted bass	168158	*Micrpoterus*	sp.	LMBS/STBS
Largescale sucker	163896	*Catostomus*	*macrocheilus*	CSUK
Longear sunfish	168153	*Lepomis*	*megalotis*	LESF
Longnose gar	161094	*Lepisosteus*	*osseus*	LNGR
Longnose sucker	163894	*Catostomus*	*catostomus*	LNSK
Mixed species		Mixed	sp.	MIXD
Mooneye	161906	*Hiodon*	*tergisus*	MNEY
Mountain whitefish	162009	*Prosopium*	*williamsoni*	MTWF
Mozambique tilapia	170015	*Oreochromis*	*mossambicus*	MTIL
Northern pike	162139	*Esox*	*lucius*	NTPK
Northern squawfish	163523	*Ptychocheilus*	*oregonensis*	NSQW
Orangespotted sunfish	168151	*Lepomis*	*humilis*	OSSF
Peamouth	163521	*Mylocheilus*	*caurinus*	PEAM
Pumpkinseed	168144	*Lepomis*	*gibbosus*	PMSD
Quillback carpsucker	163917	*Carpiodes*	*cyprinus*	QLBK
Rainbow trout	161989	*Oncorhynchus*	*mykiss*	RBTT
Redbreast sunfish	168131	*Lepomis*	*auritus*	RBSF
Redear sunfish	168154	*Lepomis*	*microlophus*	RESF

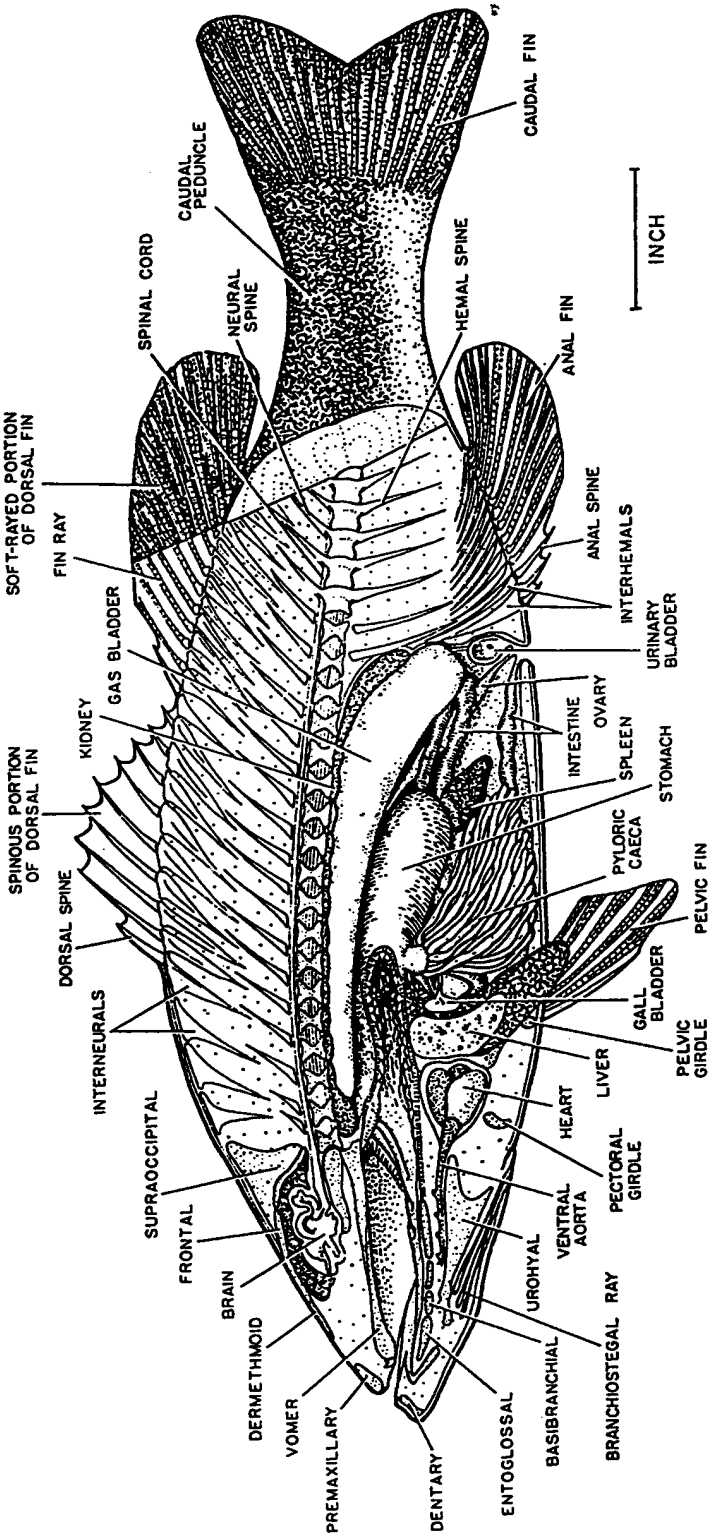
COMMON NAME	TSN	GENUS	SPECIES	SPECIES CODES
Redfin pickerel	162140	*Esox*	*americanus*	RDFP
Redhorse	163927	*Moxostoma*	sp.	REDH
Redside dace	163373	*Clinostomus*	*elongatus*	RSDC
River carpsucker	163919	*Carpiodes*	*carpio*	RCSK
River chub	163392	*Nocomis*	*micropogon*	RVCB
River redhorse	163936	*Moxostoma*	*carinatum*	RVRH
Rock bass	168097	*Ambloplites*	*rupestris*	RKBS
Round whitefish	162008	*Prosopium*	*cylindraceum*	ROWF
Sacramento blackfish	163589	*Orthodon*	*microlepidotus*	SCBF
Sacramento sucker	163908	*Catostomus*	*occidentalis*	SASK
Sauger	168509	*Stizostedion*	*canadense*	SGER
Skipjack herring	161707	*Alosa*	*chrysochloris*	SKIP
Smallmouth bass	168159	*Micropterus*	*dolomieui*	SMBS
Smallmouth buffalo	163955	*Ictiobus*	*bubalus*	SMBF
Spotted bass	168161	*Micropterus*	*punctulatus*	STBS
Spotted gar	161099	*Lepisosteus*	*productus*	SDGR
Spotted sucker	163959	*Minytrema*	*melanops*	SSUK
Striped bass	167680	*Morone*	*saxatilis*	SDBS
Striped mullet	170335	*Mugil*	*cephalus*	STMU
Tahoe sucker	163914	*Catostomus*	*tahoensis*	TASK
Threadfin shad	161738	*Dorosoma*	*petenense*	THSH
Unknown species		Unknown	sp.	UNKN
Walleye	168508	*Stizostedion*	*vitreum*	WLYE
Warmouth	168139	*Chaenobryttus*	*gulosus*	WARM
Whiper	167676	*Morone*	sp.	WHIP
White bass	167682	*Morone*	*chrysops*	WTBS
White catfish	164037	*Ameiurus*	*catus*	WHCF
White crappie	168166	*Pomoxis*	*annularis*	WTCP
White perch	167678	*Morone*	*americana*	WTPR
White sucker	163895	*Catostomus*	*commersoni*	WTSK
Yellow bullhead	164041	*Ameiurus*	*natalis*	YLBL
Yellow perch	168469	*Perca*	*flavescens*	YLPR

