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APPENDIX A.17

FIELD SAMPLING AND ANALYSIS PLAN FOR BULLFROGS

1. INTRODUCTION

1.1 BACKGROUND

Several life history characteristics make the bullfrog (*Rana catesbeiana*) a useful indicator of potential ecological effects of PCB contamination in Housatonic River sediments. The bullfrog is an aquatic species favoring permanent bodies of water (DeGraaf and Rudis, 1983; Smith, 1961), including river oxbows (DeGraaf and Rudis, 1983). In addition to being in frequent contact with water and sediment, it hibernates in the mud under water during winter months (Raney, 1940).

The home range of the bullfrog is small. In a study of a New York woodland lake, the average distance traveled in a day during the summer months ranged from 200 to 300 ft (Raney, 1940; Ingram and Raney, 1943). Male bullfrogs defend small territories during breeding season (DeGraaf and Rudis, 1983), which can extend into July in northern states (Smith, 1961). Thus, bullfrogs may act as indicators of localized contamination, should they be present.

In addition, the bulk of the bullfrog diet consists of aquatic insects and crustaceans (Smith, 1961). Bullfrogs are preyed upon by piscivorous birds such as bitterns and herons, by mammals such as raccoon (*Procyon lotor*) and mink (*Mustela vison*), and by aquatic reptiles such as snakes (Martin et al., 1951). Anecdotal information indicates that bullfrogs in the Housatonic River watershed are harvested for human consumption, posing potential human health risks that may require evaluation.

1.2 OBJECTIVES

There are two basic objectives of this field investigation:

- The whole-body frog tissue concentrations will be determined and used in the fate and effects model and the ecological risk assessment.

- To provide bullfrog leg muscle tissue for contaminant analysis that can be used to qualitatively evaluate the potential risk to human health from consumption of bullfrog leg muscle tissue, if warranted.

2. STUDY DESIGN

2.1 FIELD SAMPLING DESIGN

2.1.1 Number of Samples

Five to ten adult bullfrogs will be collected from each of four sampling areas. The number of bullfrogs sampled per area was determined on the basis of known differences in sediment PCB concentrations between areas within suitable bullfrog habitat. A power analysis was also conducted to determine an appropriate sample size based on literature data on PCB uptake in frogs (Attachment 1). However, the power analysis was not relied upon exclusively to determine sample size, given that the primary objective of the investigation is to collect tissue data for input into exposure models, as opposed to solely drawing statistical inferences between tissue data from different populations.

2.1.2 Sampling Locations

Sampling areas were selected by conducting a field reconnaissance to identify appropriate bullfrog habitat, followed by a review of available contaminant data to identify habitats both with and without detected PCB concentrations (for use as site-related and reference sampling areas, respectively). Within the Housatonic River study area, bullfrog habitat is primarily limited to Woods Pond and backwater areas within 1 mile north of Woods Pond. These two general areas have a lower water velocity, which allows deposition of contaminated sediments, as well as growth of submerged and emergent aquatic vegetation favored by bullfrogs. In general, historical data suggests that Woods Pond has higher sediment PCB concentrations than the backwater areas, which have relatively lower, but variable concentrations. The selected reference areas are Three-Mile Pond and Muddy Pond, in which PCBs have not been detected in sediment.

2.1.3 Supplemental Sediment Sampling

Because of the small home range of bullfrogs, existing sediment PCB data may not adequately characterize the range of sediment concentrations to which the frogs are most likely to be exposed. Additional sediment samples will be collected as necessary to obtain PCB concentrations in sediment where the frog is captured. Sediment sampling will occur after the frog sampling; frog capture locations will be marked with pin flags or flagging tape.

2.1.4 Collection Methods

Bullfrog sampling will occur primarily at night. From a boat, a portable spotlight will be shone along the shoreline in order to spot and blind the frogs. Frogs will be netted while blinded. If vegetation is too dense for netting, frogs may be speared using a long-handled gig or fork. Since this method could damage reproductive tissues, or potentially cause cross-contamination with

contaminated water or sediment, the gig or fork will be used only if necessary. If the gig or fork method is used, the gig or fork will be decontaminated between captures, and the captured frog will be immediately rinsed with deionized water.

Another method of collecting frogs consists of walking along the edge of the water wearing waders and capturing frogs with a large hand net either on the marsh surface or immediately after they jump in the water. This method is limited to daylight hours for health and safety reasons. As bullfrogs are often more vocal at night, this method will be used only if sufficient numbers of frogs cannot be obtained by spotlighting.

Intensive frog sampling will be conducted during a 1-week period in August (hereafter referred to as the "main sampling period").

Upon capture, bullfrogs will be given an identification number and returned to the central processing area. If possible, additional frogs at each site will be captured, so that the frogs to be retained for analysis will be of similar weight and sex distribution among the sampled areas. The frogs selected for analysis will be processed immediately.

Initial processing includes recording sex and other physical metrics (total body weight, age class, snout vent length, and leg length), followed by processing for tissue analysis. Leg muscle tissue samples will be prepared by skinning the frog, then removing each leg. The muscle tissue will then be removed from the bone, and the sample will be weighed, packaged, and labeled. The remaining carcass will be weighed, packaged, and labeled for separate analysis. After processing, each frog sample will be snap frozen with liquid nitrogen and held in a -20 to -30 ºC freezer.

2.2 ANALYTICAL REQUIREMENTS

Table 1 presents a summary of the number of frog tissue samples to be collected and the corresponding analyses to be undertaken. Each frog will be analyzed separately. Both leg muscle tissue and whole body tissue will be analyzed for the following parameters: PCBs (total, Aroclors, congeners, and homologs), percent moisture, and percent lipids. A subset of each group of samples will be analyzed for dioxins/furans and Appendix IX OC pesticides. The analytical methods to be used and the desired detection limits are specified in Appendix C of the *Quality Assurance Project Plan* (QAPP) (WESTON, 2000). A total of 10 g of tissue is required for each the whole body carcass and leg muscle tissue analyses. This tissue mass should be easily obtainable from individual adult bullfrogs, so that composite samples from different frogs will not be required.

Table 1
Analytical Summary for Bullfrogs
Housatonic River
Pittsfield, MA

Location	Number of Frogs	Number of Samples		Required Tissue Mass Per Sample (g)		Analytical Parameters				
		Leg Muscle Tissue	Whole Body Carcass	Leg Muscle Tissue[a]	Whole Body Carcass[a]	PCB	OC Pesticides[b]	Dioxins/ Furans[b]	Percent Moisture	Percent Lipids
Woods Pond	10	10	10	6	6	X	X	X	X	X
Backwater Areas Within 1 Mile North of Woods Pond	10	10	10	6	6	X	X	X	X	X
Three-Mile Pond Reference	5	5	5	6	6	X	X	X	X	X
Muddy Pond Reference	5	5	5	6	6	X	X	X	X	X
Total	30	30	30			30	12	12	30	30

Notes: [a] Required mass per sample is 10 g.
[b] Five frogs per target area, 1 frog per reference area

Actual whole-body tissue concentrations will be calculated after receipt of analytical results as the weighted average concentration in leg and carcass for each parameter. The weight of the leg muscle tissue will be multiplied by the concentration in muscle for each analytical parameter. The same will be done for the remainder of the carcass that is analyzed. The resulting sum (contaminant load in the leg muscle plus contaminant load in the carcass) will be divided by the total wet weight to obtain a wet-weight whole body concentration.

3. PROCEDURES

3.1 FIELD SAMPLING

1. Working in two-person teams, deploy boat with equipment.

2. At night a spotlight will be used to spot and blind frogs so they may be captured with a dip net, or if necessary, speared with a gig or fork.

3. Place the frogs captured at each location in a decontaminated 5-gallon polyethylene bucket filled with 2 to 3 inches of river water in the bottom. The lid of each container will be perforated to allow air exchange while the animals are held for processing.

4. After all frogs have been collected from a location, label the bucket lid and side with the location number, date/time, collector's initials, and method of collection.

5. Record a description of the location, date and time, method of collection, name(s) of collector(s), and the number of frogs collected in a field logbook.

6. Mark the exact location that each frog is collected with a pin flag or flagging, and record it on a map. Return later to record GPS coordinates at each flag point and to sample sediment.

7. Proceed to the next location and collect frogs as above. Return frogs to the central processing area.

8. If frogs are to be held for more than 3 hours, transfer them to coolers fitted with an aerator, and filled with 3 to 6 inches of river water from the same location.

3.2 PROCESSING

3.2.1 Initial Processing

1. Gather and set up equipment for two-person teams. Prepare processing table with clean plastic sheeting. One person records data, while the other processes the frog.

2. On the data sheet, record the location, date/time of collection, collector's initials, method of collection, and habitat description.

3. Decontaminate two to three pieces of aluminum foil with nitric acid/deionized water/hexane/isopropyl alcohol and air dry.

4. From the first location bucket, remove a frog while wearing Neoprene gloves and stun it with a sharp blow to the back of head with a decontaminated steel rod. The frog should then be double-pithed to ensure a humane death. This approach is deemed "conditionally acceptable" by the American Veterinary Medical Association Panel on Euthanasia. Sample processors will then tare the scale with the decontaminated aluminum foil, rinse the frog with deionized water, and weigh it.

5. Weigh the frog and record the data to the nearest 0.01g.

6. Measure frog leg length and total length (snout to vent) and record the data to the nearest 1.0 mm.

7. Identify and record the frog's age class (juvenile/adult). Male juveniles are generally less than 85 mm (females 89 mm) in length (Wright and Wright, 1949), and darker gray in color (Smith, 1961).

8. Identify the sex, and record on the data sheet. The following criteria may be used to ascertain sex:

   - Male bullfrogs have a tympanic membrane that is larger than the eye; in females it is as large or smaller than the eye.

   - Male bullfrogs may show stronger mottling near the vent.

   - Male bullfrogs are yellowish below the throat during breeding season, while females are whitish below the throat. This characteristic may not be as useful in August.

   - Males have enlarged thumbpads, which are larger and darker in color than in females.

9. Inspect the frog and note any abnormalities or deformities. Record the frog number on a resealable plastic bag.

3.2.2 Tissue Sample Processing

1. Complete a sample attribute form for each sample to be collected (leg muscle tissue and whole body carcass tissue, duplicate or MS/MSD samples) from the frog.

2. Make a small incision on lower back of frog with scissors or probe, and gently pull off skin from the legs with pliers.

3. Cut legs at the hind joint with scissors, and rinse with deionized water.

4. Remove the leg muscle tissue from the bone of each leg, and place the tissue on decontaminated foil and weigh it after first taring the scale with the foil. The weight should be at least 10 g and will be recorded to 0.01 g.

5. Label foil with: (1) location, (2) date and time, (3) collector's initials, (4) weight, and (5) tissue type (leg or body). The sample should be placed in a resealable plastic bag similarly labeled and immediately placed on ice in a cooler.

6. Place the leg bones and skin with the remainder of the carcass on a separate piece of foil and weigh it after first taring the scale. The weight should be at least 10 g and will be recorded to 0.01 g. The foil will be labeled as above and placed in a labeled resealable plastic bag on ice in a cooler.

7. Weigh and label duplicate and MS/MSD samples in the same fashion. These samples should also be labeled either "Duplicate" or "MS/MSD" as appropriate. These samples will require a minimum of 20 g and 30 g tissue mass, respectively.

8. Freeze the specimen and place it in a resealable plastic bag.

3.2.3 Sample Handling and Shipping

1. Keep samples in a -20 to -30 ºC freezer until shipment to the laboratory.

2. When ready to ship, place the samples (wrapped in labeled foil and enclosed in labeled resealable plastic bags) in a large plastic bag into a cooler lined with vermiculite.

3. Complete a chain-of-custody form listing the contents of each cooler, and place it into a resealable plastic bag. Tape the resealable plastic bag to the inside of the top lid of the cooler, or place it on top of the samples.

4. Seal the cooler with two custody seals, and label the cooler with appropriate WESTON shipping labels, including the WESTON return address, and U.S. Fish and Wildlife Service (USFWS) laboratory address.

5. Samples will be delivered by courier or overnight delivery to the USFWS. Samples sent to the USFWS should be shipped to:

Ken Carr/Ken Munney/Drew Major
USFWS
22 Bridge St., Unit 1 — Phone: 603-225-1411
Concord, NH 03301 — Federal Express Acct: 1510-1036-9

3.2.4 Sample Documentation

Use a field logbook to record the location, date and time, amount of time spent in collecting activities at each area, method of collection, name(s) of collector(s), the number of frogs collected, and any other pertinent information such as problems encountered.

Complete a specimen data sheet for each frog collected. Specimen data sheets should include: location; date and time of collection; method of collection; collector's initials; total weight, sex, total length, and leg length; sample type (whole body or legs); and analyses.

Complete a sample attribute form for each tissue sample. In most cases that will be two samples per frog (leg muscle tissue and whole body carcass). Put the sample number for each sample and the date and processor's initials on the form.

Complete a chain-of-custody form for each cooler of samples shipped to the USFWS laboratory. Provide copies to the task manager, who will retain them in the WESTON files.

4. QUALITY ASSURANCE/QUALITY CONTROL

4.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

4.1.1 Data Quality Objectives

The two primary data quality objectives of the bullfrog collection and tissue analysis were outlined in Subsection 1.2 above. In addition, as part of the larger Supplemental Investigation, the bullfrog program must support and complement applicable data quality objectives established in Subsection 4.1 of the Final Quality Assurance Project Plan (WESTON, 2000) for the project. To achieve these objectives, the following types of data and specific quality criteria will be required:

- Total Biomass (wet weight) for each specimen: Biomass must be determined accurately and recorded to 0.01 g using a calibrated balance of 0.01 g sensitivity.

- Sex for each specimen: Sex must be determined and recorded for each specimen whenever possible. Sex will be determined by examining the morphological characteristics described in Subsection 3.2.1. The morphological characteristics that distinguish male and female bullfrogs are obvious to the naked eye; therefore, the use of a low-power microscope or hand lens is not necessary.

- Age class: The age class for each specimen must be determined accurately and recorded whenever possible. Age class will be determined by size and breeding characteristics.

- Total body length for each specimen: Body length must be determined accurately and recorded to the nearest 0.1 cm (1.0 mm) using a ruler capable of accurately measuring length to 0.1 cm. Total body length will be measured as snout to vent length for each specimen.

- Leg length for each specimen: Leg length must be determined accurately and recorded to the nearest 0.1 cm using dial calipers capable of accurately measuring length to 1 mm. Leg length will be measured on the right leg. (Note, to assure that the correct leg is measured, orient the frog on its ventral surface, head facing away from investigator.) Leg length will be measured from the top of the knee joint to the bottom of ankle joint.

- Presence of abnormalities/deformities: Each specimen collected must be examined for gross abnormalities and deformities, including tumors, scars, lesions, or other abnormalities. All observed abnormalities/deformities must be recorded. This morphological examination may be conducted by eye or using a low-power microscope or hand lens.

- Leg muscle tissue biomass (wet weight) for each specimen: The thigh and calf muscle tissue will be removed from both the left and right hind legs using the procedure outlined in Subsection 3.2.2. The combined leg muscle biomass must then be determined accurately and recorded to the nearest 0.01 g for each specimen, using a calibrated balance of 0.01 sensitivity.

- Offal biomass (wet weight) for each specimen: After the leg muscle tissue has been removed from the specimen, the remaining bullfrog tissue must be accurately determined and recorded to the nearest 0.01 g using a calibrated balance of 0.01 sensitivity.

- Tissue residue concentrations for PCBs and other contaminants: Tissue residue analysis will be conducted on 1) leg muscle tissue for each specimen, and 2) offal tissue for each specimen. Quality control considerations to ensure achievement of DQOs for PCBs and other contaminants will follow the QAPP (WESTON, 2000).

- Percent moisture and percent lipids: An analysis for percent moisture and percent lipids will be conducted on each tissue sample submitted for tissue residue analysis. Quality control considerations to ensure achievement of DQOs for these parameters will follow the QAPP (WESTON, 2000).

4.1.2 Data Quality Indicators

Data developed in the bullfrog study must meet acceptable standards of precision, accuracy, completeness, representativeness, comparability and sensitivity, as defined in Section 15 of the QAPP. Each of these data quality indicators, some of which are not readily quantifiable for the bullfrog data, is discussed below.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. Rather than control and measure precision, the study design includes an increase in the number of samples to obtain sufficient statistical resolution. For this study 10 samples per target site and 5 samples per reference site will be collected and processed. Precision may also be evaluated by an assessment of the degree to which sample collection procedures are able to ensure collection of consistent sample volumes. For the measurements that are not unique to the bullfrog study, such as tissue chemistry, precision is evaluated as defined in the QAPP (WESTON, 2000).

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study (total biomass, leg tissue biomass, offal biomass, body length, leg length, sex determination, and age class determination) accuracy is defined as meaning that each specimen is correctly weighed, correctly measured, and correctly identified. The data generated by this study will be evaluated for accuracy via comparison with known and/or expected results from similar studies conducted in similar biophysical regions. Accuracy is as defined in the QAPP for abiotic parameters, such as sediment contaminants.

Completeness is defined as the percentage of the planned samples actually collected and processed. Completeness can be evaluated for all components of the bullfrog program. To ensure achieving the planned statistical resolution, it is important that completeness of 100% be achieved for all components of this study.

Representativeness refers to the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. This data quality indicator is addressed through implementation of the sampling design and sample processing methods and will be evaluated via comparison with known and/or expected results.

Comparability is a measure of the confidence with which the bullfrog data may be compared to another similar data set. Comparability will be evaluated by examination of the intra-site and inter-site (particularly target sites vs. reference sites) variability in key parameters as determined from the group of samples to be collected at each location. Comparability will also be evaluated for this data set through comparison with previous similar bullfrog studies (if located) and with known characteristics of bullfrog populations in similar stream systems in the biophysical region.

Sensitivity, the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest, will be assured for the biological parameters by using scales and measuring devices of appropriate resolution (see sensitivity discussions within each DQO above). The detection limits for chemical analysis specified in the QAPP will provide more than sufficient sensitivity for the purpose of providing insight into factors controlling abundance and distribution of the benthic taxa and populations.

4.1.3 Data Validation, Verification, and Usability

Procedures for data validation for the chemical and physical data are discussed in various sections of the project QAPP and will be used whenever applicable in this study. Usability will be largely be determined by two factors: (1) the experience of the senior investigator in establishing that the field sampling was conducted following the SOP and that accuracy and

precision were not compromised by an inability to control the sampling procedures in the field; (2) a direct comparison between the chemistry data and other data developed by the project from similar areas of the river.

The purpose of the remainder of this section of the study plan is to document the measures included in the study to ensure that the standards discussed above are met.

4.2 SAMPLING DESIGN

The rationale for selection of the four locations to be sampled in the bullfrog study was presented in Subsection 2.1.2 above. The locations are not intended to be representative of the entire river but rather are intended to encompass the range of sediment PCB concentrations in the Lower River between the Confluence and Woods Pond where bullfrog habitat occurs. Two appropriate reference locations with background PCB levels and two "target" sites will be sampled.

Bullfrog tissue residue concentration data are typically highly variable in nature. To achieve acceptable statistical resolution it is necessary to collect large numbers of samples. Data will be collected from 10 frogs at each of the target sites and from five frogs at each of two reference stations. This number of samples was selected based on a power analysis using data from bullfrog studies found in the literature and with consideration of what level of resolution would be needed to meet the objectives of this study (see Attachment 1).

4.3 SAMPLING METHODOLOGY

4.3.1 Sampling Procedures

Sampling methods, as discussed in Subsections 2.1.4 and 3.1, have been selected to ensure that the objectives of the study are met. Note that the stated objectives do not include a characterization of the distribution of frogs in the Lower Housatonic River. As a result, sampling for this study is limited to one species (bullfrogs) and sampling methodology is biased toward collecting bullfrogs with sufficient mass for tissue analysis.

All samples will be collected directly by the highly trained and experienced personnel on this subject to further promote comparability and reduce potential bias through the oversight and use of the professional opinion of the expert. Subsamples for physical and chemical analyses will be collected following procedures documented in the project QAPP, and will therefore be comparable with procedures followed for all other similar samples efforts throughout the Supplemental Investigation.

4.3.2 Quality Control Samples

Table 2 summarizes QA/QC requirements for tissue analyses.

Table 2
QA/QC Analytical Summary for Bullfrogs
Housatonic River
Pittsfield, MA

Location	Number of Frogs	Number of Samples[a]		MS/MSD Samples[b]		Duplicate Samples[c]	
		Leg Muscle Tissue	Whole Body Carcass	Leg Muscle Tissue	Whole Body Carcass	Leg Muscle Tissue	Whole Body Carcass
Woods Pond	10	10	10	0	0	1	1
Backwater Areas Within 1 Mile North of Woods Pond	10	10	10	1	1	1	1
Three-Mile Pond Reference	5	5	5	1	1	1	1
Lake Washington Reference	5	5	5	0	0	0	0
Total	30	30	30	2	2	3	3

Notes: [a] Required mass per sample is 10 g.

[b] Required total mass per MS/MSD sample is 20 g (Original sample 10 g, MS 10 g, MSD 10 g).

[c] Required total mass per duplicate sample is 20 g (Original sample 10 g, duplicate 10 g).

Duplicate analyses will be conducted for each parameter on approximately 5% of tissue samples (both whole body tissue and leg muscle tissue). An additional 10 g of tissue will be required for each set of analyses (whole body carcass and leg muscle tissue) beyond the 10 g required for each set of original analyses. Therefore, the amount of tissue required will be 20 g from the whole body carcass and 20 g from the leg muscle tissue. These samples will be submitted for analyses separately.

In addition, a matrix spike/matrix spike duplicate (MS/MSD) sample will be conducted on 5% of samples. These samples will also be collected from both leg muscle tissue and whole body tissues. An additional 20 g of tissue will be required for each set of MS/MSD analyses, beyond the 10 g required for the original analyses. Thus, the total amount of tissue required for original, duplicate, and MS/MSD analyses, if conducted on the same sample, is 40 g each from the whole body carcass and leg muscle tissue samples. This mass should still be obtainable from a single frog. The results of the analysis of these split samples will be compared for quality control purposes.

4.3.3 Sample Processing and Preservation

Detailed procedures for collection and initial processing of all samples to be collected as part of the bullfrog study are provided in Subsection 3. Decontamination between samples will follow procedures established in the project QAPP (WESTON, 2000). All specimens will be held alive in site water and returned to the field laboratory twice daily. Biological samples will be frozen after processing; sediment samples will be frozen immediately. The holding time for physical and chemical samples will follow procedures established in the project QAPP.

4.3.4 Training

All sampling will be directed in the field by senior scientists with experience in the collection of bullfrog samples. Supporting staff will receive training from the senior scientist(s) in the overall goals of the study and in techniques to be followed to ensure collection of quality data.

4.4 SAMPLE ANALYSIS

4.4.1 Biological Samples

The collection of morphometric information and dissection of all samples will be conducted by experienced staff who have received specific training in the SOP and whose work is checked periodically by their supervisors and peers. Biological samples will be processed following procedures and SOPs provided in Subsection 3.

4.4.2 Physical/Chemical Samples

Samples for sediment chemistry and tissue chemistry will be processed following procedures and SOPs provided in the project QAPP (WESTON, 2000). These samples will be submitted in catalogs and batches with other samples from the larger project and data validation will be performed on a catalog basis in accordance with procedures established and described in the QAPP.

4.5 DATA ANALYSIS AND REPORTING

The overall analytical approach for data generated under this study is described in Subsection 2.2 above. The findings will be included in the ecological risk assessment including all data, analyses, and interpretations and will be prepared with specific reference to both the data quality objectives specific to the bullfrog study (Subsection 4.1.1) and Subsection 4.1 of the project QAPP (WESTON, 2000).

5. EQUIPMENT LIST

5.1 FIELD

- First aid kit
- 4 headlamps, 16 AA batteries, extra set of 16 AA batteries
- Spotlights (2) 12-V battery
- 13 medium buckets for collection with lids and holes for ventilation
- 4 all-purpose nylon nets, 12-inch diameter and 5-ft extendable handle
- Indelible markers, duct/labeling tape
- Waders for each field technician
- Life vests
- Oars, anchor, rope for 2 jon boats, trolling motor
- Field logbook

5.2 PROCESSING AREA

- 2 folding tables
- Polyethylene plastic sheets
- 4 boxes of Nitrile gloves
- 4 boxes of gallon-sized resealable plastic bags
- Data sheets
- Pliers, probe, scissors, steel rod
- Knives/scalpels for incision or reproductive examination
- Weighing scale for up to 500 g
- 4 boxes of aluminum foil
- 2 large coolers for freezing samples

- 15 holding coolers, if necessary, with aerators
- Drill for putting hole in side of cooler
- 1 to 2 shipping coolers
- Ice to fill cooler, in plastic resealable plastic bags or free
- Dry ice for shipping
- Gloves for handling dry ice
- Indelible markers (fine and wide)
- Ballpoint pens
- Hexane in rinse bottle
- Nitric acid in rinse bottle
- Isopropyl alcohol in rinse bottle
- Distilled, deionized water in rinse bottle
- Large bucket for decontamination solutions
- Packaging tape
- Laboratory sample labels with unique sample numbers
- WESTON or USFWS QA/QC labels

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ATTACHMENT 1

POWER ANALYSES FOR BULLFROGS

Regression – Analytical

The effects of releasing PCB in the environment on the concentrations of PCB in frog tissues may be tested by a regression of this variable against the concentrations of PCB in sediments. This statistical test is recommended because concentrations of PCB in frog tissues are expected to be a function of the concentration of these chemicals in the environment. Frogs frequently have small home ranges, and uptake PCBs via absorption through the skin (particularly when hibernating in sediments) and ingestion of contaminated water and food. For a desired power (1-β) and level of significance (α), the adequate sample size *n* is

$$n = ((Z_{\beta(1)} + Z_{\alpha})^2 / \zeta_0) + 3 \qquad \text{(Zar 1984)}$$

where β (1) is the one-tailed probability of the normal deviate, α is the level of significance, and $Z_{\beta(1)}$, Z_{α}, and ζ_0 are the Fisher *z* transformations at β (1), α, and ζ_o levels, respectively, and ρ_o is the specific correlation coefficient to be tested. For α = 0.05, $Z_{0.1(1)}$ = 1.2816, $Z_{0.05(2)}$ = 1.9600, power = 0.9, and β (1) = 0.1 the required sample sizes are shown in Figure 1.

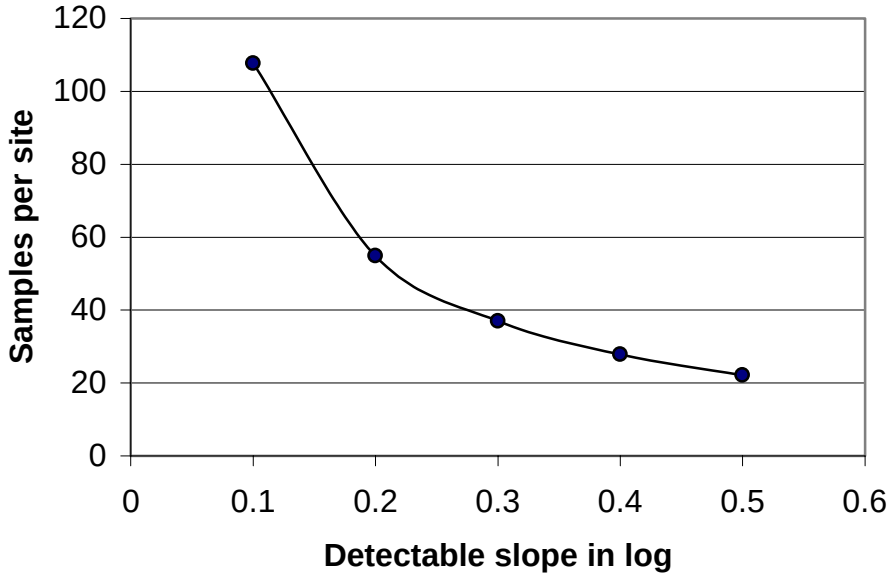

Figure 1. Estimated number of samples, for each site, required to detect a significant regression of log concentration of PCB in bullfrog tissues (wet mass) as a function of the log concentrations in sediments (dry mass). Power ≥ 0.9, α = 0.05.

Regression - Simulation

Computer simulations can also estimate the sample sizes required to achieve a desired power. In our model the log-transformed concentration of PCB in frog tissue (Y) was a linear function, with zero intercept, of the log-transformed PCB concentration in sediments (X):

$$\ln Y = \delta \ln X + \varepsilon(0,\sigma)$$

where δ represents a hypothetical value for the true slope of the regression line, and $\varepsilon(0,\sigma)$ represents a normally distributed error term with zero mean and standard deviation σ. The standard deviation σ of the error term was estimated from data in Huang (1999) on PCB concentrations in northern leopard frogs from the Green Bay, Wisconsin area (Figure 2).

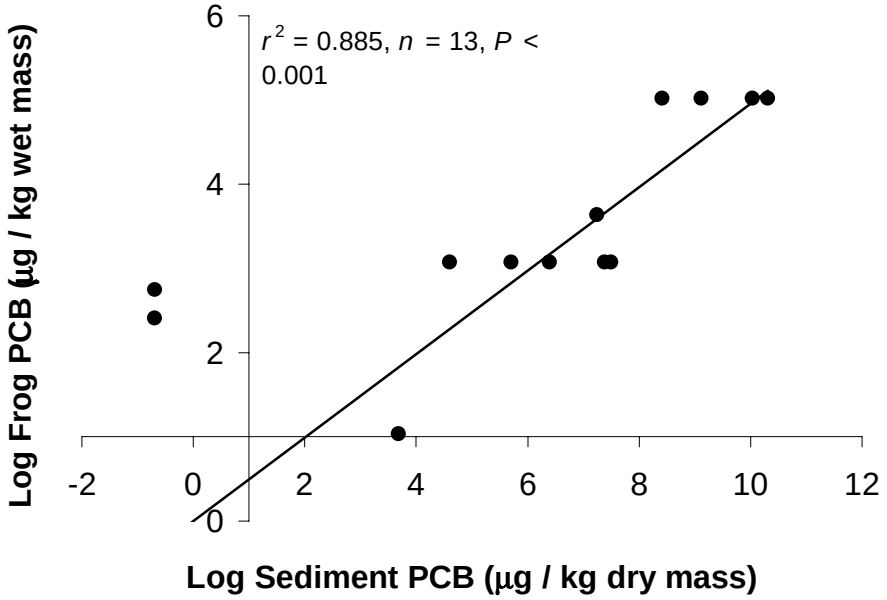

Figure 2. Linear relationship between PCB concentrations in sediments and tissues of northern leopard frogs. Data from Huang (1999).

σ is the root residual mean square of the regression line relating log concentrations of PCB in sediments and frog tissues

$$\sigma = \sqrt{\sum (Y - \overline{Y})^2 / (n-1)}.$$

In two of the sites where Huang (1999) sampled PCB concentrations in sediments and frog tissues, Deposit C and Strobe Island, there were multiple sediment samples corresponding to a single sample of frogs. Huang (1999) only reported their means and standard errors. This procedure reduces our estimate of the deviations in PCB concentrations in frog tissues in the values projected by the regression equation. We compensated for this reduction by arbitrarily generating values of PCB concentration in sediments that fit the reported mean and standard error. Based on the published data for the four sites without replicates, and the simulated data for Deposit C and Strobe Island,

$$\sigma = 0.643.$$

Sediment PCB concentrations (X values) were generated at random from lognormal distributions having the means and standard deviations given in Table 1.

Site	1	2	3	4	5	6
Mean	679	568	12,680	21,880	31,070	50,070
SD	2,312	458	14,370	9,592	29,890	58,540

Table 1. Arithmetic means and standard deviations (SD) of PCB concentrations (μg/kg dry mass) in 0–6-inch sediments at six sites of the Housatonic River (WESTON project data). The concentrations are presumed to be lognormally distributed, thus their log transformed values are likely to improve normality and homoscedasticity.

We also compensated for the reduced variance due to composite frog samples (4-8 animals per sample, Y. Huang, personal communication) by multiplying σ by a factor f, where $f = 1$, 2.5 or 5. Therefore, in the simulation, a corresponding Y value for the bullfrog concentration was then simulated for each X value according to the formula

$$Y = \exp(\delta \ln X + \varepsilon(0, f\sigma))$$

to generate 1, 2, 3, 4, 5, 7, 10, 15, 20, and 100 pairs of *X Y* values for each of the six sites in Table 1. The bivariate pairs were then log-transformed and subjected to a regression analysis. A tally was kept of the number of times the resulting regression was statistically significant at the α=0.05 level of significance. The number of significant regressions divided by the total number of regressions estimates the power for that particular sample size (under the prevailing hypothesis about the true slope). A Pascal program written for this analysis was used to conduct the simulations. The results of this analysis are shown in Figure 3, which displays the sample sizes required to achieve a power of no less than 80% as a function of the minimally detectable slope of the underlying log-log regression.

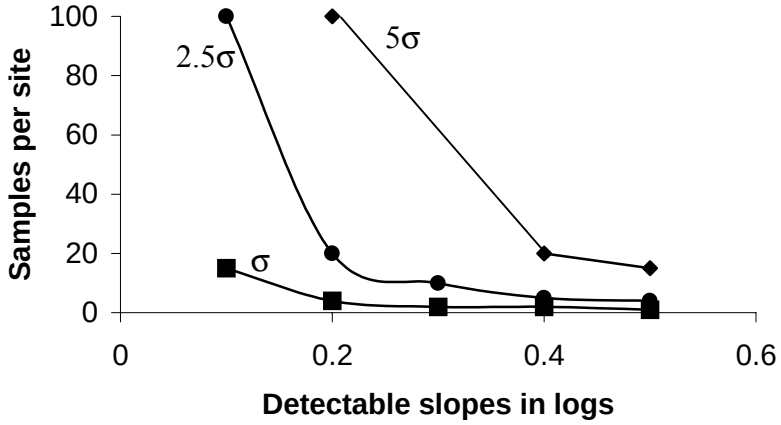

Figure 3. Estimated number of samples, for each of the six sites listed in Table 2, required to detect increasing slopes of the regression line relating log transformed concentrations of PCB in frog tissues (wet mass) and sediments (dry mass) with a power of > 0.8. Each curve has a distinct value for the standard deviation of the error term (σ = 0.643).

REFERENCES

Huang, Y. 1999. "Exposure of Northern Leopard Frogs in the Green Bay Ecosystem to PCBs, PCDDs, and PCDFs Is Measured by Direct Chemistry but not Hepatic EROD Activity." *Environmental Toxicology and Chemistry* 18:54-57.

Zar, J.H. 1984. *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, New Jersey, USA.

APPENDIX A.18

WORK PLAN FOR THE STUDY OF AMPHIBIAN REPRODUCTIVE SUCCESS WITHIN VERNAL POOLS ASSOCIATED WITH THE HOUSATONIC RIVER FROM NEWELL STREET TO WOODS POND (WOODLOT ALTERNATIVES, INC.)

APPENDIX A.18

WORK PLAN FOR THE STUDY OF AMPHIBIAN REPRODUCTIVE SUCCESS WITHIN VERNAL POOLS ASSOCIATED WITH THE HOUSATONIC RIVER FROM NEWELL STREET TO WOODS POND

Submitted to:

Roy F. Weston, Inc.
1400 Weston Way
West Chester, Pennsylvania 19380-1499

Submitted by:

Woodlot Alternatives, Inc.
122 Main Street
Topsham, Maine 04086

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1. INTRODUCTION

The United States Environmental Protection Agency (EPA) is characterizing the natural resources of the Housatonic River in portions of Pittsfield, Lenox, and Lee, Massachusetts. The study area is approximately 12 miles long and extends from Newell Street in Pittsfield to Woods Pond Dam in Lee. It includes riverine habitats, floodplain wetlands, and uplands associated with the main-stem of the river. Polychlorinated biphenyls (PCBs) that originated from the General Electric (GE) facility in Pittsfield have been found within the river and its adjacent floodplains (Blasland, Bouck, and Lee, 1996a and 1996b). This work plan is for a study of amphibian reproductive success within vernal pools that have varying levels of PCBs in their sediments.

The Housatonic River and its floodplains provide habitat for a wide variety of reptiles and amphibians (collectively called herps) and up to 40 species of snakes, turtles, frogs, toads, and salamanders potentially occur in the study area (TechLaw, 1998). Breeding amphibians (frogs, toads, and salamanders) use portions of the river and temporary (known as vernal pools) and permanent pools for courtship and egg-laying. These areas then support larval amphibians for periods ranging from several months to more than a year, depending on the species. Wood frogs (*Rana sylvatica*), for example, metamorphose into the adult form in 2 to 3 months, while green frogs (*Rana clamitans*) can take over a year (Hunter et al., 1992).

Documenting amphibian reproductive success within vernal pools is being conducted because these animals may be influenced by exposure to PCBs in contaminated sediments. They may also bioaccumulate PCBs, which can then be passed on to other animals in the food chain. In addition, several herps that could occur in the study area are listed as State-Endangered, Threatened, Special Concern, or Watch List species (Massachusetts Natural Heritage and Endangered Species Program, 1997). These include the Jefferson salamander (*Ambystoma jeffersonianum*), spotted salamander (*A. maculatum*), marbled salamander (*A. opacum*), spring salamander (*Gyrinophilus porphyriticus*), and four-toed salamander (*Hemidactylium scutatum*).

1.1 OBJECTIVE

The objective of this task is to determine if PCB contamination is potentially having an adverse effect on amphibian reproduction in vernal pools.

1.2 PROJECT APPROACH

Several steps will be taken to meet the task objectives, including: 1) literature review, 2) observing amphibian reproductive success in vernal pools that have varying levels of PCBs in their sediments, 3) data analysis, and 4) report preparation. The literature review will be used to locate information on the present and historic use of the study area by amphibians and to identify the potential effects of PCBs on amphibians.

As presented in Figure 1, vernal pools in the study area have been mapped and characterized using methods developed by Kenney (1995) for Massachusetts (TechLaw, 1998). Table 1 presents the estimated area and average depth for the vernal pools observed for the study area.

The concentration of PCBs in vernal pool sediments will be measured to identify a sample of pools exhibiting a range of contamination levels. Amphibian reproductive activity, including courtship and breeding, egg laying, hatching, larval growth and development, and metamorphosis, will then be observed in each of the sample pools to determine if PCBs may be influencing reproductive success. Carcasses of amphibians that succumb incidentally to the performance of this study will be analyzed to measure the level of PCBs in their tissues.

The data from these studies will be analyzed and presented in a report that includes results of the literature review and an evaluation of amphibian reproductive success in relation to observed PCB levels. Tasks will be performed in conjunction with EPA and Roy F. Weston, Inc. (WESTON®) scientists and other principal investigators, who will assist with study plan development and implementation, data analysis, and report preparation.

2. METHODS

Prior to conducting field work, appropriate Scientific Collecting Permits for the studies described in this Work Plan will be obtained from the Commonwealth of Massachusetts, Division of Fisheries & Wildlife. If rare amphibians, invertebrates, or plants are encountered as part of this study, appropriate Division of Fisheries & Wildlife, Natural Heritage and Endangered Species Program Rare Animal and Rare Plant Observation Forms will be completed.

2.1 LITERATURE REVIEW

The literature review will be used to develop species identification protocols for amphibian eggs, larvae, recently metamorphosed juveniles, and adults. A list of scientific and technical articles related to the known and potential effects of PCBs on amphibians will also be developed as part of the literature review. These articles will be reviewed and references will be entered into an annotated Papyrus (version 7.0.14) database that includes: 1) author(s) name(s), 2) year of publication, 3) article title, 4) journal/source, 5) abstract, and 6) annotated comments.

2.2 AMPHIBIAN REPRODUCTIVE SUCCESS IN VERNAL POOLS

Sediment samples (0 to 6 inches from ground surface) will be collected from vernal pools in the study area and analyzed for total PCB concentration. Amphibian reproductive success within four pools exhibiting a range of PCB concentrations, from no contamination up to the highest observed concentration, will then be studied. The final selection of pools, including the number and location of sample pools, will be made in consultation with EPA scientists and will be based on PCB concentration, presence of target species, and similarity of physical and hydrologic characteristics. It is believed that at least three pools will be needed to represent pools with varying levels of PCBs, i.e., one low or non-detect, one moderate, and one high. A weather station w ill be established near the study pools to measure temperature, relative humidity,

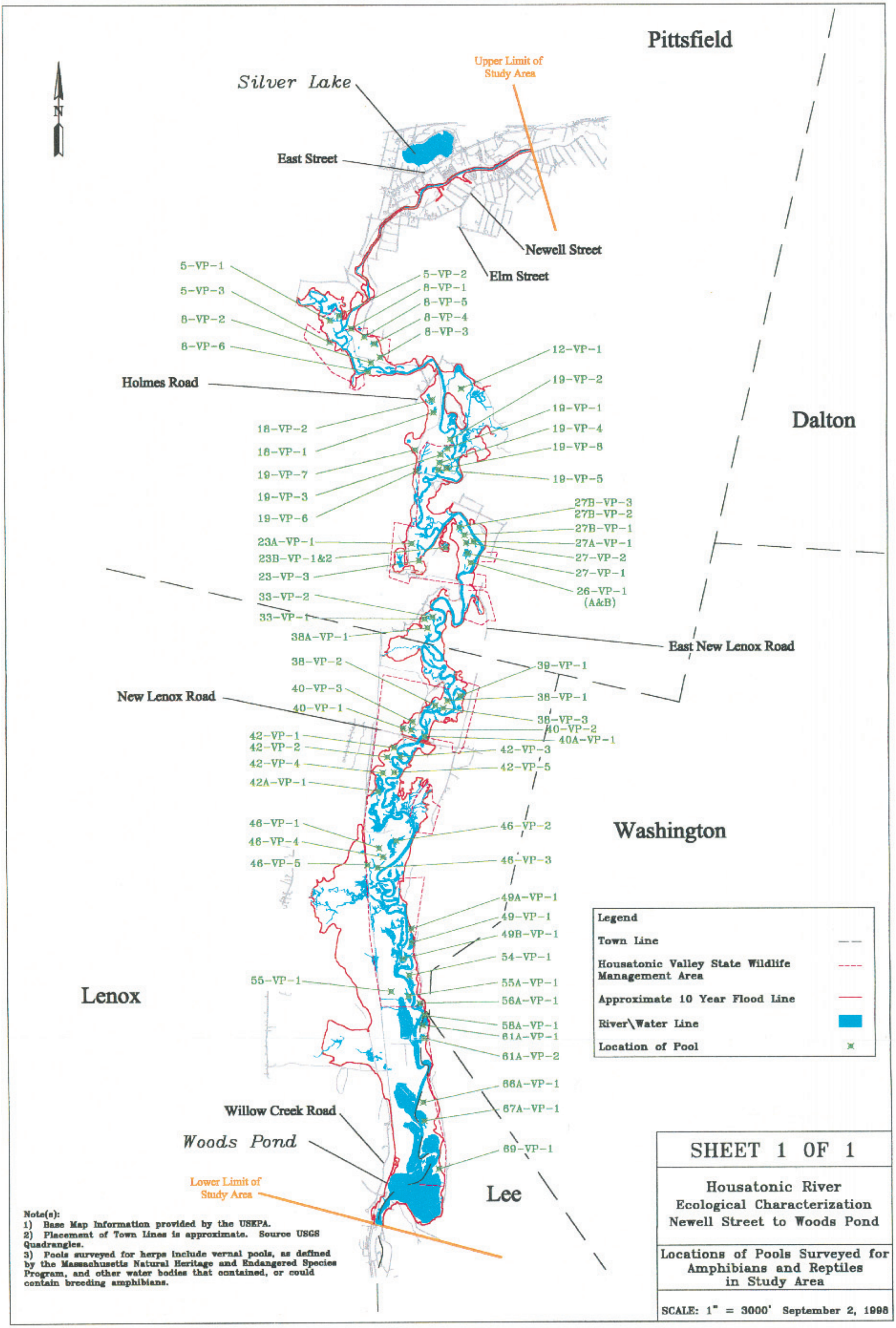

Pittsfield
Upper Limit of Study Area
Silver Lake
East Street
Newell Street
Elm Street
5-VP-1
5-VP-3
8-VP-2
8-VP-6
5-VP-2
8-VP-1
8-VP-5
8-VP-4
8-VP-3
Holmes Road
12-VP-1
19-VP-2
19-VP-1
19-VP-4
19-VP-8
19-VP-5
18-VP-2
18-VP-1
19-VP-7
19-VP-3
19-VP-6
Dalton
27B-VP-3
27B-VP-2
27B-VP-1
27A-VP-1
27-VP-2
27-VP-1
26-VP-1 (A&B)
23A-VP-1
23B-VP-1&2
23-VP-3
33-VP-2
33-VP-1
38A-VP-1
East New Lenox Road
38-VP-2
39-VP-1
New Lenox Road
40-VP-3
40-VP-1
38-VP-1
38-VP-3
40-VP-2
40A-VP-1
42-VP-1
42-VP-2
42-VP-4
42A-VP-1
42-VP-3
42-VP-5
46-VP-1
46-VP-4
46-VP-5
46-VP-2
46-VP-3
Washington
49A-VP-1
49-VP-1
49B-VP-1
54-VP-1
55-VP-1
55A-VP-1
56A-VP-1
Lenox
58A-VP-1
61A-VP-1
61A-VP-2
66A-VP-1
67A-VP-1
Willow Creek Road
Woods Pond
69-VP-1
Lower Limit of Study Area
Lee
Legend
Town Line
Housatonic Valley State Wildlife Management Area
Approximate 10 Year Flood Line
River\Water Line
Location of Pool
Note(s):
1) Base Map information provided by the USEPA.
2) Placement of Town Lines is approximate. Source USGS Quadrangles.
3) Pools surveyed for herps include vernal pools, as defined by the Massachusetts Natural Heritage and Endangered Species Program, and other water bodies that contained, or could contain breeding amphibians.
SHEET 1 OF 1
Housatonic River
Ecological Characterization
Newell Street to Woods Pond
Locations of Pools Surveyed for Amphibians and Reptiles in Study Area
SCALE: 1" = 3000' September 2, 1998

Table 1

Housatonic River Floodplain Pool Data
Berkshire County, Massachusetts

Pool Designation	Estimated Area (ft^2)	Avg. Depth (Inches)	Date Observed
5-VP-1	1,275	5	4-20-98
5-VP-2	3,150	14	5-6-98
5-VP-3	10,000	12	5-7-98
8-VP-1	800	11	4-14-98
8-VP-2	250	14	4-14-98
8-VP-3	2,000	6	4-14-98
8-VP-4	6,000	18	4-14-98
8-VP-6	1,000	4	5-5-98
12-VP-1	6,000	20	4-20-98
18-VP-1	10,000	18	4-14-98
18-VP-2	10,500	18	4-20-98
19-VP-1	6,000	14	4-14-98
19-VP-2	400	12	4-14-98
19-VP-3	750	8	5-5-98
19-VP-4	2,700	6	5-5-98
19-VP-5	12,000	14	5-5-98
19-VP-6	16,000	10	5-5-98
19-VP-7	5,000	8	5-6-98
19-VP-8	675	4	5-6-98
23-VP-3	16,000	14	5-6-98
23A-VP-1	9,000	10	4-21-98
23B-VP-1	15,000	18	4-21-98
23B-VP-2	6,000	12	4-21-98
26-VP-1 (A+B)	2,560	14	4-21-98
27-VP-1	6,000	18	5-7-98
27-VP-2	8,400	10	5-7-98
27A-VP-1	2,000	15	4-21-98
27B-VP-1	2,200	14	4-21-98
27B-VP-2	5,625	15	4-21-98

Table 1

Housatonic River Floodplain Pool Data
Berkshire County, Massachusetts
(Continued)

Pool Designation	Estimated Area (ft^2)	Avg. Depth (Inches)	Date Observed
33-VP-1	3,750	11	4-21-98
33-VP-2	18,200	9	4-21-98
38-VP-1	3,400	14	4-22-98
38A-VP-1	2,450	10	4-22-98
38-VP-2	6,000	10	4-22-98
38-VP-3	8,000	14	4-22-98
39-VP-1	30,000	25	4-22-98
40-VP-3	9,000	18	4-22-98
42-VP-1	21,000	14	4-24-98
42-VP-2	12,000	10	4-24-98
42-VP-2	12,000	10	4-24-98
42-VP-3	4,225	>36	4-24-98
42-VP-4	50,000	>36	4-24-98
42-VP-5	28,900	15	4-24-98
42A-VP-1	175,000	>36	4-24-98
46-VP-1	23,800	>36	4-24-98
46-VP-2	180,000	21	4-28-98
46-VP-3	10,000	16	4-28-98
46-VP-4	300	4	4-28-98
46-VP-5	800	12	4-28-98
49-VP-1	44,000	>36	4-22-98
49A-VP-1	1,320	8	4-22-98
49B-VP-1	240	6	4-22-98
54-VP-1	40,000	>36	4-23-98
55-VP-1	30,000	18	4-29-98
55A-VP-1	56,000	18	4-29-98
56A-VP-1	30,000	>36	4-23-98
58A-VP-1	30,000	>36	4-23-98
61A-VP-1	26,250	>36	4-23-98
61A-VP-2	50,000	>36	4-23-98

Table 1

Housatonic River Floodplain Pool Data
Berkshire County, Massachusetts
(Continued)

Pool Designation	Estimated Area (ft^2)	Avg. Depth (Inches)	Date Observed
67A-VP-1	1,800	6	4-23-98
69-VP-1	4,900	7	4-23-98

Note: Pools surveyed for herps include vernal pools, as defined by the Massachusetts Natural Heritage and Endangered Species Program and other water bodies that contained or could contain breeding amphibians.

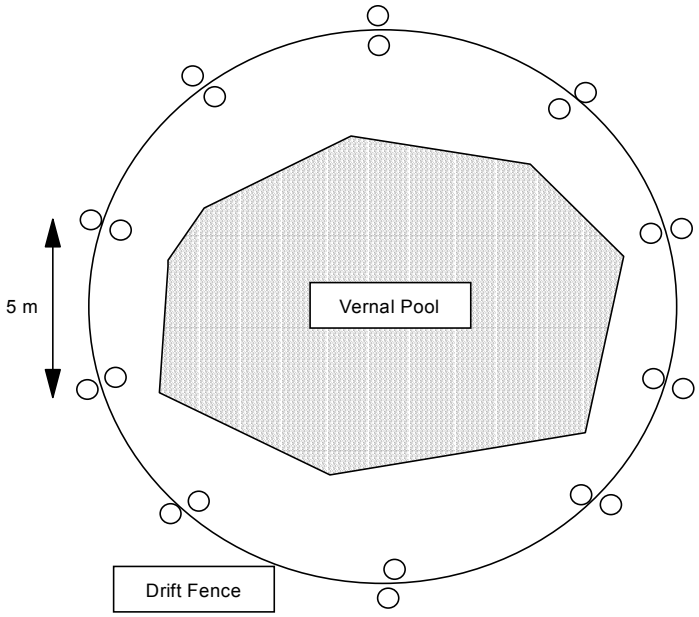
and rainfall throughout the study beginning on, or about, 1 April 1999.

2.2.1 AMPHIBIANS ENTERING VERNAL POOLS

To determine the relative abundance of amphibians entering vernal pools to breed, drift fences with pit traps (Corn and Bury, 1990; Corn, 1994) will be constructed in a circle around each of the study pools (Dodd and Scott, 1994). Pit traps will be constructed from two No. 10, or similar, metal cans (approximate diam. 61/8 in [15.6 cm], depth 14 in [35.6 cm]). Traps will be placed at approximately five meter intervals and will be paired inside and outside the fence (Figure 2). Each trap pair will be given a unique number and its position will be recorded using a Trimble ProXR™ GPS receiver. Drift fences and traps will be installed by 1 April 1999, or as soon as weather and ground conditions permit.

Figure 2

Drift Fence and Pit-Trap Configuration



Pit traps will be checked daily and captured individuals will be identified to the species level, sexed, measured, weighed (to nearest 0.1 g), marked, and released onto the pool-side of the drift fence. If large numbers of individuals are captured and marking all individuals becomes too time consuming, then a minimum random sample of 25 males and females per species will be marked. Body measurements (to nearest mm) will include head and body length (snout-vent length or SVL), tail length, total length, and the length of each hind and fore limb. Each individual will also be inspected for deformities, erosion, lesions, and tumors (DELTs) using procedures developed by the North American Reporting Center for Amphibian Malformations (Attachment 1). Individuals will be marked with fluorescent elastomers in colors and locations on the body that are unique to the individual animal, species, sex, and study pool (Donnelly et al., 1994). If necessary for identification, a collection of six voucher specimens (3 %:3 &) may be maintained

for captured species (Reynolds et al., 1994) using McDiarmid=s (1994) methods for specimen preservation. During the course of studies associated with the Housatonic River specimens will be held by Woodlot Alternatives, Inc. Following completion of work on the river, specimens will be offered to the Harvard Museum of Comparative Vertebrate Anatomy. If this museum does not accept the specimens, they will be offered to the University of Massachusetts.

2.2.2 Courtship and Breeding Behavior and Condition

Courtship activity and breeding behavior and condition within the study pools will be monitored as soon as the first amphibians are captured and placed on the pool-side of the fence. Audio surveys will be conducted to determine if male frogs in the vernal pools are calling to advertise their position to potential mates and rivals (Zimmerman, 1994). To conduct these surveys, observers will sit quietly beside a pool and note general presence/absence of frog chorus.

During audio surveys, observers will attempt to determine if frogs are engaged in breeding activities (e.g., observing amplectic embraces [Duellman and Trueb, 1986]). While searching for breeding activity in frogs, observers will also look for breeding salamanders. Salamanders do not vocalize during courtship, but instead engage in series of maneuvers designed to persuade the female to breed (i.e., a nuptial dance). With spotted salamanders, which occur in the study area, the nuptial dances of several individuals can Amake the water fairly boil@ (Bishop, 1941, *in* Hunter et al., 1992).

When marking individuals with fluorescent elastomers (see Subsection 2.2.1), unique combinations of colors and locations will be used to differentiate between species and sexes A blue light and amber goggles will be used to enhance the visibility of the elastomer marks. Adults captured in pit traps as they leave the vernal pools will also be inspected for external indicators of breeding activity. In addition to the reproductive organs and their associated tracts, external sexual differences exist in many amphibians. These can include body size, glandular development, skin texture, dermal ornamentation, vocal sacs, and coloration. Some persist throughout adult life, but others occur in response to gonadotropic hormones and can serve as indicators of reproductive activity (Duellman and Trueb, 1986). Secondary sexual characters also include nuptial excrescences (modified dermal and epidermal tissues) in salamanders and anurans (Duellman and Trueb, 1986).

2.2.3 Egg Laying, Hatching Success, and Larval Growth and Development

Each pool will be surveyed daily to locate amphibian egg masses. Within each study pool, five egg masses (if available) of wood frogs and spotted salamanders will be enclosed within a box sampler to monitor hatching success (Shaffer et al., 1994). Box samplers will be 50 cm long, 50 cm wide, and 50 cm deep and will be fitted with small mesh screens on the sides, bottom, and top to allow water to flow into the sampler while minimizing egg predation. Box samplers will be monitored daily to evaluate egg development. At this time, the temperature, dissolved oxygen (DO) concentration, pH, and conductivity of the water will also be measured from a station near the center and edge of the vernal pool. Temperature will be automatically recorded using a *HOBO*7 data logger (Onset Computer Corp., North Falmouth, MA), and the remaining

parameters will be measured with hand-held instruments (*YSI*7 *85* salinity, conductivity, DO, and temperature system, and *Omega*7 *PHH-1X* pH meter).

Hatching normally occurs over a relatively short period of time; and once hatching is complete, the remaining egg gelatin and eggs (hatched and unhatched) will be collected and tested for PCBs (total and Aroclors). Eggs, both hatched and unhatched, will first be examined and necropsied by an expert in the field of amphibian larval development and then tested for PCBs. Following hatching, larval amphibians in the box sampler will be monitored daily for 10 days, a period sufficient to measure early growth, yet not detrimental to the young larvae, which feed on algae and microorganisms (Hunter et al., 1999) that are of limited supply in the samplers. Larvae will be captured with a sweep net, and then measured for body length (SVL), tail length, and total length. Each individual will also be inspected for DELTs. After 10 days, the larvae will be released into the vernal pool to be exposed to the normal conditions of the remainder of the pool. A random sample of 25 individuals/species, or 50% of the total if less than 50 are available, in each box sampler will be retained and examined by an expert in the field of amphibian larval development and then tested for PCBs (total and Aroclors) and if sufficient tissues mass is available, PCB congeners and homologs, dioxins/furans, and select OC pesticides.

At least 10 aquatic funnel traps will be placed in each pool selected for sampling to monitor larval growth and development on a weekly basis (Shaffer et al., 1994). Marbled salamanders lay their eggs in the fall and larval forms can be active even when ice covers the vernal pool (Kenney, 1995). Traps, therefore, will be deployed during the first week of April 1999, or as soon as site conditions allow. Traps will be placed in the pools in the evening and collected the following morning. If too many individuals are being captured, resulting in trap mortality, the amount of time the traps are operating will be reduced or the time of day during which the traps are operated may be changed. At each trap location, water depth and micro-habitat information will be recorded, as will the trap entry and exit date and time.

The total number of larval amphibians of each species will be recorded for each trap, and the SVL, tail, total length, and length of each limb of up to 25 individuals of each species will be recorded. For abundant species, such as wood frogs, five measured individuals from each trap will be weighed and examined by an expert in the field of amphibian larval development. This will result in a total sample of up to 50 individuals per pool per week (i.e., 10 traps and 5 individuals/ trap). If traps contain fewer than 10 individuals, then half of the individuals will be examined. For less common species, such as spotted salamanders, only one individual will be sacrificed from each trap per week (i.e., total sample of 10 individuals per pool per week). General notes on trap mortality, condition of larvae, associated invertebrates, and any other observations will also be recorded. Aquatic macroinvertebrates (some of which eat larval amphibians) from each trap will be collected and preserved in 80% ethanol for potential identification to the lowest practicable taxonomic unit. Dipnet sampling may also be used to supplement aquatic funnel trap sampling.

2.2.4 Metamorphosis and Exodus from Vernal Pools

Drift fences and pit traps will be monitored daily to record the exodus of metamorphosed juveniles from the pools. Captured individuals will be measured, weighed, and inspected for

DELTs using the methods described in Subsection 2.2.1. Following examination, these individuals will be placed on the outside of the drift fence.

2.3 TISSUE COLLECTION FOR PCB ANALYSIS

Amphibians that succumb incidentally to the performance of this study, or that are sacrificed, may be preserved and submitted for PCB (total and Aroclors) tissue analysis (Attachment 2). If sufficient tissue mass is available, additional analyses that may be conducted include PCB congeners and homologs, dioxins/furans, and select OC pesticides.

2.4 DATA ANALYSIS

2.4.1 Amphibians Entering Vernal Pools

The goals in analyzing data from this portion of the study are to: 1) compare the relative abundance of amphibian species using each of the study pools, 2) compare the time of arrival, by species and sex, between study pools, 3) compare the proportion of the population with DELTs between pools, and 4) compare the body sizes of amphibians, by species and sex, among pools. The primary purpose is to characterize the community of breeding amphibians entering each vernal pool. Identifying an effect of PCBs on amphibians entering the vernal pools is not a primary goal of this portion of the analysis because a variety of natural factors can influence species composition and condition within vernal pools. Significant differences in species composition, sex ratios, body size, and/or the proportion of the population with DELTs between pools with high and low concentrations of PCBs, however, will be interpreted as suggesting a potential effect. Such potential effects, however, will be investigated as part of the remainder of the study.

Species richness (i.e., the number of species) will be reported for each study pool and qualitatively compared to species richness within similar pools in the region based on existing pool descriptions compiled by the Massachusetts Division of Fisheries & Wildlife, Natural Heritage and Endangered Species Program. The relative abundance of amphibian species using each of the study pools will be compared using a contingency table and chi-square (χ^2) analysis (Fienberg, 1983; Zar, 1984).

The hypotheses for this analysis can be stated as:

H_O: The species composition is similar in each of the study pools.
H_A: The species composition differs between one or more of the study pools.

Due to the inherently variable nature of species composition in vernal pools, a significance level of 10% ($\alpha = 0.10$) will be used in determining whether to accept or reject the null hypothesis (H_O) for this analysis. The actual significance level (α) and test statistic (χ^2) of the analysis, however, will be reported. All statistical tests will be run using the *Statistica* J software package (StatSoft7, Tulsa, OK). The statistical power of this test (i.e., the probability of rejecting the null hypothesis when it is in fact false and should be rejected) will be reported using the *Power and*

Precision J software package (Biostat7, Teaneck, NJ) and a power value of 0.70 will be considered acceptable for this analysis.

Since the number of amphibians entering each pool cannot be predicted, the power of this test cannot be determined before implementing the study (a priori). A likely scenario, however, is that one or two species, particularly wood frogs, will be common in each pool and one or two species will either occur in low numbers or perhaps be absent from some pools. The absence of some species, such as the blue-spotted salamander, could conceivably be due to natural causes and/or the effects of PCB contamination. In the example in Table 2, 50 individuals were captured in each of the five sample pools and four species were observed. Two species, wood frog (RASY) and American toads (BUAM), were common and spotted salamanders (AMMA) and blue-spotted salamanders (AMLA) were absent from Pool 5. In this example, with $\alpha = 0.10$, the power of the χ^2 test is 0.76 (Borenstein et al., 1997), and it can be concluded that the observed difference in species composition was statistically significant. The biological significance of this observation, however, would depend upon the results observed in the remainder of the study.

This analysis is sensitive to small changes in the proportion of rare species in the sample. Changing the proportion of spotted salamanders in Pool 5 to 5%, and decreasing the proportion of wood frogs to 65%, for example, lowers the power value to 0.49, and it could be concluded that the difference in species composition was not statistically significant. In this example, however, the test is relatively weak (power=0.37). Even collapsing the table to combine Pools 1-4 in a comparison with Pool 5, which would be done if Pools 1-4 were not statistically different, would only increase the power of the test to 0.56. Ultimately, the power of this test will depend upon the number of individuals captured for each species, the observed sex ratios, and the number of pools sampled. The number of pools to be sampled will depend upon the results of the sediment PCB sampling. Once these data are available, a power analysis for this test will be run to assist in selecting the number of study pools.

Analyzing the proportion of the sample exhibiting DELTs would be accomplished using a three-dimensional contingency table (i.e., pools *x* species *x* with/without DELTs). Multiple models are possible with this type of analysis and are too complex to explain here (see Fienberg, 1983 and Zar, 1984 for a detailed discussion). The goal of the analysis, however, is to determine if there is a difference in the occurrence of DELTs between pools, between species, or some combination of pools and species (i.e., an interaction effect). The *Power and Precision* J software package does not calculate power values for multidimensional contingency tables. As with the preceding tests, however, an α of 0.10 will be considered statistically significant for this analysis. An assumption of this test is that animals coming back to the pool were either born in the pool and/or have come to breed in it in previous years. This assumption cannot be tested as part of this study, but is, nonetheless, reasonable based on previous studies. Efts (*Notophthalmus viridescens*), for example, usually return to the pond where they hatched, even when other pools are available (Hurlbert, 1969, *in* Duellman and Trueb, 1986). Individual amphibians, in fact, even frequently enter and leave vernal pools by the same path year after year (Shoop, 1965 and 1968; Hardy and Raymond, 1980, *in* Duellman and Trueb 1986).

Table 2

Sample Power Analysis for Amphibian Captures

	Proportion falling in column				Proportion in Row
	AMMA	AMLA	RASY	BUAM	
Pool 1	15	5	50	30	0.20
Pool 2	15	5	50	30	0.20
Pool 3	15	5	50	30	0.20
Pool 4	15	5	50	30	0.20
Pool 5	0	0	70	30	0.20

Number of cases = 250

Alpha (2-tailed) = 0.10, Power=0.76

Power computation: Normal approximation

The observed sex ratio for each species will also be compared between pools using chi-square analysis. With many amphibians, males can outnumber females in vernal pools even under natural conditions. Detecting a difference in sex ratios between pools, therefore, may involve comparing already low proportions (see Table 3 for an example).

Table 3

Power Analysis for Hypothetical Sex Ratio of an Amphibian Species

Borenstein et al., 1997

	Proportion falling in column		Proportion in Row
	Female	Male	
Pool 1	20	80	0.20
Pool 2	20	80	0.20
Pool 3	20	80	0.20
Pool 4	20	80	0.20
Pool 5	5	95	0.20

Number of cases = 125

Alpha (2-tailed) = 0.10, Power=0.37

Power computation: Non-central chi-square

Body weight is undoubtedly related to body size within each species. In addition, length/weight relationships can be used as a general indicator of the health of amphibians. A salamander with a normal SVL, but low body weight, for example, might be undernourished or have other health problems. The relationship between the body length (SVL) and weight measurements, therefore, will be analyzed using simple linear regression (Zar,1984). Weight will be used as the dependent variable and SVL as the independent variable. For these tests, an α of 0.10 and a power value of 0.70 will be used to test the significance of each regression. Since the number of animals captured can=t be predetermined or influenced by the design of the experiment, an *a priori* power analysis is not reported here. The power of each regression, however, will be reported when presenting the results of the analysis.

Assuming that the regressions of body size and weight are statistically significant, the slope of the regression lines (β) for each species will then be compared using analysis of covariance (ANCOVA) to determine if there is a significant difference in the length/weight relationship between pools (Zar 1984). The hypotheses for this analysis can be stated as:

$$H_O: \quad \beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta_5$$

$$H_A: \quad \beta_1 \neq \beta_2 \neq \beta_3 \neq \beta_4 \neq \beta_5$$

If the null hypothesis is rejected, multiple comparisons among slopes will be used to determine which βs are significantly different (Zar, 1984). From a biological standpoint, the analysis will be used to determine if one or more of the pools has a subpar length/weight relationship for adults as they enter the pool.

If the null hypothesis $\beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta_5$ is not rejected, it may still be possible that body size is significantly different among study pools (i.e., the length/weight relationship is similar, but animals are smaller in one or more of the pools). This difference will be tested by comparing the elevation of the regression lines using ANCOVA and multiple comparison procedures (Zar, 1984). According to Cohen (1977, *in* Zar, 1984), the power of this analysis can=t be determined, except in very unusual cases. In addition, the *Power and Precision* J software package does not calculate power values for this analysis. As part of this study, however, we will determine if other methods of determining the power of this test have been recently developed. If they have, the power of the test will be reported along with the observed significance level (α).

2.4.2 Courtship and Breeding Behavior and Condition

Data to be analyzed related to courtship and breeding behavior and condition include: 1) surveys for chorusing frogs followed by searches for visual evidence of breeding activity (e.g., amplexus in frogs), and 2) inspecting adults entering and leaving the pool for external indications of reproductive activity.

Amphibians are attracted to breed in vernal pools in response to a variety of endogenous and extrinsic factors (Duellman and Trueb, 1986). Endogenous factors include the seasonal development and activity of male and female gonads and the development of secondary sexual characteristics in males in response to secretions from the pituitary gland. These biochemical

processes are potentially influenced by PCB contamination, but are not the subject of this study. The study, instead, is focused on outward evidence that these processes are functioning normally. This evidence includes: 1) males and females of all species are coming to vernal pools at the appropriate times, 2) secondary external sexual characteristics are present, 3) males are calling or otherwise seeking out mates, and 4) breeding is taking place.

Audio chorus and visual evidence of breeding (e.g., amplexus in frogs, nuptial dance in salamanders) can be difficult to observe because: 1) amphibians may avoid human observers, 2) many amphibians, and salamanders in particular, are difficult to observe, and 3) for the previous two reasons the data acquired through direct observation may not be random. In addition, being in the pool for the length of time needed to collect quantitative data may unnecessarily disturb activity within the pool. Therefore, reproductive evidence will be qualitatively described for the pools.

Amphibians leaving the pool that are captured in pit traps will be weighed and inspected for DELTs and external evidence of breeding activity. Individuals may gain or lose weight while in the vernal pool, and excessive weight loss may be an indication of compromised health. Change in weight, therefore, will be compared between pools for each species. Data for this analysis will come from the weight of the individual (i.e., individuals will receive unique color markings) when it leaves the pool compared to its weight when it entered the pool expressed as a percent increase or decrease. Change in weight (the dependent variable) is potentially related to the length of time the animal spends in the pool. It is also expected to be influenced by species and sex and to potentially vary from pool to pool. Multiple linear regression, therefore, will be used to evaluate weight change in relation to: 1) pool, 2) species, 3) sex, and 4) length of time in the pool (Zar, 1984 et seq.). As with other analyses, a significance level (α) of 0.10 and a power value of 0.70 will be used to determine the statistical significance of these tests.

Methods for comparing the proportion of the population in each pool exhibiting DELTs has been described in Section 2.4.1 and will be repeated for animals captured leaving the pool. Similar methods will also be used to compare the proportion of the population, by species and sex, in each pool exhibiting external evidence of breeding activity.

Since animals will be marked as they enter the pools and counted again as they leave the pools, the proportion of the population surviving within each pool can be estimated as $N_{leaving}/N_{entering}$. Adult amphibians that come to vernal pools to breed generally do not remain in the pool after breeding and egg-laying. Animals that are not recaptured in the pit-traps therefore, will be assumed to have died in the pool. The number of individuals alive and dead at the end of the study will be compared between pools using contingency tables and chi-square analysis. Since the number of animals entering the pool cannot be manipulated, an a priori power analysis is not presented here. The significance level (α), power value, and test statistic, however, will be reported and an α of 0.10 and a power value of 0.70 will be considered statistically significant.

2.4.3 Egg Laying, Hatching Success, and Larval Growth and Development

Data related to egg laying, hatching success, and larval growth and development includes: 1) numbers of egg masses per species in each pool; 2) growth and development of larval forms in

box samplers; 3) survival of larval forms in box samplers over a 10-day period; 4) PCB concentrations in larval forms; and 5) growth and development of larval forms throughout the pools.

A complete count of all egg masses in each pool is likely not possible without significantly disturbing the pool. In addition, some species' egg masses are near the bottom of the pool or hidden among plants within the pool. The number of egg masses observed in each pool, therefore, will only be reported and qualitatively compared between pools in relation to the number of individual females of each species known to be in the pool.

Within each box sampler, the number of hatched and living larvae will be counted on a daily basis. The number of larvae will be determined by sweep netting the box sampler to retrieve all live larval forms. Enumeration of larvae in the box samplers may be estimated if total numbers are excessive and holding time required for counting is thought to adversely affect the larvae. The egg mass will be retained for PCB testing, and an expert in larval amphibian development will inspect the hatched and unhatched eggs.

Larval growth and development data include the SVL, tail length, and total length as well as the environmental covariates DO, temperature, conductivity, and pH. These data will be analyzed by multiple regression techniques using SVL as the dependent variable and age (days since hatching), DO, temperature, conductivity, and pH as independent variables. The multiple regression equations for each pool will then be compared using the techniques described by Zar (1984). The hypotheses for this analysis can be stated as:

H_O: The regression functions for each pool box sampler estimate the same population regression.

H_A: The regression functions for each pool box sampler do not estimate the same population regression.

The same analysis will be used to compare growth and development throughout the pools (i.e., the data from larval forms captured with aquatic funnel traps).

Survival of the larval forms within the box samplers will be examined and compared between pools using a 10-day daily survival analysis (Caughley, 1977 and *StatSoft*7 1995). The final models to be used in this analysis, however, will be determined in association with EPA scientists.

2.4.4 Metamorphosis and Exodus from Vernal Pools

Data related to metamorphosis and exodus from the pools will include: 1) numbers per species, 2) SVL, 3) weight, and 4) proportion of the population with DELTs. The number per species in relation to the number of adult females entering the pools will be compared between pools, by species, using chi-square analysis (i.e., a 2-dimensional contingency table). These techniques and the associated hypotheses have been described in previous sections. The length/weight relationship will be compared between pools using the regression techniques described in previous sections. In addition, the proportion of the population with DELTs will be compared using the contingency table and chi-square analysis described earlier.

3. QUALITY ASSURANCE/QUALITY CONTROL

3.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

3.1.1 Data Quality Objectives

The objectives of the amphibian reproduction study were discussed in Subsection 1.1. To achieve these objectives, the following types of data will be required:

Taxonomic identification of amphibian organisms: taxa will be identified to specific level based on morphological, habitat, and distributional information. Adult individuals are generally unproblematic compared to larval stages. Knowledge of which adult species has entered the pools (i.e., distributional information) will be used in conjunction with time of appearance to assist species determinations.

Gender identification of amphibian organisms: the gender of adult individuals of each species will be determined on the basis on gender specific reproductive traits (e.g., swollen thumbs in males frogs, size of tympanum in bull frogs and green frogs, swollen venter in male salamanders).

Amphibian age: determination of age (adult, juvenile, metamorph) will be primarily performed though use of published size data (length and weight). Demonstration of sexual characteristics (as for gender determination) and date (metamorphosed individuals will not be present until early summer) will also be used as evidence for classifying the age of amphibian individuals.

Morphometrics for each individual: detailed length and weight measurements for individual amphibians will be recorded. Length measurements will be obtained to the nearest 0.1 mm using hand-held dial calipers. Weight measurements will be obtained to the nearest 0.01 grams using a calibrated balance designed to be capable of accurately measuring masses of this magnitude.

Visual inspection for deformities, erosions, lesions, and tumors (DELTs): each individual amphibian will be examined for DELTs as outlined in Subsection 2.2.1.

Unique individual indentifiers: individual amphibians will be marked through use of fluorescent elastomers that are injected under the skin, as described in Subsection 2.2.1. Elastomers are viewed through use of blue light and amber goggles. To determine the effectiveness of ingredient mixing (elastomer and curing agent), and stability and visibility of marks, captured amphibians will be cross-referenced with marking code charts to ensure that unique marking codes are applied to each individual.

Water chemistry and description data: each day, vernal pools will be measured for temperature, pH, dissolved oxygen, conductivity, and depth. Information will be acquired for the shallow edge and deeper center of the pools. Devices used to acquire the measurements will be the Hobo Data Logger, YSI 85 salinity, conductivity, dissolved oxygen, and temperature system, Omega PHHH-1X pH Meter, and staff gauges, as described in Subsection 2.2.3. Except for the Hobo

Data Logger and staff gauges, which remain in the pool for the length of the study, the remainder of the devices are hand-held units and are calibrated daily. Temperature will be measured to 0.1 Celsius, pH to 0.1, dissolved oxygen to 0.1 mg/l, conductivity to 0.1 Fs, and depth to 0.1 feet.

Weather data: each day, in the close vicinity of the vernal pool, current, daily minimum, and daily maximum temperature, rainfall, and humidity information will be recorded. Temperature will be measured to the nearest degree Celsius, rainfall to 0.1 mm, and humidity to 0.1%.

3.1.2 Data Quality Indicators

Data developed in this study must meet standards of precision, accuracy, completeness, representativeness, comparability, and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000), that are appropriate to the data quality objectives. Each of these data quality indicators that are applicable to this study are discussed below.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. Due to the type of organism involved (living organisms subject to stress from researcher handling) and its characteristics (though a plant may have multiple flowers per stem, an individual frog only has one right forelimb), repeated measurements will not be performed for many types of data collected. Rather than control and measure precision, the study design includes a large number of samples (25 or more) for each species involved in the study, when possible, to provide statistical resolution.

Accuracy is defined as the agreement of a measurement with its true value. For parameters of concern to this study, accuracy is defined as meaning individual amphibians are correctly identified (species, sex, and age) and measured (length and weight), and that physical and chemical data was collected appropriately. Accuracy will be controlled through training and careful supervision by the project manager and daily calibration of hand-held instruments.

Completeness is defined as the percentage of the planned samples actually collected and processed. For some aspects of this study, completeness is a difficult indicator to achieve. For example, during the aquatic funnel trapping effort (Subsection 2.2.3), traps will be deployed early in the season to determine the presence of marbled salamanders. During this time, other species of larval amphibians are not active. Due to the rarity of marbled salamanders, it is likely that few or no captures of amphibian larvae will result during the early trapping effort. The objective, however, is to collect 50 individuals per pool per week of trapping. Therefore, completeness must be a flexible indicator and samples will be processed as possible, given the limitations of season, pool size, species present, etc.

Representativeness is defined as the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. Representativeness for this study is ensured through establishment of an approved sampling design and through careful implementation of the sample processing and analytical methods. Additionally, results from the amphibian reproduction study can be compared to similar studies for the region to evaluate the representativeness of the data collected.

Comparability is defined as the measure of confidence with which the results from this study may be compared to data from a similar study. Comparability of this study will be limited due to potential interaction of the results and PCB concentrations. However, a thorough literature survey may reveal similar studies from the region on which to make comparisons of study results.

Sensitivity is defined as the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest. For the amphibian reproduction study, chosen instruments are capable of measuring at desired levels of sensitivity. This data quality indicator also applies to identification of species, age, and sex of individual amphibians. Techniques for assessing these data will be important in order to limit the number of unknown determinations. As the number of unknown diagnoses increases, the ability of analyses to determine differences in sex and age ratios declines. This data quality indicator can be evaluated by comparing the number of unknown to potential known identifiers (the sex of some species as juveniles cannot be determined in the field due to lack of gender specific differences at that life stage, and therefore sex was not potentially known).

3.1.3 Data Validation, Verification, and Usability

Procedures for data validation for the chemical and physical data (e.g., sediment samples) are discussed in various sections of the project QAPP and will be used whenever applicable to the study. Usability of information gathered during this study will be based on: (1) the experience of the senior investigator to competently oversee field investigations and ensure that field surveys were conducted following the established plan and to accurately identify specimens collected at survey locations; and (2) an evaluation of the taxonomic data collected in the study area compared to previous studies from the biophysical region.

3.2 SAMPLING DESIGN

Survey locations for the amphibian reproduction study will be based on rationale presented in Subsection 1.2. The locations are not intended to be characteristic of the vernal pools in the study area, but rather represent a range of sediment PCB concentrations. To facilitate comparisons for statistical analyses, pools will be selected on the criterion of species observed during 1998 field surveys.

Herpetofauna communities are typically highly variable in nature. To achieve acceptable resolution for analyses it is important that sample size be sufficiently large to capture population variability and provide necessary power. When possible, twenty-five or more samples will be collected for each parameter (e.g., tail length for larval frogs, weight of adult males).

3.3 SAMPLING METHODOLOGY

3.3.1 Sampling Procedures

Sampling methods, as described in Subsections 2.2 and 2.3, were chosen to insure the study objectives will be obtained. Sampling procedures were developed to encompass each stage of the amphibian life cycle (egg, larvae, metamorph, juvenile, adult). Project oversight and researcher training will serve to facilitate accurate and unbiased sampling.

3.3.2 Quality Control Samples

The nature of ecological studies and the type of sample involved (i.e., living organism) does not allow the incorporation of typical duplicate and blank samples as part of the study design. As well, weather and vernal pool chemistry (e.g., dissolved oxygen, pH, conductivity) data does not possess acceptable methods for obtaining samples in a manner analogous to duplicates and blanks collected for soil chemistry analysis. Quality control will be performed though use of project oversight and calibration of instruments.

3.3.3 Sample Processing and Preservation

Description of the types of samples to be collected for this study can be found in Subsections 2.2 and 2.3. Samples can be classified in the manner they will be processed: egg masses; larval frogs; and dead amphibians. Egg masses will be collected in the field in chemically clean jars with water from the site. At the field office, jars containing the egg masses will be placed in large coolers packed with vermiculite to protect contents during shipping. Each jar, as well as the cooler, will possess custody seals and chain of custody forms will accompany the shipment.

Larval frogs (i.e., tadpoles) will be collected in the field in chemically clean jars with water from the site. At the field office, jars containing living larval frogs will be placed in large coolers packed with vermiculite to protect contents during shipping. A small volume of wet ice (ca. 2 liters) will be placed in a resealable plastic bag and located in the cooler to insure the contents remain at a suitable temperature. Each jar, as well as the cooler, will possess custody seals and chain of custody forms will accompany the shipment.

Individual amphibians, regardless of the size or age, that have succumbed to incidental mortality will be collected in the field in labeled, resealable plastic bags and stored in a cooler on wet ice (enclosed in plastic containers), to prevent decay, until delivery to the field office. Amphibians will be removed from the plastic bags at the office, rinsed in distilled water, and snap frozen in liquid nitrogen in a decontaminated mortor. Samples will then be placed in labeled, chemically clean jars and stored in a freezer (maximum temperature -20 Celsius) until shipment to the laboratory. Specimens will be shipped in large coolers on dry ice with appropriate custody seals and chain of custody forms. Holding time for all samples will follow the procedures established in the QAPP.

3.3.4 Training

Sampling will be directed in the field by senior scientists with academic training and experience in the collection of amphibians and related data. Support staff will receive training from the senior scientists in the goals and techniques to be employed during the study to insure collection of quality data.

3.4 SAMPLE ANALYSIS

3.4.1 Biological Samples

The collection of taxonomic and morphometric information for samples will be processed by experienced staff who have received specific training in the SOP and whose work is directly overseen by the project manager. Determination of species, sex, age, and collection of morphometric data does not require magnification, with the exception of larval amphibians. Magnification, in the form of a 10H hand lens, will be required for identification of these samples. Collection of samples for PCB analysis and laboratory study will follow procedures outlined in Subsection 3.3.3. Analysis of biological samples for PCB concentrations will follow standards established in the QAPP.

3.4.2 Physical/Chemical Samples

Sediment and water samples will follow procedures and SOPs provided in the QAPP. Weather and water chemistry data recorded daily for the amphibian reproduction study will assessed in the field rather than through collection of physical samples.

3.5 DATA ANALYSIS AND REPORTING

The overall analytical approach to collection, analysis, and reporting is detailed in Section 2. Numerical analyses will be performed using Statistica for Windows™ (Statsoft, 1999). The study findings will be included in the ecological risk assessment, including data, analyses, and interpretations, and will be prepared with specific reference to both the data quality and objectives specific to the amphibian reproduction study.

4. EQUIPMENT LIST

Equipment that will be needed as part of the field component of the study includes:

- Camera
- Binoculars
- Field notebooks
- Rubber knee- and hip-boots and chest waders

- Heavy-duty rain gear
- Eye protection
- Rubber gloves
- GPS receiver
- D-ring dipnet
- Dissecting tray
- Funnel traps with small rope
- Box samplers
- Fluorescent pigments
- Scale
- Sample jars, both chemically and non-chemically clean
- Plastic sheeting
- Hammer
- No. 10 (or similar) cans with covers
- Duct tape
- Wooden shingles
- Wooden stakes
- Heavy-duty stapler
- Ethanol
- Survey flagging
- Resealable bags
- Wet and dry ice
- Aluminum foil
- Coolers for shipping samples
- Conductivity, DO, pH meters
- Thermometer

5. RESULTS

The results of this study will be provided in a report that includes: 1) a physical description of each study pool using methods described in Work Plan Appendix A.9; 2) PCB concentrations within the sediments of each pool; 3) a summary of the potential effects of PCBs on amphibians based on the literature review; 4) summary statistics for data collection activities (e.g., mean, standard deviation, sample size); 5) results of statistical tests (e.g., final hypotheses, test statistics, significance levels, and power values where applicable); and 6) an interpretation of the results.

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ATTACHMENT 1

NARCAM PROCEDURES FOR CHARACTERIZING DELTs

Standard Field Forms

Included in this section are field forms designed by a working group of scientists researching the amphibian malformation issue. We are awaiting standard protocols as well. In the meantime, we suggest following the protocol designed for surveys on National Wildlife Refuges.

We are making these forms available to verifiers who are interested in collecting detailed data for their own purposes. If so, completing the forms ensures that data is collected in a standardized manner and is comparable to other efforts in North America.

General Survey Information

Reporter Information

Reporter's Name:_____
E-Mail Address:_____
Postal Address:_____
City:_____
State/Province:_____
Phone Number:_____

Survey Site Information

State/Province:_____
County (if in US):_____
Town/City:_____

Date

Date of Observation:_____/_____/_____

Species

Species Name:_____

Amphibian Numbers

Number of Normal Individuals:_____ Number of Malformed Individuals:_____

Malformation Description

Please complete this form for each malformed individual

*These categories are for use with metamorphosing (4 legs) or adult frogs and toads.
*Start exam from the head and work toward the hind legs, noting any abnormalities seen by checking the boxes below.
*If a deformity is seen that does not fit into one of the categories below, please describe the deformity in the sections marked "other".
*Deformities that are difficult to describe can be drawn in on the frog diagram below.
*NOTE: "Left" and "Right" refer to viewing the frog from above as it would rest normally.

Eyes:

L	R	
___	___	eye absent
___	___	eye smaller than normal
___	___	pupil abnormally shaped
___	___	eye in unusual position(describe):_____
___	___	extra eye(s) (describe):_____
___	___	other (describe):_____

Jaws:

L	R	
___	___	lower jaw shortened
___	___	upper jaw shortened
___	___	other deformity (describe):_____

Front limbs:

L	R	
___	___	entire limb missing at shoulder
___	___	limb partially missing:(describe):_____
___	___	foot missing
___	___	complete calf (tibiofibula) present, abnormal musculature
___	___	enlarged
___	___	small (atrophied)
___	___	digits missing from foot (specify digits):_____
___	___	digits fused or clubbed
___	___	other (describe):_____

Spine:

L	R	
___	___	curved to the left or right (scoliosis)
___	___	other (describe):_____

Webbing (cutaneous fusion):

L	R

___ ___ between thigh and calf (femur and tibiofibula)
___ ___ other (describe degree):_____

Hind Limb:

L R

___ ___ entire limb present, unusual angle (twisted, rotated, etc.)
(describe):_____
___ ___ entire limb present, abnormal size (atrophied, enlarged)
(describe):_____
___ ___ digits missing from foot (specify digits):_____
___ ___ digits shortened, fused or clubbed:_____
___ ___ digits in abnormal location (describe):_____
___ ___ extra digits:(describe):_____
___ ___ foot missing (tarsal bones)
___ ___ complete calf (tibiofibula) present, abnormal musculature
___ enlarged
___ small (atrophied)
___ ___ portion of calf (tibiofibula) missing:
(estimate length of calf present):_____
___ ___ entire calf (tibiofibula) missing
___ ___ complete thigh (femur) present, abnormal musculature
___ enlarged
___ small (atrophied)
___ ___ portion of thigh (femur) missing
(estimate length of thigh present):_____
___ ___ entire limb missing
___ ___ other (describe):_____

___ **Abnormal color or pattern:**

(describe and locate:)_____

Extra Limbs:

How many extra limbs are present?_____

For each extra limb, describe location of origin (left or right, hip, knee, spine, etc.). Also specify musculature (larger or smaller than normal limb), and completeness (entire limb present, or portion of limb). If only part of an extra limb is present, try to specify which part is present (thigh, thigh and calf, foot, etc.). Draw the extra limbs on the frog diagram below.

extra limb #1
location_____

musculature_____

completeness_____

extra limb #2
location_____

musculature_____

completeness_____

___ **Retained tail**

Fully:(length)_____
Partially (length):_____

___ **Any bleeding or fresh injuries?:**
(describe):_____

___ **Other abnormalities:**
(please describe):_____

Site Characterization Form

Field Crew:_____ **Date:** ___/___/___ **Site #:**_____

Weather: Clear/Sunny:___ **Some Clouds:**___ **Overcast:** ___ **Rain:**___ **Temp.(°F):**___

Location: Map Name:_____ **Map Scale:**_____

GPS Coordinates: Latitude:_____ **Longitude:**_____

GPS Sampling Time:_____ **GPS Post-Processing?: YES / NO**

Address nearest building/residence:_____

Street/Highway address:_____

Town:_____ **County:**_____ **State:**_____

Describe how to find the site (e.g. closest intersecting roads, mileage marker, etc.)
_____
_____
_____

Stream/River Name:_____ **Pond/Lake Name:**_____

Federal/State/Reserve/Park Name:_____

LAND USE/LAND COVER:
Describe Surrounding Land Uses:_____

Describe Major Land Uses:_____

Upland

___Urban/Roads/Commercial/Industrial ___Residential/Roads ___Cropland
___Barren Land ___Deciduous Forest ___Shrub/Brush ___Orchard
___Mixed Forest ___Coniferous Forest ___Plantation ___Pasture

Wetland/Water

___Upper perennial stream ___Lower perennial stream ___Intermittent Stream
___Open Water (Describe depth and extent, if known)_____
___Emergent ___Vegetated Shallows ___Wet Meadow/Agricultural ___Scrub/Shrub
___PFO1(broad-leaved deciduous e.g. red maple swamp)
___PFO2(needle-leaved deciduous e.g., tamarack swamp)

___PFO4(needle-leaved evergreen e.g., coniferous swamp)
___PFO5(e.g., dead snags)

Comments on Classification (e.g. dominant, sub-dominant, and minor cover types)

_____
_____
_____

Wetland/Hydrology Indicators (e.g. mottled leaves, ring around trees, adventitious sprouting):

_____
_____
_____

Soil Type:

_____
_____

DOMINANT PLANT SPECIES (note **dominant** species in each strata with % cover or class):

Trees (> 20 feet in height and > 5 inches d.b.h.):

Species_____ % Cover____ Species_____ % Cover____
_____
_____

Shrubs/Saplings:(typically < 20 feet in height < 5 inches d.b.h. for saplings):

Species_____ % Cover____ Species_____ % Cover____
_____
_____

Forbs/Herbs/Emergents/Graminoids:

Species_____ % Cover____ Species_____ % Cover____
_____
_____

Ferns/Fern Allies:

Species_____ % Cover____ Species_____ % Cover____
_____
_____

Organic Litter (e.g. leaf litter, deadfall water, etc.):

% Cover_____
_____
_____

Surface Substrate (e.g. soil, bedrock cobbles, etc.):

% Cover_____
_____
_____

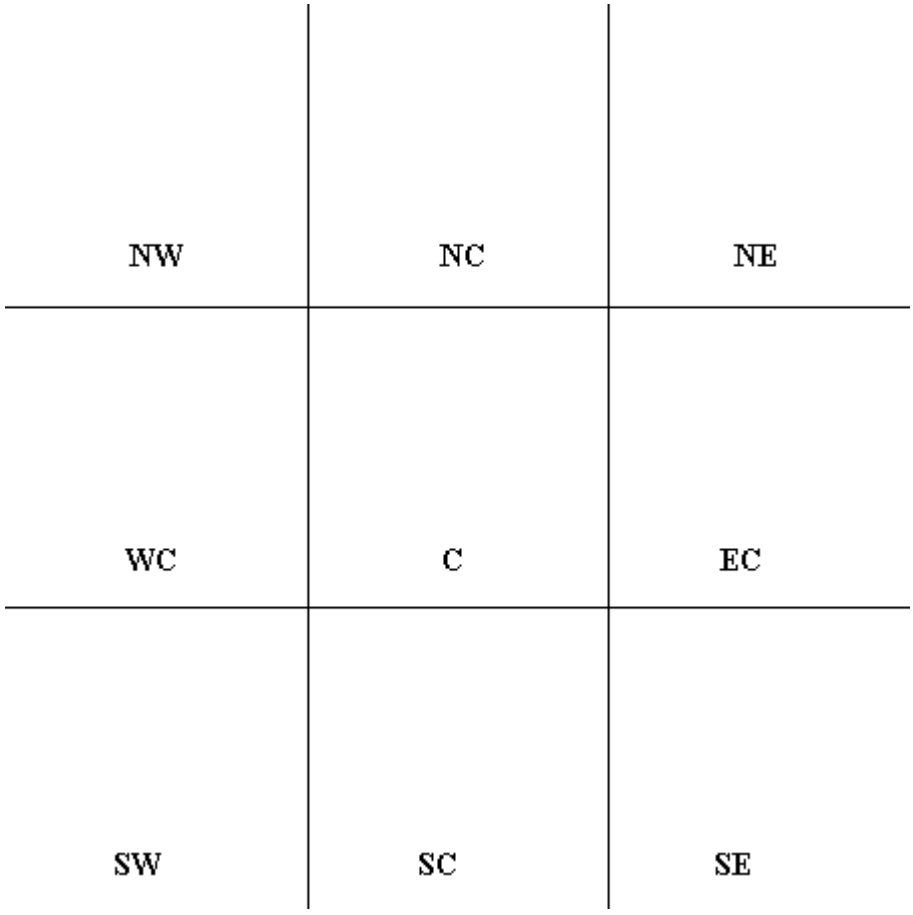
Site General Description (e.g., describe variability in the site):

_____
_____
_____
_____

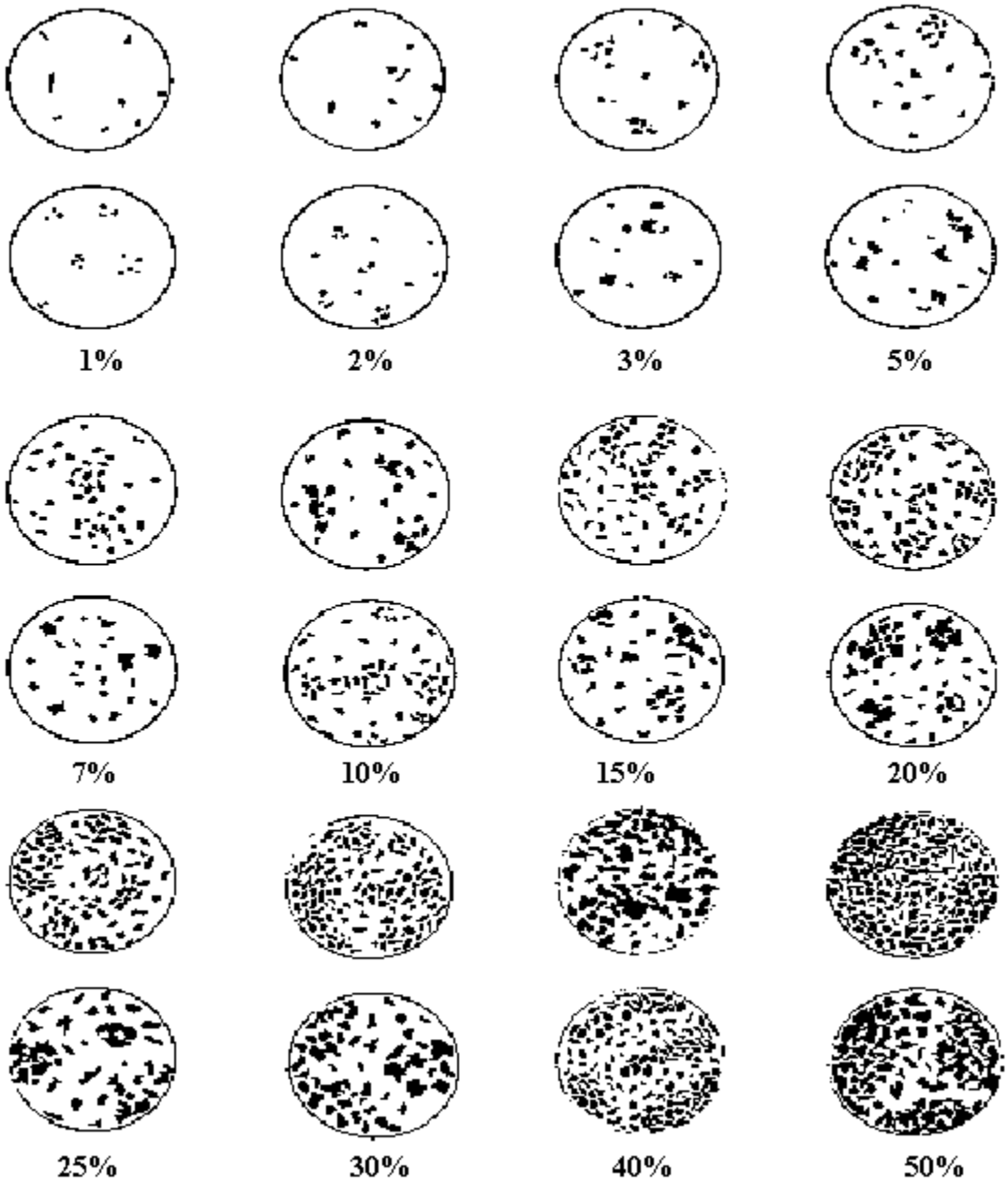
Photographs (Looking out from the center at each of the four cardinal directions. Use the chalk board for site I.D. For homogeneous sites two pictures may suffice):

Roll-Frame #	Compass Direction	Comments
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Site Map: Diagram any distinct features or boundaries present within the verification site (indicate the position and compass direction you are facing in photos taken):



Comparison Chart for Visual Estimation of Foliage



ATTACHMENT 2

PCB TISSUE SAMPLE COLLECTION STANDARD OPERATING PROCEDURES

Sample Handling for Herpetofauna - Housatonic River. Pittsfield, MA Spring/Summer 1998

1. Mortality specimens (samples) from pitfall traps should be collected on a daily basis, preferably early each morning.. The date and time of collection should be noted.

2. Samples should be retained in the field in chemically clean jars. Each jar (8, 16 or 32 ounce dependent on biomass) should be representative of one trapline area or one pitfall. Jars should be labeled with the site name, trap line/pitfall number, date and time of collection. Samples should be kept on wet ice in the field. The wet ice should be retained in double ziploc bags to avoid contact with samples. If sample biomass is too large for a 32 ounce jar then dedicated decontaminated stainless steel or aluminum buckets should be used for transport.

3. Processing of samples should be as follows: individual specimens should first be thoroughly rinsed with deionized water for removal of all external organic matter, identified to species, weighed (to the nearest 0.1 gram), measured (nose to vent, to the nearest millimeter), and examined for gross external pathology. All information should be recorded on a separate data sheet for each sample.

4. Each individual should be assigned a specific sample number, based on species, trap line and pitfall number. If samples are composited, the composite should consist of the same species and preferably the same size class. To the extent possible, the number of individuals per composite should remain consistent and be recorded.

5. Individuals should be wrapped in aluminum foil (shiny side out, dull side next to sample). Aluminum foil can be hexane-rinsed or a field blank of representative unrinsed aluminum foil from each roll used should be submitted for analysis. The field blank should be prepared in the same method as biota samples.

6. A sample label should be placed on the exterior of each individually wrapped sample or sample composite. The sample label should be securely taped with clear tape. The sample label should contain the sample number, sample type, analysis to be performed, site name, and date.

7. Aluminum-wrapped, labeled samples should be placed in a ziploc bag and sealed. That bag should be placed in another ziploc bag containing a separate sample tag with the sample number clearly showing.

8. Samples should be kept on dry ice until they are shipped (with dry ice) to the analytical laboratory (or the USFWS field office for freezer storage). For large cooler/numerous sample shipments, layering of samples with dry ice and newspapers allows for better sample preservation and longer dry ice persistence.

9. All pertinent information regarding each sample (sample number, sample type, date collected, etc.) should appear on a standard EPA chain-of-custody form and be included

in a sealed ziploc bag, taped to the lid, inside each sample cooler.

10. Samples should be sent Federal Express Overnight (Next morning delivery). Samples sent to the USFWS should be shipped to:

Ken Carr/Ken Munney
USFWS — Phone: 603-225-1411
22 Bridge St., Unit 1
Concord, NH 03301 — Fed Ex Acct #: 1510-1036-9

Shippers should call ahead to the receiving laboratory or the USFWS and notify that samples are being sent for next day delivery. Samples should not be sent to USFWS if Ken Munney, Ken Carr, or Drew Major are not available for receipt of the shipment. Samples need to be sent for arrival on a weekday only. Therefore, Thursday is the last day of the week to ship samples. Shippers should also call the receiving laboratory or USFWS the day of delivery to verify receipt of samples.

APPENDIX A.19

PROTOCOL FOR FROG REPRODUCTION AND DEVELOPMENT STUDY (FORT)

PROTOCOL FOR FROG REPRODUCTION AND DEVELOPMENT STUDY

STUDY PROTOCOL NO.: WESR01 – RSTS01 - 1

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Preface

The following protocol has been prepared for the "Supplemental Investigation Work Plan for the Lower Housatonic River" under the Technical Support Services, General Electric (GE) Housatonic Project, Pittsfield, Massachusetts, and is intended only for this specific project. The methods used to develop this protocol are available in public scientific literature and are thus non-proprietary. Potential risk associated with the use or misuse of this protocol outside the scope of this project will be assumed by future investigators.

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1. INTRODUCTION

This protocol describes a study design to evaluate reproductive performance and developmental effects in frogs potentially exposed to polychlorinated biphenyls (PCBs) in the Housatonic River study area. Frogs have been chosen as the representative amphibian species due to their presence in the Housatonic River study area, reported sensitivity to PCBs, high potential for exposure due to both aquatic and terrestrial life stages, and capacity to be evaluated for reproductive and developmental metrics in the field and laboratory, and are considered sentinel species in the environment.

The overall objective of this study is to assess the impact of potential PCB exposure on frog populations in the Lower Housatonic River area between the confluence of the East and West branches and Woods Pond Dam (target area). More specifically, the objectives of this study will focus on the potential impact that PCB contamination may have on reproduction, early development, and maturation (metamorphosis) in Northern Leopard frogs (*Rana pipiens*). These events represent critical stages in the life cycle of an amphibian and the evaluation of the effect of PCBs and potentially other organic compounds at these stages will determine the capacity of these contaminants to disrupt the life cycle of amphibians.

This study is designed to determine the effect of PCB exposure to sexually mature adult frogs on reproductive capacity and developmental fitness in their progeny using both target and reference sites. The routes of exposure and generational transport of the PCBs will also be assessed to determine the extent of maternal transfer to the oocytes and developing progeny, as well as the extent of bioaccumulation during early and later development. Reproductive performance and early developmental effects will be assessed by comparing the following endpoints:

- gravidity;
- numbers of eggs produced;
- necrosis;
- oocyte maturity (stage);
- sperm count;
- sperm morphology and viability;
- fertilization;
- early embryogenesis;
- hatching success;
- mortality; and
- morphological development (teratogenesis)

for frogs obtained from the target area, with the same endpoints in frogs originating from a uncontaminated reference area. To document potential impact on longer-term developmental processes, exposure studies will be conducted in the laboratory throughout metamorphosis. Metamorphosis, because of the array of biochemical processes occurring simultaneously, is a sensitive stage in the life cycle of amphibians and a stage that is sensitive to endocrine disruption. Since PCBs, along with organochlorine pesticides, have been shown to alter thyroid function in metamorphosing frogs (Fort et al., 1999a and 1999b), this phase of the study is of great importance.

An overview of this program is provided in Figure 1. In summary, male and female frogs will be collected from the target and reference areas and transported to the amphibian toxicology laboratory. The gravidity of the females will be recorded and the gravid females will be hormonally induced to superovulate egg masses, which will then be fertilized *in vitro*. The number of eggs produced per female will be estimated on a volumetric basis and rates of necrosis and stage determined. Sperm counts, morphology, and overall viability will also be assessed. The eggs will be monitored in the laboratory for fertilization, morphology, and coloration, while the embryos will be monitored for mortality, hatching success (including time to hatch), and morphological abnormalities. Deformities, particularly those that could directly affect juvenile survival and, therefore, the population, will be specifically documented by type of terata induced and number responding. Exposure studies will be conducted throughout metamorphosis of cultured progeny. The rate of metamorphosis, which will include an evaluation of the rate and morphology of limb development, rate and morphology of tail resorption, and development of secondary morphological characteristics, including mature skin, will be evaluated. In addition, a portion of each egg mass, as well as the ovaries of females from which egg masses are obtained, testes from the males, and whole bodies of developing embryos and larvae will undergo PCB and congener-specific analysis to allow determination of a concentration-response relationship between observed effects and PCBs. Although the number of parameters to be evaluated during this study will likely increase the experiment-wide error rate, the utility of measuring multiple parameters (endpoints) will also decrease the likelihood of overlooking a potential effect from PCB exposure.

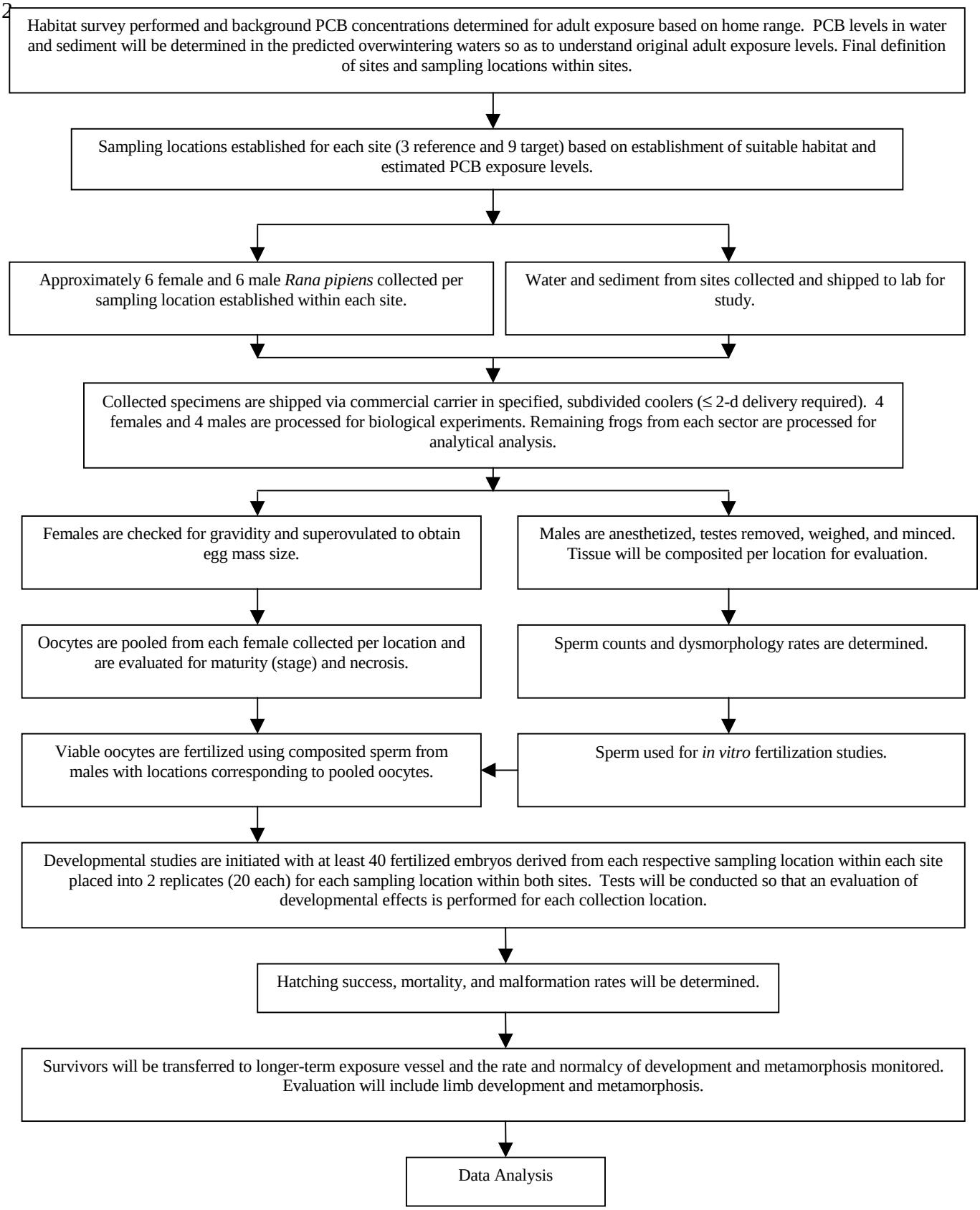
2. DEFINITION OF STUDY METRICS AND ENDPOINTS

Egg mass	Group of eggs laid by a single female
Gravid	Containing a mass of eggs in ovary
Reference area	An uncontaminated area with generally comparable flow regime, habitat, alkalinity, and hardness to the target area
Target area	The reach of the Housatonic River between the confluence and Woods Pond Dam
Viability	Capable of surviving and developing normally (in the case of embryos) or fertilizing eggs (in the case of sperm)

3. SELECTION OF TEST SPECIES

The species selected for this study is the Northern Leopard frog (*Rana pipiens*). Previous field surveys have indicated that Northern Leopard frogs are abundant in the Housatonic River study area and constitute an important component of the Housatonic River ecosystem. They have a limited home range, spending a good proportion of their life spans in aquatic environments; thus, their PCB body burdens should reflect the diet, sediment, and water column concentration in the areas from which they are collected. Also, because Northern Leopard frogs lay thousands of eggs, it should be possible to collect a sufficient number of eggs to ensure statistical power and confidence in study results. Finally, there is an established peer-reviewed methodology for *in vitro* fertilization of Northern Leopard frogs and culturing of their embryos in the laboratory

Figure 1 Overview of Frog Reproduction and Development Study



(Dickerson, 1969; Nussbaum et al., 1983; Carolina Biological Supply Company, 1993; Fort et al., 1996a; ASTM, 1998; Bantle et al., 1998).

Temporal applicability of the study is directed toward the peak breeding season for the Northern Leopard frog (Stebbins, 1995). Sample collection will ideally occur during the last week of April and the first two weeks of May.

The spatial applicability of the target area of the study is limited to the reach of the Housatonic River between the confluence and the Woods Pond Dam. This reach represents a range of PCB concentrations in sediment such that development of exposure-response relationships may be feasible. Additionally, Northern Leopard frog habitat is present in Woods Pond, and other backwater regions. Suitable locations that are representative of a reference area within the Housatonic River watershed will also be identified.

4. REQUIRED EQUIPMENT AND SUPPLIES

Equipment to be used during the field collection phase includes the following items:

- 3 ambient air thermometers
- 15 collection nets, drift fences, and funnel traps
- 2 digital GPS locators
- 45 cubic feet sphagnum moss, cured and sanitized
- 300 data forms
- 4 medium seines
- 50 fiberboard boxes
- field maps
- field notebooks and clipboards
- 25 field marking pencils
- 10 flashlights
- 150 ft bubble wrap
- 10 gallons distilled water
- 1,000 live crickets
- plastic sheeting
- 12 pairs of heavy work gloves
- 12 pairs of steel-toed boots
- polyethylene resealable plastic bags for shipping
- 12 portable cassette tape recorders and tapes
- 5 cellular 900 MHz telephones
- 250 preprinted sample labels
- 15 reusable ice packs
- liquid nitrogen
- dry ice
- 2 rolls of duct tape
- 100 sample containers for residual egg masses
- 50 sample bags for sacrificed female frogs
- 15 permanent magic markers

- Site Health and Safety Plan (HASP)
- 100 6-gallon Styrofoam coolers
- vermiculite
- 10-L 3% (w/v) MS-222 anaesthetic

5. QUALITY ASSURANCE/QUALITY CONTROL

5.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

As indicated in Section 1, the primary objective of this study is to assess the impact of PCB exposure on frog reproduction, development and maturation. Overall, this study will attempt to determine the effect of PCB exposure to sexually mature adult Northern Leopard frogs on reproductive capacity and developmental fitness in their progeny by comparing a series of biological and toxicological indicators in specimen collected from uncontaminated areas (reference sites) and contaminated areas (target sites). In order to achieve this objective, the following data and specific quality assurance criteria will be needed.

Gravidity – Reproductive capacity in female frogs will be evaluated initially by determining if egg masses are present within the specimen collected. Although gravidity is a binary response, gravidity will be determined immediately upon specimen arrival at the laboratory and will be recorded with accompanying chain-of-custody information (sample identification), and health characteristics upon arrival (general appearance and weight). Gravidity will also be used to confirm the sex of the specimen.

Numbers of eggs produced (egg mass) – Total egg counts must be determined and recorded accurately for each specimen. Volumetric determination of egg mass is not accurate or precise enough to use in this case. Therefore, manual counting of the egg masses will be required. Egg masses will be counted at least twice unless the values exceed 10% of one another. In the case of excessive variability, the process of counting will be repeated. In addition, the counts will be verified by a separate analyst using the same criteria described above.

Necrosis and oocyte stage profile – The number of necrotic eggs will be determined using the same approach and quality control measures as described for egg mass determination. The oocyte stage of development profile is one of the best indicators of reproductive status in frogs. The laborious nature of this process requires significant attention to consistency to be accurate and will require independent peer verification. Data verification using the approach described above for egg mass counting will be used to verify the results.

Sperm count, viability, and morphology – The assessment of male reproductive fitness on a gametogenesis level depends on the accurate collection and recording of the data. Total sperm cells and abnormal sperm will be counted at least three times unless the values exceed 10% of one another. In the case of excessive variability, the process of counting will be repeated. In addition, the counts will be verified by a separate analyst using the same criteria described above.

Fertilization – Fertilization will be microscopically determined based initially on the formation of a gray crescent region immediately dorsal to the animal-vegetal pole axis. The formation of the gray crescent occurs on the opposite side of sperm penetration into the egg and is the first sign of fertilization. Subsequent formation of a cleavage plane in the egg will be the unambiguous sign of fertilization and will be used to verify the number of eggs fertilized. The same quality control measures described for the previous metrics will be used to ensure the quality of the data collected and reported.

Early embryogenesis, hatching success, mortality, and morphological development – To determine the effect of PCB exposure on frog development, early embryonic development, hatching, and more advanced morphological development will be monitored. Embryolethal effects will also be recorded throughout development. As with the other metrics, close attention to accurate counting will be imperative. Counts will be verified by a separate analyst using the criteria described above.

Metamorphosis – The effect of PCB exposure on maturation of the larval frogs will be monitored since this life phase is often a sensitive indicator of potential stress. Detailed records of developmental stage, types and incidences of mal-development and the rate of limb development and tail resorption will be required. Digital photographic documentation of metamorphic events will represent an important record of maturation. Peer review by a separate analyst will be used to verify the data collected and authenticate the results.

Water and sediment PCB and other contaminant analyses – Analysis of water and sediment for the various contaminants identified in this Work Plan must result in data which is consistent with data for water and sediments collected for the other components of the entire project. Satisfactory results will be ensured by submitting samples to the same laboratories that are responsible for analyzing samples from the other studies associated with the program. Quality control specifications for these data are identified in the project QAPP (WESTON, 2000).

Tissue residue analysis – QC considerations to ensure achievement of the data quality objectives (DQOs) for this parameter will follow the QAPP (WESTON, 2000).

5.2 DATA QUALITY INDICATORS

Data developed in the frog reproduction and development study must meet acceptable standards of precision, accuracy, completeness, representativeness, comparability and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000). Each of these data quality indicators, some of which are not readily quantifiable for data associated with this study, are discussed below.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. Because of the biological heterogeneity inherent in Northern Leopard frog communities, it is not possible to take repeated independent measurements of the biological parameters. Rather than control and measure precision, the study design includes a total sample number and a number of replicates to obtain sufficient statistical resolution, as is defined in the subsequent section. Precision may also be evaluated by the assessment of the degree to which sample collection procedures are able to ensure collection of a consistent number of samples.

Endpoint or test metric precision within a given sample (frog) will be measured by ensuring an adequate number of replicates and are used to ensure adequate statistical measure of precision and significance. For measurements that are not unique to the frog reproduction and development study, such as water and sediment chemistry and tissue residues, precision is evaluated as defined in the QAPP (WESTON, 2000).

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study, accuracy is defined as meaning that the test metrics are correctly determined in each sample, correctly enumerated, and correctly recorded. Accuracy of each test metric is a function of each sample being processed, reviewed, and recorded and of consistent field sampling techniques. The data generated by this study may be evaluated for accuracy via comparison with known and/or expected results from similar studies conducted in the Housatonic River or in similar New England ecosystems, although a limited number of comparable studies are currently available. For parameters such as water and tissue residue and sediment contaminants, accuracy is as defined in the QAPP (WESTON, 2000). With respect to the study design, a rough measure of precision and accuracy may be determined by the relation or degree of fit obtained between the biological effects observed (represented by each test metric) and the body burdens measured.

Completeness is defined as the percentage of the planned samples actually collected and processed. Completeness can be evaluated for all components of the frog reproduction and development study. To ensure achieving the planned statistical resolution, it is important that completeness reasonably near 100% be achieved for all components of this study, with the exception of the tissue residue analyses. The minimum sample size required to complete this study, based on the anticipated test metric variance, is approximately 30 males and 30 females for the study. Thus, approximately 3 male and 3 female frogs will need to be collected per sampling location. For the tissue analysis study component, the number of analyses will be determined by the material available for collection, and establishment of an *a priori* completeness goal is not possible.

Representativeness refers to the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. This data quality indicator is addressed through implementation of proper sampling design, sample processing methods, and sample analysis may be evaluated via comparison with known and/or expected results.

Comparability is a measure of the confidence with which the frog reproduction and development data may be compared to another similar data set. Comparability may be evaluated for this data set through comparison with previous amphibian studies in the Lower Housatonic River, if they exist, and with known characteristics of Northern Leopard frog communities in similar ecosystems in the Northeast. Comparability may also be evaluated by examination of the sample location variability in key parameters as determined from the large numbers of replicates to be collected at each location.

Sensitivity, the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest, is difficult to apply to the biological parameters associated with this study. Frog reproduction, development, and maturation represent sensitive indicators of frog health and fecundity. The ability of the test metrics designed in this study to determine

potential changes in reproductive capacity or developmental fitness relative to corresponding tissue PCB residues or sediment PCB levels will be the primary determinant of the sensitivity of this model system. Sensitivity of analytical analyses alone is described in the QAPP (WESTON, 2000).

5.3 DATA VALIDATION, VERIFICATION, AND USABILITY

Procedures for data validation for the chemical and physical data are discussed in various sections of the project QAPP (WESTON, 2000) and will be used whenever applicable in this study. For the biological data, usability will be largely determined by three factors: (1) the experience of the principal investigators in establishing that the field sampling was conducted using appropriate techniques and that accuracy and precision were not compromised by an inability to control the sampling procedures in the field; (2) an evaluation of the toxicological data as compared with previous studies; and (3) a direct comparison between the analytical chemistry and tissue residue data and similar data collected by other studies from similar areas of the river. The purpose of the remainder of this section of the study plan is to document the measures included in the study to ensure that the standards discussed above are met.

5.4 SAMPLING DESIGN

The rationale for selection of three reference site sampling locations with non-detectable sediment PCB levels, and nine target site sampling locations with varying degrees of sediment PCB contaminant levels to be sampled is presented in Subsection 6.1. The locations are not intended to be completely representative of the entire river, but rather are intended to encompass the range of sediment PCB levels in the Lower River between the Confluence and Woods Pond. The nine target sampling locations are sufficient to achieve the study DQOs consistent with the resources available for the study.

Frog reproduction and development data may be variable. To achieve acceptable statistical resolution, it is necessary to collect an adequate number of samples from each location. Ideally, six female and six male frogs will be collected from each of the 12 (three reference and nine target) sampling locations. This number of samples was selected based on power analyses.