APPENDIX E

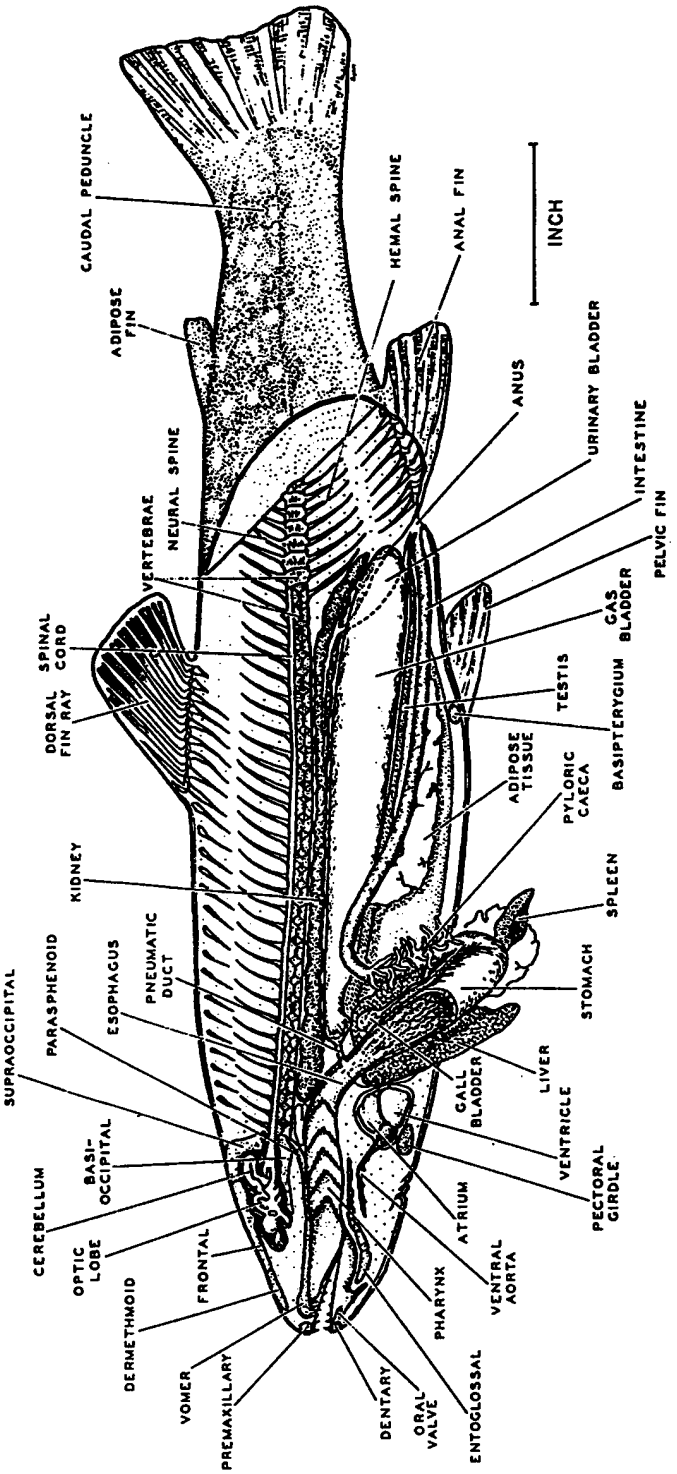
External and Internal Anatomy



External features of a composite fish. From Hunn (1988).