5.4.1 Sampling Methodology

Sampling Procedures. Sampling methods, as discussed in Subsection 6.3, have been chosen to ensure an adequate collection of specimen that will permit comparisons between target and reference sites and determine the relationship between PCB body burden and toxicological effects on reproduction, development and maturation. Frogs will be collected personally by trained WESTON and Woodlot Alternatives personnel. Samples for physical and chemical analyses will be collected following procedures documented in the project QAPP (WESTON, 2000) and will therefore be comparable with procedures followed for all other similar efforts throughout the river.

Quality Control Samples. The nature of frog specimen sampling does not allow the incorporation of blank samples as part of the study design. Although it should not be considered directly analogous to a duplicate abiotic sample (i.e., water or sediment) collected for analytic chemistry analysis, additional frogs beyond those required for statistical evaluation will be collected from each sample location if available. As a general guideline, one additional female and one additional male frog will be collected for QC analysis. The QC specimen will be processed for biological examination and whole body or tissue residue analysis in a similar manner to the other samples collected. QC sample collection and analysis of sediment and water samples are described in the QAPP (WESTON, 2000).

Sample Documentation, Preservation, and Shipping. Detailed procedures for the documentation, preservation, and shipment of all samples associated with the frog reproduction and development study are described in Subsection 6.4 This study presents no unusual issues with regard to sample documentation, preservation, or shipment. Subsampling, homogenization (water and sediment samples), and decontamination between samples will follow procedures established in the project QAPP (WESTON, 2000). Holding time for physical and chemical samples will follow procedures established in the project-wide QAPP (WESTON, 2000). Since live frogs will be shipped, detailed methods of ensuring safe delivery to the laboratory will be used and are described in Subsection 6.4.2 and Attachment 2 of this document. Holding times for preserved biological samples for residue analysis and water and sediment samples will be strictly followed and are defined in the project-wide QAPP (WESTON, 2000).

Training. All sampling will be directed in the field by senior scientists at Woodlot Alternatives and WESTON with experience in the collection of Northern Leopard frogs in the field. Supporting staff will receive training from the senior scientist(s) in the overall goals of the study and in techniques to be followed to ensure collection of quality data.

5.4.2 Sample Analysis

Laboratory Studies. Processing of the frogs for *in vitro* fertilization, early developmental monitoring and evaluation of metamorphosis will follow procedures established in Subsection 7.1. All samples will be processed by experienced staff who have received specific training in this area and whose work is checked periodically by their supervisors and peers. Methods of QC for each metric evaluated were addressed in the DQOs. Each analysis will be repeated until consistent results are obtained (i.e., two separate egg counts within a given specimen should fall within 10% of one another). Verification by a separate analyst will also be used to authenticate the results. Corrective action, including reprocessing of samples and retraining of staff, will be instituted if these QC checks produce unsatisfactory results.

Physical/Chemical Samples. Samples for water and sediment chemistry and tissue residue analysis will be processed following procedures and SOPs provided in the project-wide QAPP (WESTON, 2000). These samples will be submitted in catalogs and batches with other samples from the larger project, and data validation will be performed on a catalog basis in accordance with procedures established and described in the QAPP (WESTON, 2000).

5.5 DATA ANALYSIS AND REPORTING

Data collection, statistical analysis, and reporting for this study are described in Section 8. The study will produce a final report that will include all data, analyses, and interpretations and will be prepared with specific reference to both the DQOs provided in Subsection 5.1 of this specific protocol for the frog reproduction and development study and Subsection 4.1 of the project QAPP (WESTON, 2000).

6. FIELD PROCEDURES

Field procedures require the predetermination of sampling statistics, collection of frogs from the target and reference areas, collection of water and sediment samples for the bioassays, and all procedures associated with documentation, packing, and shipping samples. Each of these procedures is discussed in greater detail below. Target and reference area(s) will be verified Northern Leopard frog habitats, and an effort will be made to select reference area(s) that are as similar as possible to the target reach in relevant biotic and abiotic factors (apart from the presence of PCBs or other significant non-background chemical contamination). Such factors include, but are not limited to:

- topography;
- extent of industrial and residential development and habitat fragmentation;
- substrate type;
- soil type;
- level of dissolved oxygen in water;
- alkalinity;
- hardness of water;
- temperature; and
- total organic carbon (soil and water).

As noted previously, Northern Leopard frogs typically emerge from hibernation in mid-April and early May and begin breeding soon after. Unseasonably warm weather may accelerate this process such that migration of gravid females could occur prior to the planned field collection period. However, unseasonably cold weather or dry conditions may postpone breeding for several weeks. Weather conditions prior to field collection will be closely monitored to determine when to initiate collection efforts.

In the final stage of mobilization, all equipment specified previously will be assembled and packed. If any items need to be purchased, they will be ordered well in advance to ensure that the schedule is not impacted by equipment needs.

6.1 SAMPLING DESIGN

An overview of the sampling program and the anticipated number of samples to be collected for this study are provided in Figure 2 and Tables 1a and 1b. Again, the objective of this study is to evaluate frog reproductive capacity and developmental fitness by assessing gravidity, number of

Figure 2 Overview of Specimen Sampling for Frog Reproduction and Development Study

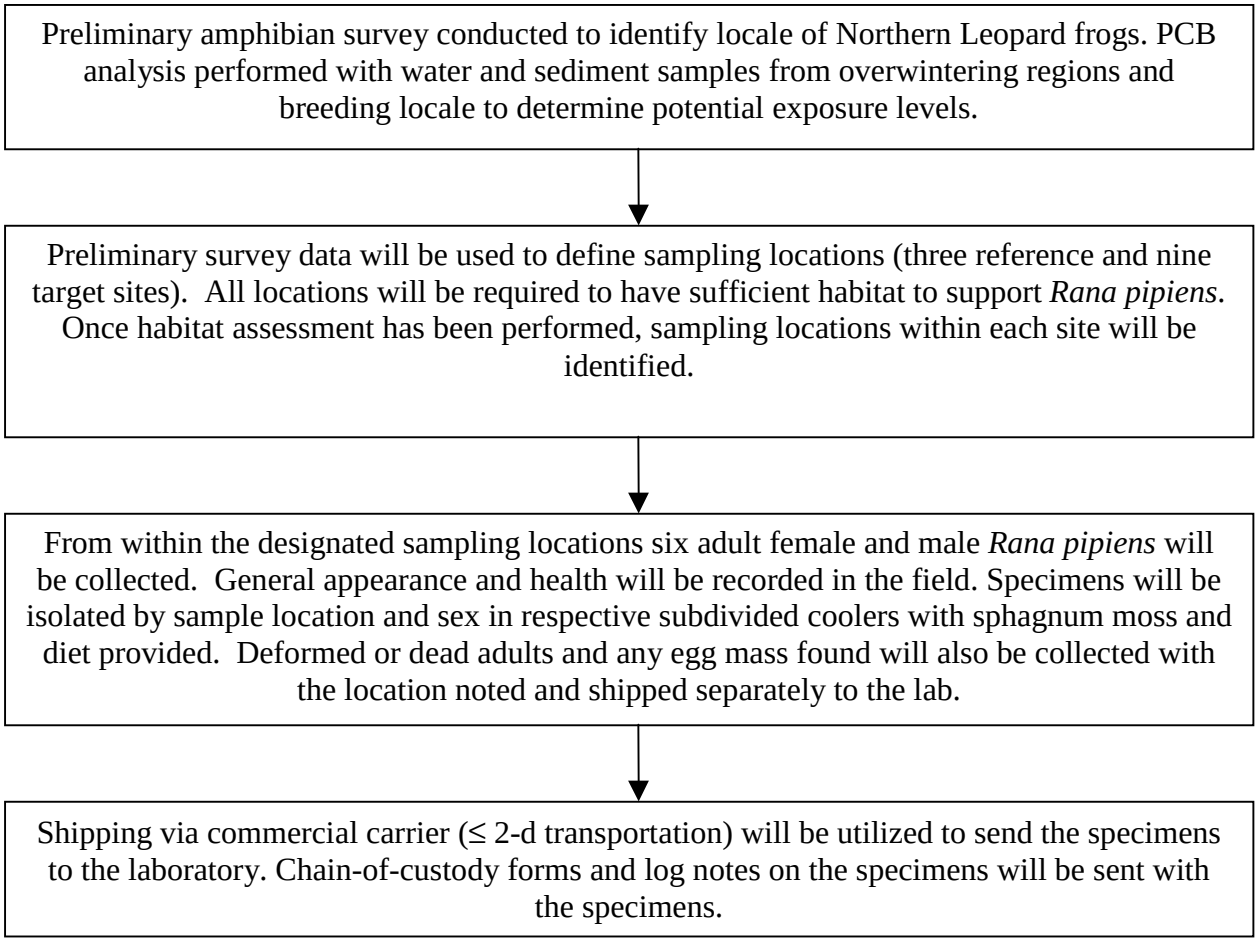


Table 1A and 1B

Proposed Sampling for Frog Reproduction and Development Study[1]

Sample	Sample Type	Grab Samples/ Sampling Location[2]	Estimated Sampling Locations/ Reference Site[3]	Estimated Sampling Locations/ Target Site	Total Samples/ Study
Water	Composite	4	3	9	12
Sediment	Composite	4	3	9	12

Sample	Sample Type	Samples/Sites	Estimated Sampling Locations/ Reference Site	Estimated Sampling Locations/ Target Site	Total Specimens or Samples/ Study
Adults (whole body)[4,5]	Individual	6 male/6 female	3	9	144
Ovary[4]	Composite	1	3	9	12
Testes[4]	Composite	1	3	9	12
Egg mass[4]	Composite	1	3	9	12
Larvae[4]	Composite	1	3	9	12

[1]One reference and one target site.

[2]Composite sample will be prepared from four grab samples collected.

[3]Anticipated 3 reference and 9 target site sampling locations.

[4]Based on availability. Does not include QA/QC specimens collected at rate of 1 male and female per sampling location.

[5]Based on individual specimen. Six adult males and six adult females will be collected per sample location within each site.

oocytes, oocyte viability, sperm viability and fertilization, mortality, developmental anomalies, growth, and maturation of embryos obtained from target area frogs as compared to results from frogs collected from paired reference sites. The study will evaluate data from sampling locations representative of a relatively uncontaminated site (referred to as a reference site) and sampling locations representative of the conditions of the target site, based on preliminary reconnaissance and a thorough evaluation of the habit and PCB concentrations. Sample locations such as those provided in Figure 3 will be considered in this study. Only regions containing suitable habitat will be used in this study. Statistical evaluation will be based on the adult frogs collected, resulting egg masses produced, and embryos produced from nine separate locations within the target area, and an additional three locations within the reference area designation. These locations will represent the sampling units for each site. In this design, three selected sampling locations within the reference area and nine selected sampling locations within the target area will be used for sample collection. These locations have been identified from habitat maps with potential PCB exposure information provided by WESTON (Figure 3). Six female and six male frogs will be collected from each sampling location. Approximately 36 frogs will be collected from within the reference area and 108 frogs from the target area. The three reference sampling locations will contain sediment PCB concentrations of <1.0 mg/Kg. Of the anticipated nine target site sampling locations, three will contain sediment PCB concentrations of <1 mg/Kg, 3 with sediment containing 1-30 mg/Kg, and 3 with sediment containing >30 mg/Kg. Additional QC specimens will be collected at each site as discussed in Subsection 5.4. Of the six frogs per sex collected per site, four frogs will be used for reproduction and development study, one for whole-body total PCB (tPCB) analysis, and one for ovary and oocyte, or testis tPCB analysis. This approach will provide a means of directly correlating a response within the sampling location to range of PCB concentrations (Steel and Torrie, 1980; Hicks, 1982; Thompson, 1992). This design will also allow statistical comparison between the reference and target areas.

A key variable with respect to ensuring adequate statistical power is the selection of appropriate sample sizes. For this study, sample size is defined as the total number of adult frogs evaluated from the target and the reference areas. A power analysis to determine minimal sample size requirements for the frog reproductive study was conducted to determine the total number of adult frogs needed per area, as well as the number of oocytes required to relate body PCB concentrations to the reproductive and developmental endpoints previously mentioned (Steel and Torrie, 1980; Hicks, 1982; Thompson, 1992). A summary of these statistical design analyses is provided in Tables 2 and 3.

Each endpoint has been evaluated to determine the sample sizes required (Tables 2 and 3). The number of organisms to be evaluated in development as recommended by power analysis is consistent with current organism loading practices used in the laboratory for each of the endpoints to be monitored (Fort et al., 1995; Fort and Stover, 1996a and 1996b, 1997a and 1997b). Based on this analysis, 6 adult female and 6 adult male frogs from each sample location will be required for the current study design and additional analytical analyses. Thus, at least six adult frogs of each sex will be collected per sampling location per site.

Table 2

Detectability, Sample Size, and Power Analysis of Respective Endpoints Associated with Reproduction and Development in *Rana pipiens* Using Hypothesis Testing[1]

Endpoint	Minimal Detectable Difference[2]	Sample Size (n/2)[3]	Power
Egg mass[4]	700	20	0.90
Egg necrosis[4,5]	20	20	0.90
Oocyte maturity[5,6]	25	10	0.90
Sperm count[4]	200,000	20	0.90
Sperm Dysmorphology[5]	5	20	0.90
Fertilization[6]	20	10	0.90
Teratogenesis[6]	20	20	0.90
Hatching Success[6]	20	20	0.90
Mortality[6]	20	15	0.90
Rate of Metamorphosis[6]	10	20	0.90
Gravidity[7]	30	15	0.90

[1]Analysis based on assumption that a t-test with normally distributed data and equal variances will be used to compare data sets.

[2]Minimal detectable difference based on anticipated standard deviation.

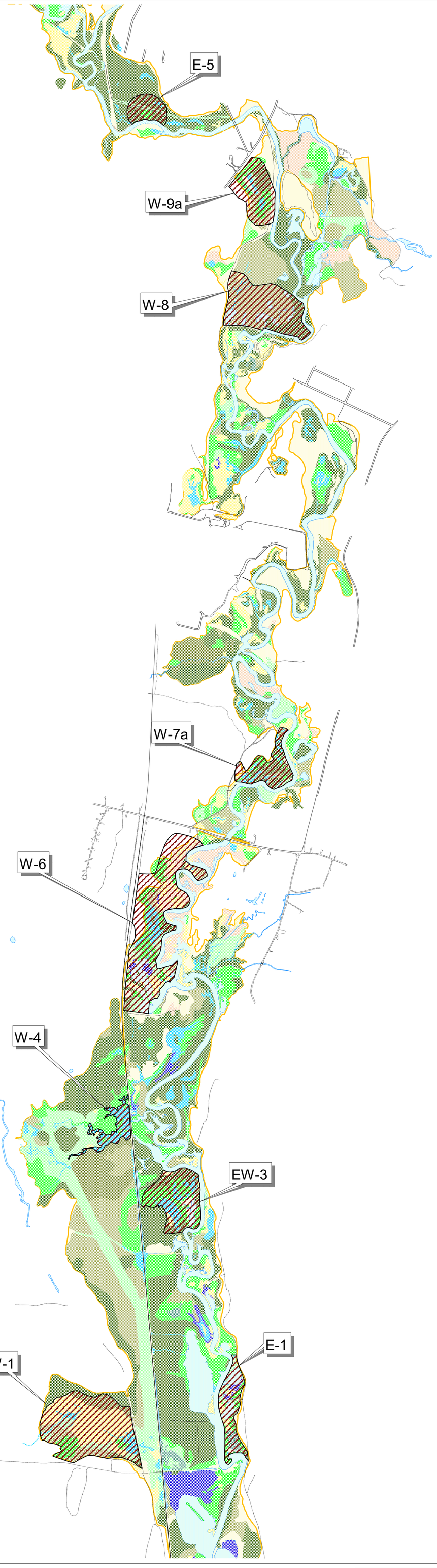
[3]Values reported as total n, but split into two equal replicates in order to evaluate variability.

[4]Expressed as total egg or sperm count, respectively.

[5]Expressed as a transformed proportion for statistical evaluation.

[6]Based on proportion of oocytes reaching advanced maturity relative to viable egg mass.

[7]Binary variable. Estimates based on assumption provided above.



LEGEND:

- Survey Locations
- Roads
- Hydrology
- 10-year Floodplain

Wetland Habitat

- WAI_PAB
- WAI_PABUB
- WAI_PEM
- WAI_PEMAB
- WAI_PFO
- WAI_PFOEM
- WAI_PFOSS
- WAI_PSS
- WAI_PSSEM
- WAI_PUB
- WAI_RIV_HLNE
- WAI_SAND
- WAI_WMEAD

N

Scale in Feet

1000 0 1000 2000 3000

SI Work Plan
Lower Housatonic River
Massachusetts

Appendix A-20
Figure 3
Leopard Frog
Survey Locations

| O:\gepitt\aprs\frog_locs.apr | layout - 1 | o:\gepitt\epsfiles\portrait24x36\in\frog_locs.eps | 4:54 PM, 2/2/2000 |

Table 3

Detectability, Sample Size, and Power Analysis of Respective Endpoints Associated with Reproduction and Development in *Rana pipiens* Using Simple Linear Regression Modeling[1]

Endpoint	Predicted Distribution (N or LN)[2]	Minimal Detectable Difference[3]	Sample Size (n)[4]	Power
Egg mass[5]	N	700	40	0.8
Egg necrosis[4,6]	LN	20	40	0.95
Oocyte maturity[5,7]	LN	25	20	0.95
Sperm count[4]	N	200,000	40	1.0
Sperm Dysmorphology[5]	LN	5	40	0.85
Fertilization[6]	LN	20	20	0.95
Teratogenesis[6]	LN	20	40	0.85
Hatching Success[6]	LN	20	40	0.85
Mortality[6]	LN	20	30	0.9
Rate of Metamorphosis[6]	N	10	40	1.0
Gravidity[8]	---	30	30	---

[1]Analysis based on normally distributed data or log normally distributed date based on anticipated data set. Paired data will be based on the reproductive effect regressed against either tissue PCB levels or sediment/soil levels.

[2]N = normal distribution; LN = log normal distribution

[3]Minimal detectable difference based on anticipated standard deviation.

[4]Values reported as total n.

[5]Expressed as total egg or sperm count, respectively.

[6]Expressed as a transformed proportion for statistical evaluation.

[7]Based on proportion of oocytes reaching advanced maturity relative to viable egg mass.

[8]Binary variable. Modeled as a Bernoulli variable.

The current sampling requirement of 144 adult frogs (72 male and 72 female), plus an additional 24 (12 male and 12 female) for QA/QC should provide a suitable cushion to allow for the following contingencies:

- all of the females collected may not be gravid;
- injury or death of frogs during transport;
- QA/QC measures.

Eggs will be collected from a minimum of 20 female frogs per site in order to allow subsequent statistical analyses. As a standard procedure, 40 eggs/embryos are typically taken from each egg mass (20 per replicate) when evaluating chemical effects on frog embryos (Fort and Stover, 1996a; Bantle et al., 1998). Thus, the sample size for each area will be 800 fertilized eggs, corresponding to 40 eggs collected from each of 20 females from each area. A larger sampling size from each egg mass will be used if practicable.

In summary, 54 female and 54 male frogs should be collected from the target area and 18 female and 18 male frogs from the reference areas, for a total of 144 frogs.

6.2 SAMPLING APPROACH

Following authorization, WESTON or its contractors will collect frogs from the target and reference areas over one or more three-day periods at the end of April or beginning of May, unless alerted earlier to the presence of migrating female Northern Leopard frogs. As previously indicated, nine sampling locations within the target area and three sampling locations within designated reference areas will be used to ensure systematic searching of the areas and, in the case of the target area, a broad distribution of PCB concentrations (including areas with elevated concentrations). As a requirement of this study, both sites to be used in this study must have sufficient habitat to support Northern Leopard frogs. The search for specimens will focus on areas known to be favored by Northern Leopard frogs, such as shallow areas on the edges of relatively deep standing waters that are sheltered by overhanging trees and brush. Optimal times to search for gravid females will be evenings in light rain. Because gravid females tend to go to areas beyond their typical daily habitat to lay eggs, roadsides and open areas adjoining favored aquatic habitats will also be searched. A key element in the search for females will be to listen for the calls of male Northern Leopard frogs, which tend to reside in the shallow regions of their aquatic environment. While females typically prefer deeper waters, they will come to the shoreline in response to male vocalization during the breeding season. Depending on the difficulty of collecting frogs, male vocalizations may be recorded and played back in an effort to lure female frogs.

The order in which the sampling locations in the target area are sampled will be flexible, yet systematic, with the objective of collecting frogs from all sampling locations. The goal in collecting frogs from multiple sampling locations is to ensure that target frogs represent a range of exposures, thus supporting the subsequent regression analysis for potential dose-response relationships. The anticipated sampling locations range in tPCB concentrations of <1 mg/Kg to > 30 mg/Kg sediment.

Ideally, all frogs will be collected within as short a time frame as possible in order to reduce stress to the frogs that are caught early in the collection phase, and to minimize the possibility of premature release of eggs while in captivity. While females can be held in captivity for a period of up to four days, it is not advisable to extend this time frame.

Since shipment of specimens to the lab will be staggered, the sampling team will continue with field collection efforts (if necessary) while the laboratory proceeds with evaluation of gravidity, sperm viability, and *in vitro* fertilization. The laboratory will then contact the project manager and advise as to the number of gravid females and the egg masses collected.

6.3 METHODS OF FROG COLLECTION

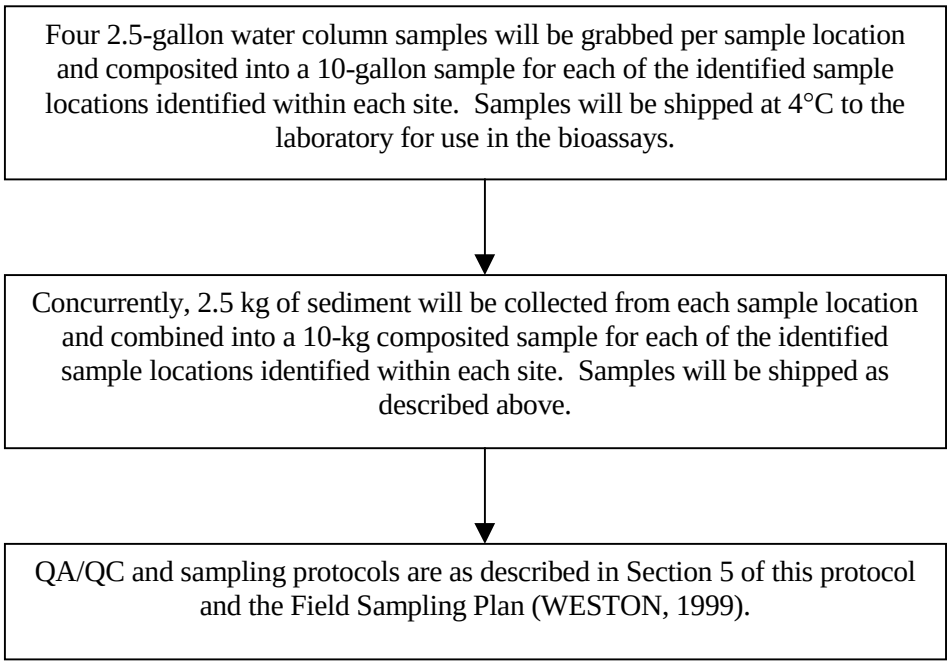
The sampling team will capture frogs using several techniques, including drift fences and pitfall traps. The frogs will be delivered to the processing area in separate containers labeled with location, sex, and date of collection. Each frog will then be placed into its own compartment into a six-gallon Styrofoam cooler lined with moist sphagnum moss for shipping to the laboratory. Perforated lids will be securely affixed to the coolers with duct tape to prevent escape and the coolers will be labeled.

Prior to delivery to the laboratory, the coolers containing the frogs will be kept in air-conditioned rooms with temperatures ranging from 10 to 15 °C. The frogs will be fed a daily diet of live crickets and water. The water used for maintaining the frogs during transportation will be collected from the locations in which the frogs are collected. Additionally, the sphagnum moss will be changed as needed and kept moist. Representatives of the laboratory will be present in the field during the collection to assist in making decisions on sampling if additional sampling locations are needed or the number of frogs requested cannot be achieved for any reason. The specimens should be shipped by a priority overnight (24-h) service which offers a ground service, or by a commercial carrier offering air transit of live specimens. Packaging of the specimens with moss, food (excess live crickets), and water will be adequate to ensure successful arrival. Laboratory representatives will provide instructions and assist in packaging for shipment.

6.4 COLLECTION OF SEDIMENT AND WATER SAMPLES

Sediment and water column samples will be collected at each of the sampling locations within both areas. At each location, four grab samples of sediment from inundated areas will be collected by WESTON in accordance with the methods specified in the Field Sampling Plan (WESTON, 1999). The four grab samples will then be composited into one sample for each of the five locations within the test areas. A similar approach will be used to collect water column samples. The water and sediment samples will be used in the embryo-larval and metamorphosis bioassay studies. Thus, for both areas composited samples of sediment and water will be collected. Duplicate samples will be collected for analytical analysis as necessary. Sample size and volume requirements are further defined in Figure 4.

Figure 4 Overview of Water/Sediment Sampling for Frog Reproduction and Development Study



6.4.1 Sample Documentation and Labeling

Field notes will be recorded in a logbook in accordance with the Field Sampling Plan (WESTON, 1999). Each frog will be identified in the logbook using a unique 16-digit sample identification number. Sample nomenclature methodology is specifically described in the QAPP (WESTON, 2000). The label coding system will not be explained to biological laboratory personnel to ensure that they remain blind as to the origin of a given animal. Global positioning system (GPS) data will be collected so that the geographical coordinates of the sampling locations are identified. Specific documentation of habitat at each location will be provided using digitally collected images and written field observations. In addition, analytical samples will be recorded in a logbook using labeling consistent with that specified in the QAPP (WESTON, 2000).

6.4.2 Sample Preservation and Shipping

Live female and male frogs will be transported in Styrofoam coolers lined with moist sphagnum moss and an excess of live crickets in accordance with the standard operating procedure, included as Attachment 2. The coolers will be labeled and sealed with perforated sides and lids. Two signed and dated custody seals will be placed on two sides of the cooler to ensure the specimens are not tampered with during shipment.

A member of the project team will transport them to the biological laboratory. Following *in vitro* fertilization, all females that had been gravid will be euthanized, frozen, and packaged for shipment to the laboratory for whole-body PCB and congener-specific analysis. Additionally, residual portions of the egg masses from which embryo groups are obtained for the bioassay will be prepared for PCB analysis as well.

Frogs, residual egg masses, and water and sediment samples will be shipped to the analytical laboratory in dry, clean, perforated sample containers (Styrofoam ice chests) that are labeled in accordance with ERT/REAC SOP #2002 (EPA, 1994). The Styrofoam ice chests will be placed into polyethylene bags (one sample per bag), which will then be sealed and placed into U.S. Department of Transportation (DOT) approved fiberboard boxes lined with plastic sheeting, bubble wrap, and sufficient vermiculite to absorb any potentially leaking material. All outer packing materials will also be perforated to allow gas exchange. One chain-of-custody form (in triplicate) will be placed into a watertight bag and taped to the inside of the lid of each cooler. Specimens to be analyzed for analytical parameters, including PCBs, will be packaged as described above following snap freezing in liquid nitrogen and inclusion of dry ice. In accordance with DOT regulations, the lids will be slightly perforated to allow for release of carbon dioxide gas as the dry ice melts. In this case, the Styrofoam coolers will then be placed into cardboard boxes that have also been perforated to allow gas release. The boxes will be securely taped and appropriately labeled according to the courier's protocols. International Civil Aviation Organization regulations stipulate that any volume of dry ice is a Class 9 Miscellaneous Hazardous Good (IATA, 1993). In order to provide a means by which the entire path of a sample can be traced, a chain-of-custody record will be maintained from the time a sample is collected through analysis or hatching, as specified in the QAPP (WESTON, 2000).

7. LABORATORY METHODS

It is anticipated that *in vitro* fertilization and monitoring of developing embryos will be performed by THE STOVER GROUP in Stillwater, Oklahoma. Tissue, surface water, and sediment chemical analyses will be conducted by laboratories under contract to WESTON or USFWS. The laboratory procedures are described below.

7.1 IN VITRO FERTILIZATION, EARLY DEVELOPMENTAL MONITORING, AND EVALUATION OF METAMORPHOSIS

After allowing female frogs to acclimate for 24 hours following receipt by THE STOVER GROUP, female frogs will be induced to ovulate and the egg masses will be stripped and fertilized *in vitro.* It is only upon stripping the eggs that a definitive determination of gravidity can be made. The number and identity of gravid versus non-gravid females will be recorded for a subsequent analysis of the differences in percent gravidity in the target and reference areas. In addition, the number of eggs produced by each female will be specifically counted, rather than using volumetric techniques.

Superovulation will be induced by injection of 100 IU (international standard units) of luteinizing hormone releasing hormone (LHRH) or pituitary extract in Amphibian Ringers solution using tuberculin syringes with ½-inch-long 26 gauge needles, in accordance with standard methods cited in Bantle et al. (1998), Fort and Stover (1995 and 1996a), and ASTM (1998). The females will be carefully immobilized prior to injection by holding them underneath an aquarium net. The solution will be injected into the dorsal lymph sac, which is bound by the lateral line that runs along the side of the frog and appears as stitching on the skin. Care will be taken to inject the frog subcutaneously by wrinkling the skin.

Approximately 24 to 36 hours after the females have been superovulated, male frogs from each location will be sacrificed and the testes will be removed. Testes from each male will be gently mashed together in a Petri dish containing 9 mL of dechlorinated tap water and 1 mL of Ringers solution that has been left standing for 48 hours. A 1-mL aliquot of the resultant sperm solution will be checked for sperm count, motility, and dysmorphology under a microscope (Fort et al., 1999c).

The concentrated sperm solution from males collected from each sample location within a site will then be divided into Petri dishes. Sperm solutions prepared from the combined testes of these males will be used to fertilize eggs from each respective location within the sample site. This approach will be important to determine effects within each site due to differing levels of PCBs within the site itself. Care will be taken to ensure that target area sperm fertilize target area eggs and that reference area sperm fertilize reference area eggs. Egg masses will be squeezed out of the females into the concentrated sperm solution by firmly grasping the frog, extending the legs back and close to each other, and applying gentle pressure on the abdomen. After standing in the sperm solution for 30 minutes, the eggs will be flushed with dechlorinated tap water and will be loosely separated to ensure adequate oxygen viability. After two hours the eggs will be checked for fertility and quality. Grey crescents that form on the opposite side of

sperm entry immediately above the equator, dividing the animal pole from the vegetal pole, will be the first sign of fertilization. Normal cleavage (indicating successful fertilization) will be determined based upon the general technique of Nieuwkoop and Faber (1994) and Dickerson (1969), in which the embryos are characterized by vegetal hemisphere cells that are creamy white in color and are always larger than the pigmented animal hemisphere cells; the pigmentation line should be at the equator of the blastula. However, because cellular development can be observed in the Northern Leopard frog, early embryogenesis will also be monitored. Egg masses characterized by significant infertility will be recorded relative to the female of origin, so that differences in fertility rates between target and reference area females can be subsequently evaluated.

Groups of 40 embryos will be separated from each fertilized egg mass (each female within each sampling location), keeping the jelly coat intact, and placed in each of two replicate dishes containing 20 embryos each. Embryos that are not perfectly round, blastula with abnormal pigmentation, or gastrula that have bleeding yolks will be excluded from the remainder of the bioassay, although such abnormalities will be recorded relative to the female of origin so that differences in viability can be subsequently evaluated.

Each group of 20 embryos from each female will be placed in specimen jars for monitoring through the seven-day post-hatch observation period in each of two replicates per female. Because we cannot assume that the primary source of PCBs to the developing embryos is via maternal transfer during egg formation, it will be necessary to add target site or reference site water and sediments to the test vessels. Furthermore, based on its composition, it is likely that the jelly coat surrounding the embryo will not completely prevent passage of PCBs to the developing embryos. Thus, testing of pre-hatch embryos will be performed in 9-oz specimen bottles equipped with a glass tube/Teflon® mesh insert as the exposure chamber. Thirty-five g of sediment (wet weight) will be placed in the bottom of the specimen jar, the exposure insert added, and the jar filled with 140 mL of dilution water (FETAX solution, reference site water, or test site water). This represents a 1:4 dilution of sediment to dilution water. Early embryos will be placed on the Teflon® mesh insert that will rest over the top of the sediment in the sediment/water interface region. This represents a fairly realistic exposure scenario in the laboratory (Fort and Stover, 1997b). Vessels containing embryos from reference and target areas will be discretely labeled and then randomly distributed within an incubator. Laboratory personnel will be blind to the origin of the embryos in each beaker. Since embryos from at least 20 females will be used per site, each site, including designated reference sites, will have a total of 40 vessels containing 20 embryos, for a total of 800 embryos evaluated per site (1,600 embryos for the study). In addition, a separate set of 40 embryos collected from 20 females (for a total of 800 embryos) obtained from a commercial source (Carolina Biological Supply, Burlington, NC) and confirmed to be devoid of PCB or dioxin contamination will be placed in each of two replicates (20 per replicate) containing clean sand and laboratory culture water. These uncontaminated embryos will be designated as laboratory controls. The purpose of including these controls in the study will be to ensure that the laboratory culture conditions are adequate to culture *R. pipiens* successfully.

In order to determine what extent of developmental effects induced in target site embryos/larvae are due to maternal PCB transfer, a separate set of experiments will be performed concurrently. In these studies, an additional set of 120 embryos from reference site females will be exposed to

water and sediment from three selected locations within each target site containing representative levels of PCBs, and developmental effects (hatching and metamorphosis) monitored as described in this section. Thus, 40 embryos will be collected from each of three randomly selected reference site females and exposed in sets of 40 (20 per replicate) to the three target site samples. The reverse experiment will also be conducted to help confirm these findings by exposing target site embryos (120 total, 40 per female) from adults collected from a representatively contaminated test site location to reference site water and sediment collected from three selected locations as described above.

A pH of 7.5 in the culture solution will be maintained at all times, as well as a temperature of 24 ±1° C. Dissolved oxygen (DO) will be monitored on a daily basis and will not be allowed to drop below 6.0 mg/L. Prior to hatching, the test chambers will be maintained on a 12-hour day/12-hour night cycle, and test solutions and sediment will be changed every 4 days using a Pasteur pipette with an enlarged fire-polished orifice. Dead embryos will be removed, counted, and recorded every 24 hours.

Time to hatch will be monitored. The embryos are expected to hatch within seven to ten days. Juvenile frogs will not be fed during the seven-day pre-hatch observation period, since the yolk sac that remains following hatching provides sufficient nourishment for the first seven to eight days. If overall mortality in the laboratory culture water controls exceeds 10 percent in the embryo-larval evaluation, results will be considered conditionally acceptable pending further review. In the case that the data is considered unacceptable, evaluation of study results would be limited to the gravidity, sperm viability, and fertilization rates.

Morphological evaluation of the embryos and juveniles will be conducted either at the end of the observation period or upon the death of the juvenile or embryo. The following specific abnormalities will be recorded:

- gut;
- hemorrhaging;
- axial malformations;
- blistering and edema;
- head, face and eye;
- heart; and
- brain.

Following hatching (longer-term evaluation), larvae will be fed Salmon Starter fish food, which has been successfully used to culture *Rana* tadpoles in the laboratory (Carolina Biological Supply Company, 1993). Post-hatch larvae will be cultured as described above in 5-L glass aquaria with underlying sediment. Five hundred g of sediment or blasting sand will be placed on the bottom of the aquaria and filled with 3 L of site water, reference site water, or FETAX solution. Mortality, limb development, and other morphological markers of metamorphosis (skin maturation and tail resorption) will be morphologically evaluated through this process. We anticipate that metamorphosis and limb development (hind limbs) will be complete within 3 months of hatch. Renewal of test solutions and sediments during this longer-term development phase will be performed every seven days (weekly).

During this phase, digital images of the developing larvae will be recorded during the renewal process both for observation of the developing limbs and resorbing tail, as well as photographic documentation of the results, in accordance with the methods for Fort and Stover (1996b, 1997a; Fort et al. 1999a).

7.2 ANALYTICAL ANALYSES

It is anticipated that the Texas A&M University Geotechnical and Environmental Research Group (GERG) will conduct PCB and lipid analyses on adult frogs, residual egg samples and testes, sediments, and water samples within ten days of receipt. A summary of the sample matrix and analytical requirements are identified in Table 4 and Figure 5. Other analyses will include, but are not limited to, Aroclor-specific PCBs, congener homologs, dioxins, dibenzofurans, and organochlorine pesticides. As discussed in the introduction to this protocol, there is a need to perform a PCB and congener-specific analysis on the male frogs, even if the majority of PCBs found in offspring may be derived from the female. This analysis will complement the male reproductive assessment. Tissues will be analyzed from at least one female and male frog per sampling location per site. Each composite and random grab sample of water and sediment will be analyzed for the parameters indicated above.

8. DATA ANALYSIS

8.1 DATA COLLECTION

For embryo-larval and limb development, mortality and malformation rates will be determined for the test and reference site using a dissecting microscope (Fort et al., 1995, 1996b and 1997a; ASTM, 1998). For monitoring the rate and extent of tail resorption (metamorphosis), video images will be captured using a Sony CCD-iris high-resolution color digital video camera. A Pentium 233 MHz computer with image processing software and a FlashPoint 128 (Integral Technologies, Inc., Indianapolis, IN) video frame grabber will be used to digitize tail lengths throughout the study. A ruler videotaped with the larvae will be used to correct for image distortion and calibrate the length-measuring program to ensure accurate measurements of the larvae. Tail lengths will be measured using Sigma Scan (SPSS, Corte Madera, CA).

Overall, data evaluation will entail:

- preparation of a database that includes chemical concentrations, gravidity, number of eggs produced, number of necrotic eggs, portion of eggs in each respective stage of development, sperm count, sperm morphology/dysmorphology, egg viability, fertilization rate, time to hatch, hatching success, incidences of mortality and abnormality for target and reference area embryos and/or frogs, rate of limb mal-development, rate and extent of the tail resorption, and morphological observations during metamorphosis;

Table 4

Proposed Analytical Analysis for Frog Reproduction and Development Study Reference Site[1]

Sample	Sample Type	Grabs or Individual Samples/ Location	Anticipated Sample Locations/ Study	Analytical Analyses				
				tPCBs	Congeners[2]	Dioxin[3]	Dibenzo-furan[3]	Organo-chlorine Pesticides[3]
Water	Composite	4/location	12	12	12	12	12	12
Sediment	Composite	4/location	12	12	12	12	12	12
Adult whole body[4,5]	---	1male/ 1 female	24	24	24	24	24	24
Ovary[4]	---	1	12	12	12	4	4	4
Testes[4]	---	1	12	12	12	4	4	4
Egg mass[4]	---	1	12	12	12	4	4	4
Larvae[4]	---	1	12	12	12	4	4	4

[1] One reference and one target area with nine sampling locations within the target area and three sampling locations within the reference area.

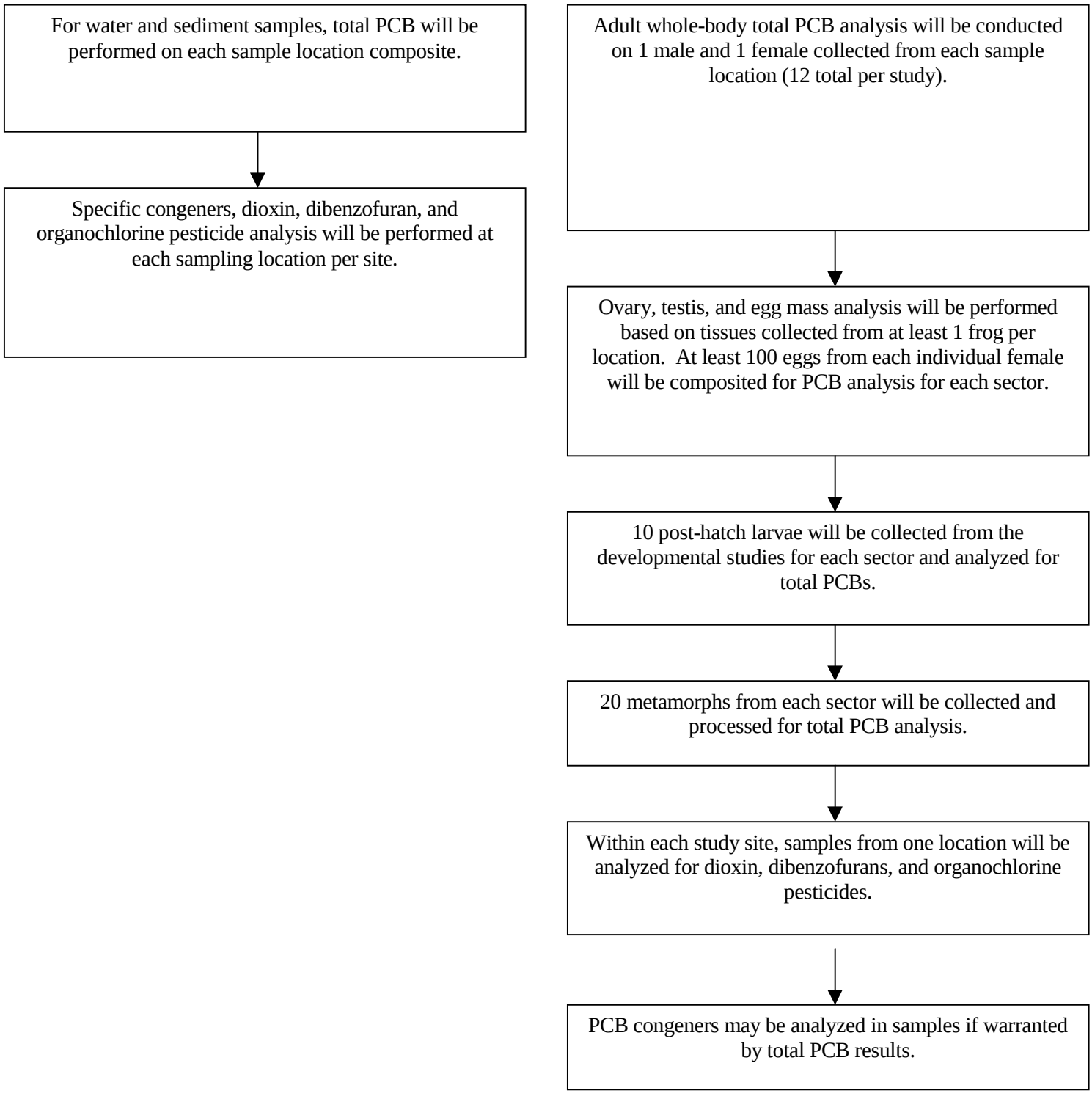
[2] Collected but only analyzed if tPCB analyses results warrant.

[3] Two randomly selected composite samples per site.

[4] Based on availability.

[5] Based on individual specimen.

Figure 5 Overview of Analytical Analysis for Frog Reproduction and Development Study



- statistical analysis of the data comparing endpoints between target and reference area frogs;
- statistical analyses relating endpoints to PCB concentrations (congener-specific, as practicable) in female frogs and residual eggs; and
- documentation of study results and supporting QA/QC data.

As an initial step in the evaluation, THE STOVER GROUP will develop a database for the target and reference areas. This database will be developed in spreadsheet format and sorted by area and by embryo groups derived from each female. The database will include the following information:

- identification number of the maternal and paternal frogs;
- gravidity;
- egg mass;
- necrosis;
- portion in respective oocyte stages;
- number of eggs;
- sperm counts;
- sperm morphology;
- fertilization rate;
- time to hatch;
- mortality incidence;
- abnormality incidence by type of deformity and total number;
- limb mal-development;
- rate of and abnormalities occurring during metamorphosis; and
- PCB concentrations in the associated fertilized egg mass and adult tissues.

Because the concentration of PCBs is often correlated with the lipid content of a tissue sample, tissue PCB data will be normalized to the lipid concentrations before statistical analyses are performed. THE STOVER GROUP will verify the accuracy of the data entry prior to statistical evaluation.

8.2 STATISTICAL ANALYSIS

8.2.1 Hypothesis Testing

Statistically significant differences in outcomes between target and reference areas will be evaluated based on a homoscedastic t-test (two-sample comparison of the means) providing the data sets are found to be normally distributed. If the homoscedasticity assumption is violated, a heteroscedastic t-test will be used to compare the data sets. If the data sets from either site do not meet normality assumptions, transformation of the data (arc sine) will be used to normalize the results. If no transformation proves useful in normalizing the data sets, non-parametric tests, such as the Mann-Whitney or Wilcoxen tests, will be used. In addition to evaluating the means of the two data sets, we will also use the Kolmogorov-Smirnov (KS) two-sample test to compare

the overall distribution of the variability in the two sites. Abnormalities will be evaluated individually, as well as cumulatively.

8.2.2 Regression Analysis

In an effort to establish a concentration-response relationship between PCB concentration and biological effects observed, adult female or male tissue and egg mass PCB concentrations and water and sediment PCB concentrations will be regressed separately against the biological endpoints. The proportions themselves can be thought of as comprising a continuous distribution in which each proportion represents a numerical data point that is matched in the regression to the PCB concentration of the group. Regression analysis will be used to determine if a relationship exists between rank (extent of biological efforts) and deviation from monotonicity. In this analysis, monotonicity will be measured by the difference in the slopes of the ranks. Although this sample size may fluctuate slightly, the approach will remain statistically powerful (Table 3) and should determine if a correlation exists between PCB levels and biological effects observed in the laboratory. As with the hypothesis test, data sets will be evaluated for normality or, in the event that the data is not normally distributed, log normality. We anticipate that some of the data will require transformation prior to simple linear regression analysis. Because records of habitat will be collected during sampling, we will also be able to determine if biological responses may be attributed to habitat or lack of habitat. Following analysis, an exposure-response model will then be fit to the data, if possible. As indicated above, standard curve fitting approaches for dose-response data will be used and may include log or arc sine transformations. Because gravidity is a binary variable, it will be evaluated as a Bernoulli variable rather than using the aforementioned methods. If the data analysis indicates statistically significant differences between target and reference areas that do not appear to be consistent with a PCB exposure-response relationship, the archived frog tissue and egg samples may be analyzed for selected other chemical constituents, and the results evaluated in order to help in understanding the differences.

The last step will be to document the methods, results, and conclusions in both text and tabular form. The methods section will state any assumptions made and any changes made to this protocol. The results will be explained in terms of the data analyses and statistical procedures described above. Uncertainties associated with the analysis, as well as their potential impacts on the results, will be incorporated into these discussions.

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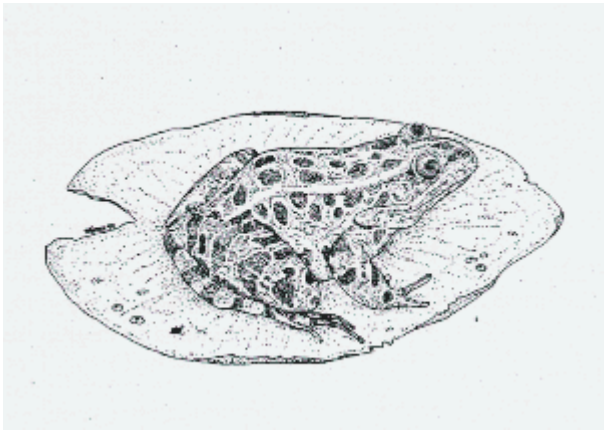
ATTACHMENT 1

HOME RANGE OF *RANA PIPIENS*

Representative Species - Canadian Great Lakes Frogs and Toads

Leopard Frog - *Rana Pipiens*



Distribution

- Found throughout Ontario but more abundant in southern and central areas.

Home Range

- Adults maintain small home ranges (up to 500 m^2) in fields or open forest during the summer. Where terrestrial habitats are quite dry, home ranges include some shoreline.
- A minimum of 4 ha of terrestrial habitat is recommended for the vicinity of breeding sites, however, individual adults may move several kilometres away.
- Most recently metamorphosed froglets stay within 20 m of shoreline although some froglets begin dispersal before metamorphosis is entirely complete.

Food

- Larvae eat algae, phytoplankton, periphyton and detritus.
- Adults eat mainly invertebrates but will also take tadpoles or very small froglets.

Reproduction

- Successful breeding sites are permanent ponds, marshes, or pools or backwaters of streams.
- Breeding occurs from mid-March to mid-May in southern Ontario, and a few weeks later further north.
- Metamorphosis occurs in 2-3 months.
- Tadpoles require minimum oxygen concentrations of 3 ppm.

Cover/Habitat

- Relative to bullfrogs and green frogs, leopard frogs use open fields more and prefer denser terrestrial vegetation.
- In aquatic habitats, submerged vegetation, detritus and soft mud are used for cover.

Lookout/Sunning

- Eggs and tadpoles require warm (prefer 18 - 28 C), shallow, sunny areas.
- Froglets require muddy shorelines, lily pads, rocks, logs or beaver dams with clear access to deeper water.
- Adults prefer unmowed fields (15 - 30 cm high, no more than 1 m high vegetation) or open forest in the vicinity of shallow open marshes.

Connectivity/Corridors

- Corridors may be required among breeding, hibernation and summeringhabitats, within 2 km. These may be either aquatic (streams or rivers) or terrestrial (field or forest, usually not cropland except

during periods of irrigation).

Hibernation

- Hibernate in deep or running water that will not freeze solid or become anoxic. Are found hibernating on muddy substrate or under rocks, sunken logs, leaf litter or vegetation.
- Oxygen levels at one known successful hibernation site were 7 ppm.
- Tadpoles metamorphose in the year of hatching.

Hydrology

- Permanent wetlands with fishless areas or near fishless (temporary) wetlands.
- Breeding requires sufficient water for metamorphosis to be completed (mid-late August).

Soils/Substrate

- In water prefer muddy bottom.
- On land prefer moist soil, leaf litter or moss.

Design Criteria

Vegetation

- Prefer egg-laying sites with emergent vegetation on about 2/3 of edge and submergent vegetation in 1/2 of surface area in May.

Structures

- Rocks, logs, floating vegetation or dams to sun on, with access to deep water.
- Submerged vegetation, logs or rocks to hide in.

Soils, Slope, & Substrate

- Prefer wetlands with gradual slope at edge.

Hydrology

- Hibernate in streams with minimum depth 90 cm, moderate mid-depth water velocity, minimal sedimentation, and rocks with average diameter of 20 cm.

Critical Periods

- Breed April-June, metamorphose July-September

Other Considerations

- Froglets are used as bait for fishing.
- Has declined in much of its western range and apparently in northern Ontario.
- Tadpoles and froglets are vulnerable to predation by large Bullfrogs and fish.

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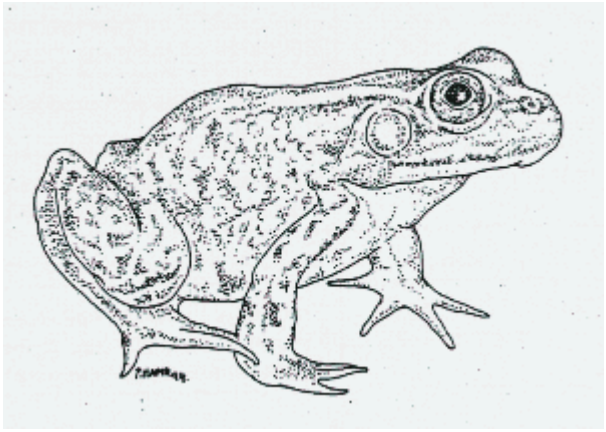
Green Frog - *Rana clamitans*



Distribution
- Widespread and abundant in Ontario.

Home Range
- Shoreline species, using the area closer to shore than bullfrogs.

Food
- Adults are carnivorous, stalking spiders, insects, snails, slugs, and aquatic crustaceans like crayfish.
- Tadpoles feed continuously on phytoplankton.

Reproduction
- Breed from June-August.

Cover/Habitat
- Edges of slow streams and rivers, ponds, lakes, reservoirs, marshes, swamps, and bogs.

Hibernation
- Tadpoles that hatch late - in small crevices between rocks, fallen logs, and branches.
- Adults - on land in leaf litter and soil pockets.

Hydrology
- Prefer a greater water depth immediately offshore and cooler water than bullfrogs.

Soils/Substrate
- In water - muddy bottom.
- On land - loose soil.

Design Criteria

Vegetation

- Prefer egg-laying sites with lots of underwater plants such as *Elodea* which make up mats on which the eggs rest.
- In water - prefer greater submergent vegetation canopy, with greater stem diameters than bullfrog.
- On land - prefer less dense vegetation than leopard frog.

Structures

- Rocks and logs to sit on. Rocks away from the shoreline are safer for froglets.

Soils, Slope, & Substrate

- Prefer a muddy bottom.
- >On land - loose soil.

Hydrology

- Slow streams and rivers, ponds, lakes, reservoirs, marshes, swamps, and bogs. Prefer greater water depth immediately offshore and cooler water than bullfrogs.

Critical Periods

- Breed June-August.

Other Considerations

- The leopard frog has similar habitat requirements, but breeds earlier.

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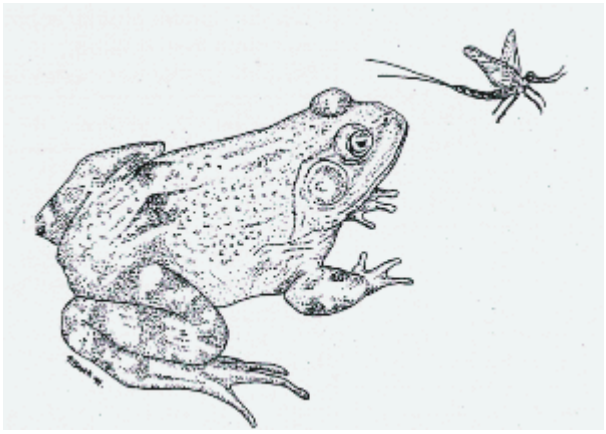
Bullfrog - *Rana catesbeiana*



Distribution

- Southern Ontario as far north as Nipissing District.

Home Range

- Adult males aggressively defend territories. Boundaries are not stationary, but males defend 3-25 m of shoreline.

Food

- Tadpoles - aquatic plant material, invertebrates, and dead fish or tadpoles.
- Adults - voracious 'sit and wait' predators - fish, mice, moles, bats, snakes, ducklings, birds, and other bullfrogs.

Reproduction

- Breed May-July.

Cover/Habitat

- Vegetated shoals, sluggish river backwaters and oxbows, farm ponds, reservoirs, marshes, and still waters with tules, dead trees, snags, and twisted roots.

Hibernation

- Bury themselves in surface mud and construct protective pits or cave-like holes underwater.
- Adults may disappear before frost begins. Bullfrogs are last ranids to emerge in spring.

Hydrology

- Permanent water bodies, and prefer warm, still, shallow waters.

Soils/Substrate

- Muddy bottom to water body.

Design Criteria

Vegetation

- May be dense - pickerel weed, lily pads, cattails, sedges, berry vines, and willows.

Structures

- Still waters with tules, dead trees, snags, and twisted roots.

Soils, Slope, & Substrate

- Muddy bottom to shallow water body with gently sloping sides.

Hydrology

- Permanent water bodies - prefer warm, still, shallow waters.
- In potentially hot water bodies, bullfrogs must find cooler areas to keep eggs from dying.

Critical Periods

- Breed May-July.

Other Considerations

- Abundant in habitats modified by humans.

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GLIMR

Date: September 5, 1995 by: Scott Monds

ATTACHMENT 2

SAMPLE SHIPPING PROCEDURE

FINAL

ATTACHMENT 2
SAMPLE SHIPPING PROCEDURE
Rana pipiens REPRODUCTION AND DEVELOPMENT STUDY
Effective 1/19/2000
Revision No. 0

1. INTRODUCTION

1.1. ROY F. WESTON, INC. has contracted THE STOVER GROUP to perform a *Rana pipiens* Reproduction and Development Study. Live biological samples and environmental water and sediment samples will be collected in the field and shipped from the WESTON Pittsfield, MA Office to THE STOVER GROUP in Stillwater, OK.

2. APPARATUS

2.1. 1-gallon amber glass containers (100 count) with Teflon® caps

2.2. 1-liter wide-mouth amber glass containers (100 count) with Teflon® caps

2.3. Wide-mouth glass specimen jars

2.4. Cardboard boxes for shipping glass bottles

2.5. Styrofoam coolers encased in cardboard boxes (20 count)

2.6. Ice chests

2.7. Sphagnum moss

2.8. Live crickets

2.9. Packing tape

3. SHIPPING ADDRESSES

3.1. THE STOVER GROUP, Attention R&D Laboratory, 5302 W. Sixth St., Stillwater, OK 74074. Telephone number is 405-743-1435, Fax number is 405-743-1489, and e-mail address is rlrogers@stovergroup.com.

3.2. ROY F. WESTON, INC., Pittsfield Field Office, Attention Scott Campbell, 10 Lyman St., Pittsfield, MA 01201. Telephone number is 413-442-4224 and Fax number is 413-442-4447.

4. WATER/SEDIMENT ENVIRONMENTAL SAMPLE SHIPPING PROCEDURE

4.1. Randomly pull 4 – 2.5-gallon water column samples from each sampling location.

4.2. Composite the 4 water samples into a single, homogenous, 10-gallon sample.

4.3. Transfer the composited 10-gallon water sample to 1-gallon glass amber containers and cap with Teflon® caps.

4.4. Concurrent to the water sampling, randomly take 4 – 2.5-kilogram sediment samples from each of the sampling location.

4.5. Composite the 4 sediment samples into a single, homogenous, 10-kilogram sample.

4.6. Transfer the composited 10-kilogram sediment sample to 1-liter, wide-mouth, glass amber containers with Teflon® lids.

4.7. Label all sample containers appropriately with the sample date, sample description, site and location designation, WESTON sample number, and container number (i.e. 1 of 10, 2 of 10, etc.).

4.8. Individually wrap each glass container with bubble wrap or equivalent packaging material to prevent breakage.

4.9. Place wrapped containers in ice chests.

4.10. Add blue ice or zip locked bags of ice to maintain a temperature of 4°C during shipping.

4.11. Ship ice chests to THE STOVER GROUP.

5. ADULT *Rana pipiens* SAMPLE SHIPPING PROCEDURE

5.1. Adult *Rana pipiens* will be collected live from each sample location.

5.2. During a collection period, store frogs in cardboard boxes lined with damped sphagnum moss. Keep specimen separated by sex and location.

5.3. Feed frogs live crickets daily (2-4 crickets per frog).

5.4. Live specimen holding times for a collection period should not exceed 48-hours before frogs are packaged for shipment.

FINAL

5.5. Shipping containers for the live biological samples are Styrofoam coolers with each cooler packaged inside its own cardboard box. Each cooler/box set will need approximately 8 – ½-inch holes drilled into the top and sides for ventilation.

5.6. Line each Styrofoam cooler with damped sphagnum moss and add live frogs separated by sample location.

5.7. A chain of custody will be attached to each cooler, documenting the sample date, sample description, sample site and location, WESTON sample number, sampler's signature, and packager's signature. Place the chain of custody in a zip lock baggy to prevent damage and tape to Styrofoam cooler inside the cardboard box.

5.8. Clearly label the outside of each cooler/box as "LIVE ANIMALS".

5.9. Ship cooler/boxes by express carrier, with a no longer than 2-day delivery time, to THE STOVER GROUP.

5.10. Any miscellaneous live biological aqueous samples, other than adult frogs (i.e. larvae, egg masses, etc.), should be collected in wide-mouth glass specimen jars, bubble wrapped and shipped to THE STOVER GROUP using an overnight express carrier. Do not lower sample temperatures for shipping.

5.11. Any miscellaneous dead biological samples should be shipped on dry ice in ice chests to THE STOVER GROUP using an overnight express carrier.

APPENDIX A.20

FIELD SAMPLING AND ANALYSIS PLAN FOR FISH COLLECTION AND PROCESSING

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APPENDIX A.20

FIELD SAMPLING AND ANALYSIS PLAN FOR FISH COLLECTION AND PROCESSING

1. INTRODUCTION

1.1 BACKGROUND

Fish are sensitive to both long-term and short-term changes in habitat, sediment, and water quality. While fish are mobile, they frequently spend most of their lives in a single location, due to their territorial behavior or when the location is subjected to impounding structures that limit fish movement. Thus fish can serve as effective indicators of environmental conditions in that location. The fish community in streams, rivers, and ponds is an important food source for instream consumers, as well as for some bird and mammal species, including man. Fish generally depend on other aquatic sources for their various life functions and are a principal component of the aquatic food chain sequence from contaminated sediments and water through benthic macroinvertebrates, to smaller consumers, such as the cyprinids; intermediate consumers, such as the yellow perch and larger sunfish; larger consumers, such as the largemouth bass; and scavengers, such as the bullhead. Each of these may itself be the prey of a higher level consumer, such as an otter, a heron or kingfisher, and ultimately, man. Predation upon fish represents an important transport mechanism for the movement of contaminants from in-stream sediment to terrestrial sources. Because of PCB contamination in fish, a fish consumption advisory in the Housatonic River has been in place since 1988.

Fish community structure and function have been used extensively to evaluate the quality of water resources and characterize causes and sources of impacts in lotic (flowing water) and lentic (standing water) freshwater ecosystems. The individual organisms that make up fish communities respond to both biotic and abiotic environmental variables; therefore, the structure of these communities reflects the integration of the influence of these variables. Biotic variables may include competition, predation, and food availability, whereas abiotic variables may include stream temperature, dissolved oxygen, flow characteristics, and pollutants.

Because of the long-recognized importance of fish community structure in evaluating the health and condition of aquatic habitats, and the importance of fish tissue as a measure of the transport of contaminants through the ecosystem, collection and assessment of fish representing the community in this system is an essential element of the ecological characterization. Fish tissue sampling and community assessment will be conducted to determine if PCB contamination from the GE facility is adversely affecting fish in the study area and accumulating in fish tissue at concentrations detrimental to human and ecological consumers, fish tissue sampling and community assessment is to be conducted.

1.2 OBJECTIVES

The principal objective of the fish collection effort is to determine the PCB and other organic contaminant concentrations in tissue for use in both human health and ecological risk assessments, to evaluate congener patterns by species for use in the fish and mink reproduction studies, and in the PCB fate and effects model. In addition, the fisheries community will be qualitatively assessed (see Attachment1) for use in the ecological characterization of the river system.

Fish tissue, whole body samples, and fillet and offal samples will be analyzed for PCBs (Aroclor and congener/homologue analyses) and for dioxin/furans and organochlorine pesticides. Fish tissue sample collection and analysis will be used to evaluate both ecological and human health endpoints. Fish tissue concentrations will be used to determine potential risks to individuals who may be catching and eating fish in violation of the fish consumption ban, as well as to determine risk to subsistence and recreational anglers in the absence of administrative or institutional controls. Ecological measurement endpoints are the comparison of tissue concentrations to Maximum Allowable Tissue Concentrations (MATCs) from literature and reference area concentrations, and incorporation in food chain models for piscivorous receptors. Tissue analyses will be conducted in accordance with the QAPP (WESTON, 2000).

All fish collection and capture will be performed by personnel from the U.S. Fish and Wildlife Service (USFWS), Office of Fishery Assistance, Laconia, New Hampshire, and Office of Fishery Assistance, Sunderland, Massachusetts. Fish capture techniques and protocols will be conducted using the USFWS standard operating procedures (SOPs) and safety requirements (Attachment 2).

2. STUDY DESIGN

2.1 FIELD SAMPLING

2.1.1 Sample Locations

Fish will be collected from the following seven locations:

Upper East Branch Housatonic River (Dalton)—This reach will serve as the non-impacted flowing reference reach. A dam separates this reach from the lower river system, prohibiting fish passage from the contaminated portion of the system into this reach; however, it does not have the extreme “impoundment” characteristics of the slower, lower river system. The upper portion of this reach is similar to the “shallow” river reach.

Housatonic River (Confluence to WWTP) “Shallow Reach”—This contaminated reach has shallow water of moderate velocity, larger sediment grain size, less total organic carbon (TOC) in the substrate, and less in-stream cover. It is also free from enrichment from the wastewater treatment plant (WWTP).

Housatonic River (WWTP to Contaminated Backwaters) "Deep Reach"—This contaminated reach is characterized by deeper, slower water and more in-stream cover. It is also downstream of the WWTP outfall.

Housatonic River - Impoundment (Contaminated Backwaters and Woods Pond)—This reach includes areas of the river where contaminated sediments have accumulated from upstream areas. This area is characterized by large impoundment areas with associated shallow and deep water habitat.

Rising Pond—This impoundment in the Housatonic River is located approximately 17 miles downstream of Woods Pond. PCBs have been detected in the sediment (although at lower concentrations than Woods Pond).

Goodrich Pond—This is a small pond located within Pittsfield adjacent to some residential properties known to contain fill from the GE facility, but is currently not posted against fish consumption. This pond was included in this study to determine if fish are contaminated with PCBs at levels adverse to human health. A tributary from this pond discharges into the East Branch of the Housatonic River, and sediments from this tributary have been found to be contaminated with PCBs.

Three-Mile Pond—This impoundment serves as the reference impoundment within the Housatonic River watershed outside of site influence. This pond reportedly has most or all of the target species selected for this investigation.

2.1.2 Target Species

- Largemouth Bass (*Micropterus salmoides*).
- Yellow Perch (*Perca flavescens*).
- Sunfish: pumpkinseed (*Lepomis gibbosus*), bluegill (*Lepomis macrochirus*) or other.
- Cyprinids: golden shiner (*Notemigonus crysoleucas*), common shiner (*Luxilus cornutus*), or other.
- Brown Bullhead (*Ictalurus nebulosus*).
- Goldfish (*Carassius auratus*).

2.2 QUALITATIVE FISH SAMPLING

Fish in the Housatonic River study area and in reference areas will be qualitatively sampled to characterize fish communities in terms of species presence and relative abundance. In each area fish will be captured or observed by electrofishing using electroshocking boats or backpacks. All fish sampling will be performed by U.S. Fish and Wildlife Service personnel in compliance with their standard operating procedures and safety requirements detailed in Attachment 2.

Timed electrofishing surveys for qualitatively characterizing fish communities will occur over 30 minute periods within representative areas in each of the above mentioned sampling sites. Starting and ending locations of each timed survey will be plotted on data sheets or located in the field using GPS equipment. During each 30-minute survey all fish that are shocked will be identified to species and enumerated by personnel in the boat. Certain fish may be netted and put into live wells, or buckets, to verify species identification. Additional information collected during each timed survey will include date, location, capture method, weather, crew members, and miscellaneous comments. Data forms will be completed during each timed survey.

2.3 FISH PREPARATION FOR TISSUE ANALYSIS

Fish will be collected in accordance with the methods identified in Attachment 2 by location and retained in live wells containing location-specific water until sample processing is initiated. Fish containers (e.g., live wells) will be labeled with capture location information and aerated to minimize fish mortality before fish processing. All fish retained for potential sample analysis will be enumerated and separated by species and size class. This information will subsequently be used to determine the number of samples and associated IDs. Fish will be sacrificed by cervical separation or sharp blow to the head with a stunning rod. All fish not retained for analysis will be released at their approximate capture location unharmed after processing.

The following metrics will be recorded for each individual fish included in any sample:

- Total Length (cm) — The greatest dimension of a fish from its anterior-most extremity to the end of the tail fin. For fish with a forked tail, the two lobes should be pressed together, and length of the longest lobe should be recorded.
- Total Weight (g) — Fish will be placed in a pre-weighed decontaminated tray and weighed to the nearest gram.
- Fillet Weight (g) — For appropriate samples (same procedures as total weight).
- Offal Weight (g) — For appropriate samples (same procedures as total weight).
- Sex (M/F) — When possible (i.e., bass), fish sex will be identified by external morphological characteristics or internal reproductive examination.
- Age — Otoliths and scale samples will be collected to determine the age of largemouth bass. Age will be determined in a laboratory setting at a later date by USFWS or designated EPA contractor.
- Physical Exam — Gross pathological examination of all fish will be conducted and documented. Special consideration will be given to gross pathological conditions on largemouth bass.

Upon completion of collection of metrics, fish samples will be either submitted for whole body, or fillet and offal analysis.

2.3.1 Whole Body Sample Processing

Fish samples for whole body analysis will be rinsed of all debris with deionized water and placed in decontaminated aluminum foil (dull side toward the fish). The sample ID labels will be placed on the outside of the aluminum foil and secured with clear tape. If more than one fish is used for a sample (composite), all fish used for the sample will be placed on one piece of aluminum foil, wrapped and labeled with the appropriate sample ID. To preserve sample integrity, samples will be placed in double resealable plastic bags with a second ID label and placed in either a cooler with dry ice or a suitable freezer until analyzed.

2.3.2 Fillet and Offal Sample Processing

Procedures for filleting fish are described below.

An initial cut should be made from the dorsal fin to the pelvic fin, just behind the opercular flap. Run the tip of the knife along the dorsal side of the fish, from the initial cut to the caudal fin. Continue making successively deeper cuts, running the knife blade as close to the neural spines and ribs as possible. After the fillet is obtained, remove the skin. Place the skin side of the fillet down on the dissecting tray, hold on to the tail portion of the fillet, and run the knife between the skin and the muscle tissue. Remove any debris from the skinless fillet by rinsing with deionized water.

After a fillet is cleaned, place the sample in a pre-weighed decontaminated tray and record the weight to the nearest gram. For composite samples, obtain all the fillets for the composite and weigh to the nearest gram. Fillet samples will be placed in decontaminated aluminum foil (dull side toward the fish). Offal samples (fish tissue remaining after fillets have been removed) will also be placed on decontaminated aluminum foil in the same manner. The sample ID label will be placed on the outside of the aluminum foil and secured with clear tape. The samples will be placed in double resealable plastic bags with a second ID label and stored on dry ice or suitable freezer until submitted to a designated laboratory.

2.3.3 Tissue Analysis

Fish tissue, whole body samples, and fillet and offal samples will be analyzed for PCBs (Aroclor and congener/homolog analyses) and for dioxin/furans and organochlorine pesticides. Tissue analyses will be conducted in accordance with the QAPP.

2.4 SAMPLE SIZE

Individual and composite fish samples will be collected for the aforementioned sample reaches and impoundments. The following provides an outline of the types of samples targeted for collection (Table 1).

- For yellow perch, bullhead, and sunfish, 10 to 25 edible size fish will be submitted for analysis as fillet and offal samples. Both sides of the fish will be filleted to obtain the minimum sample weight of 30 grams. All fillet samples will have the skin removed.

- For yellow perch, bullhead, sunfish, and cyprinids, five composite samples of five fish each, of forage size (5 to 9 cm), will be submitted. Each composite will contain fish within 75% of the total length between the largest and smallest fish of each composite.

- One size range of goldfish (23 to 28 cm) will be submitted as a whole body sample.

- A total of 25 largemouth bass of all size ranges observed, and an additional five composites of forage size fish will be submitted for analysis. Bass will be broken up into three size ranges:

    1) Bass greater than 12 inches (28.5 cm) MA legal limit.
    2) Bass less than 12 inches (28.5 cm).
    3) Bass 5 to 9 cm.

Bass greater than 12 inches will be submitted as fillet samples with the skin removed. Bass less than 12 inches will be submitted as whole body samples.

Table 1. Sample Grouping Strategy, Housatonic River, MA

Species	Largemouth Bass	Yellow Perch	Brown Bullhead	Sunfish	Cyprinid	Goldfish
Fillet	X	X	X	X*		
Offal	X	X	X	X*		
Whole Body	X					X
Forage size Whole Body	X	X	X	X	X	

* Sunfish will be sampled as fillets/offal if determined to be large enough for human consumption.

2.5 DOCUMENTATION

All sample documentation will follow project specific SOPs for field sample ID, data sheet, chain-of-custody, and custody seal procedures.

2.6 DECONTAMINATION

All dissection equipment will be decontaminated following the project-specific SOP for equipment decontamination including detergent/water wash, potable water rinse, hexane rinse,

isopropyl alcohol rinse, and deionized water rinse. All aluminum foil will be hexane rinsed prior to use.

2.7 FIELD QUALITY CONTROL SAMPLES

One field rinsate blank sample will be submitted during each day of processing. One duplicate sample (left side fillet) will be collected every 20 samples for samples large enough to produce the minimum required sample mass (approximately 30 grams) per fillet. One MS/MSD sample will be collected for every 20 samples large enough to provide triple the required sample mass.

2.8 SAMPLE SHIPPING

Samples should be sent by overnight delivery service (next morning delivery) or hand delivered. Samples sent to the USFWS should be shipped to:

Ken Carr/Ken Munney
USFWS
22 Bridge St., Unit 1 Phone 603-225-1411
Concord, NH 03301 Fed. Ex Acc# 1510-1036-9

Shippers will notify the receiving laboratory or the USFWS and notify that samples are being sent for next-day delivery. Samples should not be sent to USFWS if Ken Munney, Ken Carr, or Drew Major are not available for receipt of the shipment. Samples need to be sent for arrival on a weekday only. Therefore, Thursday is the last day of the week to ship samples. Shippers should also call the receiving laboratory of USFWS the day of delivery to verify the receipt of samples.

3. QUALITY ASSURANCE/QUALITY CONTROL

3.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

3.1.1 Data Quality Objectives

The three data quality objectives of the fish collection and evaluation are outlined in Subsection 1.2. To achieve these objectives, the following types of data and specific quality criteria will be required:

- Taxonomic identification of fish to LPIL (lowest practical identification level)—Fish must be identified to the species level whenever possible. When identification to the species level is not possible, the LPIL will be consistent with standard practice for fish. The six target species must be identified to species. Fish collected as part of an incidental take should be identified to the species level where possible.

- Enumeration (counts) for each species in each replicate sample—Counts must be made and recorded accurately. Accurate counts are readily achievable in the field.

- Total length (cm) for each fish in each of the target species collected—Total length must be measured accurately in the field using a fish board and recorded accurately. Procedures have been established (Subsection 2.2, above) to ensure that consistent length measurements are taken and recorded.

- Biomass (total weight) for each fish—Total weight must be determined accurately and recorded to 1 g using a calibrated balance designed and intended by the manufacturer to be capable of accurately measuring masses of this magnitude.

- Fillet weight (total fillet weight) for each fish—Fillet weight must be determined accurately and recorded to 1 g using a calibrated balance designed and intended by the manufacturer to be capable of accurately measuring masses of this magnitude. Adherence to the fillet sample processing procedure described in Subsection 2.2.2 is essential.

- Offal weight (total offal weight) for each fish—Offal weight must be determined accurately and recorded to 1 g using a calibrated balance designed and intended by the manufacturer to be capable of accurately measuring masses of this magnitude. Adherence to the offal sample processing procedure described in Subsection 2.2.2 is essential.

- Age for largemouth bass—Collection of otoliths and scale samples using the accepted procedures is essential. Age determinations will be made in a laboratory setting at a later date by the USFWS or a designated EPA contractor.

- Physical exam of all fish—Gross pathologies for each fish collected must be accurately recorded.

- Tissue chemistry for PCBs and selected other contaminants—Analysis of tissues (whole body, fillet, or offal samples) for chemical constituents must result in data that are consistent in all respects with other contaminant data collected as part of the larger project. Satisfactory results will be ensured by following the quality control specifications for these data as delineated in the project QAPP (WESTON, 2000).

- Qualitative fish community data including number of fish per species observed per unit effort.

3.1.2 Data Quality Indicators

Data developed in the fish community and tissue study must meet standards of precision, accuracy, completeness, representativeness, comparability, and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000) that are appropriate to the data quality objectives. Each of these data quality indicators, some of which are not readily quantifiable for fish community data, is discussed below.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. The study design includes an increase in the number of replicates to increase the statistical resolution; for this study the number of replicates (up to 25 largemouth bass

samples, for example) is used in this manner. Precision during the fish community evaluation is defined as agreement on species identification and enumeration by multiple personnel involved with collection efforts.

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study (fish taxonomy and biomass), accuracy is defined as meaning that the fish are correctly identified in each sample, correctly enumerated, and correctly measured for length and weight. Accuracy of these parameters is a function of each fish being processed by eye, and of consistent field sampling techniques. The data generated by this study will also be evaluated for accuracy via comparison with known and/or expected results from similar studies conducted in the Housatonic River or in similar New England systems. For parameters such as tissue contaminants, accuracy is as defined in the QAPP. For the qualitative fish survey, accuracy is defined as the ability to identify the fish species observed by eye, and to generate a reasonable estimate of number of individuals observed in the water during electrofishing. This is constrained by a number of factors including the selective nature of the likelihood of the electrofishing to stun different species, and the ability to accurately estimate number of individuals observed for either small fish or fish that are observed in large numbers instantaneously.

Completeness is defined as the percentage of the planned samples actually collected and processed. Completeness can be evaluated for all components of the fish program. To ensure achieving the planned statistical resolution, it is important that completeness of 100% be achieved for all components of this study with the exception of the tissue residue analyses. For this latter study component, the number of analyses will be determined by the material available for collection; therefore, establishment of an *a priori* completeness goal is not possible. For the qualitative fish survey, completeness will not be 100% because of the known fact that electrofishing will not result in an observation of all fish at a given location. It is expected that there will be a more complete response in the shallow water areas and that completeness will decline with water depth.

Representativeness refers to the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. Representativeness for this study is ensured through establishment of an approved, thorough sampling design and through careful implementation of the sample processing and analytical methods. Specific aspects of representativeness will also be evaluated via comparison with known and/or expected results based on previous investigations of the Lower Housatonic River and other similar systems. Representativeness of the qualitative fish survey will be constrained by the differential response of species to the electrofishing technique.

Comparability is a measure of the confidence with which the fish data may be compared to another similar data set. Comparability will be evaluated by examination of the in-station variability in key parameters as determined from the large numbers of replicates to be collected at each location and fish observations to be made. Comparability will also be evaluated for this data set through comparison with previous fisheries work in the Lower Housatonic River and with known characteristics of fish populations in similar stream systems in the Northeast.

Sensitivity, the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest, is related for fisheries investigations to the ability of the taxonomic analysis to resolve the various fishes into individual species. This data quality

indicator will be evaluated by comparing the number of species-specific separations against the number of unresolved larger taxonomic groups. As the number of unresolved groups increase, the community metrics such as species richness and diversity are less able to resolve differences between samples. Sensitivity is applicable and important for the chemistry parameters that will be analyzed as part of the tissue study. For these parameters, the detection limits for chemistry specified in the QAPP will provide appropriate sensitivity for the purpose of providing insight in to factors controlling abundance and distribution of the fish populations.

3.1.3 Data Validation, Verification, and Usability

Procedures for data validation for the chemical and physical data are discussed in various sections of the project QAPP and will be used whenever applicable in this study. For the biological data, usability will be largely determined by three factors: (1) the experience of the senior investigator in establishing that the field sampling was conducted following the SOP and that accuracy and precision were not compromised by an inability to control the sampling procedures in the field; (2) an evaluation of the taxonomic data both within the study and compared with previous studies in the river and in the New England area; and (3) a direct comparison between the chemistry and similar data developed from co-located samples that have been collected as part of other project components.

The purpose of the remainder of this section of the study plan is to document the measures included in the study to ensure that the standards discussed above are met.

3.2 SAMPLING DESIGN

The rationale for selection of the seven locations to be sampled in the fish study is presented in Subsection 2.1.1. The locations are not intended to be representative of the entire river but rather are intended to encompass the range of sediment PCB concentrations, and the associated fish tissue concentrations, in the Lower River between the Confluence and Woods Pond; two appropriate reference locations with background PCB levels will also be sampled.

3.3 SAMPLING METHODOLOGY

3.3.1 Sampling Procedures

Sampling methods, as discussed in Subsections 2.2 and 2.3 and Attachment 2, were chosen to ensure unbiased (i.e., accurate) samples that will facilitate comparisons with other fish data, both from the Housatonic River and from other areas. All samples will be collected by trained and experienced personnel; senior oversight of all aspects of the sampling and sample processing will further promote comparability and reduce potential bias. Subsamples for tissue chemical analyses will be collected following procedures documented in the project QAPP and will therefore be comparable with procedures followed for all other similar efforts throughout the Supplemental Investigation.

3.3.2 Quality Control Samples

The nature of fish sampling does not allow the incorporation of typical duplicate and blank samples as part of the study design. For community metrics, there is no acceptable method of obtaining such samples in a manner analogous to that for duplicates and blanks collected for chemistry analysis.

Duplicate samples for tissue chemistry will be collected in this study. Quality control of tissue chemistry analyses will be provided by the analysis of duplicate samples (including MS/MSD samples) at a rate of approximately 5% of samples collected. Duplicates will be processed in accordance with the QAPP and via comparisons with results from split samples provided to GE.

3.3.3 Sample Processing and Preservation

Detailed procedures for collection and initial processing of all samples to be collected as part of the fish study are provided in Subsection 2.3. Subsampling, homogenization, and decontamination between samples will follow procedures established in the QAPP. All samples will be held on dry ice and returned to the field laboratory daily and will be either frozen (physical, chemical samples) or preserved (taxonomic samples) at that time. Holding time for physical and chemical samples will follow procedures established in the QAPP; there is no holding time for taxonomic samples.

3.3.4 Training

All sampling will be directed in the field by senior scientists with experience in the collection of fish samples. Supporting staff will receive training from the senior scientist(s) in the overall goals of the study and in techniques to be followed to ensure collection of quality data.

3.4 SAMPLE ANALYSIS

3.4.1 Taxonomy Samples

Processing of taxonomy samples will follow USFWS procedures as documented in Attachment 1. All samples will be processed by experienced staff who have received specific training in the SOP and whose work is checked periodically by their supervisors and peers. While performing the qualitative fish community survey, any individual for which the identification to species is in question will be captured and either identified and released, or if not definitively identified the individual will be retained for identification in the laboratory. Five percent of the fish will be re-checked by someone other than the original identifier. Corrective action, including reclassification of fish samples and retraining of staff, will be instituted if these QC checks produce unsatisfactory results.

Quality of taxonomic identification will be ensured by maintaining voucher collections and requiring a consensus among all taxonomists at the processing laboratory prior to an identification becoming accepted as a type for the voucher collection. In the event that the

taxonomists are unable to agree on an identification, specimens will be sent to a third party for determination.

3.4.2 Physical/Chemical Samples

Samples for tissue chemistry will be processed following procedures and SOPs provided in the QAPP. These samples will be submitted in catalogs (sample delivery groups) and batches with other samples from the larger project and data validation will be performed on a catalog basis in accordance with procedures established and described in the QAPP.

3.5 DATA ANALYSIS AND REPORTING

The overall analytical approach for data generated under this study is described in Subsection 2.4. The study findings will be included in the ecological risk assessment including all data, analyses, and interpretations and will be prepared with specific reference to both the data quality objectives specific to the fish study (see Subsection 2.3.1 above and Subsection 4.1 of the QAPP).

4. PROCEDURES

4.1 FIELD SAMPLING

4.1.1 Collection of Taxonomy Samples

All fish collection and sampling will be conducted by personnel from the USFWS, Office of Fisheries Assistance, Laconia, New Hampshire and Office of Fishery Assistance, Sunderland, Massachusetts, following the SOP provided as Attachment 2.

4.1.2 Initial Processing of Fish for Tissue Residue Analysis

Fish preparation for tissue analysis, whole body analyses, and fillet and offal tissue samples will be conducted pursuant to the procedures outlined in Subsection 2.3.

5. REFERENCES

WESTON (Roy F. Weston, Inc.). 2000. *Final Quality Assurance Project Plan.*

ATTACHMENT 1

FISH COMMUNITY DATA SHEET

GENERAL ELECTRIC/HOUSATONIC RIVER
SUPERFUND SITE FISH CAPTURES

Date: _____ Pond/River Name: _____

Capture Method: _____ Weather/Temp: _____

Crew: _____ Photo/s: _____

_____ _____

River Section: _____ River Reach: _____

Pond Run: _____ Map Reference: _____

Time Start: _____ Time Finish: _____ Total Time: _____

Fish Species Observed	Estimated Numbers	Total

Comments: _____

_____

_____

_____

_____

_____

GENERAL ELECTRIC/HOUSATONIC RIVER
SUPERFUND SITE FISH CAPTURES

Date: 9/28/98 Pond/River Name: Housatonic River

Capture Method: Electroshocking Boat Weather/Temp: Sunny, 70's

Crew: Ev, Joe, Johanna, Ken, Doug Photo's: 2, 3, 4

River Section: Upper - EPA Desig? River Reach: Smokestack To ledge outcrop

Pond Run: If pond - Run # 1, 2, etc. Map Reference: Map 1 - "A"

Time Start: 5:30 PM Time Finish: 7:30 PM Total Time: 2 hours

Fish Species Observed	Estimated Numbers	Total
LMB	2, 6, 0, 3, 1	12
SMB	1, 0, 0, 2, 0	3
YP	10, 14, 18, 22, 3	67
CP	1, 0, 1, 2, 0	4
GF	4, 5, 3, 1	13

EXAMPLE

Comments:

ATTACHMENT 2

USFWS FISH COLLECTION PROTOCOLS

U.S. FISH AND WILDLIFE SERVICE
STANDARD OPERATING PROCEDURES

General Electric/Housatonic River Superfund Site
Pittsfield, Massachusetts
September - October, 1998

All fish sampling will be performed by personnel from the U.S. Fish and Wildlife Service (USFWS), Office of Fishery Assistance, Laconia, New Hampshire and Office of Fishery Assistance, Sunderland, Massachusetts. Reference sites will be sampled before contaminated sites. Bass, perch, bullheads, sunfish and cyprinids are the species likely to be targeted for contaminant analyses. Fish will be captured using electroshocking boats, backpack electroshockers, gill nets, minnow traps, and trot lines. Electrofishing will be conducted in compliance with USFWS standard operating procedures and safety requirements per 24 AM 13 (attached).

SITES

Contaminated

Housatonic River - Pittsfield to Lee
Goodrich Pond - Pittsfield
Woods Pond - Lee
Rising Pond - Great Barrington

Reference Sites

East Branch Housatonic River - Pittsfield
Three Mile Pond - Sheffield

FISH CAPTURE PROTOCOL

Fish captured by boat electroshocking will be held in live wells on board the boat containing water from the site until transferred to appropriate EPA or contracted personnel. It is anticipated that each boat will have a minimum of one operator, two netters and one person to record data. In riverine situations, boat operators will select a specific reach within a river section and will designate the upper and lower boundaries of the section of the river to be electrofished. Data will be recorded for each reach. In pond situations, boat operators will electrofish in one half hour intervals, recording data for each interval until such time as appropriate samples of target species are collected. Approximate locations of electroshocking runs will be designated on a map for all sites.

Fish captured by backpack electrshocking will be held in buckets containing water from the site, or in live cars placed in the site until transfers can be made to appropriate personnel. A minimum of one operator, one netter and one data recorder is anticipated at each site chosen for backpack

electroshocking.

Trot lines will be baited only with bait from the site in which the trot line is deployed. Gill nets will be closely monitored to minimize mortalities. Data sheets will be completed for all capture sites, and will include site location, date, capture method, time required for collection, fish species observed, relative abundance of all species, and other pertinent data. (See attached sampling form.)

FISH HANDLING PROTOCOL

All fish captured for contaminant analyses will be held by the USFWS until transferred to EPA personnel or contractors working under EPA supervision. Fish will be held at all times in water from the collection site prior to processing. Containers of fish will be labeled with capture location and any other information deemed approriate by EPA personnel. Aeration will be provided until fish can be processed.

CHAPTER 13

Electrofishing

TABLE OF CONTENTS

Electrofishing

13.1 Purpose. To ensure the safe conduct of electrofishing operations by establishing Servicewide competency requirements for electrofishing operations. This chapter also provides guidelines for the safe construction, modification, and operation of electrofishing equipment.

13.2 Scope. The provisions of this chapter apply to all Service activities using electricity (produced by gasoline powered generators/alternators or batteries) to sample animals in aquatic habitats.

13.3 Policy. The Service recognizes the electrofishing operation as a hazardous activity for which skills training is required in accordance with 24 AM 1.7 B (2).

It is, therefore, Service policy that all personnel serving as electrofishing team leaders demonstrate knowledge of the principles and techniques of electrofishing. Team leaders will be considered knowledgeable of the principles and techniques of electrofishing upon satisfactory completion of the National Fisheries Academy course, Principles and Techniques of Electrofishing. In lieu of course completion, Service personnel may satisfactorily complete a certifying examination by the Superintendent, National Fisheries Academy.

13.4 Authority.

A. 29 CFR 1910 - General Industry Standards.

B. Federal Boat Safety Act of 1971 as amended (46 U.S.C. 1451-89).

C. National Fire Protection Association (NFPA) 70-1981, National Electric Code (NEC).

13.5 Definitions.

A. Anode. The positive electrode.

B. Bonding. The permanent joining of metallic parts to form an electrically conductive path which assures electrical continuity, with the capacity to safely conduct current.

C. Branch circuit. The circuit conductors between the final overcurrent device protecting the circuit and the electrical load(s).

D. Cathode. The negative electrode.

E. Circuit breakers. A device designed to open and close a circuit by a non-automatic means, and to open the circuit automatically on the predetermined overcurrent without damage to itself when properly applied within its rating.

F. Deadman switch. A switch which requires constant pressure to supply electrical current to the circuit.

G. Electrofishing. The use of electricity to provide a sufficient electrical stimulus in fish to permit easy capture by netting.

H. Electrofishing team leader. The individual in charge of the electrofishing operation. Only persons demonstrating knowledge of the principles and techniques of electrofishing in accordance with 13.6D can serve as electrofishing team leaders.

I. Ground. A conducting connection, whether intentional or accidental, between an electrical circuit or equipment and the earth, or to some conducting body that serves in place of the earth.

J. Isolation transformer. A transformer inserted into a system to separate one section of the system from undesired influences with other sections.

K. Netter. The individual who nets the captured fish during electrofishing operations.

L. Power control circuit. The circuit which interconnects and adjusts the power from the pulsator or generator to the electrodes.

M. Raintight. Constructed or protected so that exposure to a beating rain will not result in the entrance of water.

N. Variable voltage pulsator electroshocker. The device used to deliver the pulsed electric current.

O. Watertight. Constructed so that moisture will not enter the enclosure.

P. Weatherproof. Constructed or protected so that exposure to the weather will not interfere with successful operation.

13.6 Responsibilities. These responsibilities supplement those found in 24 AM 1.5.

A. Chief, Office of Safety and Security. Will maintain a current listing of all Service personnel possessing an electrofishing certificate of competency, and provide regional safety managers with such listing.

B. Regional directors. Regional directors will ensure that all persons serving as electrofishing team leaders have received from the Superintendent, National Fisheries Academy, a certificate of competency for electrofishing.

C. Superintendent, National Fisheries Academy.

(1) Prepares electrofishing certifying examination for persons desiring to demonstrate knowledge of the principles and techniques of electrofishing by satisfactory completion of a certifying examination in lieu of completion of the National Fisheries Academy course, Principles and Techniques of Electrofishing. The certifying examination may be taken 3 times, in intervals of at least 30 days. Persons failing to satisfactorily complete the certifying examination in 3 attempts will be required to complete the National Fisheries Academy course, Principles and Techniques of Electrofishing, prior to serving as a team leader.

(2) Ensures sufficient scheduling of the course, Principles and Techniques of Electrofishing.

(3) Issues certificates of competency for individuals either completing the course, Principles and Techniques of Electrofishing, or satisfactorily completing the certifying examination.

(4) Provides the Office of Safety and Security with a listing of all personnel possessing an electrofishing certificate of competency and update such listing as appropriate.

D. Electrofishing team leader. Only individuals demonstrating knowledge of electrofishing techniques can serve as electrofishing team leaders. Team leaders will be considered knowledgeable of the principles and techniques of electrofishing upon satisfactory completion of the National Fisheries Academy course, Principles and Techniques of Electrofishing. In lieu of course completion, Service personnel may satisfactorily complete a certifying examination prepared by the Superintendent, National Fisheries Academy. Training and education for electrofishing operations will otherwise be in accordance with section 13.7. As the individuals in charge of electrofishing operations, the team leaders will do the following:

(1) Identify hazardous conditions associated with proposed electrofishing operations, determine measures to protect electrofishing team members, and appropriately brief team members (see section 13.7B).

(2) Ensure that employees have and utilize the proper safety equipment.

(3) Ensure adequate warning is provided to the public to avoid public exposure to the potential hazards of electrofishing operations.

(4) Ensure precautions are taken to avoid harm to pets, domestic animals, or wildlife.

(5) Ensure that all electrofishing operations cease and all crew members go ashore in the event of a thunderstorm.

(6) Ensure that only those persons necessary to conduct a safe and efficient operation, and observers being trained, engage in each electrofishing operation.

(7) Ensure the availability of a well equipped, water-tight first aid kit. Questions concerning the contents of the first aid kit may be directed to the regional safety manager.

(8) The team leader should review the electrofishing considerations checklist found in Exhibit 1, and ensure the addition of specialized items to the checklist that pertain to his/her region or operation.

E. Project leaders. Ensure compliance with the provisions of this chapter.

F. Employee. Report all potential work hazards/accidents/incidents and job related illnesses/injuries to his/her supervisor immediately.

13.7 Training and education.

A. Team leader training and education will cover the areas identified below.

(1) The basic principles of electricity and transmission of current in water.

(2) The basic concept and design guidelines for electrofishing equipment.

(3) Electrofishing equipment and the equipment's capabilities, limitations, and safety features.

(4) The safety precautions to employ while using electrofishing equipment.

(5) The team leader must have a current certification in cardiopulmonary resuscitation (CPR) training and first aid.

Completion of the course, Principles and Techniques of Electrofishing, at the National Fisheries Academy or at a field location, or successful completion of the certifying examination, will serve to satisfy competency for factors 1, 2, 3, and 4. A certificate from the Red Cross or other recognized institution will certify CPR and first aid training.

B. All members of the electrofishing crew will be briefed in the following areas:

(1) Hazards involved in electrofishing.

(2) Safe operation of electrofishing equipment.

(3) Basic emergency procedures for drowning, unconsciousness, and electrical shock.

(4) All members of the electrofishing crew will also be knowledgeable of defensive driving techniques, including towing and backing of boat trailers if an electrofishing boat is used, and safe boating operations.

13.8 Electrical equipment: specifications and operation.

A. General.

(1) Isolation transformer. AC voltage from the generator will be isolated from ground either by removing the ground strap from the generator case or by adding an isolation transformer.

(2) Voltage. Rated voltages of insulation of conductors used to deliver output current from the pulsator to the electrodes must exceed the maximum potential voltage of the pulsator or generator by the next higher rating as follows:

Pulsator/generator	Minimum insulation rating of conductor
0 - 249 volts	250 volts
249 - 599 volts	600 volts
599 - 899 volts	900 volts
900 - 12,999 volts	13,000 volts

(3) Conductor size. Conductor size (i.e., current carrying wire) will be approved for rated amperage of equipment as follows:

Maximum amperage	Minimum conductor size
10	16 AWG
15	14 AWG
20	12 AWG
30	10 AWG

(4) Conductor type.

(a) Conductors will be of the stranded type for flexibility and be suitable for use in dampness.

(b) All conductors in the boat will be enclosed in conduit or liquid-tight, flexible conduit; however, appropriate heavy duty rubber cord can be used where flexibility is desired.

(c) Connectors used in association with flexible cords will be of the locking, waterproof type.

(5) Connections.

(a) Solices in wiring will not be permitted. If connections are necessary, the rating of the connector must be the same or greater than the wire.

(b) All equipment will be turned off before making any connections or replacing parts.

(6) Junction boxes. Junction boxes will be cast iron, cast aluminum, fiberglass, plastic, or rubber. All types must either be weatherproof or raintight depending on use. All junction boxes with switching equipment must be weatherproof. Junction boxes without switches may be raintight.

(7) Circuit breakers.

(a) Power output conductors from the generator or alternator will include a circuit breaker or fuse to provide branch circuit protection.

(b) Circuit breaker or fuses used for providing branch circuit protection will be enclosed in a weatherproof enclosure or cabinet that complies with National Electric Code, Article 373-2, which states the following:

"In damp or wet locations, cabinets and cutout boxes of the surface type will be so placed or equipped so as to prevent moisture or water from entering and accumulating within the cabinet or cutout box and will be mounted so that there is at least 1/4-inch air space between the enclosure and the wall or other supporting surface. Cabinets or cutout boxes installed in wet locations will be weatherproof."

(8) Electrodes and net handles. Net handles will be constructed of a non-conductive material and will be of sufficient length to avoid hand contact with the water.

(9) Noise. Noise levels will be maintained within the acceptable exposure of 85 dba for 8-hour exposure. Personal protective measures, such as use of earplugs, are described in 24 AM 8. The purchase of sound powered headphones is authorized through station funding. This type of headphone shuts out generator and motor noise and provides clear communication between the netter and equipment operator.

(10) Exhaust from power source. The exhaust from gasoline powered engines and generator alternators will be directed away from the equipment operator. Exposed hot pipes will be enclosed in protective screening to reduce the potential of burn exposure to crew members. The use of galvanized pipe for exhaust is discouraged due to the potential release of toxic gases that are produced under extreme heating conditions.

(11) Fuel storage. Gasoline will be stored and transported in approved metal containers. Such containers when used for storage on metal hull boats will be grounded.

(12) Refueling. To refuel the generator/alternator, all equipment will be turned off. Hot surfaces will be allowed to cool. It is recommended that all tanks be filled prior to each operation to avoid the potential for explosion or fire while refueling hot gasoline engines.

(13) Instruction sheets. Instruction sheets for boat, equipment, and operational procedures will be enclosed in waterproof plastic and be readily available for reference at all times during the electrofishing operation.

(14) Preventive maintenance.

(a) All equipment used in electrofishing will be scheduled for an annual preventive maintenance inspection. In addition, all equipment will be inspected before each use.

(b) Any equipment deficiency which may present a safety hazard will be corrected before each field operation or when equipment damage occurs during actual use.

B. Portable electroshockers.

(1) Electrodes.

(a) Electrode handles will be constructed of a nonconductive material and be long enough to avoid hand contact with the water.

(b) The positive electrode (anode) used with portable electroshockers will be equipped with a pressure switch that breaks the electric current upon release.

(2) Netter position. Netters will work beside or behind the individual with the electrofishing equipment to ensure the electrical field is well in front of both workers.

(3) Standard safety equipment.

(a) All persons using portable electroshockers will wear rubber footwear which will insulate the wearer from electrical shock. All footwear will be equipped with nonslip soles.

(b) Rubber linesman gloves, rated above the voltage being used in the electrofishing operation, will be worn. These gloves will be inspected for punctures before each use and will be replaced at adequate intervals.

(c) Polaroid sunglasses will be worn when there is glare.

(4) Portable electrical power source.

(a) Batteries used as electrical power source for backpack shockers will be of the gel type that will not leak when tipped or overturned.

(b) Backpacks will be equipped with a quick release belt (hip) and shoulder straps.

(5) Power control.

(a) The operator will have a switch to the pulsator or power control unit so that the electricity can be turned off quickly in an emergency.

(b) All equipment purchased after October 1, 1985, must be equipped with a tilt switch that breaks the circuit if the operator falls. The switch must be a type that has to be manually reset after the operator has regained his/her footing.

(6) Personal flotation devices. All persons will wear U.S. Coast Guard approved personal flotation devices (Type II) (i.e., life jackets or float coats) when operating in waters that are deep, high velocity, or turbid, to prevent drowning.

Note: Flotation devices constructed of materials such as ensolite are not bulky and are light weight. This material used in float coats can provide some protection against loss of body heat if the person accidentally falls into cold water.

(7) Hazard awareness. All persons will be aware of the hazards involved in using portable electroshockers in running waters such as slippery surfaces, swift water currents, deep areas, and obstacles such as logs or similar objects.

C. Electrofishing boats.

(1) Design.

(a) Electrofishing boats will provide adequate flotation and freeboard clearance consistent with equipment, cargo, and passenger weight when being operated. The boat will be equipped to meet U.S. Coast Guard or State boating regulations.

(b) The boat deck will be painted with a nonslip or skid resistant coating.

(2) Clear working space. General boat housekeeping must provide adequate working space to conduct safe operations. Care will be exercised to prevent clutter that may result in safety hazards.

(3) Boat inspection before each use. The boat and equipment will be visually inspected for safety by the supervisor or operator in charge prior to each use. Significant deficiencies, which could result in employee injury, will be corrected prior to operation or use of the equipment.

(4) Controls for electrical equipment.

(a) Electrical amp-volt meters will be installed to provide adequate monitoring of boat electrical power equipment.

(b) The boat operator should be able to operate an electrical control or switch to cut the power in case of an accident.

(c) The netter will have a deadman switch connected to the power control circuit from the pulsator or generator source. This allows the current between the electrodes to be broken in case of an accident.

(d) Power control circuits will not exceed 24 volts.

(5) Grounding/bonding. All metal surfaces within a metal boat will be electrically connected, grounded, and bonded to the boat hull to eliminate differences in electrical potential that may result in electric shock. The metal boat hull may also be used as a cathode.

To avoid possible electrolysis problems when the metal hull is being used as a cathode, zinc strips should be attached to the hull as "sacrificial anodes." The electrolysis will occur on the zinc strips which will preserve the integrity of the hull.

(6) Battery enclosure. An acid proof, nonmetallic enclosure and holder will be provided for wet cell batteries.

(7) Conductor protection. All conductors may be installed in a common raceway (conduit) provided each conductor installed is continuous (without connectors, breaks, or splicing), is independently and correctly insulated. All low voltage (24 volts or less) circuits will be contained in separate raceways from those containing high voltage conductors.

(8) Auxiliary circuits. Lighting and other auxiliary circuits should not exceed 24 volts. Note: 110 volt lamps may be used if the lamp is shielded with a nonconductive cage.

(9) Lighting.

(a) When the boat is to be operated at night, adequate on-board lighting (12-24 volts) will be provided for working areas.

(b) Adequate lighting will also be provided while electrofishing to avoid safety hazards such as striking logs, rocks, and overhead tree branches.

(10) Safety rails. Safety rails will be provided around the outside of the netting area and will be at least 42 inches high and be constructed of at least 3/4-inch diameter heavy-walled steel pipe or 1 1/2-inch heavy wall aluminium pipe. Rails will be so designed to withstand a 200-pound side thrust. The work deck will be covered with nonskid material and sloped to allow drainage. The high gunnels of wooden draft boats are satisfactory as safety rails.

(11) Fire extinguisher. Each boat will be equipped with at least one 5-pound type ABC fire extinguisher mounted in a holder for easy access to the boat operator and away from high fire potential sources.

(12) Personal flotation devices. All occupants will wear U.S. Coast Guard approved personal flotation devices at all times. Life vests that meet the requirements of Type II are designed to turn an unconscious person in the water from a face downward position to a vertical or slightly backward position. Float coats may provide some protection against the loss of body heat if the person were to accidentally fall into the cold water.

(13) Standard safety equipment.

(a) Hip boots will be worn so they can be easily removed in case the boat capsizes.

(b) Rubber chest waders will also be worn when necessary in order to remain dry as protection against electrical shock.

(c) Rubber gloves will be worn that are rated above the voltage being used. These will be inspected before each use and replaced at adequate intervals.

(d) Polaroid-type sunglasses will be worn to reduce glare from the water.

(14) Color coding/labeling of significant hazards. To ensure visibility, the color red will be used to identify fire extinguishers, safety cans, and stop buttons for electrical equipment. The color fluorescent orange will be used to identify all other safety switches.

APPENDIX A.21

FISH HEALTH AND TOXICITY PROTOCOL

APPENDIX A.21

Fish reproductive health assessment in PCB contaminated regions of the Housatonic River, Massachusetts, USA: Investigations of causal linkages between PCBs and fish health

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APPENDIX A.21

Fish reproductive health assessment in PCB contaminated regions of the Housatonic River, Massachusetts, USA: Investigations of causal linkages between PCBs and fish health

1. INTRODUCTION

The Housatonic River is a valuable aquatic resource, both aesthetically and economically (Orciari and Leonard, 1990). Draining over 2,000 square miles, the Housatonic flows south through a series of impoundments in western Massachusetts and western Connecticut, terminating in Long Island Sound. During the past two decades there has been increasing concern regarding the threat posed to fish and wildlife inhabiting the river due to the presence of highly toxic environmental contaminants (Henning et al., 1997). The principal cause of this contamination is a polychlorinated biphenyl (PCB) point source located on the East Branch Housatonic River at Pittsfield, Massachusetts. Total PCB concentrations in both fish and surface sediments downstream of this source have been reported at levels as high as 200 ppm (Smith and Coles, 1997). Although PCBs are considered to be the major toxic input to the river, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) may also contribute to contamination of the river (Eitzer, 1993).

PCBs, PCDDs, and PCDFs are collectively referred to as planar halogenated hydrocarbons (PHHs). The toxic effects of PHHs and structurally similar compounds are thought to be mediated through contaminant binding to a cytosolic aryl hydrocarbon receptor (AhR). This ligand activated transcription factor binds with selected sites on DNA called dioxin responsive elements (DRE), which control the expression of genes that encode both Phase I and Phase II enzymes. The induction of these proteins are part of a response to dioxin-like chemicals that leads to alterations in cellular homeostasis (DeVito and Birnbaum, 1994). Binding of PHHs to the AhR has been linked to several molecular events including the production of electrophilic metabolites and oxygen radicals, reduced capacity for xenobiotic metabolism, and alteration in the rates of endogenous substrate metabolism (Stegeman and Hahn, 1994). In fish, early life stages appear to be particularly sensitive to the effects of AhR ligands (Mehrle et al., 1988; Walker and Peterson, 1991) and recent evidence indicates the involvement of cytochrome P450 enzymes, specifically in this embryotoxic response (Cantrell et al., 1996; 1998).

A detailed assessment of the toxicological impact of PHHs on fish from the Housatonic has not been conducted. Using a combination of validated field- and laboratory-based tools, this study will evaluate the potential for contaminants in the Housatonic River to elicit embryotoxic effects on fish that could alter population structure. The study would have the combined benefits of not only demonstrating any association between PCB exposure and changes in early fish development, but also would develop the foundation for the determination of causality, if such a relationship exists. The studies would provide dose-response relationships for fish embryotoxicity caused by the actual mixture of chemicals found in fish from the Housatonic River. The multiple levels of biological organization (biochemical, histological, organismal) investigated will provide various lines of evidence for the conclusions of the studies.

2. HYPOTHESES AND SPECIFIC OBJECTIVES

2.1 HYPOTHESES

1) PCBs present in fish from the Housatonic river elicit detrimental effects through an AhR-mediated mechanism of toxicity.

2) Early life stages of fish species endemic to the Housatonic River are sensitive to the amount and composition of the PCBs found in the fish.

2.2 SPECIFIC OBJECTIVES

1) Evaluate the survival and development of offspring of fish collected from the selected PCB-contaminated locations of the Housatonic River.

    a) Determine the appropriate rearing conditions for the embryos of the representative species in the model.
    b) Determine the dioxin-like effects present in embryos and early life stages of fish eggs collected from the areas of interest in the Housatonic River.
    c) Determine the concentration of PCBs and other organic contaminants present in the ovaries of fish used in the rearing studies.
    d) Determine the ability of an additive model of toxicity of dioxin-like chemicals to predict the effects observed in the embryos and fry in the rearing studies.

2) Determine the embryotoxic effects of PCBs found in fish from selected areas of the Housatonic River.

    a) Develop an organic extract of the fish from the four areas and characterize the PCBs and other hydrophobic organic chemicals in the extracts.
    b) Determine the embryo toxic effects of the extracts in a species of interest from the Housatonic River and a laboratory surrogate species.
    c) Determine the extent to which an additive model of dioxin-like toxicity accounts for the toxicity of the chemicals in the complex organic extracts taken from fish from the selected areas of the Housatonic River.

3. EXPERIMENTAL APPROACH

The first phase of the assessment of PCB impacts on fish health in the Housatonic River is the collection of brood fish from the study areas with subsequent rearing of the embryos in the laboratory. Stage-specific mortality, gross pathologies, histological examination, and biochemical measurements will be made on the developing embryos and resultant fry, and offspring of fish collected from the four study areas of the Housatonic River. This will be followed by a 15-day

growth and mortality study of the surviving swim-up fry . The information on survival, development, and growth will be augmented with exposure assessment. The brood fish for these animals and portions of the eggs collected for this phase of the study will be analyzed for organochlorine chemicals including pesticides, congener PCBs, planar PCBs, and dioxins and furans. These exposure data will be used in conjunction with the effects observed in the developing embryos to determine the ability of the laboratory models (Phase II) to predict dose-response relationships in fish eggs collected from the Housatonic River. Therefore, studies with eggs reared in the laboratory from field-collected fish will serve as a validation exercise for the laboratory generated dose-response curves.

The most direct measure of the embryo toxicity of contaminants present in fish from the Housatonic River is to use those exact chemical mixtures to develop dose-response relationships in fish eggs. This will aid in revealing the toxicological mechanisms and allow for quantitative dose-response relationships to be developed exclusive of other stressors. The studies proposed in this portion of the proposal are designed to allow controlled laboratory exposures of fish eggs and developing embryos to an extract of PCBs from the whole body of fish from the study areas. The specific objectives of these studies will be achieved through the use of egg injection techniques and subsequent monitoring of the developing embryos until a time of exogenous feeding occurs in the fry or juvenile stage of the fish. The egg injection procedures are an alternative to full-life cycle studies, in which the adults are reared on contaminated feed designed to mimic the environmental conditions (Walker et al. 1996). The egg injection procedures effectively mimic the maternal transfer of hydrophobic contaminants, such as PCBs, to developing oocytes. Moreover, the toxicity of the contaminants received from such injections has been similar to that observed in studies where the eggs obtained the contaminants through maternal deposition (Walker et al., 1994).

The extent and nature of contaminant exposure in fish from the areas of the Housatonic River will be assessed through analytical measurements (OCs, PCBs, PCDDs and PCDFs as in previous phases of this project). As additional lines of evidence, biological indicators of contaminant exposure including ethoxyresorufin-*O*-deethlyase induction (EROD, a measure of cytochrome P450 induction) will be assessed in adult fish. Physiological and biochemical measures of effect in these fish may be assessed through the measurement of plasma concentrations of estrogen and testosterone, observation of the ratios of these steroid hormones (E/T ratios), plasma concentrations of vitellogenin, or selected other indicators of effect based on observations in the laboratory studies. Again, the appropriate selection of endpoints in the field-laboratory studies will allow the characterization of any causal linkages among contaminant exposure and adverse effects through a comparison with the laboratory-based results of these studies.

The results of the field-laboratory (Phase I) and laboratory (Phase II) studies will elucidate the extent, if any, to which PCBs are adversely affecting the early life stages of fish in the study areas of the Housatonic River. The two phases of the study will support or controvert the findings of each other and allow for stronger conclusions regarding the contaminant species involved in observed embryotoxicity. The laboratory studies serve as a standard curve of the effects expected to occur in the field. The studies proposed in Phase I offer a bridge between the controlled laboratory studies and the realistic findings of the field studies.

3.1 FIELD COLLECTION OF BROOD FISH

The first phase of these studies calls for fish to be collected from the study locations on the Housatonic River during or near spawning. The fish are to be transported to the CERC in Columbia, MO, where they will be bred. The intent of this portion of the study is to observe the development of the offspring and determine if any effects are observed in the offspring of species of fish collected at the areas of concern in the Housatonic River. The same endpoints of dioxin-like toxicity will be measured and assessed in the developing embryos and fry of adult fish taken from the Housatonic River as will be assessed in the subsequent egg injection studies. The egg injection studies will, as such, serve as a standard curve for calibration of any effects observed in the field-collected fish. Additionally, the rearing of field-collected eggs will serve as a validation exercise of the model developed from the egg injection studies described above.

The collection of brood fish will be performed by U.S. Fish and Wildlife Service personnel under its collection protocols. CERC personnel will assist in the collections and transport as needed. Any fish not used as brood fish will be processed in accordance with the protocols set forth in the Biomonitoring of Environmental Status and Trends (BEST) program of the USGS (Schmitt et al. CERC SOP P.326). The carcasses will be logged in the centralized sample tracking system and stored at -20°C until extracted and analyzed.

3.2 EMBRYO REARING

Wild-caught largemouth bass will be stocked in ponds (six males and six females) for spawning (two ponds per Housatonic River collection site) supplied with spawning mats. After the largemouth bass have completed spawning, the largemouth bass will be removed from the ponds and the bluegill will be stocked in enclosures in the ponds for spawning. Ten enclosures for each Housatonic River collection site will contain two females and two males of the wild-caught bluegill. The 40 enclosures that contain fish from each of the four sites (10 enclosures/site) will be randomly distributed among four ponds. Eggs collected from each of the spawning mats will be handled separately and will be treated as a separate spawning event. Fertilization rates will be checked by clearing of the eggs in glacial acetic acid and observation of a germinal disc. Each spawning event will be split into six replicates in the laboratory and each replicate will have 50 to 100 eggs. An additional set of two batches of eggs will be designated for temporal sampling for histological examination and immunohistochemical analysis (see below).

Developing embryos will be incubated according to optimal procedures to be developed in the first phases of the study. The bluegill, bass, and surrogate warmwater fish will be held in incubation chambers and the embryos gently rolled during development (Piper et al., 1982). Cold water species, used as surrogates in the injection studies (e.g., rainbow trout), will be held in vertical incubator trays with a constant flow of chilled water (8-12°C). Water quality is maintained within acceptable limits for embryo development, and water quality parameters are monitored periodically during the experiment, including dissolved oxygen, pH, water hardness, ammonia, and alkalinity.

Offspring of Housatonic River collected fish will be reared and observed for PCB-related effects. The endpoints that are to be monitored in these studies and the subsequent injection studies are those

consistent with a Ah-R mediated mode of action (Peterson et al., 1993) and include: mortality (daily); time to hatch (daily); gross pathology (weekly measurements of edema, hemorrhage, and craniofacial anomalies); histopathology (weekly sampling); weight and length (end of study); and in selected samples, apoptosis (programmed cell death) and cytochrome P4501A induction. The incidence of gross physical abnormalities will be quantified weekly for all experiments.

3.3 EGG INJECTIONS

The treatments in the experimental design matrix call for fish collected from the study locations to be extracted with organic solvents (see below) and the extract be used in fish egg injection studies to determine the toxicity of the chemical mixtures found at each of the sites. The extracts will be injected in graded doses into freshly fertilized fish eggs (Wilson and Tillitt, 1996; Walker et al., 1996; Tillitt and Wright, 1997; Wright and Tillitt, 1999). The concentrations of the extracts employed will be based on an estimated percent of adult body burden predicted to be transferred maternally to the eggs naturally. The eggs will be incubated under appropriate conditions and various endpoints associated with dioxin-like toxicity will be measured in addition to stage-specific mortality (see above).

The egg injection procedure to be used in these studies allows the accurate injection of nano- to picoliter amounts of various liquids into fish eggs to determine early life stage toxicity. The injection equipment allows small volumes of liquid to be delivered precisely with pulled-glass micropipettes, a regulated gas pressure system, and a digital control device. Pressure is applied pneumatically (compressed nitrogen gas) to the micropipettes with digital control of the dwell time, which allows delivery of a range of volumes. Eggs of various sizes, such as Atlantic salmon (*Salmo salar*), fathead minnow (*Pimephales promelas*), sea trout (*Salmo trutta trutta*), and northern pike (*Esox lucius*) can be used with this method with only minor modifications (Wilson and Tillitt, 1996; Walker et al., 1996). This procedure may be applied to various life stages of fish embryos, but was developed for use with newly fertilized eggs prior to completion of epiboly.

The species that are to be used for injection include one representative of the areas of interest and another species that is a routine surrogate used in toxicity testing. The decision about which species will be used in the laboratory portions of these studies will depend on the ability to culture the organism in the laboratory or obtain their eggs from a hatchery. It is anticipated that largemouth bass will be the species representative of the study area. The fathead minnow (*Pimephales promelas*) is a possibility for the laboratory surrogate species. The laboratory surrogate species is important to use in addition to the representative warmwater species because it will allow the results to be directly compared with the results from other laboratory examinations of PCBs and their mixtures. Additionally, the use of fathead minnows ensures that there will be a ready supply of eggs for use in the injection tests. Inclusion of the laboratory surrogate species in the experimental design ensures results from the laboratory egg injection studies for assessment and comparison to the other portion of the overall fish health assessment in the circumstances that difficulties are encountered in culturing the "endemic" species.

The experimental design for these studies calls for dose-response curves to be developed in the representative warmwater species and the surrogate species. Additionally, rainbow trout will be used

in dose-response studies because of the large amount of PCB-related reference information available for this species (Walker and Peterson 1996) and the fact that while not a dominant species in the study areas, salmonid species are present in the Housatonic River. The chemicals that will be used as standards include 2,3,7,8-TCDD and PCB 126 (3,3',4,4',5-pentachlorobiphenyl). The dose-response of embryo toxicity produced by the organic extracts of Housatonic River fish will be compared to the response of the test species to the chemical standards. The relative potencies of the various extracts may then be calibrated against these two standards. TCDD was chosen because it is the hallmark chemical for Ah-receptor (Ah-R) related responses. PCB 126 was chosen as the other standard because it often accounts for the majority of the dioxin-like potency of PCB mixtures in the environment and has the greatest toxic equivalency factor (TEF) of all of the 209 PCB congeners in fish (Van den Berg et al., 1998).

The specific objectives of these studies will be achieved through the use of egg injection techniques and monitoring of the developing embryos until exogenous feeding begins. The treatments in the experimental design matrix are: fish species to be used as source of eggs (N=3; bass/bluegill, fathead minnow or medaka, and rainbow trout); type of chemical or mixture of chemicals (N=6: PCB 126, TCDD, and four environmental extracts of Housatonic River fish); and dose of the chemical or chemical mixture (N=6, control and five graded doses). The combinations of these treatments are described below (Table 1).