Anatomy of a spiny-rayed bony fish, the largemouth bass, *Micropterus salmoides*. From Hunn (1988).



Anatomy of a soft-rayed bony fish, the brook trout, *Salvelinus fontinalis*. From Hunn (1988).

APPENDIX F

Bulletin on Shipping Liquid Nitrogen

Federal Aviation Administration

Civil Aviation Security

Dangerous Goods Advisory Bulletin

Information of Concern to Air Carriers

Subject: Liquid Nitrogen in Dewars

Date: Aug 25, 1998

Number: DGAB-98-03

INFORMATION: The Federal Aviation Administration (FAA) is issuing this Advisory Bulletin to alert air carriers to the hazards associated with mishandling authorized packagings utilizing liquid nitrogen as a refrigerant. These packagings are "non-pressurized" flasks and specially designed flasks known as "dry shippers", which are used to transport refrigerated biological specimens. Liquid nitrogen is a regulated material subject to 49 CFR Parts 100 – 180, Hazardous Materials Regulations, (see 173.320(c)) and the International Civil Aviation Organization's Technical Instructions for the Safe Transport of Dangerous Goods by Air (see Packing Instructions 202).

Safe Handling:

The closure of the container is designed to allow venting to the atmosphere, through the fill opening, in order to prevent the build up of pressure within the package. Packages are designed to be transported in an "upright" position at all times. These containers release of liquid nitrogen through the venting system when handled adversely to the orientation markings and package design. Therefore, it is important that personnel that handle, load and unload flasks that contain liquid nitrogen maintain the flask in the upright position at all times. Failure to do so may result in the release of liquid nitrogen and cause injury.

Non-pressurized flasks: (*other than "dry-shippers"*)

These types of containers are authorized for liquid nitrogen (Division 2.2, cryogenic), in the ICAO Technical Instructions (TI), and must meet the packing instructions in 202. Non-pressurized flasks are similar in design and appearance to "dry shippers", except they are filled with liquid nitrogen, and the biological specimens are suspended in it.

Dry Shippers:

Dry shippers, when properly prepared (see the Note at the end of Packing Instructions 202) are not subject to the requirements of the regulations. However, if the dry shippers are offered with free liquid nitrogen present, they would be subject to the regulations when offered for transportation by aircraft (see 49 CFR 173.320) and must be offered in

accordance with the ICAO Technical Instructions. These packagings use liquid nitrogen (Division 2.2, cryogenic liquid) as a refrigerant. A dry shipper consists of an outer metal jacket and an inner shell, with the space between filled with insulation and vacuum-sealed. The interior of the packaging contains a cylindrical void, which holds the material requiring refrigeration, surrounded by absorbent material. The absorbent material is saturated with the liquid nitrogen. The FAA has found shipments where the nitrogen is not completely absorbed so when handled adverse to the orientation markings, results in a loss of liquid nitrogen.

FAA Enforcement:

The FAA will actively pursue enforcement actions against all parties who violate the Hazardous Materials Regulations or the ICAO Technical Instructions. Violators are subject to civil penalties of $27,500 and criminal prosecution with penalties of $250,000 and up to five years in prison.

/s/

Bruce Butterworth

Director, Office of Civil Aviation Security Operations

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REPORT DOCUMENTATION PAGE

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Biomonitoring of Environmental Status and Trends (BEST) Program: Field Procedures for Assessing the Exposure of Fish to Environmental Contaminants

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6.AUTHOR(S)
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12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*) This document describes procedures used by biologists to collect information, tissues, and fluids useful for determining the exposure of fish to environmental contaminants. Fish are to be captured and held alive (generally <1 h), then weighed, measured, and examined for grossly visible external lesions. A blood sample is collected by caudal veinipuncture. The fish is subdued, and its abdominal cavity is opened with a mid-ventral incision. The internal organs are dissected from the fish for examination. Selected organs are weighed, and tissues are collected for laboratory analyses. All remaining tissues and fluids are then returned to the carcass, which is prepared for chemical analysis. Individual fish are composited by station, species, and gender; frozen; and shipped to the analytical laboratory. Procedures are also described for record keeping; processing blood to obtain serum and plasma; flash-freezing samples; cleaning equipment; and preventing the transport of living organisms among waterways. A list of necessary equipment and supplies is also provided.

14. SUBJECT TERMS

contaminants, fish health, biomarkers, chemical analysis, histopathology

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