Table 1

Experimental Design Matrix for the Egg Injections

Chemical/Mixture	Bluegill/Bass	Fathead Minnow	Rainbow Trout
2,3,7,8-TCDD	5 doses and sham	5 doses and sham	5 doses and sham
3,3',4,4',5-PCB	5 doses and sham	5 doses and sham	5 doses and sham
Extract Source			
Three-Mile Pond (H9)	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group
H. River Deep (H3) (RM 7-11)	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group
Woods Pond (H4)	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group
Rising Pond (H5)	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group	5 doses, sham, & 2 histological group

A complete dose-response relationship will be developed for each of the treatment combinations as described in the matrix. There will be five doses and the control (sham-injected) in each of these treatment combinations. The actual amount to be injected will be defined to bracket the concentrations observed in fish from the study sites. The doses will be defined as gram-equivalents (g-EQ) in which one g-EQ will equal the amount of the extract that corresponds to one gram of fish and will be lipid normalized based on the lipid content of the species of interest (Tillitt and Wright,

1997). The dosing range will be defined as one-tenth to one hundred times the concentration in the field collected samples. Each dose will be replicated three times and there will be 20 eggs injected at each of the doses, in each of the dose-response curves. There will also be a separate set of doses (at or near the expected ED50 of each of the mixtures and a sham-injected group) that will be incubated and sampled temporally for histopathological and biochemical lesions. The dose to be used in each case will be defined to bracket the LD50 values generated for bluegills using 2,3,7,8-TCDD and PCB 126 standards. An estimate of the LD50 for the extracts will be based on the PCB content of each extract.

The details of the egg injection procedures, reagents, and preparatory step are given in Walker et al. (1996). Briefly, the injections are conducted on freshly fertilized eggs, prior to epiboly. Eggs are held in a petri dish by agarose and the injections are conducted with drawn glass needles (micropipettes). Injections of graded doses of the chemicals or extracts are delivered accurately and precisely through the use of a regulated gas pressure system, and a digital control device. Pressure is applied pneumatically (compressed nitrogen gas) to the micropipettes with digital control of the dwell time, which allows delivery of volumes of 0.1% of the egg volume or less (often 0.5-20 nL). Following injection, the eggs of the various species will be incubated according to the appropriate procedures for that species.

The exposure concentration of the graded doses of the extracts that are to be injected into the eggs will be assessed with the toxic equivalency approach. This approach assumes an additive model of toxicity for the dioxin-like congeners (see Van den Berg et al. 1998 for further details on this approach). From this approach, the contribution of the planar PCBs (non-ortho-chloro-substituted congeners) to the overall dioxin-like toxicity may be estimated. The toxic equivalency factors (TEFs) that we will use are those developed from fish embryo mortality (Van den Berg et al., 1998). The dioxin toxic equivalents (TEQs) estimated to be each dose of the extracts allows calibration of the dose-response relationship of the complex extracts against the model compound for this class of chemicals, TCDD. The slopes of the extract dose-response curves may then be compared with that of the standard, TCDD. If the prevailing mode of toxic action of the extract is through an Ah-R mediated pathway, then it would be expected that the slope of the dose-response curves of the extract and TCDD would be parallel. Additionally, comparison of the median values for mortality (LD_{50}) between the extract and TCDD dose-response curves will also allow us to evaluate if an additive model of toxicity is appropriate for the various complex mixtures that we will test. If the slopes of the extract dose-response curves are parallel to TCDD and the toxicity is largely additive, then the contribution of PCBs to the overall dioxin-like toxicity can be estimated from this set of data.

3.4 PREPARATION OF EXTRACTS

Whole body samples of fish collected from the Housatonic River will remain frozen until the start of the sample preparation process. An extract will be developed for each of the study areas from the same species of fish used in the survival and development study of Phase I. The organic extracts will be used for the egg injection studies and will represent the complex mixture of organochlorine chemicals found in the fish at each location. The extraction and cleanup procedures are described below in detail, but generally will follow the methods described in Meadows et al. (1993; 1996). The fish will be processed individually through the grinding process. Fish will be sliced in 2- to 3-cm

slices while frozen and passed twice through a Hobart meat grinder. Approximately 15 kg of the ground tissue from each site will be weighed and thoroughly homogenized, individually by site. An aliquot equal to 1% of the total weight of each fish composite will be removed and set aside for analytical characterization of the chemical composition of the fish used to make the extract from each study area. Additionally, 20 to 50 g of each individual fish used to make the composites for extraction will be archived frozen at -20°C.

The remaining 99% of each composite will be chemically dehydrated by mixing with four times the tissue weight of anhydrous sodium sulfate. After dehydration, the tissue will be extracted by column percolation with methylene chloride. A set of five glass columns, 6 cm i.d. x 80 cm, with 1L reservoirs, will be used for this purpose. Each column can accommodate approximately 400 g of tissue with its attendant sodium sulfate. The columns will be filled in a serial fashion with the dehydrated tissue. The tissue composite of a given site will be exhausted before proceeding to the homogenate from the next site. Each column will be extracted with 1800 mL of methylene chloride. The tissue extracts from each site will be pooled and the extraction solvent removed from the extracted lipids by rotary evaporation.

The environmentally incorporated contaminants will be separated from the lipids by large-scale dialysis with polyethylene membranes (PM). Aliquants of 50 mL volume will be placed in 32-inch by 2-inch layflat tubing (0.005-inch wall thickness), heat-sealed, and dialyzed with 1800 mL of 80% hexane/20% methylene chloride. Each extract-filled PM will be dialyzed twice. The first dialysis will be for 4 days, followed by a change of dialytic solvent and additional dialysis for 3 days. Two 50-mL aliquants of clean salmon oil containing ^{14}C-2,5,2',5'-tetrachlorobiphenyl recovery spikes will be processed with each wash of each group as QC samples to monitor recoveries. The dialysates of each wash of each group of PMs will be composited and a portion equal to 0.1% of each removed for gravimetric lipid determination. Residual lipid removal will be through reactive cleanup with acid- and base-treated silica gels (Meadows et al., 1996). The effluents of the columns will be combined and placed at a volume of 40 mL. The combined extract will then be passed through high-performance gel permeation chromatography (HP-GPC) in 1-mL aliquots. The separation system consists of a 50-mm by 7.8-mm Phenomenex Phenogel® guard column and a 250-mm by 21.5-mm Phenomenex Phenogel® HP-GPC column both with a pore size of 100 A and particle size of 10 microns.

The purified sample extracts from each site will be composited and placed at a volume of 2 mL and the relative concentration (g-equivalents/mL) determined. A gram-equivalent is the amount of the extract equivalent to 1 gram of the original fish composite on a lipid normalized basis, relative to the eggs that are being injected. Three portions of 5 nL (approximately 40-g-equivalents each) each will be removed for analysis. The triplicate samples will be each brought to a volume of 5 mL (~ 8 g-equivalent/mL) and a 1-mL portion of each (~8-g-equivalents) will be used for chemical analysis. Internal standards for quantification of recovery and determination of the chemical content are to be added to each of the triplicate 1-mL portions prior to further processing (carbon fractionation on PGC and alumina cleanup for the dioxin/furan fraction). Comparison of the results of these three aliquots with analytical data from the original tissue will be used to indicate the recovery efficiency through the procedure as well as verify that no contaminants have been added during the procedure.

3.5 CHEMICAL CHARACTERIZATION OF PCBs

PCB exposure assessment in fish from the Housatonic River is the initial step for the determination of toxicological risk associated with these chemicals. Therefore, our first objectives in chemical characterization will include determination of the PCB congener profiles and dioxin-like chemicals in the ovaries of the fish collected from the study areas on the Housatonic River and used in the first phase of this study. The purpose of this will be to characterize the relationship between exposure to PCBs and dioxin-like chemicals and any effects observed in the rearing and growth studies described above. The carcasses of the adult fish collected from the four study areas on or near the Housatonic River will be the source of fish for development of extracts that will be used in the egg injection portion of the study. Thus, the same fish collected from the Housatonic River as a source of embryos to study survival and development will be used to make the chemical extract for use in the egg injection studies. The organic extracts will be used for the egg injection studies and will be representative of the complex mixture of organochlorine chemicals found in the fish at each location. The toxicity of each organic extract of fish will be determined from their ability to cause embryo toxicity in the subsequent portions of these experiments. Therefore, the second objective of the chemical exposure assessment portion of this work will be to characterize the extracts for PCBs, other dioxin-like chemicals, and organochlorine pesticides. Characterization of the fish egg dosing solutions prepared from the organic extracts will include quantification of congener PCBs, planar PCBs, chlorinated dioxins and furans, and organochlorine pesticides. The chemical analytical procedures that will be used are briefly described below.

Congener-specific PCB analysis for determination of PCB profiles in the field-collected fish from the study locations will proceed as follows. Sample preparation and analysis will generally follow the methods described by Schwartz and Stalling (1991). A 5-g portion from each sample will be dried with 20 g of anhydrous sodium sulfate (Merck, USA, 99 %) and ground. The samples will be homogenized with sodium sulfate and column extracted with CH_2Cl_2. A portion of each sample will be used to gravimetrically determine the lipid content and the remainder of each extract will then be treated by two stages of reactive column cleanup, followed by high-performance gel permeation chromatography. PCB congeners will be analyzed with a Hewlett-Packard 5890A Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a ^{63}Ni electron capture detector (ECD) and a Hewlett-Packard 7673 autosampler. The detector temperature will be 330°C and the injector set to follow the oven temperature. Injections will be 1-nL cool on-column onto a 30-m by 0.25-mm by 0.25-μm DB-5 capillary column (J & W, Folsom, CA, USA) with a 1-m x 0.53-mm deactivated retention gap connected to the column, with H_2 carrier gas maintained at 12 psig, linear velocity 60 cm/s. The oven temperature program will be as follows: 60°C, 10°C/min to 120°C, 2°C/min to 240°C, and then 10°C/min to 320°C with a 5-minute hold. Data will be collected with PC-based PE Nelson chromatography software (Perkin Elmer, Norwalk, CT, USA). Quantitation of approximately 100 PCB congeners will be based on an internal standard calibration.

Characterization of the chemical composition of the organic extracts will include chemical measurements of the OC pesticides, PCBs, PCDFs, and PCDDs found in the extracts (Feltz et al., 1995; Peterman et al., 1996). The determination of the exact amounts of dioxin-like chemicals in the fish composites and dosing solutions will proceed as follows. The raw fish composites will be homogenized, dried with sodium sulfate, spiked with the appropriate standards and column-

extraction with methylene chloride (Feltz et al., 1995). All the concentrated extracts will then be treated by a two-stage reactive cleanup; using first a sulfuric acid silica gel/potassium silicate column, and second, a column of sulfuric acid silica gel/potassium silicate/silica gel. High-pressure gel permeation chromatography (HP-GPC) cleanup will follow to remove residual lipids (Feltz et al., 1995).

The fish tissue extracts and aliquants of the final extracts will be fractionated using high-performance porous graphitic carbon chromatography (HP-PGC) into fractions containing: 1) bulk through mono-*ortho* chlorine substituted PCB congeners, 2) non-*ortho* chlorine substituted congeners, and 3) 2,3,7,8-PCDDs and PCDFs according to the procedures in Echols et al. (1997). The instrumental analysis for the determination of the congener-specific PCBs (fraction 1) will be as described above by GC/ECD. Non-ortho PCBs (fraction 2) will be analyzed by gas chromatography/high-resolution mass spectrometry (GC/HRMS) (Peterman et al. 1996). Finally, PCDD/PCDFs (fraction 3) will be eluted through basic alumina (according to CERC SOP C5.152) for removal of potential co-contaminants such as polychlorinated diphenyl ethers (PCDEs) and residual polychlorinated naphthalenes (PCNs) and PCBs. The instrumental internal standard, ^{13}C-labeled 1,2,3,4-PCDD, will be added to each semiconical autosampler vial prior to transferring the PCDDs/PCDFs (fraction 3). PCDFs and PCDDs will be determined by GC/HRMS by monitoring five sequential mass windows of selected ions during the chromatographic separation (according to CERC SOP C5.183 and Peterman et al. 1996). GC/HRMS analysis will be performed using a HP 5890A capillary gas chromatograph interfaced to a VG 70-250S high-resolution mass spectrometer. An HP 7673 autosampler will be used to introduce 2 of 25 nL of the enriched extract from a conical vial through a spiral uniliner onto a 5-m by 320-µm deactivated fused silica retention gap via a heated (285 °C) direct inlet. The analytes of interest will be separated on a 50-m by 200-µm by 0.11 µm Ultra-2 (Hewlett Packard) capillary column with an initial hold of 1 min at 120 °C followed by a ramp to 200 °C at 20 °C/min, another ramp to 300 °C at 2.3 °C/min, and a final hold of 5 mins. The He carrier gas is maintained at 44 psig with an initial linear velocity of 25 cm/s. All column-to-column connections were made using fused silica press-tight connectors.

The VG GC/HRMS system is tuned to 10,000 R.P. and calibrated using perfluoro-tetradecahydro-phenanthrene, and mass windows are established for five ion groups to measure Cl_{4-8} PCDFs and PCDDs. These windows are monitored sequentially during the temperature program. Within each mass window, the two most abundant ions are measured for positive identification and quantitation of each analyte. The ion responses are quantitated and averaged, unless interferences occur. Within each mass window, additional ions monitor any responses from Cl_{5-9}_PCDEs, Cl_{5-7}_terphenyls, Cl_{6-7}_PCNs, Cl_{3-8} dibenzothiophenes, and Cl_{3-8} phenanthrene/anthracenes.

Determination of non-ortho PCBs (planar PCBs) in fraction 2 above is conducted by GC/HRMS analysis and performed with a HP 5890A capillary gas chromatograph interfaced to a VG 70-250S high resolution mass spectrometer. An HP 7673 autosampler is used to introduce 2 nL of the enriched extract from a conical vial onto a 2.5 m x 530 µm deactivated fused silica retention gap via a cool on-column injection technique. A 50-m by 200-µm by 0.11-µm Ultra-1 capillary column (Hewlett-Packard's equivalent to DB-1) is used to resolve most non-*ortho*-PCBs from interferences. The GC oven is held at 120°C for 1 min, programmed to 240°C at 2.2°C/min, then ramped to 310°C at 5°C/min, for a final hold of 5 mins. Helium carrier gas is maintained at 48 psig with an initial

linear velocity of 25 cm/s. The analytical column is put into the MS interface, heated to 310°C. All column-to-column connections are made using fused silica press-tight connectors.

The VG GC/HRMS system is tuned to 10,000 R.P. and calibrated using perfluorodecalin, and mass windows are established for two groups of non-*ortho*-PCBs. Group 1 from 23-48:00 min included ions for Cl_4-biphenyls 77 and 81 and Cl_5-biphenyl 126; Group 2 from 48:05-65 min included ions for Cl_6-biphenyl 169. Within each mass window, the two most abundant ions are measured for positive identification and quantitation of each analyte. The ion responses are quantitated and averaged, unless interferences occur. Within each mass window, additional ions monitor the responses of higher chlorinated, potential interfering PCB congeners, Cl_{4-8} naphthalenes (PCNs), Cl_{3-5} terphenyls (PCTs), Br_5- and Cl_6-diphenyl ethers, and Cl_4-PCDF (to ensure no breakthrough of PCDFs).

The amount of each analyte detected is inherently self-corrected for losses through the whole analysis (extraction, isolation of analytes, and instrumental analysis). A calibration curve describing the response of each native congener to that of a isotope-labeled congener is used directly in the calculations and its range of values is determined in the calibration procedure. Concentrations of the native PCB congeners in standards ranged from 0.25 to 2,500 pg/nL. Each calibration curve is specifically matched to the range of analyte responses in the sample set.

3.6 HISTOLOGICAL EXAMINATIONS

Preparation of embryos for histological analysis will be performed according to procedures in Cantrell et al. (1998). Developmental stages will be chosen based on the embryology of the selected surrogate and representative species. Key stages for analysis will include the development of the vitelline vasculature, gill and digestive organs, and hatching of embryos from the chorionic membrane. Embryos collected from the incubation chambers will be assessed for the presence of a heartbeat, an intact pericardial sac, and circulating blood to assess viability. Embryos not meeting all criteria will be scored as nonviable and will not be used for histological analysis. Eggs and fry will be collected and preserved in buffered neutral formalin, washed in 10 mM HEPES (pH 7.4), and dehydrated by immersion in solutions containing ethanol from 50 to 100%. This is followed by immersion in xylene and infiltration with paraffin. The paraffin-embedded samples will be placed into plastic molding cassettes, sectioned into 10-µm sections, placed onto silanized slides, and stored at room temperature until analysis. For histochemical staining, tissue sections will be dewaxed and rehydrated by immersion in solutions containing decreasing concentrations of ethanol (100 to 0%). Gross morphological alterations (e.g., deformities and hemorrhaging) will be determined by hematoxylin/eosin staining at each developmental stage.

3.7 BIOCHEMICAL ANALYSIS

3.7.1 Apoptotic Cell Death in Embryonic Fish

Previous work in our lab demonstrated DNA degradation and morphological changes characteristic of apoptosis in cells of the embryonic vasculature in Japanese medaka exposed to 2,3,7,8-TCDD

(Cantrell et al., 1996, 1998). To determine if PCBs in Housatonic fish extracts exert toxic effects through a similar mechanism, apoptosis will be examined in the paraffin-embedded tissue sections prepared above. Sections will be dewaxed and rehydrated by immersion in solutions containing decreasing concentrations of ethanol (100 to 0%). The rehydrated tissue sections will be analyzed for the presence of apoptotic cells by using terminal transferase-based assay, which tags 3´OH DNA strand breaks with a fluorescein-conjugated antibody. The presence of numerous 3´OH DNA strand breaks is a hallmark of apoptosis (Compton 1992). Apoptosis detection will be accomplished using a commercial apoptosis detection kit (Oncor, Gaithersburg, MD). The rehydrated tissue sections will be washed in phosphate-buffered saline (PBS) and digested with 20 µg/mL solution of proteinase K. Slides will be washed in PBS and incubated with a digoxigenin-conjugated nucleotide in the presence of terminal deoxynucleotidyl transferase (TdT) followed by incubation with a fluorescein conjugated anti-digoxigenin antibody (Compton, 1992; Cantrell et al., 1998).

Image analysis will be performed using a Nikon inverted Diaphot-TMD microscope along with a PT/LPS-210/250 DC power supply and Xenon arc lamp. The fluorescein-based DNA labeling assay and the low-light amplification system allows for sensitive quantitative analysis of individual cells in the tissue sections of the embryos. To quantitate apoptotic cell death, the total number of apoptotic cells in a defined area will be counted from each image. This number will be expressed as a percentage of the total cells in the same defined area of tissue. Three separate embryos from at least two independent dosing experiments will be used to obtain a mean ± SD for each extract/standard dose.

3.7.2 Cytochrome P450 1A Induction in Embryonic Fish

The presence of compounds, such as PCBs, that can exert effects through the aryl hydrocarbon receptor (AhR) is commonly inferred from the level of cytochrome P450 1A induced following contaminant exposure. Immunodetection of induced cytochrome P450 1A in the paraffin-embedded tissue sections will be accomplished using an indirect peroxidase-labeling method. The tissue sections will be deparaffinated and hydrated in 1% bovine serum albumin/PBS containing 1% bovine serum albumin. The hydrated slides will be incubated in 0.5% H_2O_2 in methanol for 45 min to block endogenous peroxidase. The hydrated tissue sections will be immunochemically stained using monoclonal antibody (mAb) 1-12-3 made against scup P450E as the primary antibody (Park et al., 1986). The tissue samples will be observed for peroxidase staining (red-brown deposit) using light microscopy. Companion sections will be incubated with non-specific monoclonal immunoglobulin G2 (Smolowitz et al., 1991). All sections will be counterstained with Mayer's hematoxylin.

3.7.3 Adult Fish

The extent and nature of contaminant exposure in fish from the areas of the Housatonic River will be assessed through analytical measurements (OCs, PCBs, PCDDs, and PCDFs as in previous phases of this project). As an additional line of evidence, biological indicators of contaminant exposure including ethoxyresorufin-*O*-deethlyase induction (EROD) will be assessed in adult fish. Physiological and biochemical measures of effect in these fish may be assessed through the measurement of plasma concentrations of estrogen and testosterone, observation of the ratios of these

steroid hormones (E/T ratios), and plasma concentrations of vitellogenin. The appropriate selection of endpoints in the field studies will allow the characterization of any causal linkages among contaminant exposure and adverse effects through a comparison with the laboratory-based results of these studies.

3.7.4 7-Ethoxyresorufin-O-deethylase Induction

Induction of cytochrome P450 1A in adult hepatic tissue will be inferred from the catalytic activity of 7-ethoxyresorufin-*O*-deethylase (EROD). This assay is based on the work of Pohl and Fouts (1980). Preparation of hepatic microsomes and EROD analysis will be performed according to CERC SOPs P.123 and P.124. Microsomal preparation involves homogenization of livers in cold phosphate buffer (pH 7.4) with two passes of a hand-held tissuemizer (Omni International, Warrenton, VA). After centrifugation for 25 min at 9000 g and 4°C, the supernatant fraction will be centrifuged at 105,000 g for 50 min at 4°C. Microsomal pellets will be resuspended in phosphate buffer and then recentrifuged at 105,000 g for 50 min. The resulting pellet will be resuspended in phosphate buffer. The EROD assay will be performed on the same day as the microsomal preparation.

Enzymatic activity of CYP1A in triplicate samples per fish will be measured as the conversion of 7-ethoxyresorufin to resorufin (EROD) (Pohl and Fouts, 1980). Microsomal samples (5 nL) added to 96-well microtitre plates will be mixed with 50 nL of 10 µM ethoxyresorufin, 50 nL of 4.3 mM NADPH and 50 nL of phosphate buffer (all reagents at 25°C). Plates are incubated for 10 min at 25°C and then scanned on a Cytofluor 2300 plate reading fluorometer (Perseptive Biosystems, Framingham, MA) with the following settings: emission filter (590 nm), excitation filter (530 nm), sensitivity 3, and 10 scans at a scan cycle of 60. All resorufin concentrations will be calculated based on a resorufin standard curve. Immediately following the EROD scan, protein content of the mixture will be determined fluorometrically using the method of Kennedy et al. (1994).

3.7.5 Steroid Hormone Analysis

Planar halogenated hydrocarbons can affect natural steroid hormone levels, potentially affecting reproduction in fish (Munkittrick et al., 1992). To determine if PCBs in the Housatonic are interfering with steroid hormone regulation, we will measure levels of 17ß-estradiol and testosterone in plasma from fish collected at the four studies sites. The radioimmunoassay (RIA) technique described in Van der Kraak et al. (1984) will be used. Briefly, plasma samples (50 nL) will be combined with 1-3 mL of phosphate buffer (0.05 M, pH 7.6, containing 0.1% gelatin) and heated at 70°C for 1 hour. 17ß-Estradiol and testosterone will be measured using rabbit anti-17ß-estradiol and anti-testosterone serum. Diluted, heated plasma or appropriate standards (200 nL each) will be combined with 200 nL of tritiated estradiol or testosterone and 200 nL of diluted antiserum. Samples are then incubated for 16 to 20 h at room temperature, cooled on ice for 15 min prior to the addition of 200 nL of phosphate buffer containing 0.5% Norit A charcoal and 0.05% Dextran T-70. Samples will then be incubated 10 min on ice followed by a 10-min centrifugation at 4,000 rpm and 4°C. The resulting supernatant will be poured directly into scintillation vials and combined with 6 mL of scintillation fluid for counting. The antibody concentrations in the RIA procedure will be adjusted

so that 45 to 55% of the radiolabeled steroids will be bound in the absence of competitor. All plasma samples will be analyzed in duplicate.

3.7.6 Vitellogenin Determination

To ascertain the presence of estrogenic contaminants in the Housatonic river, induction of vitellogenin synthesis in male fish will be compared to that in females. Since it is the major source of protein-bound phosphate in fish plasma, the amount of vitellogenin can be quantified accurately using a phosphoprotein-phosphate assay (Ng and Idler, 1983). Plasma vitellogenin will be estimated in duplicate plasma samples as described in Mount et al. (1988). Five mL of cold 10% trichloroacetic acid (TCA) will be added to 20-40 nL of undiluted plasma in a 12-mm by 75-mm glass test tube and incubated overnight at 4°C to precipitate plasma proteins. After 24 h, tubes will be centrifuged at 2,000 g for 10 min and the supernatant discarded. To remove lipids, the pellet will then be washed twice with 2 mL of acetone followed by centrifugation. After the second wash, the pellet will be blown to dryness under air. The dried pellet will be dissolved in 1 mL of 2 N NaOH and incubated in water at 90°C for 15 min, liberating protein-bound phosphate. Tubes will then be cooled in an ice bath and neutralized with 167 nL of concentrated HCl. Four mL of cold 10% TCA will be added and incubated overnight at 4°C to precipitate any remaining proteins. Tubes will then be centrifuged and the supernatant decanted into 13-mL by 150-mL tubes. Duplicate standards of 0, 1, 2, 3, 4, 5, and 6 mg PO4/L in 5 mL TCA will be made and 5 mL of 3% (v/v) HCl will be added to both standards and samples. Phosphate content is then determined colorimetrically by reaction with ammonium molybdate.

3.8 DATA ANALYSIS

Mortality and gross pathologies exhibited during the egg and sac-fry stages of development will be recorded and evaluated for contaminant-related increases by chi-square analysis (Snedecor and Cochran, 1980). Data that exhibit treatment-related increases will be used to generate continuous dose-response curves and 95% fiducial limits using a probit procedure (SAS, 1988). The probit procedure corrects for control mortality analogous to using Abbott's formula. In addition, this procedure uses chi-square goodness of fit, estimates the slope and intercept, and is based on the assumption that mortality/effect is independent for fish within a dose group and among dose groups.

The slopes for the apoptosis-response curves will be compared with the mortality-response curves as described in Cantrell et al. (1998). Determination of statistical differences in slopes will be accomplished using analysis of covariance with interaction of the data curves (SAS 1988). Confidence level will be set to 95% (type I error set at 5% or $p < 0.05$).

Individual dose groups for egg injections will be tested against controls using ANOVA and a multiple post-hoc comparison, the least-significant difference (LSD) test. This method will also be used to ascertain differences among sites for EROD induction, vitellogenin synthesis, and steroid hormone levels in adult fish from the Housatonic. Relationships between measured parameters will be determined by linear regression and Pearson's pairwise correlations. Significance levels are set at $p \leq 0.05$.

The statistical analysis of the analytical chemistry data will consist of mean concentration comparisons among sites. Concentrations of total PCBs and TEQs generated from the analytical chemistry and an additive model of toxicity will be compared among sites by ANOVA (SAS, 1988) if assumptions of normality and homogeneity of variance are met, or Kruskul-Wallace one-way analysis of variance (Snedecor and Cochran, 1980) if assumptions are not met.

4. QUALITY ASSURANCE /QUALITY CONTROL

4.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

4.1.1 Data Quality Objectives

The data quality objectives for the proposed study are: 1) to ensure that the analytical measurements, biological/toxicological assays, and biochemical analyses are accurate and precise measurements of the samples collected in the field or laboratory portions of the study, and 2) to ensure that the potency of the mixtures measured in these toxicological tests of embryo development are reflective of the toxicity expected to be observed in feral fish.

To meet these objectives, a quality assurance plan has been designed whose general approach includes:

- Replication of various stages of the study,
- Comparison and calibration of analytical results against known standards,
- Proper maintenance and calibration of equipment,
- Accurate sample tracking and custody,
- Proper documentation at all steps of sample processing,
- Other considerations of Good Laboratory Practice (GLP).

The specific aspects of the QA Plan for this study are given below.

4.1.1.1 Fish Sampling Procedures

Brood fish will be collected from three study locations on the Housatonic River that are expected to be representative of the PCB exposure of fish throughout the study area. A reference location also will be selected that is expected to be representative of the background PCB levels in the Housatonic River watershed. The collection of brood fish will be performed by the US Fish and Wildlife Service according to the fish collection standard operating procedure (SOP) provided in Appendix A.20. The quality assurance procedures for the collection, holding and shipping of brood stock fish are further supplemented in CERC's *Biomonitoring of Environmental Status and Trends (BEST) Program: Field Procedures for Assessing the Exposure of Fish to Environmental Contaminants* (CERC SOP P.326 [Schmitt et al., 1999]).

4.1.1.2 Fish Processing and Preservation Procedures

Fish collected but not used as brood stock will be processed in accordance with the protocols set forth in CERC SOP P.326. All fish (brood stock and fish collected but not used as brood stock) carcasses will be logged in a centralized sample tracking system and stored at –20 ºC until extracted and analyzed. Sample tracking forms will be completed and retained with the samples to provide exact information on the samples. These forms are the Sample Inventory Listing form and the Sample Batch History Information form (both forms can be found in CERC SOP P. 326 - Appendix 1).

The Sample Inventory Listing form is a list of all the samples to be transmitted and has the sample label (identifier with year, project #, study #, and personal sample ID #) and a brief description of each sample. The Sample Batch History Information form contains information on sample collection dates and location, how samples were collected, where they were collected, how they were preserved, sample transmission dates and modes, and other pertinent information about the samples and how they have been handled.

All samples will be given an independent identification number for internal tracking and all of the information is computerized on a central sample tracking system. A complete description of the sample tracking system is provided in CERC SOP P. 326 - Appendix 1.

4.1.1.3 Extraction and Analytical Procedures

The matrices for analysis include whole fish tissue and extracts of fish tissue. Detailed methods for the extraction and subsequent chemical analysis to be conducted by CERC laboratory on fish tissue and fish tissue extracts and their respective SOPs include:

Lipid analysis	*Extraction of Animal Tissues for Residue Analysis* (CERC SOP P. 461)
Organochlorine pesticide analysis	*Organochlorine Pesticide Analysis: Fractionation of Complex Mixtures on Silica Gel/ODS* (CERC SOP P. 460)
Total & congener-specific PCB analysis	*Capillary Gas Chromotography with Electron Capture Detection Procedure for Congener Specific Polychlorinated Biphenyl Analysis* (CERC SOP P.195)
Non-ortho PCB analysis	*Analysis of Selected Non-O-Chloro-substituted Polychlorinated Biphenyls by Gas Chromotography – High Resolution Mass Spectrometry* (CERC SOP P. 481)
Chlorinated dioxin and furan analysis	*Analysis of Tetra- through Octa- Substituted Polychlorinated Dibenzo-p-dioxins and Dibenzofurans by Gas Chromotography – High Resolution Mass Spectrometry* (CERC SOP P. 482).

Ethoxyresurofin-O-deethylase (EROD) Analysis	*Microsomal Preparation of Liver Tissue* (CERC SOP P. 123).
	Procedure for the determination of 7-Ethoxyresurofin-O Deethylase (EROD) Activity on Microsomes from Liver Tissue Using 96-Well Microtiter Plates (CERC SOP P. 124)

Analyses will be performed by GC/ECD and/or GC/MS. Method limits of detection will be 1-5.0 ppt (pg/g) for dioxins and furans and <1 ppb (ng/g) for the PCB congeners. These detection limits were selected to ensure the detection of chemicals at concentrations that may cause adverse effects.

QA/QC procedures for the analyses presented above will include at a minimum, analyses of spiked samples with appropriate standards, analysis of replicates, analysis of procedural and matrix blanks, and the demonstration of correct chemical identifications. The general QA procedures for chemical analyses that will be followed by CERC for this project are provided in CERC's *Minimum Quality Assurance Standards for Trace Organic Residue Analysis.*

4.2 DATA QUALITY INDICATORS

Data developed in the Fish Reproductive Health Assessment must meet standards of precision, accuracy, completeness, representativeness, comparability and sensitivity that are appropriate to the data quality objectives. Each of these data quality indicators is discussed below.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. Precise measurements of the various chemical analyses, as well as measures of effect are crucial to the interpretation of study results. To ensure adequate precision, replication and subsequent performance checks are performed throughout a number of stages of this study. Quality control considerations to ensure precision of chemical analyses will follow those performance criteria outlined in each of the aforementioned CERC SOPs. For measuring effects in the offspring development phase of the study, the study design includes an increase in the number of replicates to increase the statistical power. For offspring development phase of the study, each spawning event will be split into six replicates and each replicate will contain 50 to 100 eggs. For the egg injection phase, the performance criteria for precision will include the replication of each delivered dose (3 replicates/dose level) and a targeted number of individuals in each replicate (20 eggs/replicate). Each chemical or chemical mixture (extract) will be tested at five separate doses.

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study, accuracy is defined as meaning that the chemical and biochemical analyses represent the true measure of that chemical and that the effects are correctly characterized and measured. To ensure accuracy of analytical results, calibration of equipment and calibration of chemical results against known standards will be performed throughout the performance of the study. Quality control considerations to ensure accuracy of chemical and biochemical analyses will follow those performance criteria outlined in each of the aforementioned CERC SOPs. To ensure accuracy of effects measurements, QA procedures include replication, the use of "sham" doses, as well as

vehicle controls. Moreover the egg injection phase will incorporate surrogate species for which a historical database will provide a measure of comparison. In addition, all effects monitoring will be directed in the laboratory by senior scientists. Technical support will include only those staff that are properly trained to ensure the collection of quality data. All equipment used in these studies is routinely inspected and preventive maintenance is performed. A logbook is kept for each instrument to document its use, performance, and maintenance

Completeness is defined as the percentage of the planned samples actually collected and processed. Completeness can be evaluated for all components of the Fish Reproductive Health Assessment. An accurate record of information on sample transmittal, receipt and inventory will be ensured by following CERCs SOP C5. 162. Completeness also refers to the percentage of the planned number of bioassays and bioassay design elements (e.g. proposed doses) that have been proposed in the study design. Completeness of this information will be ensured by the recording of all procedures and results in the study notebooks. All completeness goals will be as defined in the project QAPP (WESTON, 2000).

Representativeness refers to the degree to which the data accurately reflect the characteristics present at the sampling location at the time of the sampling. Representativeness for this study is ensured through the establishment of an approved sampling design and through careful implementation of the sample processing, handling, and maintenance measures as well as the consistent use of standardized chemical and biochemical methods. The use of chemical extracts provides a direct measure of embryo toxicity to exact chemical mixtures found in the fish in the Housatonic River. Additional aspects of representativeness will be evaluated by comparing the study results with known and/or expected results from previous studies of the chemicals and chemical mixtures being evaluated.

Comparability is a measure of the confidence with which comparisons of data within and among stations can be made. Comparability will be evaluated by examining the in-station variability as well as the dose-response variability in key measurements as determined from the large numbers of replicates that will be monitored relative to fish species type, the location from which those fish were harvested and the number of laboratory treatments. Comparability will also be evaluated for this data through comparison with known and/or expected results from previous studies of the chemicals and chemical mixtures.

4.3 DATA VALIDATION, VERIFICATION, AND USABILITY

Validation and verification of chemical and biochemical analyses data will follow the general procedures of validation and verification outlined in CERC's *Minimum Quality Assurance Standards for Trace Organic Residue Analysis*. Specific procedures for validation, verification, and usability of chemical and biochemical analyses are presented in each of the CERC SOPs (referred to previously) and will be used wherever applicable in this study.

Data on the measurement effects (assay endpoints) will be collected and reported as specified by the appropriate SOP (e.g., CERC P.326). These data will be review and verified by the senior investigator who will judge the data against data quality indicators appropriate to that data. In many

cases, the senior investigator will use his professional judgment in the determination of data usability.

All experimental information will be recorded on datasheets specified in the various CERC SOPs, signed, and copies maintained in a separate secured area. Instrument printouts and computerized data tables are uniquely labeled and cross-referenced to the project notebook as appropriate. The accuracy of all such measurements will be independently checked. Copies of the computerized data files are maintained in a project notebook and file, on floppy disk in the project file and by archived tape back up.

4.4 QA AUDITS

Internal audits are continuously performed by the Principal Investigators with routine checks by independent CERC personnel.

4.5 CORRECTIVE ACTION

Problems will be identified as they occur or through weekly staff meetings. Remedial actions will be taken as deemed appropriate and in accordance with the QA performance criteria. All such problems and corrective actions will be recorded in the project notebook(s) and reported to management or the Project Officer, if necessary.

4.6 DATA ANALYSIS AND REPORTING

The overall analytical approach for data generated by this study is described in the previous section "Data Analysis." Routine analyses will be performed and an allowance for Type I errors will be set at 5% ($\propto = 0.05$). Outliers will be determined as described by Gill (1978). Reporting of the data will initially be in draft form to the appropriate collaborators. Following review and approval, an internal review of the draft report will be made and a final report sent to the Project Officer. The study findings will be incorporated in the ecological risk assessment including all data, analyses, and interpretation and will be prepared with specific reference to both the data quality objectives specific to the fish reproductive health assessment and CERC SOP P.326 *Biomonitoring of Environmental Status and Trends (BEST) Programs: Field Procedures for Assessing the Exposure of Fish to Environmental Contaminants.*

4.7 EXPECTED BENEFITS

Results from the proposed research will significantly contribute to the risk assessment of the Housatonic River through: (1) characterization of the PCB exposure to fish in the four selected areas; (2) development of dose-response models for fish embryotoxicity of the mixture of chemicals present in fish from the study areas; (3) confirmation and validation of the dose-response models with fish eggs from the Housatonic River; and (4) elucidation and evaluation of any causal relationship for PCB-related effects on fish health in the Housatonic River.

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APPENDIX A.22

FIELD SAMPLING AND ANALYSIS PLAN FOR SOIL INVERTEBRATES

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APPENDIX A.22

FIELD SAMPLING AND ANALYSIS PLAN FOR SOIL INVERTEBRATES

1. INTRODUCTION

1.1 BACKGROUND

Soil invertebrates, particularly earthworms, have proven to be useful indicators of the environmental effects of contaminants, including PCBs (Edwards and Thompson, 1973; Goats and Edwards, 1988; Fitzpatrick et al., 1992; and Beyer and Stafford, 1993). Being in nearly constant contact with the soil, soil invertebrates are continually exposed to soil contamination. In addition, they account for the majority of animal biomass in soil, and are preyed upon by a variety of secondary consumers. Thus, soil invertebrates form a pathway by which soil contamination may be passed on to receptors such as short-tailed shrews (*Blarina brevicauda*), American robin (*Turdus migratorius*), and American woodcock (*Scolopax minor*) that rely on earthworms for a major portion of their diet.

For the purposes of this Work Plan, soil invertebrates are divided into two separate groups of interest based on their availability to receptors and their degree of exposure to contaminated soils. These two groups consist of: (1) those invertebrates living in the soil itself, as represented by earthworms, and (2) those living primarily in the litter or detritus layer, as represented by adult beetles and other arthropods, hereinafter referred to as litter invertebrates.

Invertebrates will not be sampled from the surface of terrestrial vegetation, since these invertebrates form a relatively small proportion of the diet of American robins and short-tailed shrews. For example, Whittaker and Ferraro (1963) reported that summer short-tailed shrew diets consisted of only 4.3% lepidopteran larvae (found primarily on vegetation), while earthworms, slugs, and snails (found in litter or soil) accounted for over 58.5% of the diet.

Robins may forage on invertebrates in terrestrial vegetation to a larger degree than shrews or woodcock, but these invertebrates are not a dominant item in their diet. For example, Howell (1942) found that lepidopteran larvae accounted for less than 25% of the robin's summer diet based upon stomach content data. In addition, the large proportion of earthworms and similar soft-bodied ground-dwelling invertebrates that robins ingest are likely to be greatly underrepresented in stomach content data because these invertebrates are easily digested (Wheelwright, 1986).

1.2 OBJECTIVES

The principal objective of this study is to collect representative soil invertebrate samples for the Lower Housatonic River Supplemental Investigation (SI) for analysis of PCBs, dioxins/furans, and organochlorine (OC) pesticide concentrations in tissue. Results will be used in the ecological risk assessment to model exposure through the food chain of higher trophic level consumers such

as robins, woodcock, and shrews. In addition, the results of tissue analyses and co-occurring soil analyses will be used to determine the relationship between earthworm tissue concentrations and corresponding soil concentrations.

2. STUDY DESIGN

2.1 FIELD SAMPLING DESIGN

2.1.1 Sampling Locations

Soil invertebrates will be collected at three different sampling areas corresponding to where small mammals were collected, including a reference site with soil and habitat characteristics similar to the contaminated sites.

The total PCB concentration ranges of the three sites are:

- < 1 mg/kg (reference location)
- 1 mg/kg to 30 mg/kg
- > 30 mg/kg

All three sampling areas represent suitable habitat for potential ecological receptors (e.g., robins, shrews, woodcock) for which risk will be assessed. Soil chemistry data are also available from these areas, and will be used to select specific collecting locations. In selecting sampling locations for soil invertebrates at each site, particular weight will be given to prior analytical results from surficial floodplain soils (0 to 6 inches below ground surface [bgs]), since soil invertebrates feeding within this zone are most likely to be preyed upon by the ecological receptors that may be modeled.

2.1.2 Number of Samples

A target goal of 30 earthworm samples and 9 litter invertebrate samples is proposed for tissue analysis. Ten earthworm samples (individual worms or, if necessary, composites) and three composite samples of litter invertebrates will be collected in each of the three sampling areas.

The number of proposed samples for earthworms is based primarily upon a power analysis (Attachment 1) conducted after a literature review. The power analysis focused on variability in tissue uptake in depurated worms, since that was the most applicable study found in the literature. In this investigation, however, the worms will not be depurated since their consumers (e.g., robins and shrews) ingest the entire worm. The number of samples for litter invertebrates is based on the anticipated difficulty in collecting sufficient biomass for tissue analysis.

2.1.3 Collection Methods

2.1.3.1 Field Reconnaissance/Pilot Study

A field reconnaissance and brief pilot study will be conducted in the spring of 2000 in order to refine the proposed study design. The primary objectives of the field reconnaissance are to:

- Determine the sampling plot size that will provide sufficient biomass for both earthworm and litter invertebrate tissue analysis.
- Determine the dominant species of earthworms present in different parts of the study area. (If possible, only the single dominant species of earthworm in the study area will be sampled and analyzed, in order to minimize potential interspecific variation in PCB uptake or alimentary tract content).
- Evaluate and confirm the usefulness of the different soil invertebrate sampling methods proposed.
- Evaluate plot locations based on existing surface soil PCB concentrations.

2.1.3.2 Plot Selection and Soil PCB Screening

Sample plots will be established within each of the three sampling areas to be compared. A pilot study using an initial plot size of 1 m^2 will be conducted to determine the size of plots required for the collection of sufficient tissue mass for chemical analysis of earthworms. The plot size needed to obtain a minimum of 10 g (wet weight) of earthworm tissue per plot will be determined. Individual plots will be selected on the basis of considerations identified in Subsection 2.1.1.

In addition, one set of drift fences with pit traps will be constructed in each of the three sample areas. These traps will be used to determine the sampling effort required to obtain at least 10 g of litter invertebrates. Pit traps will be constructed from No. 10, or similar, metal cans paired inside and outside the drift fence. Pit traps will be checked daily and captured individuals placed in resealable plastic bags labeled with the litter invertebrate sample plot number and transported to a central processing area.

Approximately three composite soil samples (0 to 6 inches bgs) will be collected from each potential sampling area to ensure that plot locations are representative of the desired range of soil PCB contamination. Surface soils will be screened for total PCBs. Once identified, plots and pit traps will be marked with pin flags, labeled with a plot number, and the location surveyed using global positioning system (GPS) equipment.

Pilot plots will be sampled for earthworms by excavating and sorting through soil collected to a depth of approximately 0.5 ft until at least 10 g (wet weight) of tissue mass is obtained. This information will be used to determine the appropriate plot size for earthworm sampling. The earthworm samples will be placed in resealable plastic bags labeled with the sample plot number and transported to a central processing area (see Subsection 2.1.4.1) for taxonomic identification.

2.1.3.3 Sample Plot and Pit Trap Locations

Using the results of the pilot study to determine plot size, 10 sample plots and three sets of drift fences with pit traps will be established in each sampling area. Plots will be marked with pin flags, labeled with a plot number, and the location surveyed using GPS equipment. Pit traps will be placed at select locations in each sampling area and located using GPS.

2.1.3.4 Soil Invertebrate and Soil Sampling

All sampling will be conducted in spring 2000. It is best to conduct this sampling during spring, since that is when invertebrates form the majority of the diet of the American robin. Other potential receptors such as woodcock and shrews forage on invertebrates from spring through fall.

The two methods discussed in Subsection 2.1.3.2 will be used to sample for soil invertebrates. One involves the collection of earthworms by removing and sorting through soil collected to a depth of approximately 0.5 ft. The second involves the establishment of pit traps and drift nets to collect litter invertebrates. Litter invertebrates collected daily from pit traps will be combined as necessary to meet the minimum sample size requirements for tissue analysis. Litter invertebrates will be collected from sample plots only if less than 10 g of litter invertebrates are collected from a pitfall trap.

Individual sampling plots will be sampled for earthworms. Surface litter and detritus from the sample plots will only be collected and sorted if less than 10 g of litter invertebrates are collected from a pit trap.

Each plot that is established will be sampled for earthworms according to the following approach. After the plot is delineated with pin flags, soil samples will be collected at three locations in the plot, minimizing any disturbance to the litter layer. The soil collected will be homogenized in a dedicated stainless-steel pan, and apportioned into appropriate laboratory glassware labeled by plot number. The soil will be submitted for chemistry analysis to confirm the PCB concentration range in the plot.

When it is time to sample the plot for earthworms, all surface litter and detritus will be removed and placed into one or more decontaminated 5-gallon buckets labeled with the plot location if less than 10 g of litter invertebrates have been collected from an adjacent pit trap. After litter removal, the 5-gallon buckets will be covered with cheesecloth held in place by a large rubber band. The buckets will be transported to a refrigerator in a central processing area. Earthworms will be collected by removing soil from the plot to a depth of approximately 6 inches bgs using a decontaminated shovel, and removing earthworms by hand. If necessary, worms will also be screened from the soil through standard 1/8- to 1/4-inch mesh sieves or through a larger decontaminated screen constructed from 2-inch by 4-inch lumber and 1/4-inch hardware cloth. The earthworm samples will be placed in resealable plastic bags labeled with the sample plot number and transported to a central processing area for processing and taxonomic identification (see Subsection 2.1.4.1).

If sufficient numbers of worms are not located by digging and soil conditions appear suitable, a mustard-based extraction technique may be used (Stair et al., 1995). A mustard/water suspension will be applied to each plot and surfacing earthworms will be collected by hand. The earthworm samples will be placed in resealable plastic bags labeled with the sample plot number and transported to a central processing area for processing and taxonomic identification (see Subsection 2.1.4.1).

Earthworm collection at each plot will continue until at least 10 g (wet weight) of tissue mass is collected, preferably of the same species of earthworm. If sufficient earthworm populations are still not found at a location, the professional judgment of field personnel will be used to determine if sampling at a location should be suspended or if a reduced sample volume will be collected (the analytical laboratory is able to analyze as little as 0.1 g of tissue, but with somewhat higher detection limits). Additional earthworm samples will be collected from plots for duplicate and matrix spike/matrix spike duplicate (MS/MSD) samples.

The primary sampling method for litter invertebrates will be pit traps, supplemented by hand sampling as necessary. Pit traps and drift nets will be installed adjacent to soil invertebrate sampling plots and checked daily over a 1-month period until at least 10 g of tissue mass (wet weight) is collected, plus any additional sample required for duplicate and MS/MSD samples. Invertebrates collected from the traps will be placed in resealable plastic bags labeled with the litter invertebrate sampling plot number and transported to a central processing area. Processing procedures are described in Subsection 2.1.4.

2.1.4 Invertebrate Field Sample Processing

2.1.4.1 Earthworm Sample Processing

Earthworms will not be depurated prior to processing. Specific processing steps are described in Subsection 3.2.1.

Earthworm processing will consist of:

1. Rinsing the worms with distilled water.
2. Segregating the worms into adult and juvenile age classes.
3. Identifying and segregating the species of the worms.
4. Weighing the worms and recording their individual and combined composite sample weights.
5. Freezing the worms pending shipment to the analytical laboratory.

Each sample will be placed in aluminum foil that has been rinsed with hexane and air dried (dull side toward the sample) and then labeled by plot number. Samples will be frozen in resealable plastic bags at -10°C until they are shipped to the analytical laboratory.

If possible, a single species and size class composite will be submitted for analysis. Efforts will be made to match the composition of any reference samples collected with those collected in contaminated areas. The age and size distribution and species of earthworms collected will be recorded for each composite sample collected.

A representative sample of individuals (i.e., voucher specimens) will also be retained in isopropyl alcohol for subsequent species identification.

2.1.4.2 Litter Invertebrate Sample Processing

Litter invertebrate samples retained for analysis will be processed as follows. Invertebrates collected from pit traps will be taxonomically identified to order, then weighed, returned to the plastic bag, and held in a freezer at -20° to -30°C until shipment. Samples collected from ensuing weeks will be added to the same bag after taxonomic identification and weighing. Cumulative sample weights will be recorded on sample data sheets; before shipment the final sample weight will be recorded to ensure sufficient tissue mass is available for analysis. If necessary, the sample will be supplemented with invertebrates collected from the leaf litter of the closest sampling plot to form a composite sample.

Soil invertebrates collected from litter and detritus will be processed as follows. The litter and detritus will be hand-picked until at least 10 g of invertebrate tissue is collected. No effort will be made to remove all invertebrates; rather, sampling will be biased toward larger food items since these are most likely to be taken by shrews (Churchfield, 1990) and robins (Howell, 1942). Invertebrates within these samples will be taxonomically identified before the samples are snap frozen in petri dishes, placed in aluminum foil that has been rinsed with hexane and air dried (dull side toward the sample), and bagged in a labeled resealable plastic bag.

2.2 ANALYSES

Each composite earthworm and composite litter invertebrate sample will be submitted to a contract laboratory for analysis of PCBs (total, Aroclors, congeners, and homologs), percent lipids, and percent moisture. A subset of the samples (at least one per location) will also be analyzed for dioxin/furans and select OC pesticides.

Composite soil samples will be analyzed for PCBs (total and Aroclors), total organic carbon (TOC), and grain size. A subset of all composite samples (at least one per group per location) will also be analyzed for dioxin/furans and Appendix IX OC pesticides.

The analytical results will be used in the ecological risk assessment to model exposure to higher consumers such as robins, woodcock, and shrews. The results of tissues analyses and co-occurring soil analyses will be used to determine earthworm concentration factors.

2.3 QUALITY ASSURANCE/QUALITY CONTROL SAMPLES

Duplicate analyses will be conducted for each parameter on 5% of the earthworm and litter invertebrate samples. Duplicate samples will be collected from the sample plot location as the

original sample; an additional 10 g of tissue will be required for each set of analyses beyond the 10 g required for the original analyses.

In addition, a matrix spike/matrix spike duplicate (MS/MSD) sample is required for every 20 samples. An additional 20 g of tissue will be required for each set of MS/MSD analyses, beyond the 10 g required for the original analyses.

3. QUALITY ASSURANCE/QUALITY CONTROL

3.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

3.1.1 DATA QUALITY OBJECTIVES

The two primary data quality objectives of the soil invertebrate evaluation are outlined in Subsection 1.2. To achieve these objectives, the following types of data and specific quality criteria will be required:

- Taxonomic identification of earthworms to LPIL (lowest practical identification level): earthworms must be identified to the species level whenever possible. When identification to the species level is not possible, the LPIL will be consistent with standard practice for invertebrate taxonomy. Of equal importance is that the level of taxonomy is consistent for all samples. Other soil invertebrates will be identified to the level of Order.

- Biomass (wet weight) for each taxon or larger taxonomic group: Biomass must be determined accurately and recorded to 1 mg (.001 g) using a calibrated balance designed and intended by the manufacturer to be capable of accurately measuring masses of this magnitude. Accurate determination of biomass is also partly determined by following the field sampling methodologies discussed above.

- Soil chemistry for PCBs and selected other contaminants: Analysis of soil for chemical constituents must result in data that are consistent in all respects with other sediment/soil contaminant data collected as part of the project. Satisfactory results will be ensured by submitting samples to the same laboratories that are analyzing samples for other components of the program. Quality control specifications for these data are delineated in the project QAPP (WESTON, 2000).

- Soil grain size distribution: Quality control considerations to ensure achievement of DQOs for this parameter will follow the QAPP.

- Tissue residue concentrations for PCBs and other contaminants of soil invertebrate samples: Quality control considerations to ensure achievement of DQOs for this parameter will follow the QAPP.

3.1.2 Data Quality Indicators

Data developed in the soil invertebrate study must meet standards of precision, accuracy, completeness, representativeness, comparability and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000), that are appropriate to the data quality objectives. Each of these data quality indicators, some of which are not readily quantifiable for soil invertebrate data, is discussed below.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. Because of the small-scale spatial heterogeneity inherent in soil invertebrate communities, it is not possible to take repeated independent measurements of the biological parameters. Rather than control and measure precision, the study design includes an increase in the number of replicates to increase the statistical resolution; for this study the large number of replicates (10) is used for earthworms in this manner. Precision may also be evaluated by assessment of the degree to which sample collection procedures are able to ensure collection of consistent sample volumes. For the measurements that are not unique to the soil invertebrate study, such as soil chemistry and grain size, precision is evaluated as defined in the QAPP.

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study (soil invertebrate taxonomy and biomass), accuracy is defined as meaning that the taxa are correctly represented and identified in each sample, and correctly weighed. The data generated by this study will also be evaluated for accuracy via comparison with known and/or expected results from similar studies conducted in the Housatonic River area or in similar New England systems. For parameters such as soil contaminants and area grain size, accuracy is as defined in the QAPP.

Completeness is defined as the percentage of the planned samples actually collected and processed. Completeness can be evaluated for all components of the soil invertebrate program. To ensure achieving the planned statistical resolution, it is important that completeness of 100% be achieved for all components of this study with the exception of the tissue residue analyses. For this latter study component, the number of analyses will be determined by the material available for collection; therefore, establishment of an *a priori* completeness goal is not possible.

Representativeness refers to the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. Representativeness for this study is ensured through establishment of an approved sampling design and through careful implementation of the sample processing and analytical methods. Specific aspects of representativeness will also be evaluated via comparison with known and/or expected results based on previous investigations of the Lower Housatonic River area and other similar systems.

Comparability is a measure of the confidence with which the soil invertebrate data may be compared to another similar data set. Comparability will be evaluated by examination of the variability in key parameters as determined from the large numbers of samples to be collected at each sample site.

Sensitivity, the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest, is related for soil invertebrate investigations to the ability of the taxonomic analysis to resolve the various soil invertebrates into individual species and/or

orders. Sensitivity is applicable and important for the chemistry parameters that will be analyzed as part of the soil invertebrate study. For these parameters, the detection limits for chemistry and grain-size parameters specified in the QAPP will provide appropriate sensitivity for the purpose of providing insight into factors controlling abundance and distribution of the soil invertebrate taxa and populations.

3.1.3 Data Validation, Verification, and Usability

Procedures for data validation for the chemical and physical data are discussed in various sections of the project QAPP and will be used whenever applicable in this study. For the biological data, usability will be largely be determined by three factors: (1) the experience of the senior investigator in establishing that the field sampling was conducted following the SOP and that accuracy and precision were not compromised by an inability to control the sampling procedures in the field; (2) an evaluation of the taxonomic data both within the study and compared with previous studies in the Housatonic River and in the New England area; and (3) a direct comparison between the chemistry and grain-size data and similar data developed from co-located samples that have been collected as part of other project components.

The purpose of the remainder of this section of the study plan is to document the measures included in the study to ensure that the standards discussed above are met.

3.2 SAMPLING DESIGN

The rationale for selection of the three locations to be sampled in the soil invertebrate study is presented in Subsection 2.1.1. The locations are not intended to be representative of the entire area but rather are intended to encompass a range of sediment PCB concentrations typical of the area; one of the locations with near-background PCB levels will be used as a reference.

Soil invertebrate community data are typically highly variable in nature. To achieve acceptable statistical resolution for earthworms it is necessary to collect large numbers of samples from each sampling site. Data will be collected from 10 samples at each of 3 sampling sites. This number of replicates was selected based on power analyses for this sampling plan (Attachment 1).

3.3 SAMPLING METHODOLOGY

3.3.1 Sampling Procedures

Sampling methods, as discussed in Subsection 2.1.4, were chosen to ensure unbiased (i.e., accurate) samples that will facilitate comparisons with other soil invertebrate data, both from the Housatonic River and from other areas. Steps taken to ensure that sampling does not unnecessarily induce bias include: visual inspection of each sample to confirm satisfactory collection, and confirmation of visual similarity of soil type within a location. All samples will be collected by trained and experienced personnel; senior oversight of all aspects of the sampling and sample processing will further promote comparability and reduce potential bias. Subsamples for physical and chemical analyses will be collected following procedures documented in the

project QAPP (WESTON, 2000) and will therefore be comparable with procedures followed for all other similar efforts throughout the Supplemental Investigation.

3.3.2 Quality Control Samples

The nature of soil invertebrate sampling does not allow the incorporation of typical duplicate and blank samples as part of the study design.

Duplicate and MS/MSD samples for chemistry will collected in this study. Quality control of chemistry analyses will be provided and processed in accordance with the QAPP.

3.3.3 Sample Processing and Preservation

Detailed procedures for collection and initial processing of all samples to be collected as part of the soil invertebrate study are provided in Section 4. Subsampling, homogenization, and decontamination between samples will follow procedures established in the QAPP. All samples will be held on wet ice and returned to the field laboratory daily and will be either refrigerated, frozen (physical, chemical samples), or preserved (taxonomic samples) at that time. Holding time for physical and chemical samples will follow procedures established in the QAPP; there is no holding time for taxonomic samples.

3.3.4 Training

All sampling will be directed in the field by senior scientists with experience in the collection of soil invertebrate samples. Supporting staff will receive training from the senior scientist(s) in the overall goals of the study and in techniques to be followed to ensure collection of quality data.

3.4 SAMPLE ANALYSIS

3.4.1 Taxonomy Samples

Processing of taxonomy samples will follow standard procedures established for both earthworms and other soil invertebrates. All samples will be processed by experienced staff who have received specific training in the SOP and whose work is checked periodically by their supervisors and peers. Depending on sample volume and other factors, samples will be processed by eye or under low-power microscopes.

Quality of taxonomic identification will be ensured by maintaining voucher collections and requiring a consensus among all taxonomists at the processing laboratory prior to an identification becoming accepted as a type for the voucher collection. In the event that the taxonomists are unable to agree on an identification, specimens will be sent to a third party for determination.

3.4.2 Physical/Chemical Samples

Samples for soil grain size, soil chemistry, and tissue chemistry will be processed following procedures and SOPs provided in the QAPP. These samples will be submitted in catalogs (sample delivery groups) and batches with other samples from the larger project and data validation will be performed on a catalog basis in accordance with procedures established and described in the QAPP.

3.5 DATA ANALYSIS AND REPORTING

The overall analytical approach for data generated under this study is described in Subsection 2.4. The study findings will be included in the ecological risk assessment including all data, analyses, and interpretations and will be prepared with specific reference to both the data quality objectives specific to the soil invertebrate study (Subsection 2.3.1, above) and Subsection 4.1 of the QAPP.

4. PROCEDURES

4.1 FIELD SAMPLING PROCEDURES

Working in two-person teams, identify and mark plot locations using four pin flags (one for each plot corner). The plot area is assumed for planning purposes to be 1 m^2, but may be adjusted based on the results of the pilot study. Label the flags with a sequential location number, and note the location on a field map and in a bound logbook. Survey the plot location using GPS equipment.

Scrape sufficient leaf litter from the plot area to collect three random soil grab samples using a decontaminated stainless-steel trowel. Be careful to minimize removal of surface litter and detritus that may be used by invertebrates. Homogenize the sample in a dedicated stainless steel tray or on a clean plastic sheet, and apportion it into a 4-ounce glass jar. Label the sample jar with an indelible marker and fill out a sample data sheet. The soil sample will be submitted for confirmatory analysis following successful collection of earthworms and other invertebrates from the plot.

Invertebrates should be removed daily from the pit traps during a 1-month period, or until sufficient tissue mass is obtained for analysis. If, after 1 week, it is apparent that insufficient tissue mass will be available from the pitfall traps over a 1-month period, the sample will be supplemented by invertebrate sampling of leaf litter and detritus within sampling plots.

If possible, plots should be sampled for earthworms after a heavy precipitation event, when earthworms are closest to the surface. All leaf litter and detritus will be removed from the plot area by hand, while wearing protective gloves. New gloves will be donned before sampling at a new plot to avoid cross contamination of samples. Leaf litter will be placed into decontaminated 5-gallon plastic buckets (or equivalent). The buckets will be labeled by plot number, and cheesecloth will be placed over the top and secured with a large rubber band. The buckets will be

returned to the central processing area and refrigerated until they are processed (only if necessary; see Subsection 2.1.3.4).

Plots will then be sampled for earthworms. Worms will be removed from the plot by digging to a maximum depth of approximately 0.5 ft with a decontaminated shovel. If sufficient sample mass is not achievable by this method, the soils may be screened using a standard 1/4-inch sieve or equivalent.

An alternative means of sampling earthworms is to apply a mustard/water solution to the ground surface. The solution is prepared by mixing approximately 1 tablespoon of dried mustard to 5 gallons of distilled water. Apply 5 gallons per 1 m^2 of plot area, or until the ground is fully saturated with the solution. Wait until the earthworms surface and collect them from the surface.

Individually rinse each worm with distilled water, using a spray or squeeze bottle. Then place all the worms from the plot into an appropriate precleaned sample container. Label the container with the plot location, the date, time, and collector's initials.

Place the containers in a cooler with ice and transport them to the central processing area.

All sampling equipment will be decontaminated following the project-specific SOP for equipment decontamination, including detergent/water wash, potable water rinse, hexane rinse, isopropyl alcohol rinse, and deionized water rinse.

4.2 SAMPLE PROCESSING PROCEDURES

4.2.1 Earthworm Sample Processing

1. At the central processing area, place earthworms in the refrigerator in their labeled locations until ready to process.

2. Segregate and taxonomically identify earthworm species to determine the dominant species collected within the study area.

3. Once the dominant species is determined, process each container individually. Segregate the species by placing them on decontaminated aluminum foil or paper toweling. Group the largest individuals into a composite sample and weigh the group to ensure that 10 g of tissue are available for analysis. Then weigh each earthworm separately. Record all data on a sample data sheet.

4. Note any external lesions or other abnormalities, such as a "pinched" appearance caused by constriction of the coelom.

5. Place the sample in aluminum foil that has been rinsed with hexane and air dried (dull side toward the sample), add a label with the sample number, and double bag it using resealable plastic bags. Label the outer bag with the sample number, and place in freezer at -10 °C.

6. Complete a sample attribute form for each sample.

4.2.2 Invertebrate Sample Processing

1. At the central processing area, store invertebrates in sampling containers until ready to process.

2. For litter samples from sample plots, pick the invertebrates from the soil litter from each location/container, and segregate live individuals by taxonomic order into decontaminated petri dishes with lids. The lid of the petri dish should be labeled with the sample location number.

3. Taxonomically identify individuals for both pit trap and sample plots to the level of order, and record the number of individuals per order on a data form for each sample collected. Obtain a wet weight for each order and sample.

4. If sufficient sample mass is present, group the largest individuals into a composite sample for that location, and weigh it. Record the weights, which should be at least 10 g per sample. If a composited pit trap sample weighs less than 10 g then add the litter invertebrates from the closest sample plot. If the total biomass is still less than 10 g then add the litter invertebrates from the next closest sample plot.

5. Wrap samples in decontaminated aluminum foil that has been rinsed with hexane and air dried (dull side toward the sample), and place in resealable plastic bags. Attach a label to each sample indicating the sample number and place in the freezer at -10 °C.

6. Complete a sample attribute form for each sample.

4.2.3 Sample Handling and Shipping

1. Keep samples in a -10 ºC freezer until shipment to the laboratory.

2. When ready to ship, place the samples (wrapped in labeled foil and enclosed in labeled resealable plastic bags) in a large plastic bag into a cooler lined with vermiculite.

3. Complete a chain-of-custody form listing the contents of each cooler, and place it in a resealable plastic bag. Tape the resealable plastic bag to the inside of the top lid of the cooler, or place it on top of the samples.

4. Seal the cooler with two custody seals, and label the cooler with appropriate WESTON shipping labels, including the WESTON return address, and U.S. Fish and Wildlife Service (USFWS) laboratory address.

5. Samples will be delivered by courier or overnight delivery to the analytical laboratory. Earthworm tissue samples should be sent by overnight delivery service

(next morning delivery) or hand delivered. Samples sent to USFWS should be shipped to:

Ken Carr/Ken Munney/Drew Major
USFWS
22 Bridge St., Unit 1 Phone: 603-225-1411
Concord, NH 03301 Federal Express Acct: 1510-1036-9

Shippers should notify the receiving laboratory or USFWS that samples are being sent for next-day delivery. Samples should not be sent to USFWS if Ken Munney, Ken Carr, or Drew Major are not available for receipt of the shipment. Samples need to be sent for arrival on a weekday only. Therefore, Thursday is the last day of the week to ship samples. Shippers should also call the receiving laboratory of USFWS the day of delivery to verify the receipt of samples. Composite soil samples collected will be shipped to the appropriate contract laboratory following procedures outlined in the FSP (WESTON, 1999).

4.2.4 Sample Documentation

All sample documentation will follow project-specific SOPs for field sample ID, data sheet, chain-of-custody form, and custody seal procedures.

Use a field logbook to record the location, date and time, amount of time spent in collecting activities at each area, method of collection, name(s) of collector(s), the number of earthworms collected, and any other pertinent information such as problems encountered.

Complete an earthworm specimen data sheet for each location sampled. Specimen data sheets should include location; date and time of collection; method of collection; collector's initials; earthworm species; total weight of earthworm composite sample; and total weight of individual earthworms retained for analysis. Numbers of individuals of other earthworm species collected should also be noted.

An invertebrate community data sheet should also be completed for each location sampled. Data recorded should include the location; date and time of collection; method of collection; collector's initials; species collected and number of each per sample; and weight of the litter invertebrate sample.

Complete a sample attribute form for each tissue sample (earthworm and litter invertebrates). Put the sample number for each sample and the date and processor's initials on the form.

Complete a chain-of-custody form for each cooler of samples shipped to the USFWS laboratory. Provide copies to the task manager, who will retain them in the WESTON files.

5. EQUIPMENT LIST

5.1 FIELD

- First aid kit
- 5-gallon (or equivalent) buckets for litter/detritus collection with lids and holes for ventilation or cheesecloth and rubber bands to secure the cheesecloth to the top of the container
- Plastic buckets (1-gallon or less) for collection of earthworms
- 6 ¼-inch standard soil sieves and/or a 2-ft by 2-ft sieve constructed from hardware, cloth, and 2-inch by 4-inch lumber
- Indelible markers, duct/labeling tape
- Pin flags
- Wooden stakes
- Heavy duty stapler
- Hammer
- Plastic sheeting (or other appropriate material) to use for drift fences
- No. 10 (or similar) cans with covers
- Field logbook
- Rubber gloves
- Resealable plastic bags
- Dry mustard
- Distilled water (5 gallons per 1 m^2 plot) to mix with mustard and pour on sampling plots, if necessary
- GPS receiver
- Wet and dry ice
- Coolers for sample storage and transport
- Soil sampling equipment: stainless-steel trowels, bowls, glassware for soil sample

5.2 PROCESSING AREA

- 2 folding tables
- Polyethylene plastic sheets
- 4 boxes of Nitrile gloves
- 10 boxes of gallon-size resealable plastic bags
- Data sheets
- Four sets of forceps
- 200 plastic petri dishes
- Invertebrate taxonomic keys
- 2 dissecting scopes, each 2X minimum and illuminated
- Weighing scale for up to 100 grams
- 4 boxes of aluminum foil
- 2 large coolers for freezing samples
- 1 to 2 shipping coolers
- Ice to fill cooler, in plastic resealable bags

- Dry ice for shipping
- Gloves for handling dry ice and/or liquid nitrogen
- Indelible markers (fine and wide)
- Ballpoint pens
- Hexane in rinse bottle
- Isopropyl alcohol in rinse bottle
- Distilled, deionized water in rinse bottle
- Large bucket for decontamination solutions
- Packaging tape
- Laboratory sample labels with unique sample numbers
- WESTON or USFWS QA/QC labels

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ATTACHMENT 1

POWER ANALYSES FOR EARTHWORMS

ATTACHMENT 1

POWER ANALYSES FOR SOIL INVERTEBRATES

Earthworms consume large quantities of soil and may thus accumulate polychlorinated biphenyls (PCBs) that occur in soils of contaminated areas. A comparison of PCB concentrations in earthworm tissues among three areas with different degrees of exposure to these chemicals will be tested with analysis of variance (ANOVA). The anticipated power of such a test was studied both analytically and by means of simulation.

Analytical Approach

For the smallest difference δ between means that we wish to detect and desired power, the adequate sample size n is a function of the non-centrality parameter, which is related to

$$\phi = \sqrt{\frac{n\delta^2}{2k\,s^2}}$$

where k is the number of groups being compared and s^2 is an estimate of the error mean square (variation within groups). Using published charts (Zar, 1984) that relate the power of ANOVA to ϕ, for different degrees of freedom (v) and levels of significance (α), it is possible to solve the above equation for n by guessing its value, calculating ϕ, and from the chart obtain the corresponding power. With a few iterations of this process, it is easy to find n for the desired power. For this earthworm study, the number of groups k=3. The within-group variance was estimated from Diercxsens et al. (1985) to be s^2=8110. Figure 1 displays the required sample sizes to detect a wide range of differences between means. Ten samples per site would be sufficient to detect a difference of 150 mg/kg wet mass. Detection of a difference of 100 mg/kg wet mass would require almost twice the sample size.

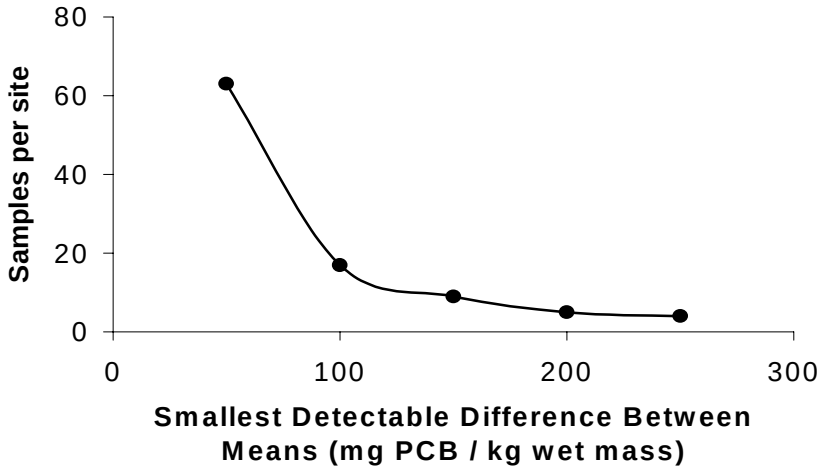

Figure 1. Number of samples required to detect the smallest difference between mean concentrations of PCB in earthworm tissues from *k*=3 sites with distinct concentrations of these chemicals in the soil. Power ≥ 0.8, α = 0.05, s^2 = 8110 (estimate based on data from Diercxsens et al. 1985).

Simulation Approach

Implicit in the analytical approach is the assumption that Housatonic earthworms have the same response to variation in PCB concentrations in the soil as earthworms from a nature reserve and a vineyard in Switzerland, the sites from which the estimate of s^2 was obtained (Diercxsens et al., 1985). We relax this assumption somewhat by modeling variable responses of PCB concentrations in earthworm tissues we might expect for the Housatonic. Log-transformed concentrations of PCB in earthworm tissues can be modeled as a linear function of log-transformed PCB concentrations in soils. Figure 2 shows an apparently good fit to this regression on the available empirical data pooled from Diercxsens et al. (1985) and Kreis et al. (1987).

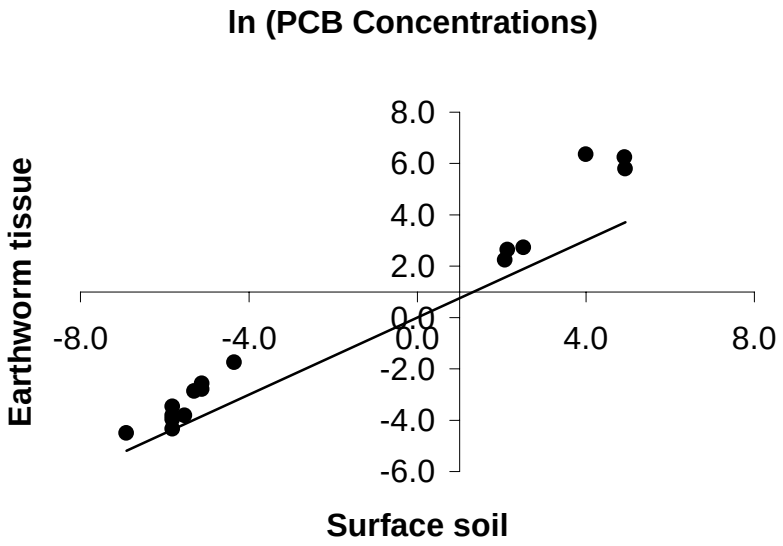

Figure 2. Linear relationship between log-transformed PCB concentrations in soils and earthworm tissues collected at agricultural fields (Kreis et al. 1987, points in the lower left quadrant), a vineyard, and a nature reserve (Diercxsens et al. 1985, points in the upper right quadrant) in Switzerland.

Monte Carlo simulations were used to estimate statistical power for an analysis of variance for each of several sample sizes. In these simulations, the anticipated means and dispersions of earthworm PCB concentrations among the three Housatonic sites were modeled with the regression function above. Because the sites are expected to have low, moderate, or high contamination, a soil concentration for each site was first simulated by randomly sampling from a loguniform distribution, having the range [0.01, 1], [1, 30], or [30, 100] mg/kg dry mass, respectively. These loguniform deviates correspond to variation due to differences in soil

concentrations. Each deviate is an X value from which a concentration Y in worm tissue is simulated using the expression

$$Y = \exp(\delta \ln(X) + \varepsilon(0,\sigma))$$

where $\varepsilon(0,\sigma)$ represents a normally distributed error term with zero mean and standard deviation estimated as the root residual mean square

$$\sigma = \sqrt{\sum (Y - \overline{Y})^2 / (n-1)} = 1.47$$

observed in the regression on the pooled data in Figure 2, and δ (the slope in log-log relationship) represents the magnitude of differences due to any bioaccumulation in earthworm tissues. The simulated Y values were log-transformed and subjected to an ANOVA test. A tally was kept of the number of times the ANOVA was statistically significant at the α=0.05 level of significance. The number of significant results divided by the total number of tests constitutes an estimate of the power for a particular sample size (under the prevailing hypothesis about the true difference among means). A Pascal program was used to conduct these simulations. The results of this analysis are shown in Figure 3, which displays the sample sizes required for a power of no less than 80% as a function of δ (the slope of the underlying log-log regression).

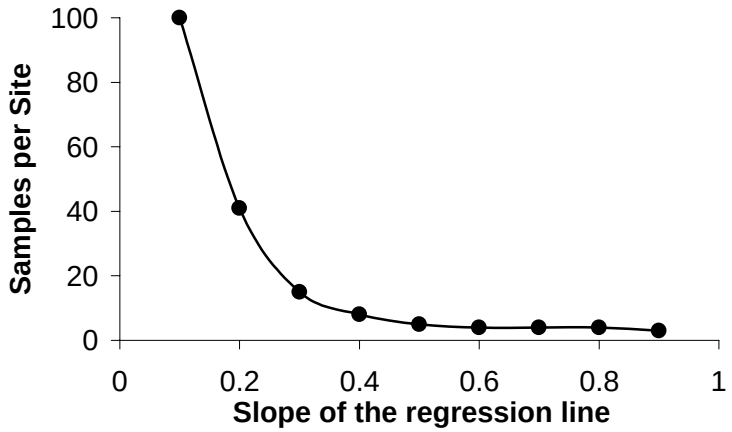

Figure 3. Estimated number of samples, for each of the three sites, required to detect significant differences in mean concentration of PCB in earthworm tissues as a function of the slope of the regression line relating log transformed concentrations of PCB in earthworm tissues (wet mass) and soils (dry mass). Power ≥ 0.8, α = 0.05.

Once the sample sizes that achieve the desired power (≥ 0.8) are identified, the selection of a specific sample size should balance costs against the ability to detect differences. Both the analytical and simulation approaches indicate that above ~10 samples per site sharp increases in sample sizes are required to detect smaller differences.

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APPENDIX A.23

WORK PLAN FOR WATERFOWL COLLECTION AND TISSUE SAMPLING (TECHLAW, INC.)

APPENDIX A.23

WORK PLAN FOR WATERFOWL COLLECTION AND TISSUE SAMPLING

Submitted to:

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1.0 Sample Site and Size Selection

Elevated levels of PCBs have been detected in sediment and floodplain soils in the Housatonic River downstream from the GE facility in Pittsfield, MA. Waterfowl, including mallards and wood ducks, have been observed using Woods Pond and upstream floodplain wetlands for breeding, brood rearing, and feeding, and waterfowl hunting is a common activity along this reach of the river. Therefore, these areas were chosen as collection sites to evaluate the potential for risk to the waterfowl directly and to humans consuming the waterfowl due to PCB accumulation in the tissue.

To determine a potentially suitable sample size for waterfowl tissue analysis, *Power and Precision: A computer program for statistical power analysis and confidence intervals* was used (Borenstein et al., 1997). PCB tissue levels from waterfowl collected at the Fox River in Wisconsin (U.S. Fish and Wildlife Service, T. Custer, pers. comm., 1998) were used for the study area population mean and standard deviation. An assumed off-site control mean of 0.5 and a similar standard deviation of 5.5 was used in a power analysis for determining sample size. A sample size of 30 was expected to yield a statistically significant result; however, a permit was issued for a sample size of 20 birds by the MADEP, restricting the sample size below that desired after the power analysis. Because the objective of determining if a risk to humans was posed through waterfowl consumption was critical to address, the decision was made to proceed with a smaller sample size to assess if a concern exists, in which case follow-up studies can be conducted if necessary.

To identify an appropriate reference site outside of the study area for the two target species, the Massachusetts Department of Fisheries and Wildlife (MDFW) was consulted for information on waterfowl habitat near the study area. MDFW suggested the Three-Mile Pond Wildlife Management Area (WMA) in Sheffield as a potential reference area. Three-Mile Pond WMA, approximately 15.6 miles south of the study area, consists of a shallow pond dominated by submerged aquatic vegetation with pockets of emergent and scrub-shrub wetlands on the periphery of the pond. This habitat contains similar water levels, similar amounts of submerged aquatic vegetation, emergent marsh, and surrounding undeveloped habitat as that of the lower portions of the study area near and upstream of Woods Pond. These factors, in combination with the fact that MDFW had conducted waterfowl banding at the site in the past because the area was known to contain populations of both local and migratory waterfowl, made it a suitable potential candidate as a waterfowl collection reference area. In addition, wood ducks and mallards were observed using the WMA during site surveys, and were known to breed at the WMA (T. Keefe, pers. comm., 1998).

A large cove with shallow (less than 1 ft deep) water and some exposed sediment at the north end of the pond was chosen as the potential trapping location due to the similarity in the habitat to that chosen for collection areas in the river study area. Samples will be collected and analyzed the sediment in the Three-Mile Pond WMA trapping location to verify that it is an appropriate reference location uncontaminated by PCBs.

Captured waterfowl were identified to species, sex, and age, therefore allowing comparisons between samples. Because waterfowl are migratory it was important to age the birds to determine if they were raised in or near the capture location.

2.0 Capture Methods

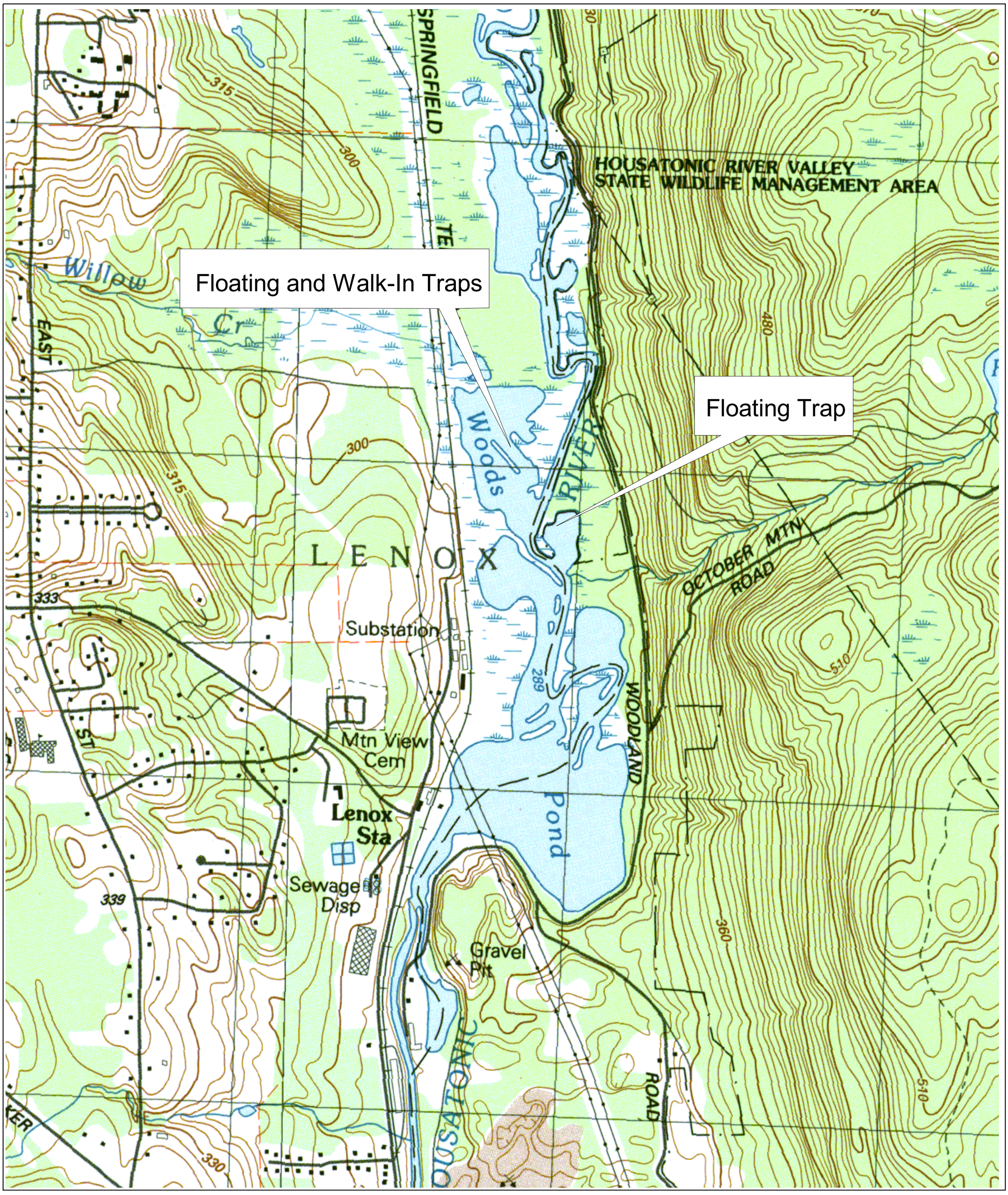
Air boating and bait trapping were used to capture wood ducks and mallards. Air boating efforts were conducted by MDFW as part of annual banding operations, which typically take place in late summer or early fall in the Woods Pond and upstream floodplain wetland areas. During this effort an air boat equipped with high-beam halogen headlights was used on the night of August 28, 1998 to locate waterfowl. Two people captured birds with hand nets while the boat was driven through non-persistent emergent vegetation and in open water areas. Birds were placed in crates and transported to the Woods Pond boat ramp, where they were aged, sexed, and banded. Eleven wood ducks and four mallards were placed in crates for tissue analysis while the banded birds were transported back to their capture location and released.

Baited traps were then used to capture the remainder of the birds needed for analysis. Two floating box traps designed by H. Heusemann, of MDFW, and one walk-in clover trap were each placed in the greater Woods Pond area and in the Three-Mile Pond reference area. Traps were deployed from August 30 to September 16 in the greater Woods Pond area and from August 27 to September 15 at Three-Mile Pond. Approximate trap locations are shown in Figures 1 and 2.

Trap sites were baited with whole and cracked corn for approximately one week before traps were set to capture birds. While in operation, traps were checked one to two times each day and all captured ducks were removed from the traps and placed in crates for transportation to the dissection area. Ducks that were kept overnight before being dissected were placed in a pet travel kennel with hay (for bedding), corn, and water. Composite samples of the corn and water fed to the ducks were collected for PCB analysis.

3.0 Dissection Methods

Dissection methods, performed using chemically clean instruments, were based on necropsy procedures outlined in Friend (1987) and Wobeser and Spraker (1980). Sample birds were aged, sexed, and weighed prior to dissection. Birds were euthanized by severing the head from the body with sharp shears and breast feathers were removed. The brain, liver and breast were removed for analysis. The brain was removed by cutting the top of the skull between the anterior end of each eye, intersecting the front end of the brain case. Brain tissue will be retained for future potential use.Two more incisions were made, one on each side of the brain, back to the foramen magnum. The top of the skull was then peeled back and the brain was removed. The breast was removed with the skin on. Finally, the ribs were cut and the breast bone was removed to expose the liver and other organs. The liver was removed whole.



SOURCE: USGS East Lee quadrangle map.

N

Scale In Feet

700 0 700 1400

SI Work Plan
Lower Housatonic River
Massachusetts

FIGURE 1
WATERFOWL TRAP LOCATIONS
AT WOODS POND

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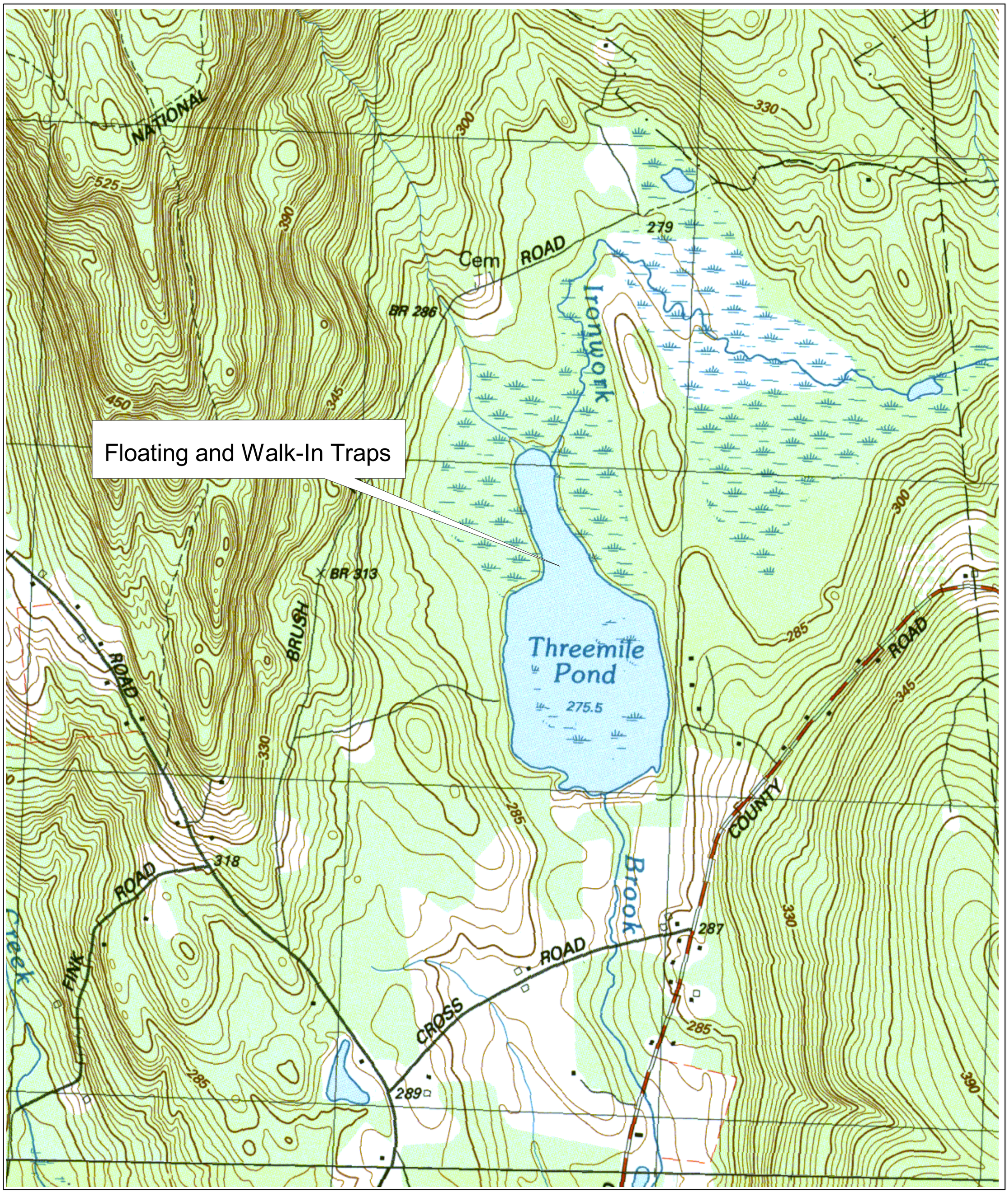

SOURCE: USGS Great Barrington quadrangle map.

N

Scale In Feet

700 0 700 1400

SI Work Plan
Lower Housatonic River
Massachusetts

FIGURE 2
WATERFOWL TRAP LOCATIONS
AT THREE-MILE POND

All sample tissues were wrapped in aluminum foil (dull side in contact with the sample), tagged, and double bagged in resealable bags with a second sample ID tag. All dissection tools, stainless steel dissection trays, and aluminum foil were decontaminated according to EPA standards and consisted of a four part process: 1) a wash with a mild detergent, 2) a rinse with potable water, 3) a spraying wash with hexane, and 4) a spraying rinse with deionized water. Samples were placed on dry ice and shipped to the USFWS for cataloging and analysis of PCBs (total and Aroclors), PCB congeners and homologs, dioxins/furans, select OC pesticides, percent lipids, and percent moisture.

4.0 Quality Assurance/Quality Control

4.1 Data Quality Objectives, Indicators, and Assessment

4.1.1 Data Quality Objectives

The objectives of the waterfowl collection and tissue sampling effort were described in Section 1.0. To achieve these objectives, the following types of data will be required:

- Collection and taxonomic classification of organisms--Mallards and wood ducks must be captured from the study area and a reference area and accurately identified to species, sex, and age.

- Concentrations of PCBs in tissues of waterfowl--Quality control considerations for PCB concentration analysis for biological samples will follow those identified in the QAPP.

4.1.2 Data Quality Indicators

Data developed in the waterfowl collection and tissue sampling study must meet standards of precision, accuracy, completeness, representativeness, comparability, and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000). For all waterfowl tissue residue analyses, data quality indicator values and standards will be in accordance with those defined in the QAPP.

Precision is defined as the level of agreement of repeated independent measurements of the same characteristic. For this study, laboratory equipment will be used that has the capability to provide measurements that fall within the allowable precision limits identified in the QAPP.

Accuracy is defined as the agreement of a measurement with its true value. Standard sample processing, preservation, and laboratory analysis will be conducted using standards and equipment identified in the project QAPP. Those standards have been established in order to eliminate sample contamination and reduce researcher bias, which results in accurate measures of PCB concentrations in tissue.

Completeness is defined as the percentage of the planned samples actually collected and processed. A power analysis was conducted to determine the sample sizes needed for this study. Completeness, therefore, will be measure of number of samples collected compared to the number of planned samples.

Representativeness is defined as the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. Representativeness for this study will be ensured through establishment of an approved, thorough sampling design and through careful implementation of the sample processing and analytical methods.

Comparability is defined as the measure of confidence with which the results of this study may be compared to another similar data set. Comparability will be attained through use of laboratory procedures that provide data in units that can be compared to other similar studies.

Sensitivity is defined as the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest. For data specific to this study, sensitivity will pertain to the ability to identify waterfowl to species and age from the study area. Sensitivity of non-biological parameters is defined in the QAPP.

4.1.3 Data Validation, Verification, and Usability

Procedures for data validation for the analysis of waterfowl tissue PCB concentrations are discussed in various sections of the project QAPP and will be used whenever applicable in this study.

4.2 Sampling Design

Two study sites were chosen for the assessment of PCB concentrations in waterfowl tissue and included the Newell Street to Woods Pond study area and one site located outside the formal study area but within the Housatonic River drainage that represents a control. Within the Newell Street to Woods Pond study area, waterfowl collection took place in floodplain and backwater wetlands from the downstream half of the study area: from New Lenox Road, south to Woods Pond.

Sample sizes were determined by using PCB concentration data from a waterfowl tissue PCB analysis study conducted in Wisconsin and *Power and Precision: A computer program for statistical power analysis and confidence intervals* (Borenstein et al., 1997); however, they were later restricted by the MADEP in issuing the collection permit.

4.3 Sampling Methodology

4.3.1 Sampling Procedures

Sampling methods were chosen to ensure unbiased samples that will facilitate comparisons with other studies on waterfowl tissue PCB concentrations and will be suitable for the evaluation of the potential ecological risk to waterfowl directly and the risk to humans consuming waterfowl from the study area.

Steps taken to ensure that sampling does not unnecessarily induce bias include: adopting relatively short holding times for live waterfowl specimens planned for sampling, establishment of a relatively undisturbed (controlled climate, reduced noise, shaded from direct sunlight) holding location for sample specimens prior to tissue collection, and nourishment for specimens prior to tissue collection.

4.3.2 Quality Control Samples

Duplicate samples for the chemical analysis of waterfowl tissues will be in accordance with the QAPP and through comparisons with results from split samples provided to GE.

4.3.3 Sample Processing and Preservation

Detailed procedures for the collection of waterfowl used for tissue analysis are provided in Section 2.0. Biological sample dissection methodology are described in Section 3.0. These procedures are in accordance with measures identified in the QAPP. Holding time for biological samples (processed and preserved tissue samples after animal dissection) will follow procedures established in the QAPP.

4.3.4 Training

Animal collection will be directed by two senior scientists with experience in waterfowl collection methodologies and identification, measurements, and dissection will be directly conducted by the two senior scientists, again with experience in animal identification and dissection.

4.4 Sample Analysis

Samples for chemical analysis were processed following procedures and SOPs provided in the QAPP. These samples will be submitted to the appropriate analytical laboratory and data validation will be performed in accordance with procedures established and described in the QAPP.

4.5 Data Analysis and Reporting

The study findings will be included in the ecological risk assessment including all data, analyses, and interpretations.

5.0 Literature Cited

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APPENDIX A.24

TREE SWALLOW STUDY PROTOCOL AND PROCEDURES

APPENDIX A.24

RESEARCH PROTOCOL

Bioaccumulation and effects of PCBs on tree swallows nesting along the Housatonic River, Massachusetts

Testing Facility

Upper Mississippi Science Center
Biological Resources Division, U.S. Geological Survey,
P.O. Box 818
La Crosse, Wisconsin 54602-0818

Study Number	WE-98-Cont-08
Study Director	Christine M. Custer, Ph.D. Wildlife Biologist (Research)
Proposed Starting Date	April 1998
Proposed Ending Date	September 2001

Reviewed and Approved by:

Christine M. Custer
Christine M. Custer, Ph.D.
Study Director

April 29, 1998
Date

Carl E. Korschgen
Carl E. Korschgen, Ph.D.
Section Leader, Wildlife Ecology

11/3/98
Date

Guy R. Stehly
Guy R. Stehly, Ph.D.
Animal Care and Use

June 30, 1998
Date

Steve Gutreuter, Ph.D.
Statistician

11/10/98
Date

Leslie Holland-Bartels, Ph.D.
Center Director

11/12/98
Date

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APPENDIX A.24

TREE SWALLOWS RESEARCH PROTOCOL

1. INTRODUCTION

The U.S. Environmental Protection Agency (EPA) Region 1, Boston, Massachusetts, and the U.S. Fish and Wildlife Service (USFWS), Region 5, Concord, New Hampshire Ecological Services Office, have requested assistance from the Upper Midwest Environmental Sciences Center (UMESC) in an evaluation of the effects of PCBs on wildlife downstream from the General Electric (GE) facility located on the Housatonic River near Pittsfield, Massachusetts.

The GE facility has contaminated the main stem and many of the backwaters of the Housatonic River with polychlorinated biphenyls (PCBs) (ChemRisk, 1997). Contamination originated from numerous sources including direct discharge to the river, a spill of liquid PCBs in the late 1960s at Building 68 that contaminated the riverbank and river sediments, and from contaminated fill that was placed in former oxbows. The extent of PCB contamination in the floodplain coincides roughly with the 10-year floodplain of the Housatonic River. The Housatonic River has been closed to all but catch and release fishing from Pittsfield, Massachusetts extending 80 miles downstream because of high concentrations of PCBs in fish tissues. The USFWS will be deciding whether additional management actions are warranted. To make this determination, data on PCB concentrations, accumulation, and injury to wildlife are required.

Little published information is available on concentrations of PCBs in avian tissues of birds nesting along the Housatonic River and no information is available on possible effect levels. In 1993 the nest success of 8 passerine species, within and outside the 10-year floodplain of the Housatonic River, was monitored (Henning et al., 1997). No data, however, on PCB concentrations in their eggs or food were collected during that study. Sample sizes were generally small in that study; however, for barn swallows (*Hirundo rustica*), clutch size was reduced at nests within the 10-year floodplain compared to reference sites at $P = 0.06$. Probably because of the reduced clutch size, mean number of young hatched in barn swallow nests from nests within the 10-year floodplain was also reduced compared to the mean number of young hatched per nest at reference sites. Again this difference may be considered significant ($P = 0.06$). Of the 4 species studied that Henning et al. (1997) had sufficient sample size to conduct statistical analyses on ($N > 5$ nests), the barn swallow would be the most likely to consume aquatic insects and hence be affected by PCBs. The food of American robins (*Turdus migratorius*), red-winged blackbirds (*Agelaius phoeniceus*), and wood thrushes (*Hylocichla mustelina*), the other 3 species studied, are terrestrial insects, and hence are not as likely to be exposed to the high concentrations of PCBs that are found in aquatic sediments (Ankley et al., 1993).

Swallows, especially tree swallows (*Tachycineta bicolor*) are now being more widely used as indicators of local sediment contamination (Shaw, 1983; DeWeese, 1985; Kraus, 1989; Ankley et al., 1993; King et al., 1994; Bishop et al., 1995; Nichols et al., 1995; Custer et al., 1998). Tree

swallows will readily use nest boxes, so study sites can be established at specific locations of interest. They feed near their nest box (± 400 m, Quinney and Ankney, 1985) on emergent aquatic insects (Blancher and McNicol, 1991) so residues in their tissues reflect sediment contamination for those chemicals that transfer into the biota (Fairchild et al., 1992). They also will nest relatively densely so that adequate sample sizes can be obtained. Data are now available on contaminant levels in tree swallows at a number of locations across the U.S. for PCBs (Bishop et al., 1995; Custer et al., 1998), other organochlorines (Shaw, 1983; DeWeese et al., 1985; Elliott et al., 1994), and metals (Kraus, 1989; King et al., 1994).

2. OBJECTIVES

This study has three primary data quality objectives. In addition, the data also will support the DQOs outlined in Subsection 4.1 of the Final Quality Assurance Project Plan (QAPP) (WESTON, 2000). The three primary objectives of the study are to:

- Determine PCB concentrations in tree swallow eggs and/or just-hatched young in the Lower Housatonic River.
- Determine PCB accumulation rates in nestlings.
- Determine whether PCBs are impairing reproduction in tree swallows.

3. STUDY DESIGN

3.1 TEST ORGANISM AND LOCATION

The test organisms are tree swallows. Identification of bird species will be made by the study director according to National Geographic Society *Birds of North America* or similar book. The study area is along the Housatonic River between Pittsfield, Massachusetts and Woods Pond (see map). The reference site will be a nearby tributary or river, such as the West Branch of the Housatonic, or the Housatonic River itself above the suspected contamination source. Other reference sites will be added if appropriate, such as Three-Mile Pond.

3.2 SAMPLE COLLECTION

Approximately 120 swallow boxes, 30 at each of 4 sites, will be attached to posts, trees, or other suitable structures in suitable habitat. Predator guards will be used as needed. Boxes will be placed approximately 20 - 30 m apart, but this can vary depending on the structure of the habitat. Additional boxes may be added in subsequent years of the study. Three sites (upper, middle, and lower reaches) will be below the GE facility on the Housatonic River and reference sites will be on a nearby waterbody such as the West Branch of the Housatonic River (Fig. 1). Location of boxes and specific study sites will be determined during the first visit to the area, but may be

modified in subsequent years of the study based on the professional judgment of the Study Director.

Each nest box will be visited approximately once per week until egg laying begins. After that time, nests will be visited up to 2 - 3 times per week, or more often as needed to collect egg or just-hatched young samples. After the eggs have hatched, boxes will be visited at least once per week until the young reach 12 days of age. Once the majority of eggs at a site have hatched, spot checks of selected nests are permissible and can be made at an appropriate interval to assess hatching and fledging success. Whether eggs or young are present in the nest box and the number of eggs and young present will be recorded on data sheets (SOP WE 408, Attachment 3).

A sample of 2 - 3 eggs and/or just-hatched eggs (hereafter termed pippers) and a sibling 12-day-old tree swallow nestling will be collected from a minimum of 5 - 10 boxes at each site. All sample collections will be covered by appropriate Federal and State collecting permits. The exact number of samples collected at each site will be dependent on the number of tree swallows that nest at each site and the analytical dollars available. If sufficient analytical dollars are available pipper samples will be collected and analyzed from all active boxes at each site. The exact number of pippers to be collected from each nest box will depend on the clutch size. Clutches $\leq$ 5 eggs will have only 2 pippers collected; clutches $\geq$ 6 eggs may have 3 pippers collected. Pippers and the 12-day-old nestlings will be collected from the first 5 - 10 clutches that are initiated at each site, however, unforeseen circumstances, such as nest predation or flooding, may preclude this.

Food samples from the stomachs of tree swallow nestlings will be removed at the time they are collected and dissected. A pooled food sample, from each site, will be analyzed for organochlorine chemicals, including total PCBs and perhaps PCB congeners. Nestling tree swallows may be ligatured to obtain additional food samples for insect species identification and for chemical analysis of their food. Adult and nestling tree swallows may be banded with standard USFWS aluminum bands.

In addition, WESTON will collect sediment samples within the tree swallows' expected foraging radius around the nest boxes to support this study.

3.3 SAMPLE PREPARATION

Pippers or 12-day-old nestlings will be removed from the nest box and weighed. Pippers and nestlings will be visually examined for gross abnormalities and this information noted. Nestlings will be decapitated with a sharp pair of scissors (Anonymous, 1993), contents in the upper gastrointestinal tract removed with forceps after an incision is made along the length of the stomach, and the carcass remainder placed in a chemically clean jar, which has been purchased in that condition. For in-depth methodology see SOP WE 409. The above will be done within 2 hours after removal from the nest box. The carcass remainders and food samples will be maintained frozen until transported to the Upper Midwest Environmental Sciences Center for storage in a freezer. Samples will be shipped to the analytical laboratory following chain of custody procedure in SOP WE 410.0.

3.4 SAMPLE ANALYSIS

Carcass remainders and pooled food samples will be analyzed for total PCBs, Aroclors, homologs, and congeners, with a subsample of pipper and nestling samples pooled by site being analyzed for polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDDs/PCDFs), organochlorine pesticides, and trace elements. Analyses will be performed according to standard operating procedures of a contract lab approved by the U.S. Fish and Wildlife Service Patuxent Analytical Control Facility (PACF), Laurel, Maryland. Quality assurance and control for chemical analysis will be the responsibility of the PACF. The samples will be analyzed for the following specific contaminants:

PCBs

- Total PCBs
- Aroclors
- PCB homologs
- PCB congeners (including but not limited to the 12 congeners with dioxin-like activity)

PCDDs/PCDFs

- 2,3,7,8-TCDD
- 1,2,3,7,8-PentaCDD
- 1,2,3,4,7,8,-HexaCDD
- 1,2,3,6,7,8-HexaCDD
- 1,2,3,7,8,9-HexaCDD
- 1,2,3,4,6,7,8-HeptaCDD
- OCDD
- 2,3,7,8-TCDF
- 1,2,3,7,8-PentaCDF
- 2,3,4,7,8-PentaCDF
- 1,2,3,4,7,8-HexaCDF
- 2,3,4,6,7,8-HexaCDF
- 1,2,3,6,7,8-HexaCDF
- 1,2,3,7,8,9-HexaCDF
- 1,2,3,4,6,7,8-HeptaCDF
- 1,2,3,4,7,8,9-HeptaCDF
- OCDF

Organochlorines

- aldrin
- α-, β-, γ- and δ-benzene hexachloride (BHC)
- α- and γ –chlordane
- o,p'-DDD; o,p'-DDE; o,p'-DDT; p,p'-DDD; p,p'-DDE; and p,p'-DDT

- dieldrin
- endrin
- hexachlorobenzene (HCB)
- heptachlor epoxide
- mirex
- *cis*- and *trans*-nonachlor
- oxychlordane
- toxaphene

Trace Elements

- Aluminum
- Arsenic
- Cadmium
- Chromium
- Mercury

The nominal limit of detection will be 0.1 ng/g wet weight for PCB congeners, 0.05 µg/g wet weight for total PCBs, and 0.01 µg/g wet weight for the remainder of the organochlorines. The number of spikes, duplicates, and blanks will be at least 5% of the total number of samples analyzed for PCB congeners and organochlorines. Total PCBs and p,p'-DDE will be confirmed by GC/mass spectrometry in at least 10% of the samples. From congener data, toxic equivalents (TEQs) will be calculated with a variety of methods including those of Safe (1990) and Kennedy et al. (1996).

PCDDs/PCDFs will be analyzed with matrix-specific extraction, analyte-specific cleanup, and HRGC/HRMS analysis techniques by a method of internal standard quantification with [$^{13}C_{12}$]PCDD surrogates. The nominal limit of detection for PCDDs/PCDFs will be 0.05 ng/g wet weight. Concentrations will not be adjusted for recovery.

Tissue samples will also be analyzed for lipid and moisture content. Sediment samples will be analyzed for PCBs (total and Aroclors), TOC, and grain size, with a subset being analyzed for modified Appendix IX parameters including PCB congeners.

4. SAFETY CONCERNS

Wasp and bees will often use nest boxes, so field personnel should look and listen for bee or wasp activity before opening a box. Personnel should be aware of whether they are allergic to bee or wasp stings and prepare accordingly. Bee or wasp nests in the nest boxes may be dispatched as necessary either by smashing them, burning them with a butane lighter, or other method.

5. STATISTICAL ANALYSIS

Prior to any statistical analysis, the data will be verified for accuracy. Any unduly influential observations that may be outliers will be identified and further checked. Contaminant levels in biota will be compared among the 4 study areas with 1-way analysis of variance. Conformation of the data to model assumptions, specifically homogeneity of variances, will be checked with Bartlett's test, and remedial measures will be implemented where necessary (Neter et al., 1990). Comparisons of chemical concentrations, TEQs, and accumulation rates, between contaminated sites and the reference site, will be made with Bonferroni's multiple comparison or other appropriate procedure. Principal component analyses of the PCB congeners, PCDF, and PCDD concentrations may be done. Reproductive success will be quantified with the Mayfield method (Mayfield, 1961, 1975) and compared among sites according to Hensler and Nichols (1981). Regression techniques, including logistic regression, will be used to correlate levels of PCB contamination and TEQs with nest success measures. Bioaccumulation factors (average concentrations in nestlings divided by concentrations in their food) will be calculated at each site. Type-I error rate of 0.05 will be used for all statistical analyses.

6. STUDY RECORDS

All data generated in the study will be recorded in bound laboratory data books or kept in file folders (SOP No. GEN 008). All data sheets and laboratory data books will be encoded with the study number when the data are generated and stored in secure files. Raw data, laboratory data books, computer disks, and the completion product will be filed in the archives (SOP No. GEN 023) of the Upper Mississippi Science Center, La Crosse, Wisconsin.

7. GOOD SCIENTIFIC PRACTICES

Data collection, storage, and retrieval procedures for the study will be conducted in compliance with good scientific practices. The Study Director has the responsibility of ensuring that all procedures used in conjunction with the study conform to good scientific practices.

All changes from the research protocol will be documented in the laboratory data book or on appropriate data sheets and reviewed by the Study Director, who will make a judgment on the impact of the deviations.

8. QUALITY ASSURANCE/QUALITY CONTROL

8.1 DATA QUALITY OBJECTIVES, INDICATORS, AND ASSESSMENT

8.1.1 Data Quality Objectives

The three primary data quality objectives of the tree swallow study were delineated in Section 2. To achieve these objectives, the following types of data and specific quality criteria will be required:

- Tissue concentrations for PCBs and selected other contaminants in food samples of emergent insects and tree swallow pippers and 12-day-old nestlings: Quality control considerations to ensure achievement of the Data Quality Objectives (DQOs) for these parameters will follow the QAPP (WESTON, 2000).

- Sediment chemistry for PCBs and selected other contaminants: Analysis of sediment for chemical constituents must result in data that are consistent in all respects with other sediment contaminant data collected as part of the larger project. Satisfactory results will be ensured by submitting samples to the same laboratories that are analyzing samples for other components of the program. Quality control specifications for these data are delineated in the project QAPP (WESTON, 2000).

- Sediment grain size distribution: Quality control considerations to ensure achievement of DQOs for this parameter will follow the QAPP (WESTON, 2000).

- Condition of nests: Assessments of nest conditions must be made and recorded accurately. Accurate assessment of nest conditions is readily achievable with proper field techniques.

- Number of eggs and young produced: Counts must be made and recorded accurately. Accurate counts are readily achievable with proper field techniques.

- Weight of pippers and 12-day-old nestlings: Tissue weights must be determined accurately and recorded to 0.1 g using a calibrated balance designed and intended by the manufacturer to be capable of accurately measuring masses of this magnitude.

8.1.2 Data Quality Indicators

Data developed in the tree swallow study must meet standards of precision, accuracy, completeness, representativeness, comparability, and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000), that are appropriate to the data quality objectives. Each of these data quality indicators, some of which are not readily quantifiable for the tree swallow study data, is discussed below.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. Because of the small number of eggs in a tree swallow clutch, it is not possible to take repeated independent measurements of the biological parameters. Rather than control and measure precision, the study design includes a large number of what may be called replicates (based on samples from 30-50 nest boxes within an area), to increase the statistical resolution. For the measurements that are not unique to the tree swallow study, such as sediment chemistry and grain size, precision is evaluated as defined in the QAPP (WESTON, 2000).

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study (e.g., tree swallow nest completeness, enumeration and weight of eggs and young), accuracy is defined as meaning that the nest completeness scores are correctly assigned; numbers of eggs and nestlings within each box are correctly enumerated and weighed (as in SOPs WE 409 and 410 and recorded on data sheets following SOP WE 408); correct assessment of live versus dead embryos and nestlings; and developmental status of embryos correctly assessed. Accuracy is a function of consistent field techniques and proper training. The data generated by this study will also be evaluated for accuracy via comparison with known and/or expected results from similar studies. For parameters such as tissue residue and sediment concentrations and grain size, accuracy is as defined in the QAPP (WESTON, 2000).

Completeness is defined as the percentage of the planned samples actually collected and processed. Completeness can be evaluated for only the sediment collection portion of this program. To ensure achieving the planned statistical resolution, it is important that completeness of 100% be achieved for sediment collection. For the tissue residue study component, the number of analyses will be determined by the weight of the material available for collection; therefore, establishment of an *a priori* completeness goal is not possible.

Representativeness refers to the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. Representativeness for this study is ensured through establishment of an approved thorough sampling design and through careful implementation of the sample processing and analytical methods. Specific aspects of representativeness will also be evaluated via comparison with known and/or expected results based on previous investigations of the Lower Housatonic River and other similar systems.

Comparability is a measure of the confidence with which the tree swallow data may be compared to another similar data set. Comparability will be evaluated by examination of the in-station variability in key parameters as determined from the large numbers of replicates to be collected at each location. Comparability will also be evaluated for this data set through comparison with data among the three years of this study in the Lower Housatonic River and with tree swallow data in similar systems.

Sensitivity, the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest, is related for this tree swallow study to the ability of the field personnel to examine nests without causing abandonment. This data quality indicator may be evaluated by comparing the initial number of active well-defined nests to the number of well-defined nests that remain active throughout a study season. As nest abandonment of well-defined nests increases, the availability of young decreases, and the ability to tell differences among reproductive effects from different areas decreases. In addition, sensitivity is applicable and

important for the chemistry parameters that will be analyzed as part of the tree swallow study. For these parameters, the detection limits for chemistry specified in the QAPP (WESTON, 2000) will provide appropriate sensitivity for the purpose of providing insight into factors contributing to the exposure of and reproductive effects in tree swallows.

8.1.3 Data Validation, Verification, and Usability

Procedures for data validation for the tissue and sediment chemistry and physical (e.g., sediment grain size, lipid content in tissue) data are discussed in the project QAPP (WESTON, 2000) For the biological data, usability will be largely be determined by two factors: (1) the experience of the study investigator in establishing that the field sampling was conducted following the SOP and that accuracy and precision were not compromised by an inability to control the sampling procedures in the field and (2) a comparison of the data both within the study and with previous tree swallow studies.

Sediment subsamples for physical and chemical analyses will be collected following procedures documented in the project QAPP (WESTON, 2000) and will therefore be comparable with procedures followed for other similar efforts throughout the Supplemental Investigation.

The purpose of the remainder of this section of the study plan is to document the measures included in the study to ensure that the standards discussed above are met.

8.2 SAMPLING DESIGN

The selection of the four general swallow box locations is based on (1) the location containing sufficient contiguous area of tree swallow habitat on the banks of the Housatonic River to accommodate 30 to 50 nest boxes; (2) the desire that each site be owned by the state or other governmental entity to facilitate access for the duration of the study; and (3) the sites covering a range of sediment PCB contamination in the Lower River between the confluence and Woods Pond. Appropriate reference locations in a nearby tributary or other waterbodies, such as the West Branch of the Housatonic River, Three-Mile Pond, or the river itself upstream of suspected contamination will also be sampled. The three “target” locations are sufficient to achieve the study data quality objectives consistent with the resources available for the study.

To achieve acceptable statistical resolution, data will be collected from a minimum of 5-10 boxes at each of the 4 stations, with the number of pippers and 12-day-old nestling samples varying depending upon the clutch size as described in the “Sample Collection” subsection of the “Study Design” (planned minimum of 2 individuals collected/box). Power analyses for logistic regressions indicated that a sample size of 75 is adequate to have a >80% probability of detecting a relationship between PCBs and hatching success if one exists. In addition, power analysis to detect differences among sites in PCB concentrations as small as 2 µg/g was 99% with as few as 5 samples per site.

8.3 SAMPLING METHODOLOGY

8.3.1 Sampling Procedures

Nest box checking procedures and sampling procedures are discussed extensively in Attachment 2, SOP WE 409 and 410, respectively. Procedures have been selected to minimize disturbance and nest abandonment. In addition, sample processing procedures have been set to avoid cross contamination between the site and reference areas, and among the site areas.

8.3.2 Quality Control Samples

The nature of tree swallow biological data does not allow the incorporation of typical duplicate and blank samples as part of the study design. For field observations, there is no acceptable method of obtaining such samples in a manner analogous to that for duplicates and blanks collected for chemistry analysis.

Duplicate samples for chemistry will be determined in the laboratory based upon the availability of tissue mass, with a target goal of 5% of the samples. Quality control of chemistry analyses for sediments will be provided by taking larger volumes of sediments at a number of locations and processing in accordance with the QAPP (WESTON, 2000).

8.3.3 Sample Processing and Preservation

Detailed procedures for collection and processing of all tissue samples to be collected as part of the tree swallow study are provided in SOP WE 410. All samples will be processed at the field laboratory within 2 hours of removal from the nest box. Food samples and carcasses will be frozen after initial processing. Holding time for samples undergoing physical and chemical analysis will follow procedures established in the QAPP (WESTON, 2000).

8.3.4 Training

All tree swallow data collection and sampling will be under the direction of the Study Director. Senior scientists experienced in sediment sample collection will direct those sampling efforts. Supporting staff will receive training from the Study Director and senior scientists (for the collection of tree swallow data and sediment samples, respectively) in the overall goals of the study and in techniques to be followed to ensure collection of quality data.

8.4 SAMPLE ANALYSIS

Samples for tissue and sediment chemistry and physical parameters (e.g., grain size and tissue lipid content) will be processed following procedures and SOPs provided in the QAPP (WESTON, 2000). These samples will be submitted in catalogs (sample delivery groups) and batches with other samples from the larger project and data validation will be performed on a

catalog basis in accordance with procedures established and described in the QAPP (WESTON, 2000).

8.5 DATA ANALYSIS AND REPORTING

The overall analytical approach for data generated under this study is described in Section 5. The study findings will be included in a stand-alone report including all field observations, tissue chemical data, analyses, and interpretations. This report will be prepared with specific reference to both the data quality objectives specific to the tree swallow study as presented in Section 2. This data will, in turn be incorporated in the ecological risk assessment. All associated sediment chemistry data will be presented in the risk assessment report with specific reference to both the data quality objectives specific to the tree swallow study and Subsection 4.1 of the QAPP (WESTON, 2000).

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ATTACHMENT 1.

KEY STAFF:

Christine M. Custer
Thomas W. Custer

ATTACHMENT 2.

Standard operating procedures (SOPs) to be used in study WE-98-Cont-05

SOP	Title
GEN 008	Laboratory data books and recording data
GEN 023	Archives management for nonregulated studies
WE 409	Nest box checking procedure
WE 410	Tree swallow sample collection and processing procedures

ORIGINAL

Upper Mississippi Science Center
2630 Fanta Reed Road, P.O. Box 818
La Crosse, Wisconsin 54602

GENERAL OPERATING PROCEDURE

PROCEDURE TITLE: Laboratory Data Books and Recording of Data

AREA OF APPLICABILITY: All research staff of Upper Mississippi Science Center-La Crosse (UMSC).

PURPOSE: To describe the organization of laboratory data books and the procedures for properly recording daily activities for studies. The laboratory data book is the key document that will facilitate replication of a study.

PROCEDURES:

A. Laboratory Data Books

1. A separate data book(s) shall be maintained for each study.

2. Data books shall be bound so that pages cannot be removed or replaced without leaving clear evidence that this has occurred. Data books that use carbon paper to make duplicate copies and have perforated pages for easy removal of the carbon copy are encouraged.

3. The front cover of the laboratory data book shall be labelled with the study number, the title of the study, the date on which the study was begun, and the name of the study director.

4. The printed name, written signature, and written initials of anyone assigned to the study shall be placed inside the front cover of the book.

5. The page opposite the front cover shall list recording error codes (see B.4). Commonly used abbreviations for the study can also be written on this page. Example BNZ = benzocaine. Do not use abbreviations in the data book unless they are defined on this page or found on the equipment list in the study guide (SOP GEN 002).

6. The first three pages of the data book may be left blank so that a table of contents can be entered.

B. Recording of information

1. Entries to laboratory data books can be made only by personnel assigned to the study.

2. Entries should be made chronologically, beginning on or before the day on which the study begins and ending on the day on which the study ends. All interim events should be entries in the laboratory

data book.

3. Entries into the data book must be made in black or blue ink.

4. Errors in recording are corrected by making a single line through the incorrect entry; the original entry must be legible. The strike-out must be initialed and dated by the individual making the correction and an explanation for the correction must be written in the margin. The explanation must be sufficient for an auditor to clearly determine what has occurred. When applicable, it is permissible to use the following error codes:

SP= spelling error	example bo~~n~~k o SP GmK 3/May 96
GE= grammatical error	example ~~was~~ were GE GmK 3 May 96
TE= transposition error	example ~~97~~ 79 TE GmK 3 May 96
NL= not legible	example ~~[illegible]~~ was NL GmK 3/May 96

5. Each entry into the data book shall begin with the date the entry was made and, if appropriate, the time.

   Next, a brief outline of the procedure or methods -- referencing the approved protocol or SOP's (whenever possible) and what is to be accomplished -- shall be given. Any materials used to generate the data must be listed. If these items are listed in an approved study guide, it can be referenced; otherwise, the items must be listed. This information must be given in sufficient detail to allow the experiment to be repeated. This description shall include the following information:

   a. The identity, name, model number, serial number, FWS property number (if available), and present location of specific pieces of equipment used to produce numerical data. Such equipment includes balances, pH and dissolved oxygen meters, spectrophotometers, gas chromatographs (GC), high performance liquid chromatographs (HPLC), osmometers, physiographs, sample oxidizers, liquid scintillation counters, and computers.

   b. The identity, name, model, serial number, FWS property number (if available), and present location of any appliance or equipment used to store or prepare samples, including refrigerators, freezers, special storage cabinets, temperature-controlled ovens or rooms, water baths, and special chromatography columns.

   c. Types and sizes of glassware, plasticware, vials, filters, and syringes.

6. Results of the study shall be recorded neatly and sequentially. All entries should be well thought out before being made.

7. If the form or volume of data being obtained makes it impossible to

enter all data into the data book, a file folder can be made to store this information. The file folder and each page of data shall contain the study number (a rubber stamp can be used), which irrefutably links the data sheets and associated information to the study. Reference should be given in the laboratory data book to the location of the study file. The file then becomes part of the data book and must be safeguarded accordingly.

a. Examples of data that might be in this category could include printouts from analytical instrumentation or absorbance traces from GCs or HPLCs, autoradiographs, original copies of thin layer chromatography traces, preprinted forms for recording mortality, and water quality data from acute and chronic toxicological testing, and spreadsheets (e.g. Lotus).

8. Anyone making an entry in a data book must initial and date the end of the entry.

   If the form or volume of data is such that the provisions of B.7. of this SOP are being used, the first and last pages of the data set must be signed and dated by the individual producing the data. In addition, the total number of pages involved shall be indicated.

9. When all entries for a given date have been made and signed in the bound data book, a heavy blue or black line shall be drawn horizontally across the page directly below the last entry for each date. One blank line may be left between the black line and the next day's entries. No blank areas of more than one line are to be left in the bound data book, except after the final entry.

10. The Study Director must review and certify all entries and must countersign the bottom of each page of the bound data book. Multiple page data sets (see B.7. and B.8.) only need to be signed and dated on the first and last pages by the study director. These reviews shall be conducted at least every two weeks; however, more frequent reviews are recommended.

11. If more than one laboratory data book is required for a study, the last entry in a full book should indicate that the study data is continued in another laboratory data book. At the end of the study, the front cover of each laboratory data book must indicate the number of laboratory data books used during the study (e.g. laboratory data book 2 of 3).

12. The last entry made in the laboratory data book(s) shall denote the ending date of the study.

ORIGINAL

C. Security: Laboratory data books and raw data files must be secured at the end of each working day in a locked file cabinet, desk drawer, storage container or vehicle. Only personnel assigned to the study shall have access to these files. Certified copies of the bound data book shall be made at least every two weeks after reviews by the study director (see B.10. above) (carbon copies are acceptable). The certified copies shall be placed in a secured location separate from the location of the laboratory data books.

Electronic data files that have been printed do not have to be copied to produce another paper copy if the electronic file and disk name are placed on the front page of the printed data set. The paper file and the electronic file shall be stored in separate locations.

D. Archives: All laboratory data books and associated raw data files are to be archived according to the UMSC Standard Operating Procedure on archiving at the end of each study.

APPROVED BY: David M. Kennedy DATE 8 March 1996
Quality Assurance Officer

APPROVED BY: [signature] DATE 3/8/96
Center Director

Upper Mississippi Science Center
2630 Fanta Reed Road, P.O. Box 818
La Crosse, Wisconsin 54602-0818

GENERAL OPERATING PROCEDURES

PROCEDURE TITLE: Archives Management for Nonregulated Studies

APPLICABILITY: All Center personnel

PURPOSE: To securely maintain all raw data, supporting documentation and ancillary information generated at the Upper Mississippi Science Center (UMSC), La Crosse, WI during nonregulated studies. Nonregulated studies are defined as studies not intended to support applications for research or marketing permits for products regulated by the U.S. Food and Drug Administration (FDA) and pesticide products regulated by the U.S. Environmental Protection Agency (EPA).

PROCEDURES:

A. Scope - All raw data and supporting documentation generated during a nonregulated study, referred to as the "study package" (Attachment #1, Section A), will be stored in UMSC archives managed by the Center's Quality Assurance Unit (QAU). Ancillary information in support of the study package (Attachment #1, Section B) will be stored as described in Section E.2., "Filing System".

B. Location and Characteristics of Archives - USMC archives for nonregulated study packages and for ancillary information such as training files of former employees, closed-out instrument log books and fish culture records are locked file cabinets adjacent to the southeast stairway (C-2) and in Room 22 in the Administration Building (Segment C, lower level). These cabinets are kept locked at all times except when accessed by a member of the QAU.

C. Access and Return Procedure - Only members of the QAU are authorized to access to retrieve items from the archives, but UMSC personnel can request access to archived materials via the QAU. The QAU will arrange a time to escort the requesting party to the archives to retrieve the requested materials and will record the transaction in the Archives Access Log Book (Form GEN 007a). The requesting party must sign for receipt of archived materials in the Archive Access Log Book. After use, item(s) must be returned to a member of the QAU, who will sign and date the Archive Access Log Book, verifying the item(s) have been returned.

D. Responsibilities

1. The QAU is responsible for managing the Center archives.

2. The Study Director is responsible for indexing and submitting a study package to the QAU for placement into the archives. Study packages will not be accepted by the QAU unless accompanied by completed index forms (Form GEN 023.1a), signed and dated by the Study Director. In addition, a Master Index for each study must be prepared on Form GEN 023.1a. This index will be placed in the archives as the first file folder for each study.

3. The Study Director is responsible for timely submission of ancillary information, such as closed-out instrument logbooks and completed temperature recording charts, to the QAU for placement in UMSC archives.

4. The Center fish culturist is responsible for submitting receipt and rearing records from the Center fish culture facility to the QAU annually, in January, for the previous year.

5. The person signing the Archive Access Log Book for receipt of archive items is responsible for those items until they are returned to the QAU and the Archive Access Log Book is signed and dated, verifying their return.

E. Filing System

1. Study packages will be filed in the archives by study number.

2. Ancillary information will be filed as follows:

   a. Instrument Use, Calibration and Maintenance Log Books and records that are no longer in use will be maintained in archive file cabinets assigned by the QAU.

   b. Temperature-recording charts from temperature-recording devices will be archived annually, in January, for the previous year.

   c. The index of nonregulated studies, copies of protocols, quality assurance inspection reports and personnel training records will be secured in locked QAU file cabinets. Training records of personnel leaving the Center's employment will be transferred to the archives.

   d. Personnel position descriptions will be stored with other facility employment records in a secure area by the Administrative Officer.

   e. Receipt and rearing records of all aquatic invertebrates and vertebrates entering the UMSC fish culture facility will be archived annually, in January, for the previous year.

F. Storage Time

1. Study packages and ancillary information will be securely maintained indefinitely at UMSC. In the event this Center closes, study packages will be transferred to the study sponsors, or if the study sponsor and the Center are the same, will become the property of the parent organization, the U.S. Department of the Interior.

2. Biological specimens will be retained by the Study Director only as long as he/she feels the quality of the preparation affords evaluation. Disposition of biological specimens will be performed at the discretion of the Study Director[1] (**Note:** regulated studies must follow SOP No. GEN 007 for disposal of biological specimens).

G. Transfer of Raw Data - Under no circumstances except closure of this Center will original study packages, or any parts thereof, be permanently transferred outside of the UMSC, La Crosse. If a sponsor requests a study package or specific study data, copies of the originals will be sent.

REFERENCES:

1. UMSC SOP No. GEN 001, *Responsibilities and Procedures of the Quality Assurance Unit.*
2. UMSC SOP No. GEN 007, *Archives Management for Regulated Studies.*
3. UMSC SOP No. GEN 132, *Care, Maintenance and Disposal of Aquatic Vertebrates*
4. UMSC SOP No. GEN 133, *Care and Maintenance Record Keeping for Aquatic Vertebrates.*
5. UMSC SOP No. GEN 134, *Approval of Housing and Care of Vertebrate Animals During Experiments.*

APPROVED BY: David M. Kennedy DATE 4 Sept 1991
Quality Assurance Officer

APPROVED BY: [signature] DATE Sept 5,
Center Director

[1] See Step F.1. for required procedures to transfer study packages if the Center ever closes permanently.

A. Study Package includes all applicable items in A.1.-A.11.: the study package must be archived at the conclusion of the study, after the study completion report is signed by the Study Director.

1. A Master Index to all files being archived (on Form GEN 023.1a)
2. Protocol, protocol amendments and protocol deviations.
3. Study guide:
   a. Verification statement signed by all study personnel.
   b. List of instruments used during the study.
   c. List of TOPs and IOPs used during the study.
4. Test chemical & test substance origin, purity, stability & storage information.
5. Test animal origin, Daily Care and Maintenance Records, Vertebrate Use and Approval (Form GEN 132a), Approval for Housing and Care (Form GEN 134a), Record of Fish Transfers (Form GEN 133d), necropsies and other raw data items associated with test animals.
6. Identity and source of test animal feed.
7. Laboratory notebooks.
8. Raw data generated from direct observation during the study.
9. Reduced data generated from analysis of raw data.
10. Significant correspondence related to the study, excluding QA inspection reports (SOP No. GEN 001).
11. Directory of computer-generated file information associated with raw and reduced data generated during the study.
12. Final report and any amendments to final report and/or other completion products (see GEN No. 014) (**Note:** Publications resulting from a study can be added to the study package at a later date by submitting them to the QAU).

B. Ancillary Information includes all applicable items in B.1.-B.4.:

1. Instrument Calibration, Use and Maintenance Log Books will be retained with the instrument until the log book is full, has been in use for five years, or the instrument is no longer in use. When any of the preceding conditions are met, the log book will be closed-out. A final entry should be written regarding the closure of the log book and the entry must be signed and dated. A line should be drawn diagonally on the remainder of the page, invalidating additional entries. The log book should then be submitted to the QAU for archiving.
2. The index of nonregulated studies, copies of protocol, QAU inspection records, final report reviews and personnel training records will be secured in locked cabinets by the QAU. Training files of personnel leaving the Center's employment will be transferred to the archives.
3. Personnel position descriptions will be retained in a secure location by the Administrative Officer with other facility employment records.
4. Receipt and rearing records of aquatic invertebrates and vertebrates entering the UMSC fish culture facility will be archived annually, in January, for the previous year.

ARCHIVES RECORDS INDEX
Upper Mississippi Science Center

Study Number: _____ Study Director: _____
Study Title: _____
_____ Test Substance: _____

GIVE TITLE OF FILE & THEN LIST ALL ITEMS THAT ARE TO BE ARCHIVED (BE SPECIFIC)

_____ _____
Study Director Date

ARCHIVES RECORDS INDEX
Upper Mississippi Science Center-La Crosse

Form GEN 023.1a
Page ___ of ___

Study Number ____________________ (Continued)

GIVE TITLE OF FILE & THEN LIST ALL ITEMS THAT ARE TO BE ARCHIVED (BE SPECIFIC)

ORIGINAL

Upper Mississippi Science Center
2630 Fanta Reed Road, P.O. Box 818
La Crosse, Wisconsin 54602-0818

TECHNICAL OPERATING PROCEDURE

PROCEDURE TITLE: Nest Box Checking Procedure

APPLICABILITY: All personnel in the Section of Wildlife Ecology who are checking tree swallow nest boxes

PRINCIPAL: To describe nest box checking procedures.

PRECAUTIONS: None

PROCEDURE:

1. Prior to beginning the nest checks, fill in the top two lines of the data collection form (Form WE 409.0a) with your name, the names of anyone assisting you during the nest check, the date, the time of day, and a brief description of the weather. For example, temp. in the mid-70s, partly cloudy with a slight breeze would be an adequate description of the weather.

2. Proceed to visit each nest box and note the contents on the data sheet (Form WE 409.0a) after the appropriate nest box number.

3. To check a box, open the door by lifting up on the nail located on the top of the door. Look inside. It may help to stand on an overturned bucket or small stool, or use an automotive inspection mirror on the end of a short pole to clearly see inside the nest.

4. If feathers obscure the nest contents move them gently aside to get a clear count of the nest contents.

5. **Do not touch** or remove the eggs from the nest. The eggs are extremely fragile and can be easily damaged or broken.

6. If a bird is on the nest gently remove her being careful not to roll eggs or young out of the nest. Her feet (it is usually always a female on the nest) can often be clutching nest material that can cause eggs or young to be displaced. Release her by gently tossing her into the air towards an area without obstructions, such as tree branches.

7. Record the quality of a nest or beginning of a nest in the "Nest" column. The choices are 0, small, medium, or good. Also note if feathers are present. 0 means no nest present, small means just a few grass blades are present, medium means there is 1/2 inch (plus or minus) of grass blades forming the nest, and good means there is a well defined nest. Even though we are categorizing the "quality" of the nest in categories, the amount of nest material is a continuum so use your best judgement as to which category the nest fits into. This column just assists us in anticipating when eggs might be laid.

8. The next 2 columns on the data sheet, "Prev. Data (#eggs and #yg)", will be transcribed from the previous nest check data form before going into the field. Unless otherwise instructed, these 2 columns will be filled out by the Study Director. This information is used to cross check the new data. If the contents are different than what was recorded on the previous check, the number of eggs or young should be recounted to verify the egg or young count or to look more carefully for missing or broken eggs or dead young. **It is very easy to miscount**. For example, if you count 6 eggs on this visit and there were 7 eggs on the last visit then you would want to recount to verify the correct number. If an egg really is missing, determine whether it might have been rolled to the back of the box, is crushed in the nest, or if it really is missing.

9. In the "New Data" columns, write in, for each box number, the number of eggs or young which are present. Draw a short horizontal line in the "#egg" column if no eggs or young are present. Be sure that either a number or horizontal line is written in the "#egg" column. Initial the data sheet for each nest you check. This ensures that we know that a nest box was check and not inadvertently missed.

10. If there are damaged eggs or dead young then include those in the total count in the "#eggs" or "#yg" columns and then note in the Comments section that dead young or damaged eggs were present. For example, if there were 3 live young and 1 dead young in the box, record it as 4 young in the "#yg" column and then put in the comments column that 1 young was dead. See attached sample for examples.

11. Note in the comments section any other item of interest for that nest.

12. **Be sure and check that the box door is securely closed** before moving on to the next box. Once the nail has been replaced, give a light tug on the door to be sure it is secure. If a door flops open, the nest will usually be abandoned.

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13. After all boxes have been checked and the data recorded, photocopy the original data sheets. Keep the photocopy and fax, mail or give the original to the Study Director.

14. Electronic versions of the data form will be used containing the updated information from the previous visit. Prior to each visit update the Prev. Data columns with the current number of eggs and young, save the file, and print-off a clean copy to use in the field. Use the following naming convention for files. Begin with the two letter state abbreviation and the date of the data in the Prev. Data columns. Use .dat as the file extension. For example, data from a May 5 check from Agassiz would be called mnmay4.dat. Data from a May 11 nest check in Massachusetts would be mamay11.dat.

APPROVED BY: Carl E Korschgen DATE: 8/6/98
Leader, Section of Wildlife Ecology

APPROVED BY: David R. Kennedy DATE: 7 Aug 1998
Quality Assurance Officer

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Form WE 409.0a
Page 1 of 1

Colony: Investigator(s): Study Protocol #

Date: Time: Weather:

		Previous Data		New Data		
Box #	Nest[1]	# Eggs	# yg	# Eggs	# yg	Comments

[1] Choices: 0 = no nest
Small = a few blades of grass blades
Medium = ½ inch of grass
Large = well defined nest

ORIGINAL

Upper Mississippi Science Center
2630 Fanta Reed Road, P.O. Box 818
La Crosse, Wisconsin 54602-0818

TECHNICAL OPERATING PROCEDURE

PROCEDURE TITLE: Tree Swallow Sample Collection and Processing Procedures

APPLICABILITY: Any UMSC study personnel responsible for collecting and processing tree swallow samples from nest boxes.

PRINCIPLE: To describe tree swallow sample selection, collection, processing and chain-of-custody procedures

PRECAUTIONS: Safety

A. Wear latex or similar gloves while dissecting tree swallow samples.

B. Adhere to Material Safety Data Sheets (MSDS) for any chemicals used during sample processing

PROCEDURE:

A. Equipment, Reagents and other Supplies Needed

1. Plastic compartment boxes and containers
2. Styrofoam or other type of cooler or ice chest
3. Frozen cooler pack(s) ("blue ice")
4. Aluminum foil
5. Latex or similar gloves
6. Jars for cleaning reagents
7. Tap water
8. Hexane
9. Acetone
10. Labels
11. Indelible marker
12. Chemically clean jars, various sizes
13. Portable field balance
14. Plastic weigh boats or weigh paper
15. Dissection scissors
16. Forceps
17. Paper towels

B. General Sample Collection Information

1. Two types of tree swallow samples are targeted for collection from each nest box: just-hatched young or unhatched eggs (both referred to as pippers) and 12 ± 1 day old young (nestlings).

2. Pippers should be collected on the day they are hatching. A nestling will be collected 12 ± 1 days later from the same nest box, whenever sample numbers permit. Other collection schedules may be implemented if a nest box has insufficient numbers of either pippers or nestlings, or as hatching rate dictates (Section C). When the collection schedule is altered, the change and reason will be documented in the field notebook and on Form WE 410.0a or Form WE 410.0b.

C. Selection of Pipper Samples

1. Two or three pippers will be collected from each nest box; two pippers from clutches of ≤ 5, 3 pippers from clutches ≥ 6. Because the eggs hatch over a 12 - 24 hour period, a variable number of eggs may be hatched when samples are collected from the nest. If all eggs are hatched, select the smaller young for the sample.

2. If there are more just-hatched young than eggs present in the nest, collect one just-hatched young and two eggs for the sample. **For example, if there are four just-hatched young and two eggs, collect one just-hatched young and both eggs.**

3. If there are fewer just-hatched young than eggs, collect three eggs only, for the sample. **For example, if there are two young and three eggs, collect the three eggs.**

4. **Important!** Once eggs have started to hatch, <u>never</u> collect all of the young and leave only eggs in the nest because this will cause the parents to abandon the nest.

D. Selection of Nestling Samples - Count the number of nestlings in the nest. Number them clockwise or linearly, depending on how they are arranged in the nest. Randomly select one nestling using a list of random numbers between 1 and 4 generated from a random number table.

E. Sample Collection

1. Before collecting samples, place a small amount of grass or other vegetation in the bottom of the pipper compartment box or plastic

nestling containers to cushion the sample(s). For nestlings, be sure the container is aerated either with air holes punched into the container or by leaving the lid cracked slightly. Use an indelible ink marker to label each sample container with the nest box number.

2. Pipper collection - Gently transfer the pippers, by hand, from the nest box to the corresponding, labeled compartments of a compartmentalized plastic box.

3. Nestlings - Gently transfer the nestling, by hand, from the nest box into the corresponding, labeled plastic container.

4. Sample containers must be handled carefully to prevent injury or damage to the pippers or nestlings. If samples are stored in a vehicle before being dissected, they should be placed out of the sun and kept as cool as possible. If the temperature inside the vehicle is uncomfortable to the occupants without venting or air conditioning then the samples can be stored in an ice chest with ice packs ("blue ice") to provide a cooler environment. Samples should be processed within 0.5 - 2 hours of collection to preserve the stomach contents.

F. Sample Processing - **NOTE**: If the samples being processed are from both reference and contaminated sites, process the reference site samples first to avoid cross contamination.

1. Field Laboratory Set Up

   a. Place a sheet of aluminum foil on top of an ice chest or other flat surface.

   b. Fill three glass jars with one each of the following: clean tap water, hexane, and acetone.

   c. Place the balance on a flat surface and verify its accuracy according to the balance Instrument Operating Procedure (IOP).

2. Data Collection Form - Prepare Form WE 410.0a or 410.0b for data collection by listing the study number, location and date of collection, and species collected.

3. Label Sample Vials - Each label must include the:

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a. Nest box number followed by a dash and the letter "P" for pippers or "N" for nestling

b. Current date

c. Initials of person performing the sample processing or the Study Director's initials; and

d. Collection site including state if state is not part of nest box numbering system. Other information may be included.

Example: This label indicates that the sample was a nestling collected at Lenox Road for study number WE-98-CONT-08 from nest box ML 845 by Christine Custer

ML845-N 6/10/98 CMC, Lenox Road

e. The study protocol number will be on the sample label or on the container box, ice chest, etc., that contains the samples.

G. Processing Pipper Samples

1. Label plastic weigh dishes alphabetically (A,B,C,..), one each for the number of pippers collected from a specific nest box.

2. Write the nest box number, the alphabet designation and the pipper type, "egg" or "young (yg)" on Form WE 410.0a.

3. Tare the first plastic weigh boat. Place the pipper in the weigh boat and record the pipper's weight (0.0 g) on Form WE 410.0a. Repeat steps E.2 and E.3 for each pipper from the specified nest box.

4. Label one chemically-clean vial to contain all pippers from a single nest box. Process the pippers beginning with just-hatched young followed by unhatched eggs.

5. Unhatched egg processing - Tare the sample vial. Hold the egg over the tared vial and cut along the air cell of the egg with a small pair of scissors. Allow the contents of the egg to drop into the tared vial.

If a 1/2 or greater developed embryo is present in the egg, it may have to be removed from the shell with a pair of forceps and placed in the tared vial. If the embryo is alive and has a visible head, snip the head off with a sharp scissors into the tared collection vial then place the remaining embryo into the vial. Discard the egg shell. Record the weight of the embryo and the stage of embryonic development on Form WE 410.0a. **Note**: Development options are: no visible development (none), 1/4, 1/2, 3/4, or full term embryo. Also record if the embryo is alive or dead.

6. Repeat the process for each unhatched egg from a single nest box. The weight on Form WE 410.0a refers to the sample weight. Either cumulative or individual sample weight may be recorded.

7. Just-hatched young processing - While still in the plastic weigh dish, euthanize the bird by cutting off its head with a sharp pair of scissors. Next make an incision in the bird's abdominal wall and remove the stomach using a forceps and small scissors. Make a slit along the stomach, then use a forceps to remove any stomach contents (food) onto a clean weigh dish. Set the food sample aside. Food samples from all just-hatched young from a specific nest box will be pooled, but can be weighed individually. See section I for additional information and labeling requirements for food samples.

8. Place the head, carcass and stomach (without contents) into the tared, chemically clean vial for that nest box and record the weight on the data sheet.

9. Repeat steps G.6 and G.7 for each pipper until all pippers from a nest box have been processed.

10. Screw the vial lid tightly onto the sample vial and double check that the nest box number is the same as that on the data sheet (Form WE 410.0a).

11. If individual food weights are not taken, then tare a clean plastic weigh boat. Transfer the pooled food sample into the clean, tared weigh boat. Record the sample weight on Form WE 410.0a. Place the food into the sample jar for the corresponding site and type (pipper or nesting).

12. Complete the form by making any other notes or comments as necessary and be sure that all data have been recorded.

13. After processing all pippers from a single nest box, clean and rinse all dissecting tools in the tap water jar to remove residual blood or tissue. Next rinse tools in the acetone then hexane before laying the tools on a clean surface to dry. Discard the foil covering the dissection surface and replace with clean foil. These cleaning procedures will prevent cross contamination between samples collected from different nest boxes and should be repeated after the sampling from each nest box is completed.

H. Processing Nestling Samples

1. Prepare Form WE 410.0b by entering the nest box number, current date and other information required on the form.

2. Tare a medium-sized plastic weigh boat or other container and weigh the young. Record the weight (0.0 g) on Form WE 410.0b.

3. Label a sample jar, then tare it.

4. Euthanize the bird by cutting off its head with a sharp pair of scissors. (Note: it works well to have one person hold the body with the head stretched out slightly and have the second person wield the scissors). Next, make an incision in the bird's abdominal wall and remove the stomach using a forceps and small scissors. Slit the stomach and use a forceps to remove any stomach contents (food) onto a clean, weigh dish. Weigh and record. See Section I. for additional information and labeling requirements for food samples.

5. Place the head, stomach (without contents), and carcass of the nestling in the tared sample jar and record the weight on the data sheet.

6. Screw the lid tightly onto the sample jar and double check that the nest box number and other information on the label corresponds to the data collection Form WE 410.0b.

7. Transfer the nestling food sample into the labeled, vial for that site and record the weight on Form 410.0b if not done previously. Cap the vial tightly and verify the label information against the data sheet.

8. Complete Form WE 410.0b by making other notes or comments, as needed, and be sure the data and other required information have been completed on the form.

9. After processing each nestling, clean all tools thoroughly and place clean aluminum foil on the dissecting surface (see G.13).

I. Food Samples.

1. Food samples from a single site will be composited to provide enough mass for chemical analysis. Food samples from the pippers will be composited separately from the food samples from the nestlings, but may be combined at a later time, as needed and decided by the Study Director.

2. The label on the food sample vials should contain the following information: site designation (Example: West Branch, Lenox, Canoe Meadows, Roaring Brood, or other study site names), state, whether it is a composite for pippers or nestlings, the year, initials of person collecting and processing the sample or the Study Director's initials, and the words tree swallow food. The study protocol number will be included on the sample label or on the container, box, ice chest, etc., that contains the sample. For example, a food sample from nestlings collected at Lenox Road, in 1998 would have the following information on the label:

tree swallow food, Lenox Road, MA, Pipper, 1998, CMC

J. Transportation and Storage of Processed Samples - Samples will be kept as cool as possible during transportation from the field to a freezer. As soon as feasible, samples will be placed in a standard freezer using the following chain of custody procedures.

K. Chain of Custody

1. Samples to be place in the freezer will first be placed in a plastic bag or box. The bag or box will be labeled, at a minimum, with the study number, type of samples, and current date and initials of person packaging them or the Study Director for freezer storage. Other information may be included as desired.

2. The bag or box will be locked or sealed with security tape to allow detection of unauthorized tampering. The security seal will be signed and dated by authorized study personnel.

3. The Study Director or other designated study personnel are personally responsible for the care and custody of the samples until they are transferred to the chemical laboratory. A sample or set of samples is considered "in the custody" of an individual if any of the following apply:

   - the sample is in the individual's possession.
   - the sample is in the individuals residential freezer.
   - the sample is within view after being in possession.
   - the sample is in a locked or sealed area or container that prevents tampering after being in possession.
   - the sample is in a designated secured area.
   - the sample is in a security-sealed bag or box.

4. A chain of custody form (Form WE 410.0c) or similar form should accompany samples when shipped to analytical laboratories. The chain of custody form should include the following information: a title indicating that the document is a Chain of Custody Record; the Study Protocol title and number; where the samples are being shipped to; the method(s) of shipment (FEDEX, UPS, hand, etc.) And the sample number(s). The form should also contain a minimum of two signature lines containing (1) signature of person releasing samples and date samples were relinquished, and (2) signature of person accepting samples and date that samples were received. This form may be computer generated.

REFERENCE: EPA/FDA GLPs At a Glance. Society of Quality Assurance.

APPROVED BY: Carl E. Korschgen DATE 8/6/98
Leader, Section of Wildlife Ecology

APPROVED BY: David A. Kennedy DATE 7 Aug 1998
Quality Assurance Officer

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Egg/Pipper Dissection Data

Location _____ Date _____

Species _____ Investigator(s) _____ Study Number _____

Nest Number	Pipper Desig[1]	Pipper Type[2]	Whole Pipper Weight(g)	Embryo Develop[3]	Sample Weight(g)	Food Weight(g)	Comments

Comments:

[1] Pipper Desig = a, b, c, etc.
[2] Pipper Type = egg or young (yg)
[3] Embryo Develop = none, 1/4, 1/2, 3/4, full

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Tree Swallow Nestling Dissection Data

Location _____ Date _____

Species _____ Investigator _____ Study Plan _____

Nest Box Number	Weight in Grams (0.0) Live Bird	Carcass Remainder	Food	Comments

Comments:

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Form WE 410.0c
Page 1 of 1

Chain of Custody Record

Study Protocol Title & Number: _____

_____

_____

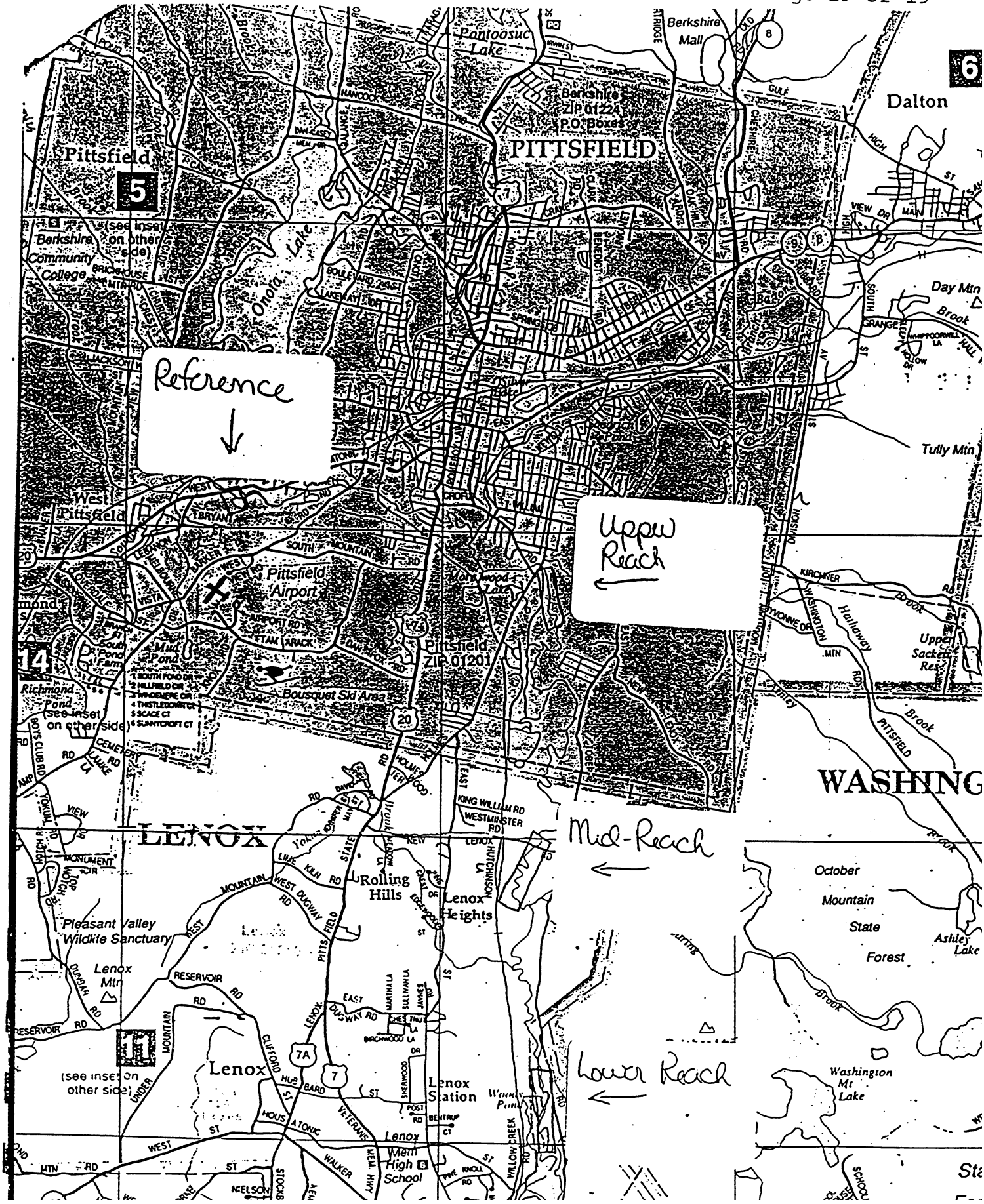
Principal Investigator: _____

Samples Shipped to (Name of Laboratory or Individual & Address): _____

_____

Item No.(s)

From: (print name) To: (print name)	Release Signature Receipt Signature	Release Date Receipt Date	Delivered via: O Federal Express O U.S. Mail O By hand O Other
From: (print name) To: (print name)	Release Signature Receipt Signature	Release Date Receipt Date	Delivered via: O Federal Express O U.S. Mail O By hand O Other


Reference
Upper Reach
Mid-Reach
Lower Reach
Pittsfield
PITTSFIELD
Berkshire Mall
Dalton
Pontoosuc Lake
Onota Lake
Berkshire Community College
West Pittsfield
Pittsfield Airport
Pittsfield ZIP 01201
Bousquet Ski Area
Richmond Pond
Mud Pond
Tully Mtn
Day Mtn
Hallaway Brook
Upper Sackett Res
LENOX
Rolling Hills
Lenox Heights
Pleasant Valley Wildlife Sanctuary
Lenox Mtn
Lenox
Lenox Station
Lenox Mem High School
October Mountain State Forest
Ashley Lake
Washington Mt Lake
WASHING
(see inset on other side)

ATTACHMENT 3.

Colony: Investigator: WE-98-Cont-08
Date: Weather:

Box	Nest?	Prev. data		New data		Comments
		#eggs	#yg	#eggs	#yg	

APPENDIX A.25

WORK PLAN FOR THE STUDY OF SMALL MAMMALS OF THE HOUSATONIC RIVER FROM NEWELL STREET TO WOODS POND (WOODLOT ALTERNATIVES, INC.)

FINAL

APPENDIX A.25

WORK PLAN FOR THE STUDY OF SMALL MAMMALS OF THE HOUSATONIC RIVER FROM NEWELL STREET TO WOODS POND

Submitted to:

Roy F. Weston, Inc.
1400 Weston Way
West Chester, PA 19380-1499

Submitted by:

Woodlot Alternatives, Inc.
122 Main Street
Topsham, Maine 04086

FINAL

TABLE OF CONTENTS

1.0 Introduction

The United States Environmental Protection Agency (EPA) is characterizing the natural resources found in and adjacent to the Housatonic River in portions of Pittsfield, Lenox, and Lee, Massachusetts (hereinafter referred to as the study area) (see Figure 1 in Appendix A.6). This reach of river is approximately 12 miles long and extends from Newell Street in Pittsfield downstream to the Woods Pond Dam in Lee. Elevated levels of polychlorinated biphenyls (PCBs) that originated from the General Electric (GE) facility in Pittsfield have been found in this reach of the Housatonic River and the adjacent floodplains (Blasland, Bouck, & Lee, Inc., 1996).

The study area is inhabited by a variety of mammals that travel through, breed, and feed in the river, floodplain wetlands, and adjacent uplands. Large mammals that have recently been sighted in the study area include black bear (*Ursus americanus*), white-tailed deer (*Odocoileus virginianus*), coyote (*Canis latrans*), beaver (*Castor canadensis*), muskrat (*Ondatra zibethicus*), red fox (*Vulpes vulpes*), raccoon (*Procyon lotor*), and striped skunk (*Mephitis mephitis*) (TechLaw, Inc., 1999). Coyote and red fox scat observed in 1998 contained small mammal fur and bones, an indication that a portion of their diet consists of mice and voles (TechLaw, Inc., 1999). Small mammals known to occur in the study area include the white-footed mouse *(Peromyscus leucopus)*, southern red-backed vole (*Clethrionomys gapperi*), meadow vole (*Microtus pennsylvanicus*), smoky shrew (*Sorex fumeus*), short-tailed shrew (*Blarina brevicauda*), masked shrew (*Sorex cinereus*), meadow jumping mouse (*Zapus hudsonius*), and star-nosed mole (*Condylura cristata*) (TechLaw, Inc., 1999).

Several studies have investigated the effects of exposure to PCBs on small mammals including laboratory mice (Marks et al., 1989; Murk et al., 1991; Kholkute and Dukelow, 1997), Mongolian gerbils (*Meriones unguiculatus*) (Phillips and Batty, 1995), little brown bats (*Myotis lucifugus*) (Clark and Stafford, 1981), and white-footed mice (Linzey, 1987). Linzey (1987) reported white-footed mice exposed to food treated with PCBs at a rate of 10 parts per million (ppm) weaned significantly smaller numbers of young, suggesting potential population impacts from exposure. Another study indicated that PCBs can bioaccumulate in small mammals (Watson et al., 1985), and therefore become available to higher trophic level predators.

1.1 Objectives

The primary objective of this study is to collect small mammals for tissue residue analysis for PCBs, and possibly dioxins/furans and organochlorine pesticides, in order to provide data on the bioaccumulation of these materials in the food web. Analytical results will be used in food chain models to estimate potential risks to higher consumers, such as large mammals or raptors that feed upon small mammals. Another study objective is to determine if there are differences between numbers of placental scars and embryos in females of reproductive age from areas with varying concentrations of PCBs. These data will be considered in the ecological risk assessment.

1.2 Project Approach

To accomplish the objectives of the study, small mammals will be captured in several areas of floodplain forest habitat within the study area with varying PCB concentrations (i.e., less than one part per million [ppm], from 1 to 30 ppm, and greater than 30 ppm). Small mammal whole body tissue will be analyzed for PCBs (totals, Aroclors, congeners, and homologs), percent moisture, percent lipids, and possibly organochlorine pesticides and dioxin/furans, and the placental scars of adult females will be examined.

2.0 Study Design

2.1 Literature Review

The scientific and technical literature, including museum records, will be reviewed to determine the historic distribution of mammals in the Housatonic River drainage system. As part of this effort, local and regional experts will be consulted to obtain unpublished records regarding the historic and current distribution of mammals in the Housatonic River drainage. The Massachusetts Natural Heritage Program and the U.S. Fish and Wildlife Service (USFWS) will also be consulted to determine if any records of mammals from the Housatonic River drainage are available from surveys sponsored or conducted by these agencies.

2.2 Field Sampling Design

2.2.1 Small Mammal Trapping Locations

Preliminary surveys in 1998 indicated that several species of small mammals live in forested floodplains in the study area. White-footed mice were the most abundant small mammal captured (64% of all captures), followed by meadow voles, short-tailed shrews, southern redback voles, and masked shrews (TechLaw, Inc., 1999). Two of these species are suitable for whole body tissue analysis because they are primarily insectivorous during the summer months, and certain insects are known to take up PCBs from soil in a relatively short time period (Paine et al., 1993). Short-tailed shrews eat earthworms, slugs, snails, lepidopterous larvae, spiders, and centipedes, while white-footed mice, although omnivorous, primarily eat insects in the summer months (Whitaker and Hamilton, 1998). Meadow voles and southern redback voles seldom eat insects (Whitaker and Hamilton, 1998), and because of this, they are unlikely to bioaccumulate PCBs at the same rate as more insectivorous species.

In 1998 small mammals were captured in floodplains dominated by overstories of boxelder (*Acer negundo*) and silver maple (*Acer saccharinum*), and understories of wood nettle (*Laportea canadensis*) and ostrich fern (*Matteuccia struthiopteris*) (TechLaw, Inc., 1999). This type of habitat is common in the forested floodplains of the study area. Based on these findings, similar habitat in the study area will be selected for potential trapping locations. These areas will be identified using aerial photos and cover type maps of the study area to locate forested floodplain habitat similar to that used by small mammals in 1998. After areas of similar habitat are identified, maps showing PCB concentrations in floodplain soils will be reviewed to identify

places in the floodplain with varying levels of PCBs. Potentially suitable trap sites will then be surveyed on the ground to verify habitat suitability based on the similarity with habitat where small mammals were captured in 1998.

Only sites where signs of small mammal use including scat, tracks, and browsing are observed will be considered for selection. Sampling sites that are selected will be described both quantitatively and qualitatively using data collected on Natural Community Survey Forms (Maine Natural Areas Program, 1997). Detailed quantitative information on plant community characteristics, soils, microtopography, and hydrology will be collected for describing habitat within small mammal home ranges, which can vary from 20 to 50 m for meadow voles (Getz, 1982).

2.2.2 Data Evaluation and Statistics

Small mammal trapping data are typically normalized to captures per 100 trap nights. For less common species, or species that are more difficult to trap, such as masked shrews, captures can range from less than one animal to a few animals per 100 trap nights. Captures of abundant species, such as meadow voles or white-footed mice, can range up to 10 captures per 100 trap nights. Assuming a mean capture rate of 10 individuals (the sum of all species) per 100 trap nights, a mean of approximately 50 individuals may be captured per site under the proposed sampling regime of 500 trap nights per site.

Data available for statistical analysis are expected to include the total number of captures and the captures per 100 trap nights by species for each site; descriptive statistics (e.g., mean, variance, and range) for all measurements of continuous variables, such as weight and length; and counts of categorical variables (e.g., sex). Placental scars and embryos are technically discrete data (i.e., a female may have 1 or 2 embryos but never 1.5 embryos), but they are often reported as continuous variables. For example, many reports in the literature refer to the mean number of placental scars. Placental scars and the number of embryos, therefore, will be reported using both frequency tables (i.e., the number of females with 0 placental scars, the number with 1 scar, etc.) and descriptive statistics, including the mean, variance, and range.

Reproductive data (placental scars and number of embryos) will be primarily analyzed as discrete variables. Treating the data as discrete variables, when using a chi-square analysis, will require correction for continuity. A contingency table and chi-square analysis will be used to compare the number of placental scars per female between sites for each species (Fienberg, 1983; Zar, 1984). This is a Poisson sampling model (Fienberg, 1983:15) with a set of Poisson processes, one for each cell in the cross-classification, over a fixed period of time, and with no *a priori* knowledge regarding the total number of observations to be taken. Each process yields a count for the corresponding cell (e.g., number of females with 2 placental scars at Site 1). All analyses will be run using the *Statistica*^{TM} software package (Steiger, 1999).

For most species in the study area, the mean number of young per litter typically ranges from 4 to 6 (Whitaker and Hamilton, 1998). It is likely, however, that the number of young per litter is not normally distributed around the mean. It is more likely that most adult females have 4 to 6 young,

very few have greater than 6 young, and somewhat more have less than 4 young (i.e., the distribution function is skewed to the right). Based on this assumption, and assuming captures of 25 individuals per species at each site and 3 sites (total N = 75), a power analysis (Borenstein, et al., 1997) for the chi-square analysis was developed using hypothetical data (Table 1). This analysis is based on $\alpha = 0.1$ and a desired power ($1-\beta$) of at least 0.8. For this analysis, it was assumed that observing a mean number of placental scars roughly half the expected mean was biologically significant[a]. The results of this analysis ($\beta = 1.00$) indicate that this test would be a suitably powerful indication of statistically significant results. When the data are analyzed, the observed power will be reported. In the event that cells within the contingency table contain no observations (e.g., no females had a count of 1 placental scar), Fienberg's (1983) guidelines for fixed and random zeroes will be followed.

Once the PCB tissue data are available, regression analysis will be used to determine whether there is a relationship between PCB concentration in female small mammal tissues and the number of placental scars or embryos. Data from all sites will be pooled for this analysis and covariates will include age of the female (immature versus adult) and weight. Assuming that the covariates explain 20 percent of the increment to R^2 and the tissue PCB level explains 40 percent, which would be considered biologically significant, a sample size of 10 would be needed to achieve a power greater than 0.8 with an α of 0.1 (Borenstein, et al., 1997). The actual power of the test and the increment to R^2, however, will be reported for the data that are collected.

Table 1

Power Analysis for Hypothetical Small Mammal Placental Scars

	Proportion falling in column								Proportion in Row
	0	1	2	3	4	5	6	>7	
<1 ppm PCBs	5	5	5	5	15	45	15	5	0.33
1-30 ppm PCBs	5	5	25	40	10	5	5	5	0.33
>30 ppm PCBs	10	15	40	15	5	5	5	5	0.34

Number of cases = 75

Alpha (2-tailed) = 0.10, Power=1.00

Power computation: Non-central chi-square

[a] The actual significance of reducing the mean litter size for each species may be modeled as part of this study. For some small mammal populations, which may turn over completely on an annual basis, a modest decrease in mean litter size may be biologically significant and result in unstable or declining populations.

2.3 Analytical Requirements

2.3.1 Analyses

Based on the average size of short-tailed shrews and white-footed mice collected during preliminary surveys in 1998, sufficient tissue mass (about 10 g) for the required tissue residue analyses will be obtainable from each individual collected. In addition, preliminary surveys (TechLaw, Inc., 1999) indicated that approximately 10 individuals per target species should be captured during the approximately 500 trap night surveys. Each small mammal submitted for analysis will be analyzed for PCBs (total, Aroclors, congeners, and homologs), percent moisture, and percent lipids. A subset of samples may be analyzed for organochlorine pesticides and dioxin/furans if there is sufficient sample weight. The analytical methods and detection limits to be used are specified in Appendix C of the *Quality Assurance Project Plan* (QAPP) (WESTON, 2000).

2.3.2 Quality Assurance/Quality Control Samples

Duplicate analyses will be conducted for each parameter on approximately 1 of every 20 samples. An additional 10 g of tissue will be required for each set of analyses beyond the 10 g required for each set of original analyses, with 20 g as the total amount of tissue required. In addition, a matrix spike/matrix spike duplicate (MS/MSD) sample is required for approximately every 20 samples. An additional 20 g of tissue will be required for each set of MS/MSD analyses, beyond the 10 g required for the original analyses. Thus, the amount of tissue required to conduct the original analyses and MS/MSD analyses on a single sample is about 30 g.

3.0 Procedures

3.1 Field Sampling

At each trap site, following established protocol (Clough, 1987; Lortie and Pelletier, 1987), 100 snap-traps baited with peanut butter will be placed in an "X" pattern (when possible, some sites may require that the direction of an axis be changed due to the configuration of the habitat area), with each axis being approximately 500 feet in length. One trap will be placed at each trapping station on the line and every tenth trapping station will include a pit-trap, which is more effective for capturing shrews. Where small mammal runways are apparent, traps will be placed on the runways to increase capture efficiency. Trapping will be conducted for 5 days, for a total of approximately 500 trap nights per site. Sampling intensity of 500 trap nights per site is based on the anticipated size of the small mammal population in the habitats being sampled. For example, Getz (1982) recommends using 450 trap nights when estimating relative abundance per habitat for meadow voles and additional sampling is unlikely to result in capturing significantly more small mammals.

Because pit traps tend to be more efficient at capturing shrews (Kirkland, 1982), pit-trap arrays will also be used at each trapping site. These arrays consist of four drift fences 25 feet long and 2.5 feet high, arranged in an "+" formation with a 50-foot gap in the center of the "+." Individual

pit traps were installed on both sides and at either end of each of the four drift fences, for a total of 16 pits per array. The pit-fall trap arrays will be situated near one of the axes of the larger snap trap arrays.

3.2 Processing

3.2.1 Initial Processing

Captured small mammals will be removed from traps and placed in clean, unused, individually labeled resealable plastic bags for transport to the processing area. Bags will be labeled with date, trap site, and individual trap location. Prior to transport to the processing area, bagged small mammals will be placed in a cooler containing bagged wet ice.

Two-person teams will prepare a processing table with clean plastic sheeting. All processing equipment and supplies including aluminum foil will be decontaminated with nitric acid/hexane/ isopropyl alcohol/deionized water as specified in the QAPP (WESTON, 2000). On the data sheet for each small mammal, the sample location, date, initials of collector(s), individual small mammal identification number, WESTON sample identification number, species, sex, weight (g), total length (mm), tail length (mm), hind foot length (mm), and ear length (mm) will be recorded. Each individual small mammal will also be sexed, aged (adult versus immature), and inspected for abnormalities or deformities, which will be described on data forms. Age will be determined by comparing body size measurements, pelage color, and reproductive status (e.g., size of testes) with those in the literature for known age individuals.

Uterine tissues will be removed from female small mammals using dissecting equipment decontaminated using the process described above. The number of placental scars and embryos will be counted for captured females with the aid of a dissecting microscope. Placental scars will be grouped and counted based on the size, shape, and opacity (Harder and Kirkpatrick, 1996). A sketch will be prepared and a photograph taken of placental scars. All small mammals will then be packaged for preservation for tissue analysis.

3.2.2 Tissue Processing

After morphometric and placental scar (females) information has been collected for each small mammal, the specimen will be individually wrapped in nitric acid/hexane/isopropyl alcohol/ deionized rinsed aluminum foil. The foil will be labeled with sample identification number, location, date, collector's initials, weight, and tissue type. The foil-wrapped sample will then be placed into a resealable plastic bag, similarly labeled, and then placed immediately into a freezer. Sample attribute forms will be completed for each sample (whole body tissue, duplicate, or MS/MSD samples). The above procedures summarize those outlined in the project QAPP.

3.2.3 Sample Handling and Shipping

The procedures summarized here follow those in the project QAPP. Samples will be kept in a freezer at -22° C until shipment to the laboratory. When ready to ship, the samples (wrapped in labeled foil and enclosed in labeled resealable plastic bags) will be placed in a large plastic bag

and then into a cooler lined with vermiculite. Chain-of-custody forms listing the contents of each cooler will be completed and placed into a resealable plastic bag. The resealable plastic bag will be taped to the inside of the top lid of the cooler, or placed on top of the samples. The coolers will be sealed with two custody seals, and labeled with appropriate WESTON shipping labels, including the WESTON return address, and USFWS laboratory address. Samples will be delivered by courier or overnight delivery to the analytical laboratory.

3.2.4 Sample Documentation

Specimen data sheets for each small mammal will be completed to include: location; date of collection; method of collection; collector's initials; total weight (g), sex (if known), total length (mm), tail length (mm), hind foot length (mm), ear length (mm); and analyses. Sample attribute forms will also be completed for each tissue sample, which will include the sample number for each sample and the species Latin name, date, and individual small mammal identification number. Complete chain-of-custody forms for each cooler of samples shipped to the USFWS laboratory will be provided to the task manager, who will retain them in the WESTON files.

4.0 Quality Assurance/Quality Control

4.1 Data Quality Objectives, Indicators, and Assessment

4.1.1 Data Quality Objectives

The objectives of the study are outlined in Subsection 1.1. To achieve these objectives, the following types of data will be required:

- **Natural community characterization**: accurate descriptions of habitat characteristics in the vicinity of small mammal trapping locations must be collected.

- **Taxonomic** data: accurate species identifications of captured small mammals must be obtained.

- **Enumeration:** counts of placental scars and embryos in female small mammals of reproductive age must be accurately recorded using standard procedures.

- **Morphometric data**: accurate measurements of small mammal morphometrics (weight, length, etc.) must be acquired.

- **Concentrations of PCBs in small mammal tissues**: Quality control considerations for PCB concentration analysis for biological samples will follow those identified in Subsection 3.2, and in the project QAPP.

4.1.2 Data Quality Indicators

Data developed in this study must meet standards of precision, accuracy, completeness, representativeness, comparability, and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000), that are appropriate to the data quality objectives.

Precision is defined as the level of agreement of repeated independent measurements of the same characteristic. For this study, repeated independent measurements of species identification will be made as mammals are collected and processed using taxonomy keys. Agreement between surveyors regarding species identification must be obtained for verification. Precision may also be evaluated by assessing the degree to which surveys are consistent among sites. For measurements that are not unique to small mammals, precision is evaluated as defined in the QAPP.

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study (small mammal taxonomy), accuracy means that: 1) the specimens are correctly identified and counted; 2) morphometric data are measured using methods and instruments capable of providing accurate, reliable measures; and 3) tissue analysis is conducted using laboratory procedures established in the QAPP (WESTON, 2000).

Completeness is defined as the percentage of the planned samples actually collected and processed. A power analysis was conducted to determine the sample sizes needed for statistically significant sample results. Completeness for this study, therefore, can be interpreted as the percentage of the desired sample size that was acquired.

Representativeness is defined as the degree to which the data accurately reflect the characteristics present at the sampling location at the time of sampling. Representativeness for this study will be ensured through establishment of an approved, thorough sampling design and through careful implementation of the sample processing and analytical methods. Specific methods will be used to collect target organisms in an unbiased fashion and should not select for certain components (only males, females, adults, juveniles, etc.) of the source population.

Comparability is defined as the measure of confidence with which the small mammal collection data may be compared to another similar data set. Comparability will be attained through use of standard sampling procedures for small mammals. These methods are well established and are repeatable at the sampling location or other areas, which results in comparable data. In addition, trapping results are measured on a per unit effort basis that can be compared to other studies.

Sensitivity is defined as the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest. For data specific to this study, sensitivity will pertain to the ability of measurement instruments to measure animal morphometrics, optics to magnify tissues well enough to identify and count placental scars, and laboratory instruments to detect and measure PCBs in animal tissues.

4.1.3 Data Validation, Verification, and Usability

Procedures for data validation for the analysis of tissue PCB concentrations are discussed in Subsection 2.3, above, and in the project QAPP and will be used whenever applicable in this study.

4.2 Sampling Design

Trapping sites will be chosen based on 1998 background trapping data and floodplain soil PCB data acquired in 1999. Sites will contain varying levels of PCBs in the soil and will represent areas with no to high levels, as identified in Subsection 1.2.

4.3 Sampling Methodology

4.3.1 Sampling Procedures

Field investigations for this study consists of standard methods for the capture of small mammals. Lab procedures will ensure that accurate tissue concentrations and scar counts are achieved and will be based on methods identified in the compilation of observational data to create species-habitat associations. No physical samples of site soil or water will be collected for this study; therefore, QA/QC procedures for physical samples are not required.

4.3.2 Quality Control Samples

Quality control samples (routine duplicates and MS/MSD duplicates) will be collected in accordance with standards identified in Subsection 2.3.2 and in various sections of the QAPP.

4.3.3 Sample Processing and Preservation

Processing and preservation of samples will be in accordance with Subsection 3.2 and in various sections of the QAPP.

4.3.4 Training

All field work will be directed by senior field scientists with considerable experience in small mammal trapping techniques. Supporting staff will have some experience in these types of surveys but will be trained, as needed, with respect to methodology by the senior scientists.

4.4 Sample Analysis

4.4.1 Tissue Samples

Samples will be analyzed according to the analytical methods and detection limits specified in Appendix C of the project QAPP.

4.4.2 Physical/Chemistry Samples

Physical and chemical samples were collected for use in this study in order to select study sites. These samples were collected, preserved, and analyzed in accordance with the project QAPP.

4.5 Data Analysis and Reporting

The study findings will be included in the ecological risk assessment including all pertinent data, analyses, and interpretations, particularly within the PCB fate and effects component of the modeling effort. Rare species, if found, will be reported on MNHESP Rare Animal Reporting Forms. Statistical analysis will be preformed using the *Statistica*^{TM} software package (Steiger, 1999).

5.0 Equipment List

5.1 Field

Equipment that will be needed as part of the field component of the study includes:

- Camera
- Pit traps
- Field notebooks
- Snap traps
- Binoculars
- Hand lens
- Rubber knee and hip boots
- Nitrile gloves
- Resealable plastic bags
- Peanut butter
- Flagging and pin flags
- Permanent markers

5.2 Processing Area

- Microscope slides
- Dissecting scope with light
- Dissecting kit (scissors, forceps, pins)
- Petri dishes
- Razor blades or scalpel
- Rinse bottle
- Dissecting trays
- Labels, permanent markers, resealable plastic bags, paper towels
- Coolers, ice
- Aluminum foil
- Nitric acid, deionized water, hexane, isopropyl alcohol
- Taxonomy reference books

- Garbage bags
- Disposable gloves
- Work table

6.0 Results

Numbers of small mammals captured per site per species will be compared between sites and to numbers reported in the literature. Site-specific tissue concentrations from small mammals from each trapping site will be compared between sites and with residue effect levels from the literature. Numbers of placental scars observed will be compared between trapping sites and with those present in the literature. The interpretation and use of placental scar data will take into account stochasticity, differences between habitat in sampling areas, the difficulties of identifying individual placental scars and scars per litter (i.e., some small mammals can have several litters in rapid succession and separating sets of scars can be difficult [Martin et al., 1976]), and the difficulties of aging small mammals. These factors may limit the extent of use of placental scar data.

A table of mammal species known or suspected to occur in the study area per habitat type will be prepared. This table will include the species of mammals observed in the study area during this study. Maps showing the location of survey points in relation to natural community types will be produced. Morphometric data for each trapped animal will include location, species, date of capture, sex, weight, total length, hind foot length, ear length, and miscellaneous comments. Data forms (Natural Community Survey Data Forms, Small Mammal Data Forms) will be appended to draft and final narrative reports. The reports will describe the methods used to survey mammals and the results of surveys. This information will be in the ecological characterization report, and will be used in the Ecological Risk Assessment.

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APPENDIX A.26

DIETARY EXPOSURE OF MINK TO FISH FROM THE HOUSATONIC RIVER: EFFECTS ON REPRODUCTION AND SURVIVAL (AULERICH, BURSIAN, YAMINI, AND TILLITT)

APPENDIX A.26

DIETARY EXPOSURE OF MINK TO FISH FROM THE HOUSATONIC RIVER: EFFECTS ON REPRODUCTION AND SURVIVAL

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1. INTRODUCTION

During the last two decades, there has been considerable concern regarding the presence of environmental contaminants, especially polychlorinated biphenyls (PCBs) and, to a lesser extent, other contaminants, such as polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), in the biota and sediments of the Housatonic River that flows through western Massachusetts and Connecticut into Long Island Sound. PCB concentrations as high as 200 ppm have been reported in sediments and fish taken downstream from a point source of PCBs located on the East Branch of the Housatonic River at Pittsfield, Massachusetts (Smith and Coles, 1997).

Because consumption of fish containing elevated levels of PCBs might pose a serious health risk to humans, parts of the Housatonic River were closed to all but catch and release fishing in 1982 (EPA, 1999). A similar concern has been raised for piscivorous wildlife that inhabit the margins of that river. The Housatonic River flows through habitat that has historically sustained viable populations of piscivorous species, such as mink. Recent field studies have failed to observe these species at expected frequencies, either directly or by sign, in suitable habitat (Woodlot Alternatives, 1999) along the more highly contaminated sections of the river, while viable populations inhabit nearby reference areas, suggesting that PCBs potentially have an adverse effect on these species. Thus, this study will evaluate whether farm-raised mink fed diets containing PCB-contaminated fish from the Housatonic River will exhibit impaired reproductive performance and/or offspring (kit) growth and survival.

Mink (*Mustela vison*) is the species of choice for testing this hypothesis because: (1) they are a semiaquatic piscivorous species native to the area; (2) they are among the most sensitive mammalian species to PCBs (Aulerich and Ringer, 1977) and PCDDs (Hochstein et al., 1988, 1998); (3) their nutritional requirements are well documented (National Research Council, 1982); (4) stock of known genetic origin is readily available; (5) all stages of their life cycle can be successfully perpetuated in the laboratory; and (6) mink have a large biological database (Shump et al., 1976; Scientifur, 1987, 1992; Sundqvist, 1989; Aulerich et al., 1999).

The Michigan State University (MSU) Experimental Fur Farm has the facilities and experienced personnel to conduct mink toxicity studies. MSU researchers have developed guidelines for mink toxicity tests (Ringer et al., 1991) and have conducted numerous mink studies involving environmental contaminants similar to the proposed study (Heaton et al., 1995a, 1995b; Restum et al., 1998; Halbrook et al., 1999).

2. STUDY DESIGN AND METHODS

2.1 COLLECTION OF FISH

Fish will be collected from the Housatonic River from New Lenox Road to Woods Pond, which have good habitat for mink. Collection and transport of fish will be handled by the U.S. Fish and Wildlife Service (USFWS), Laconia, NH, and Roy F. Weston, Inc. (WESTON®) and in general

will follow fish shipping and handling procedures presented in Appendix A.20 of this Work Plan. When fish arrive at the MSU Experimental Fur Farm, East Lansing, MI, they will be identified, sorted, and weighed by species. All fish from each site or source will be ground and blended into a homogeneous mixture. Six "grab" samples (300 to 500 g each) of the homogenized fish tissue mixture will be collected randomly, labeled, and frozen for subsequent analysis for organochlorines (OCs), PCBs, PCDDs, PCDFs, and potentially toxic or bioaccumulative inorganics. Ocean fish will be processed and analyzed in a similar manner. Results of these analyses will determine the proportions of Housatonic River and ocean fish to be incorporated into the experimental mink diets to achieve the desired doses of contaminants in each treatment.

2.2 DIETARY TREATMENTS

The diets will be conventional mink diets formulated to meet the nutrient requirements of mink (National Research Council, 1982) as described by Ringer et al. (1991; Attachment 1). There will be six dietary treatments, each containing the same percentage of fish (for example, 30%). The control diet will contain 30% "clean" ocean fish. The remaining five diets will contain a mixture of ocean fish and the homogenized fish from the test site(s) such that targeted concentrations of total PCBs in the five treatment diets composed of fish from the test site(s) will be 0.25, 0.50, 1.0, 2.0, or 4.0 ppm (Table 1). Reproductive impairment has been reported in mink fed diets containing PCB concentrations lower than 4.0 ppm (Heaton et al., 1995a; Restum et al., 1998); however, it should be noted that the congener makeup and non-PCB chemical composition of fish used in those studies differs from fish collected from the Housatonic River.

2.3 PREPARATION OF DIETS

It is anticipated that dietary treatments will be prepared two or three times during the trial. Procedures for sampling and analysis will be identical for each batch of feed mixed. After thorough mixing of the dietary ingredients for 30 minutes, three random "grab" samples from each dietary treatment will be collected and frozen for subsequent chemical contaminant analysis and an additional sample from each dietary treatment will be collected for nutrient (proximate) analysis. Feed will be placed in sealed containers and stored frozen as described by Ringer et al. (1991). Food will be thawed slowly at room temperature within coolers, or if conditions require, under mild heat suspended above the material to be thawed both for feeding and for analysis. Each feed sample submitted for nutrient analysis will be analyzed for the following:

- Moisture
- Dry matter
- Fat
- Crude protein
- Crude fiber
- Ash
- Total digestible nutrients
- Ca, K, Mn, Mg, Fe, Na, Cu, Zn, P

Table 1

Approximate Quantity of Fish Required for Test Assuming Test Site (New Lenox Road to Woods Pond) Fish Contain 150 ppm PCBs

Type of fish	Control	0.25 ppm PCBs	0.50 ppm PCBs	1.0 ppm PCBs	2.0 ppm PCBs	4.0 ppm PCBs	Total fish (30% of diet)
Ocean fish							
kg	270	269	267	264	258	244	1572
lb	594	591	587	581	568	537	3458
Test site fish							
kg	0	1.49	2.97	5.94	11.9	23.8	46
lb	0	3.27	6.53	13.1	26.1	52.3	101

All nutrient analysis will follow standard proximate analyses SOPs presented in the Final Quality Assurance Project Plan (QAPP) (WESTON, 2000). Six random "grab" samples of feed (one from each dietary treatment) will also be analyzed for organochlorines (OCs), PCBs, PCDDs, PCDFs, and potentially toxic and bioaccumulative metals each time a batch of feed is mixed. Three samples of drinking water (one taken during the acclimation period, the study midpoint, and the end of the study) will be analyzed for OCs, PCBs, PCDDs, PCDFs, and potentially toxic and bioaccumulative metals.

Because the fish species used in the diets are known to contain thiaminase, supplemental thiamine will be provided to prevent Chastek's Paralysis (National Research Council, 1982).

2.4 ANIMALS

There will be 12 uniquely identified, first-year (virgin), natural dark, female mink (*Mustela vison*) from the MSU Experimental Fur Farm herd randomly assigned to each dietary treatment, except that litter mates will not be placed in the same treatment group to minimize genetic predisposition to PCB toxicity. If randomization results in any one treatment group being significantly larger (on a mass basis), then additional randomization within groups prior to treatment will be conducted until group masses are comparable. This procedure will ensure that any effects potentially observed are not attributable to treatment group mass differences. Male mink (untreated) will be used for breeding purposes only. All mink will have been immunized against canine distemper, viral enteritis, hemorrhagic pneumonia, and botulism.

2.5 MINK FACILITIES

Mink will be caged individually in an open-sided shed in a manner described by Ringer et al. (1991) that exceeds guidelines specified in the Standard Guidelines for the Operation of Mink Farms in the United States (Fur Commission USA, 1995). As such, mink will be exposed to ambient conditions, which, based on experience, yield superior reproductive performance compared to raising mink in a more controlled indoor environment.

2.6 ACCLIMATION PERIOD

The mink will be acclimated for 7 days prior to the initiation of the definitive trial as described in Ringer et al. (1991). They will be weighed at the beginning of the acclimation period and an attempt will be made to determine food consumption at the end of the acclimation period as described by Ringer et al. (1991), if weather permits.

2.7 DEFINITIVE TRIAL

Three females from the breeding stock will be sacrificed and livers will be analyzed for OCs, PCBs, PCDDs, PCDFs, and potentially toxic and bioaccumulative metals.

After the 7-day acclimation period, the definitive test will begin on or around 1 January 2000, which is 8 weeks prior to the initiation of breeding. Test diets will be fed daily for approximately

150 days throughout the pre-breeding, breeding (March 1 to March 21), gestation, parturition (April 21 to May 15), lactation, and weaning (June 15 to July 1) periods, at which time 12 adult females and 6 kits randomly selected from each treatment will be euthanized by asphyxiation (CO_2) and necropsied for analysis. Twelve kits from each treatment group will be maintained on their respective diets through November to assess possible effects of PCBs on developmental parameters. Although Aleutian disease has not been observed in the MSU mink breeding stock over the last several years, during the necropsy stage of the study, all individuals will be examined for histopathological abnormalities typically associated with this disease. Should any individual mink be diagnosed with Aleutian disease, it and all its associated data will be removed from the study analysis.

Husbandry and experimental procedures during the pre-breeding through lactation periods are as described in Ringer et al. (1991). These will include daily observation of mink and determination of body weights every 2 weeks and feed consumption weekly. Feed consumption will be assessed on a weekly basis by measuring food consumption for 2 days during this period. Any adult mink that loses greater than 30% of its initial (acclimation) body weight will be euthanized by asphyxiation (CO_2) and necropsied, as recommended by Michigan State University's All University Committee on Animal Use and Care. Individuals removed from the study for weight loss reasons will not be included in the subsequent analyses. Breeding of treated females with untreated males will begin on or around 1 March 2000 and will follow procedures outlined in Ringer et al. (1991). A ratio of 1 male for every 4 females will be used. To ensure that successful mating has occurred, each adult female mink will have a vaginal aspiration performed after copulation to confirm the presence of sperm and sperm motility. If any male is unsuccessful in inseminating the females, the male will be replaced with another from the herd. Attempts will be made to ensure that females will have two or more confirmed matings during the breeding period. Determination of body weights and feed consumption will be discontinued at the initiation of breeding. All other procedures related to breeding, gestation, parturition, and lactation are as described in Ringer et al. (1991). Kits will be weighed within 24 hours post-partum and at 3 and 6 weeks of age.

When the last litter whelped is 6 weeks old, the adult females and six kits (non-litter mates) from each treatment will be euthanized by asphyxiation (CO_2) and necropsied. Organs (brain, liver, kidneys, spleen, heart, thyroid gland, and adrenal glands) will be removed and weighed. Samples of organs will be stored in a 10% formalin-saline solution for subsequent histological examination. Additionally, liver samples from six adults and six kits per treatment will be frozen for subsequent contaminant and cytochrome P450 analysis by the Columbia Environmental Research Center (CERC). The P450 analysis will provide an additional line of evidence in the conformation of exposure to the dioxin-like compounds. All collected materials will be appropriately labeled (type of tissue, identification of the individual animal the tissue came from, date of collection, and project identification). As previously discussed, water provided *ad libitum* throughout the study will be sampled at the beginning, midpoint, and end of the study, each water sample will be analyzed for organochlorines, PCBs, PCDDs, PCDFs, and potentially toxic and bioaccumulative inorganics.

Twelve kits from each treatment group will be maintained on their respective diets through November. These kits will be immunized against canine distemper, viral enteritis, hemorrhagic pneumonia, and botulism at 10 weeks of age. Body weights will be determined every 4 weeks.

At the end of the growth period in November, six kits from each of the six treatment groups will be euthanized by asphyxiation (CO_2) and necropsied with tissues being handled as described above. Any mink (except unweaned kits) that die during the trial period will be submitted to MSU's Animal Health Diagnostic Laboratory for necropsy by a veterinary pathologist.

3. CHEMICAL ANALYSIS

Congener-specific PCB analysis for determination of PCBs will proceed as follows. Sample preparation and analysis will generally follow the methods described by Schwartz and Stalling (1991). Samples will be homogenized in a blender. A 5-g portion from each sample will be dried with 20 g of anhydrous sodium sulfate (Merck, USA, 99%) and ground. The samples will be homogenized with sodium sulfate and column-extracted with CH_2Cl_2. A portion of each sample will be used to gravimetrically determine the lipid content and the remainder of each extract will then be treated by two stages of reactive column cleanup, followed by high-performance gel permeation chromatography. PCB congeners will be analyzed with a Hewlett-Packard 5890A Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a ^{63}Ni electron capture detector (ECD) and a Hewlett-Packard 7673 autosampler. The detector temperature will be 330 °C and the injector set to follow the oven temperature. Injections will be 1 µL cool on-column onto a 30-m by 0.25-mm by 0.25-µm DB-5 capillary column (J & W, Folsom, CA, USA) with a 1-m by 0.53-mm deactivated retention gap connected to the column, with H_2 carrier gas maintained at 12 psig, linear velocity 60 cm/s. The oven temperature program will be as follows: 60°C, 10°C/min to 120°C, 2°C/min to 240°C, and then 10°C/min to 320°C with a 5-minute hold. Data will be collected with PC-based PE Nelson chromatography software (Perkin Elmer, Norwalk, CT, USA). Quantitation of approximately 100 PCB congeners will be based on an internal standard calibration.

Characterization of the fish, diets, adult livers, and kit livers will include chemical measurements of the OC pesticides, PCBs, PCDFs, PCDDs, and potentially toxic and bioaccumulative inorganics found in the extracts (Feltz et al., 1995; Peterman et al., 1996). The determination of the exact amounts of dioxin-like chemicals in the samples will proceed as follows. All samples analyzed for organics will be homogenized, dried with sodium sulfate, spiked with the appropriate standards and column-extraction with methylene chloride (Feltz et al., 1995). All of the concentrated extracts will then be treated by a two-stage reactive cleanup, using first a sulfuric acid silica gel/potassium silicate column, and second, a column of sulfuric acid silica gel/potassium silicate/silica gel. High-pressure gel permeation chromatography (HP-GPC) cleanup will follow to remove residual lipids (Feltz et al., 1995). All samples analyzed for inorganics will follow procedures presented in the QAPP (WESTON, 2000).

Aliquots of the final extracts will be fractionated using high-performance porous graphitic carbon chromatography (HP-PGC) into fractions containing: 1) bulk through mono-*ortho* chlorine substituted PCB congeners, 2) non-*ortho* chlorine substituted congeners, and 3) 2,3,7,8-PCDDs and PCDFs according to the procedures in Echols et al. (1997). The instrumental analysis for the determination of the congener-specific PCBs (fraction 1) will be as described above by GC/ECD. Non-ortho PCBs (fraction 2) will be analyzed by gas chromatography/high-resolution mass spectrometry (GC/HRMS) (Peterman et al., 1996). Finally, PCDD/PCDFs (fraction 3) will be eluted through basic alumina (according to ECRC SOP C5.152) for removal

of potential co-contaminants such as polychlorinated diphenyl ethers (PCDEs) and residual polychlorinated naphthalenes (PCNs) and PCBs. The instrumental internal standard, ^{13}C-labeled 1,2,3,4-PCDD, will be added to each semiconical autosampler vial prior to transferring the PCDDs/PCDFs (fraction 3). PCDFs and PCDDs will be determined by GC/HRMS by monitoring five sequential mass windows of selected ions during the chromatographic separation (according to ECRC SOP C5.183 and Peterman et al., 1996). GC/HRMS analysis will be performed using a HP 5890A capillary gas chromatograph interfaced to a VG 70-250S high-resolution mass spectrometer. An HP 7673 autosampler will be used to introduce 2 of 25 µL of the enriched extract from a conical vial through a spiral uniliner onto a 5-m by 320-µm deactivated fused silica retention gap via a heated (285°C) direct inlet. The analytes of interest will be separated on a 50-m by 200-µm by 0.11-µm Ultra-2 (Hewlett Packard) capillary column with an initial hold of 1 min at 120 °C followed by a ramp to 200°C at 20°C/min, another ramp to 300°C at 2.3°C/min, and a final hold of 5 min. The He carrier gas is maintained at 44 psig with an initial linear velocity of 25 cm/s. All column-to-column connections are made using fused silica press-tight connectors.

The VG GC/HRMS system is tuned to 10,000 R.P. and calibrated using perfluoro-tetradecahydro-phenanthrene, and mass windows are established for five ion groups to measure Cl_{4-8} PCDFs and PCDDs. These windows are monitored sequentially during the temperature program. Within each mass window, the two most abundant ions are measured for positive identification and quantitation of each analyte. The ion responses are quantitated and averaged, unless interferences occur. Within each mass window, additional ions monitor any responses from Cl_{5-9}-PCDEs, Cl_{5-7}-terphenyls, Cl_{6-7}-PCNs, Cl_{3-8} dibenzothiophenes, and Cl_{3-8} phenanthrene/anthracenes.

Determination of non-*ortho* PCBs (planar PCBs) in fraction 2 above is conducted by GC/HRMS analysis and performed with a HP 5890A capillary gas chromatograph interfaced to a VG 70-250S high-resolution mass spectrometer. An HP 7673 autosampler is used to introduce 2 µL of the enriched extract from a conical vial onto a 2.5-m by 530-µm deactivated fused silica retention gap via a cool on-column injection technique. A 50-m by 200-µm by 0.11-µm Ultra-1 capillary column (Hewlett-Packard's equivalent to DB-1) is used to resolve most non-*ortho*-PCBs from interferences. The GC oven is held at 120°C for 1 min, programmed to 240°C at 2.2°C/min, then ramped to 310°C at 5°C/min, for a final hold of 5 min. Helium carrier gas is maintained at 48 psig with an initial linear velocity of 25 cm/s. The analytical column is put into the MS interface and heated at 310°C. All column-to-column connections are made using fused silica press-tight connectors.

The VG GC/HRMS system is tuned to 10,000 R.P. and calibrated using perfluorodecalin, and mass windows are established for two groups of non-*ortho*-PCBs. Group 1 from 23-48:00 min included ions for Cl_4-biphenyls 77 and 81 and Cl_5-biphenyl 126; Group 2 from 48:05-65 min included ions for Cl_6-biphenyl 169. Within each mass window, the two most abundant ions are measured for positive identification and quantitation of each analyte. The ion responses are quantitated and averaged, unless interferences occur. Within each mass window, additional ions monitor the responses of higher chlorinated, potentially interfering PCB congeners, Cl_{4-8} naphthalenes (PCNs), Cl_{3-5} terphenyls (PCTs), Br_5- and Cl_6-diphenyl ethers, and Cl_4-PCDF (to ensure no breakthrough of PCDFs).

The amount of each analyte detected is inherently self-corrected for losses through the whole analysis (extraction, isolation of analytes, and instrumental analysis). A calibration curve describing the response of each native congener to that of an isotope-labeled congener is used directly in the calculations and its range of values is determined in the calibration procedure. Concentrations of the native PCB congeners in standards ranged from 0.25 to 2,500 pg/µL. Each calibration curve is specifically matched to the range of analyte responses in the sample set. All water sample analyses will follow procedures presented in the QAPP (WESTON, 2000). Dietary treatment samples will also be shipped to Litchfield Analytical Services, Litchfield, MI, for subsequent nutritional analysis (see Subsection 2.3). All nutritional analyses will follow standard analytical procedures documented in SOPs provided by Brookside Laboratory (under contract to Litchfield Analytical Services) and presented in the QAPP (WESTON, 2000).

4. BIOCHEMICAL ANALYSIS

Samples of mink livers will be flash frozen and stored in liquid nitrogen or in an ultracold freezer at -80°C until they are to be sent to the CERC for analysis. Upon arrival at the CERC, the mink liver samples will be stored in an ultracold freezer at -80°C until they are analyzed for CYP1A or CYP2B activity. The biochemical assays of CYP1A and CYP2B activity will follow previous methods (Burke and Mayer, 1975; Burke et al. , 1985; Lubet et al., 1985; Ullrich and Weber, 1972). Briefly, portions of the frozen livers will be broken off, thawed, and homogenized and microsomes will be prepared from the homogenates by differential centrifugation. Optimal assay conditions will be determined for the assays of ethoxyresorufin *O*-deethylase (EROD), ethoxycoumarin *O*-deethylase (ECOD), pentoxyresorufin *O*-deethylase (PROD), and benzyloxyresorufin *O*-deethylase (BROD). Microsomes will be prepared on the same day the catalytic activity is assayed. Protein will be measured using the fluorescamine-based assay (Undenfriend et al., 1972; Bohlen et al., 1973) calibrated against bovine serum albumin (BSA). Triplicate assays will be performed, if sample volumes allow.

All biochemical analysis conducted by CERC will follow procedures and SOPs identified in Section 8 of this study plan.

5. SUMMARY OF ENDPOINTS

Adult body weights:	At beginning of the acclimation period; at beginning of the definitive trial; every other week thereafter until initiation of breeding; at whelping; at necropsy (Ringer et al., 1991)
Adult feed consumption:	During the acclimation period; weekly during the definitive trial (if temperature above 0°C) until initiation of breeding (Ringer et al., 1991)
Number of females mated:	(Ringer et al., 1991)
Length of gestation:	(Ringer et al., 1991)
Number of females whelping/	(Ringer et al., 1991)

not whelping:		
Total newborn/female whelped:	(Ringer et al., 1991)	
Live newborn/female whelped:	(Ringer et al., 1991)	
Average kit birth weight:	(Ringer et al., 1991)	
Average litter weight:	(Ringer et al., 1991)	
Percent kit survival to 3 weeks:	(Ringer et al., 1991)	
Average 3-week body weight:	(Ringer et al., 1991)	
Percent kit survival to 6 weeks:	(Ringer et al., 1991)	
Average 6-week body weight:	(Ringer et al., 1991)	
Average adult and 6-week kit organ weights:	(Heaton et al., 1995a)	
Histopathology of adult and 6-week-old kit organs:	(Heaton et al., 1995b)	
Total PCB and congener and planar PCB, PCDD, and PCDF analyses of adult and 6-week-old kit livers:	(Tillitt et al., 1996)	
Cytochrome P450 enzyme analysis of adult and 6-week-old kit livers:	(Burke and Mayer, 1975; Burke et al. , 1985, Lubet et al., 1985)	
Average body weight (monthly) of 7-month-old kits:	(Heaton et al., 1995a)	
Average organ weights of 7-month-old kits:	(Heaton et al., 1995a)	
Histopathology of 7-month-old kit organs:	(Heaton et al., 1995b)	
Total PCB and congener and planar PCB, TCDD, and PCDF concentrations in livers of 6-week-old and 7-month-old kits:	(Tillitt et al., 1996)	
Cytochrome P450 enzyme concentration in livers of 7-month-old kits:	(Burke and Mayer, 1975; Burke et al., 1985; Lubet et al., 1985; Ullrich and Weber, 1972)	

6. STATISTICAL ANALYSIS

The data generated in this study will be evaluated by analysis of variance or by contingency tables. Significant differences will be tested by Dunnett's method for comparison with a control or by Bonferroni's Chi square test (as described by Ringer et al., 1991).

7. QUALITY ASSURANCE/QUALITY CONTROL

All work will be conducted in compliance with the project QAPP (WESTON, 2000) and in compliance with the study-specific QA plan as discussed below.

The objectives of the quality assurance (QA) plan for the proposed study are: 1) to ensure that the analytical measurements, biological/toxicological assays, and biochemical analyses are accurate and precise; and 2) to ensure that the mink reproductive toxicity tests are conducted according to protocols and SOPs of the MSU Experimental Fur Farm, and in accordance with animal use and care requirements of MSU's Department of Animal Science and All University Committee on Animal Use and Care. The general protocol includes replication of various stages, comparison and calibration against known standards, proper maintenance and calibration of equipment, accurate sample tracking and custody, proper documentation at all steps of sample processing, and other considerations of Good Laboratory Practice (GLP).

7.1 DATA QUALITY OBJECTIVES

The data quality objectives for the mink dietary exposure study are directly linked to endpoints presented in Section 7 and study objectives discussed in Section 1. In summary, the measurement endpoints in the study will be evaluated to determine if the assessment endpoints of survival, reproduction, or development of mink are being impacted by dietary exposure to PCBs. In addition, as part of the overall Housatonic River Project, the mink dietary exposure study must support and complement applicable data quality objectives established in Subsection 4.1 of the QAPP (WESTON, 2000). To achieve these objectives, the following types of data will be required:

- Reproduction, growth, and survival data for controls and treatment groups.
- Dietary exposure chemistry.
- Mink liver chemistry.
- Biomarker and pathological evaluations.

The data developed as part of the mink dietary exposure study must achieve acceptable standards of accuracy, completeness, representativeness, and comparability. The purpose of this section of the study plan is to further document the measures being taken to ensure that these standards are met.

7.2 DATA QUALITY INDICATORS

Data developed in the mink dietary study must meet acceptable standards of precision, accuracy, completeness, representativeness, comparability, and sensitivity, as defined in Section 15 of the QAPP (WESTON, 2000). Each of these data quality indicators, some of which are not readily quantifiable, are discussed below with specific reference to the mink dietary study.

Precision is defined as the level of agreement among repeated independent measurements of the same characteristic. Rather than control and measure precision, the investigator increases the number of replicates to obtain sufficient statistical resolution; for this study the several replicates (12) per treatment group is used in this manner. For the measurements that are not unique to the mink dietary study, such as tissue chemistry, biomarkers, and water chemistry, precision is evaluated as defined in appropriate SOPs presented the QAPP (WESTON, 2000).

Accuracy is defined as the agreement of a measurement with its true value. For the parameters unique to this study (tissue weights, reproductive effects, and pathology), accuracy is defined as meaning that tissue are correctly weighed, and reproductive effects and tissue pathology were correctly assessed. The data generated by this study may be evaluated for accuracy via comparison with reference organisms, and results observed in similar dietary studies. For parameters such as tissue and water chemistry, biomarker levels, and dietary nutrient content, accuracy is as defined in the QAPP (WESTON, 2000).

Completeness is defined as the percentage of the planned samples actually evaluated and processed. Completeness can be evaluated for all components of the mink dietary study. To ensure that the desired statistical resolution is achieved, it is important that a high level of completeness be achieved for all components of this study. Mink toxicity studies have been conducted by the Department of Animal Sciences at MSU for over 20 years. During this time no studies have been discontinued or significantly impacted by non-treatment-related mortalities or sample exclusions (e.g., >30% weight loss) to such a degree that the remaining data were deemed incomplete or unacceptable for use in accessing treatment-related effects. The current statistical design of this study (i.e., 12 replicates per treatment) is adequate to account for typical non-treatment-related losses while still maintaining sufficient sample size required for a high level of data completeness.

Representativeness refers to the degree to which the data accurately reflect the effects that would be observed if a wild mink would ingest a similar diet. This data quality indicator is addressed through implementation of proper experimental design and sample processing methods and may be evaluated via comparison with expected results.

Comparability is a measure of the confidence with which the study data may be compared to another similar data set. Comparability may be evaluated for this data set through comparison with previous mink dietary studies with similar contamination levels.

Sensitivity, the ability of a measurement technique or instrument to operate at a level sufficient to measure the parameter of interest, is largely not applicable to the biological parameters. The detection limits for chemistry, biomarkers, and nutritional parameters specified in the QAPP or associated SOP, in conjunction with reproductive and pathological effects, will provide more

than sufficient sensitivity for the purpose of providing insight into factors potentially impacting resident mink populations.

7.3 SAMPLING AND ANALYTICAL PROCEDURES

Samples of mink, diets, livers, and original fish will be taken at MSU and sent to the CERC and/or WESTON's contract lab for subsequent analysis. Samples for biochemical analysis will be stored at -80°C prior to shipment, while those for chemical analysis will be stored at -20°C prior to shipment. Samples will be shipped from MSU to CERC frozen on dry ice for biochemical or chemical analysis. Prior to receipt of any samples, forms are sent to provide exact information on the samples. These forms are the Sample Batch History Information form and the Sample Inventory Listing form (see Attachment 2). The Sample Inventory Listing is a simple list of all the samples to be transmitted and has the sample label (identifier with year, project #, study #, and personal sample ID #) and a brief description of each sample. The Sample Batch History Information form contains information on sample collection dates, how samples were collected, how they were preserved, transmission dates and modes, and other pertinent information about the samples and how they have been handled. Upon receipt of the samples, they are assigned an independent identification number for internal tracking and all of the information is computerized on a central sample tracking system. A complete description of the system is attached (see Attachment 2). This approach is analogous to the chain-of-custody requirements specified in the project QAPP (WESTON, 2000).

The methods for extraction and subsequent chemical analysis are presented in Section 3. SOPs of these methods are included in Attachment 2. The matrices for analysis include homogenates of whole fish that will be used to form the diets, time-weighted sub-samples of the actual diets, adult mink liver samples, and kit livers/tissue. The measurements to be made by the CERC include lipid analysis (CERC SOP P.461), organochlorine pesticide analysis (CERC SOP P.460), total and congener-specific PCB analysis (CERC SOP P.195), non-*ortho* PCB analysis (CERC SOP P.481), and chlorinated dioxin and furan analysis (CERC SOP P.482). Analyses will be performed by GC/ECD and/or GC/MS. Method limits of detection will be 1 to 5.0 ppt (pg/g) for dioxins and furans and <1 ppb (ng/g) for the PCB congeners. QA/QC procedures will include analyses of spiked samples with appropriate standards, analysis of replicates, analysis of procedural and matrix blanks, and the demonstration of correct chemical identifications. The general QA procedures for chemical analysis by the CERC for this project are presented in the QAPP (WESTON, 2000).

Tissue samples submitted to WESTON's contract lab for inorganic residue analysis will follow QA/QC procedures presented in the QAPP (WESTON, 2000). Nutritional analyses of dietary treatment samples will follow QA/QC procedures provided in the SOPs submitted by Brookside Laboratory, which are presented in the QAPP (WESTON, 2000).

7.4 DATA REDUCTION, VALIDATION, AND REPORTING

All experimental information is recorded in bound notebooks and signed. Copies are maintained in a separate, secured area. Instrument printouts and computerized data tables are uniquely labeled and cross-referenced to the project notebook. The accuracy of all such measurements will

be independently checked. Copies of the computerized data files are maintained in a project notebook and file, on floppy disk in the project file, and by archived tape backup.

Reporting of the data will initially be in draft form to the appropriate collaborators. After their review and approval, an internal review of the draft report will be made and a final report sent to the Project Officer. The results of this work will be coordinated with that of the other collaborators into one or more articles to be submitted to peer-reviewed journals.

7.5 SAMPLING METHODOLOGY

7.5.1 Sampling Procedures

Fish sampling in the Housatonic River will be conducted in Woods Pond and associated upstream backwater areas below New Lenox Road. Fish sampling will follow techniques and procedures presented in Appendix A.20. Carp *(Cyprinus carpio)* and goldfish *(Carassius auratus)* are the primary target fish for this sampling activity because previous fish sampling activities identified populations of sufficient size and number so that collecting these species at these locations would have minimal impact on the resident populations and could be accomplished in a time-efficient manner.

Samples will be collected by a team led by trained USFWS personnel supported by WESTON, EPA, and Woodlot Alternatives team members.

7.6 QUALITY CONTROL SAMPLES

The nature of the pathological and reproductive effects portion of the mink dietary study does not allow the incorporation of typical duplicate and blank samples as part of the study design. For effects endpoints there is no acceptable method of obtaining such samples in a manner analogous to that developed for duplicates and blanks collected for chemistry analysis; however, reference samples and evaluations will be collected from study control treatment mink.

The number of quality control samples such as duplicates and blanks for chemistry, nutrient, and biomarker analysis are presented in the QAPP (WESTON, 2000).

7.7 EQUIPMENT

All equipment used in these studies is routinely inspected and preventive maintenance performed. A logbook is kept for each instrument to document its use, performance, and maintenance.

7.8 STATISTICAL ANALYSIS OF DATA AND SAMPLING DESIGN

The statistical treatment of the data is described in Section 6 of the study plan. Routine analyses will be performed and an allowance for Type I errors will be set at 5% ($p = 0.05$). Outliers will

be determined as described by Gill (1978). Performance criteria for MLOD, MLOQ, precision, and accuracy are given above. Sampling design in general follows procedures described by Ringer et al., 1991 (Attachment 1).

7.9 QA AUDITS

Internal audits are continuously performed by the Principal Investigator and are performed quarterly by the CERC QA Officer. Quality assurance procedures outlined in the QAPP (WESTON, 2000) will be followed for any analyses conducted by WESTON's contract laboratories.

7.10 CORRECTIVE ACTION

Problems will be identified as they occur or through weekly staff meetings. Remedial actions will be taken as deemed appropriate and in accordance with the QA performance criteria. All such problems and corrective actions will be recorded in the project notebook(s) and reported to management or the Project Officer, if necessary.

7.11 TRAINING

All sampling and analyses will be directed by senior scientists with experience in the collection and shipping of samples, the analyses of tissue and diet chemistry, biomarker analysis, and the evaluation of mink reproductive endpoints or mink pathology. Supporting staff will receive training from the senior scientist(s) in overall goals of the study and in techniques to be followed to ensure collection of quality data.

8. STANDARD OPERATING PROCEDURES

Standard Operating Procedures (SOPs) and Documents of Procedure for the portion of the study conducted at the Columbia Environmental Research Center, Department of Interior, Columbia, MO are provided in the QAPP (WESTON, 2000).

Contents

SOP Number	Title of Standard Operating Procedure
SOP P.461	Extraction of Animal Tissues for Residue Analysis and Percent Lipid Determination
SOP C5.162	Sample Transmittal, Receipt, and Inventory
SOP P.123	Microsomal Preparation of Liver Tissue

SOP Number	Title of Standard Operating Procedure
SOP P.475	Calibration Check of 96 Well Microplate Absorbance and Fluorescence Readers
SOP P.124	Procedure for the determination of 7-Ethoxyresorufin-O-Deethylase (EROD) Activity in Microsomes from Liver Tissue Using 96-Well Microtiter Plates
SOP P.270	Preparation of Sulfuric Acid/Silica Gel (SA/SG)
SOP P.271	Preparation of Potassium Hydroxide-Treated Silica Gel
SOP P.193	Alumina Cleanup of PCDD/PCDF Fractions from HPLC-Carbon
SOP P.186	Tissue Analysis for PCBs and Low-Level Planar Halogenated Hydrocarbons
SOP P.460	Organochlorine Pesticide Analysis: Fractionation of Complex Mixtures of Silica Gel/ODS
SOP P.482	Analysis of Tetra- through Octa-Substituted Polychlorinated Dibenzo-p-dioxins and Dibenzofurans by Gas Chromatography-High Resolution Mass Spectrometry
SOP P.481	Analysis of Selected Non-O-Chloro-Substituted Polychlorinated Biphenyls by Gas Chromatography-High Resolution Mass Spectrometry
SOP P.195	Capillary Gas Chromatography with Electron Capture Detection Procedure for Congener Specific Polychlorinated Biphenyl Analysis
General Analytical	Minimum Quality Assurance Standards for Trace Organic Residue Chemistry QA Analysis

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ATTACHMENT 1

MAMMALIAN WILDLIFE (MINK AND FERRET) TOXICITY TEST PROTOCOLS

PB91-216507

EPA/600/3-91/043
July 1991

MAMMALIAN WILDLIFE (MINK AND FERRET) TOXICITY TEST PROTOCOLS (LC50, Reproduction, and Secondary Toxicity)

by

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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before complete)

1. REPORT NO. EPA/600/3-91/043	2.
	3. PB91-216507
4. TITLE AND SUBTITLE Mammalian Wildlife (Mink and Ferret) Toxicity Test Protocols (LC50, Reproduction, and Secondary Toxicity)	5. REPORT DATE July 1991
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) R. K. Ringer, T. C. Hornshaw, and R. J. Aulerich	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Animal Science, MSU, East Lansing, MI	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS US Environmental Protection Agency Environmental Research Laboratory 200 SW 35th Street Corvallis, OR 97333	13. TYPE OF REPORT AND PERIOD COVERED Published Report
	14. SPONSORING AGENCY CODE EPA/600/02

15. SUPPLEMENTARY NOTES 1991. U.S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, OR

16. ABSTRACT

Protocols describing guidelines for conducting dietary LC_{50} and reproduction toxicity tests and for assessing the primary versus secondary toxicity of a test substance using carnivorous mammalian wildlife, specifically mink (Mustela vison) or European ferrets (Mustela putorius furo) are presented. These protocols go beyond the procedural steps and include the rationale behind each recommendation. Appropriate statistical procedures for assessing the dat are presented for each protocol.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
toxicity tests, carnivorous mammalian wildlife, mortality		

18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 84 pgs
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

EPA Form 2220-1 (9-73)

DISCLAIMER

The information in this document has been funded wholly or in part by the United States Environmental Protection Agency under IAG #13 F 28800 to the National Oceanographics and Atmospheric Administration. It has been subjected to the Agency's peer and administrative review, and it has been approved for publication as an EPA document. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ABSTRACT

Protocols describing guidelines for conducting dietary LC_{50} and reproduction toxicity tests and for assessing the primary versus secondary toxicity of a test substance using carnivorous mammalian wildlife, specifically mink (Mustela vison) or European ferrets (Mustela putorius furo) are presented. These protocols go beyond the procedural steps and include the rationale behind each recommendation. In the LC_{50} test, test species are fed diets that contain several concentrations of a test substance for 28 days in which signs of toxicity and mortality are recorded and toxicity is expressed as the mean lethal concentration of the test substance. The reproduction protocol contains guidelines for determining the reproductive toxicity of a test substance administered to males and females at several concentrations in their daily diet prior to and during the breeding period and through gestation and lactation. Adverse effects on adult survival, oogenesis and/or spermatogenesis, reproductive indices, embryo or fetal development, and offspring growth and survival are measured. In the third protocol (primary vs secondary toxicity), the toxicity and lethality (LC_{50} value) of a test substance, in the form of the parent compound, administered via the diet in several concentrations to males and females (primary toxicity test) is compared with the toxicity and lethality of the same test substance fed at identical concentrations but contained in animal tissue (prey) contaminated by previous exposure to the same parent test substance (secondary toxicity test). Appropriate statistical procedures for assessing the data are presented for each protocol.

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FIGURE

MAMMALIAN WILDLIFE (MINK AND FERRET) DIETARY LC_{50} TEST[1]

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[1] This work was supported by the U.S. Environmental Protection Agency under contract Number U.S. EPA/NOAA Interagency Agreement #AD 13 F 28800.

INTRODUCTION

The U.S. Environmental protection Agency (EPA) has been charged, under several laws, with the regulation of many toxic or hazardous substances. The key to regulation of these substances is toxicological testing of the compounds with appropriate test species. The species of choice for testing the toxicity of a compound to man and domestic animals, as specified in federal guidelines (U.S. EPA, 1975, 1978, 1982a,b), have been common laboratory animals such as rats, mice, rabbits, guinea pigs, and hamsters. No representative mammalian wildlife species have been designated in the federal guidelines. Adequacy of risk assessment for wildlife mammals has normally been based on toxicity data for evaluating hazards to humans and domestic animals (U.S. EPA, 1978; Ringer, 1988).

Under special circumstances of hazard assessment, such as secondary toxicity poisoning or evaluation of certain chemicals that bioconcentrate in the food chain, special tests involving wildlife mammals may be required. Carnivorous animals are generally the species of choice in toxicological testing for secondary poisoning (Schitoskey, 1975; Townsend et al., 1984; Aulerich et al., 1986, 1987) and represent the top of the food chain for chemicals that bioconcentrate.

Prompted by these concerns, the mink (Mustela vison) (U.S. EPA, 1975; Aulerich and Bleavins, 1981) and European ferret (Mustela purotius furo) (Thornton et al., 1979; Hoar, 1984) were suggested as representative mammalian carnivores for toxicological testing. The mink may be preferred because it is indigenous to the U.S. and Canada and occurs in the wild throughout much of North America. Its nutritional requirements are well known (National Research Council, 1982), stock of known genetic origin is available, all stages of its life cycle can be successfully perpetuated in the laboratory, and it has a large biological

data base (Shump et al., 1976; Scientifur, 1987; Sundqvist, 1989). The European ferret, although not indigenous to North America, has been examined as a laboratory model for toxicology (Willis and Barrow, 1971; Thornton et al., 1979; Anon., 1981; Hoar, 1984). The mink has been shown to be among the most sensitive, if not the single most sensitive, mammalian species to polychlorinated biphenyl (PCB) toxicity (Aulerich and Ringer, 1977). Research performed with mink was instrumental in setting US water quality standards for PCBs (Aulerich and Bleavins, 1981). Subsequent studies have shown this species to be similarly sensitive to polybrominated biphenyls (Aulerich and Ringer, 1979), hexachlorobenzene (Bleavins et al., 1984), aflatoxins (Chou et al., 1976; Bonna et al., 1991), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Hochstein et al., 1988). Additional toxicological studies with mink and ferrets have been reported in the literature (Frederick and Babish, 1985; Scientifur, 1987; Sundqvist, 1989).

The following protocol for conducting dietary LC_{50} tests with mink and ferrets was developed based on procedures used in previous toxicology studies from our laboratory and refined by additional studies on four chemicals (Hornshaw et al., 1986a,b,c; 1987) providing a range of solubilities, volatilities, toxicities, and modes of action. If a measure of secondary toxicity is also needed, see protocol entitled "Mammalian Wildlife (Mink and Ferret) Dietary LC_{50} Tests to Assess Primary and Secondary Toxicity".

1. Scope

1.1. This protocol describes a method for determining the subacute dietary toxicity of a test substance (that can be mixed uniformly into the diet) administered to animals in their daily diet. Toxicity is expressed as the median lethal concentration of the test substance (LC_{50}) and the slope of the dose-response curve.

1.2. This protocol is intended for use with carnivorous species, such as the mink (Mustela vison) and the European ferret (Mustela putorius furo). Other carnivorous species may be used with appropriate modifications.

2. Summary

2.1. Both males and females of the same species and age are fed diets containing a test substance to determine a subacute dietary toxicity (LC_{50}). Concentrations of the test substance are administered in a geometric series of doses for 28 days to measure lethality. This exposure period may be followed by a withdrawal period during which lethality is also measured.

2.2. Daily observations for signs of toxicity and mortality are reported.

2.3. Data derived from treatment and control groups are compared statistically to detect changes in body weight, feed consumption, and an index of toxicity.

3. Significance

3.1. This protocol provides a means of measuring the toxicity of a test substance in the daily diet of a carnivore under controlled conditions. The use of a 28-day dietary exposure period allows metabolic transformations of the test substance to occur. It is recommended that, when possible, tests be conducted indoors. Indoor tests allow greater control of environmental test conditions, and therefore, greater reproducibility. Indoor facilities, if heated, allow conducting tests at any time of the year. Indoor facilities also make the possibility of escape of test animals less likely.

3.2. This protocol provides data for assessing the potential adverse effects of chemicals to mammalian carnivores exposed through dietary intake, the normal exposure route in the environment. The mammalian carnivore occupies a position high on the food chain, thus it may be subject to the

effects of bioaccumulation of chemicals.

3.3. This protocol permits collection of data on signs of toxicity in addition to mortality.

3.4. The dose-response curve provides additional information about the susceptibility of carnivores to a test substance.

3.5. This test provides a basis for deciding whether additional toxicity testing should be conducted. Results from the 28-day test may indicate the need for subsequent reproduction or chronic tests with the test species. These results might also indicate the need for other types of tests, such as aquatic, inhalation, secondary toxicity, etc., or tests designed for a target organ or organ system.

4. Definitions

4.1. LC_{50}: The calculated concentration of a test substance which causes 50 percent lethality of a test animal population under the conditions of the test.

4.2. Test Substance: The element, chemical compound, formulation, known mixture, or material mixed in diets and fed for the purpose of determining an LC_{50}.

4.3. Concentration: The weight of the test substance per unit weight of the diet (expressed as mg/kg of diet).

4.3.1. Theoretical (nominal) concentration: The targeted concentration of test substance mixed into the diet.

4.3.2. Measured concentration: The concentration of test substance in the diet determined by analysis.

4.4. Acclimination period: A period of at least 7 days immediately preceding the exposure period when the test animals are housed in the test

facilities under test conditions and fed the untreated diet and drinking water to be used during the exposure period.

4.5. Range-finding test: Test conducted on a few animals to determine the concentrations of the test substance to be used in the definitive test.

4.6. Palatability test: Test where the highest proposed dietary concentration of a test substance for use in the definitive test is fed to a few animals to determine if they will consume the diet containing this concentration of the test substance.

4.7. Definitive test: Test where a substance is fed to a prescribed number of animals at several geometrically-spaced concentrations for a specified period of time (exposure period may be followed by a withdrawal period) when parameters of toxicity, including lethality, are measured.

4.8. Exposure period: The 28-day period when the test animals are fed diets containing the test substance.

4.9. Withdrawal period: The period following an exposure period when all animals are fed an untreated diet to allow for observation of delayed mortality.

4.10. Conventional diet: Feed consisting of both fresh and dried ingredients with water added to provide a semi-solid (hamburger-like) consistency.

4.11. Dry diet: Feed consisting of only dried ingredients fed in pelleted form.

5. Precautions

5.1. Contact with all test substances, solutions, and mixed diets should be minimized with appropriate protective clothing, gloves, eye protection, etc. The use of fume hoods and increased ventilation in test rooms is necessary when handling volatile substances. Information on other mammalian toxicity

and special handling procedures should be known before this protocol is used.

5.2. Disposal of excess test substances, solutions, mixed diets, etc., and treated animals should be done with consideration for health and environmental safety, and in accordance with all federal, state, and local regulations.

5.3. Cleaning and rinsing of glassware, feeders, and other equipment with volatile solvents should be performed only in well-ventilated areas.

5.4. Because this protocol addresses the use of carnivores, appropriate precautions should be used in the handling of test animals. Mink are by nature extremely aggressive and may attack a handler if given the opportunity. Appropriate precautions, such as leather gloves and arm-coverings, should be used. Ferrets are normally much less aggressive than mink but precautions should also be taken when they are handled. Mink and ferrets can transmit certain diseases, most importantly tetanus, and also tuberculosis (TB) and rabies if contacted from outside sources, so researchers should take precautions for these diseases. The possibility of TB infection can be reduced by avoiding the use of pork products in the diet. Proper caging and isolation of test facilities from wild animals can similarly reduce the possibility of rabies infections.

6. Test Animals

6.1. This protocol is intended for use with carnivorous species, such as the mink (Mustela vison) and European ferret (Mustela putorius furo). Other carnivorous species may be used with appropriate modifications, such as housing and diet.

6.2. All animals for a given test must come from one source and strain and

be of approximately the same age to minimize variability. Test animals may be obtained from commercial sources or may be reared in laboratory colonies, but they must not have been used in a previous test. It is recommended that test animals be immunized against diseases commonly afflicting the test species. Immunization for mink should include canine distemper, virus enteritis, infectious pneumonia, and botulism (Joergensen, 1985). It is recommended that ferrets be vaccinated against canine distemper and botulism (Fox, 1988). Mink and ferrets are also susceptible to Aleutian Disease (AD). Since no vaccine exists for this viral disease, testing and certification of the animals as AD-free is recommended. Animals that are deformed, injured, emaciated, or phenotypically different from normal animals must not be used as test subjects. The population of animals from which the test subjects (treated and control) are selected should be considered unsuitable for testing if mortality exceeds 5% during the acclimation period.

6.3. It is preferable to use animals that have approached their mature body size (for mink and ferrets that is about 18-20 weeks of age). Older animals can also be used to determine the LC_{50}. The use of younger animals may yield a distorted LC_{50} value because the change in body weight far exceeds the change in feed consumption resulting in a decreased amount of test substance consumed per unit of body weight over the 28-day period. Because of the sex difference in size of mink and ferrets, the two sexes should be treated as separate sub-groups.

7. Facilities

7.1. Space requirements for most carnivores have not been standardized. However, adherence to the guidelines of the Fur Farm Animal Welfare

Coalition (1988) should provide a basis for adequate space and husbandry requirements. This space requirement is currently 32,774 cu cm or 2,000 cu in. Cages measuring 61 (L) x 76 (W) x 46 (H) cm (24 x 30 x 18 in) have proven adequate for housing individual mink or ferrets for tests performed in conjunction with the development of this protocol. Cages must be constructed to prevent both cross-contamination of treatment groups and contact between individual animals. To prevent aggressive mink from attacking neighboring animals, use solid dividers between adjoining cages or provide adequate space between adjoining cages if wire mesh cage material is used throughout the cage. Species not conducive to colony rearing, such as mink, must be caged individually.

7.2. Construction materials in contact with animals should not be toxic, nor be capable of excessively adsorbing or absorbing test substances. Stainless steel, galvanized steel, or materials coated with perfluorocarbon plastics are acceptable materials.

7.3. Adequate ventilation should be provided at all times. The Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals, 1985) recommends 10 to 15 room air changes/hour for animal facilities.

7.4. If the animals have been reared outdoors and the test is conducted indoors, the photoperiod should simulate ambient daylight conditions because altered photoperiods may subject mink and ferrets to slight adjustments in normal body rhythms with respect to feeding, sleeping, fat mobilization or deposition, coat development, and hormonal activity. It should be noted that the effects of the length of the light/dark cycle on toxicity have not been investigated in these species. If the animals are reared indoors, the

photoperiod should not be altered.

8. Diets

8.1. Diets must be formulated in accordance with the nutrient requirements of the test species (National Research Council, 1982). Suggested ranges of composition of conventional diets for mink are shown in Table 1. Any unmedicated commercial diet that meets the minimum nutritional requirements of the test species is acceptable.

8.2. Fresh diets and water must be provided daily and fed ad libitum.

9. Diet Preparation

9.1. Knowledge of the physical, chemical, and biological properties of the test substance is important in test diet preparation.

9.2. Test diets can be prepared by mixing the test substance directly into the feed or by dissolving or suspending the test substance in an appropriate solvent or carrier prior to mixing with the feed. The use of solvents or carriers may be necessary to achieve uniformity. If solvents or carriers are used, they must also be added to the control diet in equivalent volumes. It is very important to assure uniform distribution of a test substance in the diet. In many instances, this will be more easily accomplished using a conventional diet, since many substances can be mixed into a diet more uniformly if the diet is semi-solid and capable of being machine-mixed. For some test substances, especially water soluble ones, this may be the only method of assuring uniform distribution, since pelleted diets are not conducive to being coated with aqueous solutions, but rather tend to become a mash. No matter which type of diet is used, it is recommended that innocuous solvents or carriers, such as distilled water or corn oil, be used whenever possible. (It is recommended that, unless the amount of test

substance to be added is large, solvents or carriers be used to introduce the substance to the diet to ensure uniform distribution). If an innocuous solvent or carrier cannot be found, it is recommended that a volatile solvent, such as acetone or hexane, be used to dissolve the test substance, if possible, and the resultant solution added to a small amount of either a dry diet or a dry ingredient (e.g., cereal) of a conventional diet. After the solvent is evaporated, the pre-mix can be uniformly mixed with the rest of the diet. (If this procedure is used, it must likewise be used on the control diet).

9.3. When conventional diets are used, sufficient diet should be mixed to provide adequate feed for the 28-day exposure period. The amount of feed to be mixed may be based upon the feed consumption data obtained during the acclimation period. When mixed, the diet should be stored frozen in containers large enough to hold 1 to 2 day's feed. It is important not to freeze the diets in containers too large, because the diets will not remain fresh under refrigeration for more than 2 or 3 days. In testing volatile substances, sealable containers must be used and stored upside down. The feed should fill the container allowing no head space. The amount stored in a container should be equal to one day's volume of feed. When dry diets are used, they should be stored to maintain the stability of the test substance in the diet. The frequency with which the feed is mixed is dependent upon the physical/chemical properties of the test substance.

9.4. All diets must be analyzed to determine the measured concentration of the test substance in the diet. Analysis of several samples should be conducted to determine homogeneity of the test substance in the diet.

10. Procedure

10.1. Range-finding test:

10.1.1. In most cases, LD_{50} estimates for mink or ferrets will not be available to aid in setting dietary concentrations for the LC_{50} test. Therefore, range-finding procedures can be used to save both time and animals by reducing errors or miscalculations in setting these concentrations. LD_{50} estimates for other species may be helpful in setting dietary concentrations, although, in general, mink and ferrets are more sensitive to toxic compounds that other mammals. For this reason, if LD_{50} estimates are available for other species, these values can be used as the upper limit of doses in the range-finding procedure. If range-finding is to be conducted, a geometrically-spaced series of doses (e.g., in multiples of 2 or ½) administered by gavage to 2 animals per dose can be used, in which case the approximate LD_{50} is the dose at which 1 or 2 animals die after an appropriate period of observation (often one week). It is suggested that, when administering an oral dose to mink or ferrets by gavage, a piece of plastic large enough to force the animal's mouth open, with a small hole in the center, be used. The tube can then be inserted through the opening without the animal biting it (see Figure 1). A three inch, 14 gauge, curved, stainless steel animal feeding needle can also be used to administer the test substance. If LD_{50} estimates are not available for other species, widely-spaced doses (e.g., 1, 10, 100, and 1000 mg/kg) can be administered to one animal per dose to find a lethal dose. The range-finding procedure described above can then be employed, centering on the lethal dose. If range-finding procedures yield an approximate LD_{50} value, the highest dietary

concentration should then be set to ensure that an animal will consume the equivalent of an LD_{50} dose in one day's feed. If a lethal dose is not found, the highest dietary concentration should be set at 5000 mg/kg because concentrations above this value are assumed to be nontoxic. Palatability tests should also be employed to save time and animals. It is recommended that the highest proposed dietary concentration be fed for several days to 2-4 animals to determine if they will eat the diet at this concentration of the test substance. If not, it may be necessary to reduce the highest concentration in the diet to a level that will be eaten.

10.2 Acclimation period:

10.2.1. All animals should be conditioned to the test facilities, including: photoperiod, temperature, and caging for a minimum of 7 days. A minimum of 7 days is required, but a longer period may be necessary, especially if the animals to be used in a test are changed from outdoor to indoor housing (or vice-versa) or if the diet or water to be used in a test is different from that which the animals are accustomed to consuming. During this period all animals should be given the untreated (control) diet and drinking water, as used during the definitive test. It is important to measure feed consumption during this period as these measurements can provide an indication of how much feed the animals should consume once the test starts, how much diet to mix for the test, and can also serve as a control value for each group. Test animals should be weighed at the start of the acclimation period.

10.3. Definitive test:

10.3.1. Each test animal should be randomly assigned according to weight

class to a specific test diet concentration and be uniquely identified.

10.3.2. The test diets must be fed for 28 days. For some test substances, it may be necessary to include a withdrawal period, during which the test diets are replaced with untreated feed, in order to observe prolonged or delayed toxicity. A withdrawal period is recommended when animals are still exhibiting signs of toxicity at the end of the exposure period. This period provides a more accurate estimation of the true toxicity of a test substance, especially if the substance causes delayed or cumulative injury. By observing the animals and measuring feed consumption during this period, the permanence of the injury can also be estimated. It is recommended that a withdrawal period not exceed 14 days.

10.3.3. Individual body weights must be recorded at the initiation of the definitive test and at weekly intervals thereafter, and on the day of death. Feed consumption must be measured weekly for the exposure and withdrawal periods, and should be based on a minimum of two consecutive day's feed consumption. In estimating feed consumption by mink or ferrets, several precautions are necessary. Since feed consumption can be adversely affected on a short-term basis by temperature, weather, and other factors, estimates should be based on at least two consecutive day's consumption. These days should also be days when the animals are not handled (e.g., during weighing, moving, etc.), since handling can produce a temporary reduction in feed consumption.

10.3.4. Mortality, behavioral abnormalities (lethargy, nervousness, etc.), and other signs of toxicity (unthrifty appearance, convulsions, incoordination, unusual vocalizations, etc.) should be recorded daily

during the test.

10.3.5. For tests conducted indoors, the photoperiod should be maintained at the same schedule in effect at the conclusion of the acclimation period because a changing photoperiod may subject mink and ferrets to slight adjustments in normal body rhythms with respect to feeding, sleeping, fat mobilization or deposition, coat development, and hormonal activity. It should be noted that the effects of the length of the light/dark cycle on toxicity have not been investigated in these species.

10.3.6. A minimum of eight animals for each test concentration should be used. The test concentrations should be geometrically spaced so as to result in at least 2 dietary concentrations yielding 10-90% mortality. These results usually can be obtained with 4-6 dietary concentrations, including a control. It is possible to conduct an LC_{50} test with as few as 3 dietary concentrations and 8 animals per concentration if a good estimate of the LD_{50} for the test species is available. In many instances, however, accurate results can be achieved with 5 dietary concentrations and 10 animals per concentration if a good estimate of the LD_{50} is available from range-finding procedures.

10.3.7. The prescribed length of the mammalian dietary LC_{50} test is 28 days for several reasons. A 28-day test allows time for absorption, distribution, metabolism, enzyme induction, redistribution, bioconcentration, and elimination to occur, similar to that which might occur in animals subacutely exposed to a substance via diet in the environment. A 28-day test also allows testing of slow-acting or bioaccumulating substances. Such tests could prove negative or

misleading in a test of shorter duration. For example, prolonged mortality patterns were observed in 28-day tests with mink (Table 2) and ferrets (Table 3) fed Compound 1080, in which mortalities were observed up to the end of the test (Hornshaw et al., 1986b). Delayed mortalities were observed in a 28-day test with mink fed Aroclor 1254 (Table 4), in which mortalities were observed during a 7-day withdrawal period as well as during the exposure period (Hornshaw et al., 1986c). In some instances, it may be possible to achieve satisfactory results with a test of shorter duration using higher concentrations of the test substance, but the possibility of feed rejection or avoidance becomes greater with increasing concentrations. For example, in the 1080 tests already noted, signs of feed avoidance appeared in the first week of both tests in a dose-related manner. Increasing the concentration in these tests may have resulted in nearly complete avoidance of the feed and subsequent removal of the highest dietary concentrations from the test for humane reasons (Hornshaw et al., 1986b). Also, certain substances cause delayed mortality, whether administered as a single dose or multiple dosages. Increasing the concentration of the substance does not necessarily shorten the time to death. An example of this phenomenon is seen in the Aroclor 1254 test (Table 4; Hornshaw et al., 1986c). A test should be considered invalid if more than 12.5% of the control animals die during the definitive test.

10.3.8. It is strongly recommended that a dietary concentration group be removed from testing when food consumption measurements indicate that 10% or less feed, compared to controls and/or acclimation period values, is consumed daily for the first two week's feed consumption measurements

or the animals lose 30% of their original body weight.

10.3.9. Necropsies should be performed on all mortalities. At the termination of the test, all surviving test animals should be killed by accepted humane methods (AVMA Panel on Euthanasia, 1986) and necropsies performed. It is suggested that necropsies be performed on all test animals, either on the day of death or at the termination of the test. Valuable information on the mode of action of the test substance and its effect on target organs or organ systems can sometimes be gained from gross observation of the test animals at necropsy. Histopatho-logical examination of organs and tissues can often provide more information. Weights of internal organs of control and treated animals can be compared statistically to determine effects of the substance, although the effects of starvation can sometimes be confounded with effects of the substance.

10.4. Statistical analysis:

10.4.1. Body weight changes and feed consumption may be analyzed by analysis of variance (Sokal and Rohlf, 1969) and significant differences may be located by Dunnett's method for comparison with the control (Dunnett, 1964).

10.4.2. An LC_{50} value, including confidence limits and slope of the dose-response curve may be calculated by the method of Litchfield and Wilcoxon (1949).

10.4.3. Other valid statistical procedures may also be used to analyze the data.

11. Quality Assurance

11.1. In order to assure the quality and reliability of data developed

using this protocol, testing facilities should have a quality assurance unit that is responsible for monitoring the test along with the investigator, to assure that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with good laboratory practice standards (U.S. EPA, 1989) and are legally defensible and appropriate for the intended use.

12. Reporting Requirements

12.1. All data and information required by this protocol should be recorded in black ink on appropriate forms and/or bound laboratory notebooks. A duplicate set of records should be kept at a location other than the test site. All data should be maintained in a secure location to prevent tampering or destruction of the records. The following information should be reported:

12.1.1. Name of the investigator(s), laboratory, laboratory address, location of raw data, and date of initiation and termination of test.

12.1.2. Name of species tested, including scientific name, source, and age of the animals at the beginning of the test.

12.1.3. A detailed description of the test substance including its chemical name, synonyms, structure, formulations, purity, source, batch, lot number, and physical/chemical properties and name and source of solvent or carrier, if used.

12.1.4. Description of the test facilities and housing conditions, including test cages, temperature, and photoperiod.

12.1.5. Name and source of feed, including description and proximate analysis of diet.

12.1.6. The theoretical and measured dietary concentrations; number of

animals per concentration; body weights; feed consumption; signs of toxicity; behavioral changes; % mortality for each concentration; significant necropsy findings; calculated LC_{50} values and 95% confidence limits, slope of the dose-response curve and 95% confidence limits, and the name and reference of the statistical methods used; highest dietary concentration at which no signs of toxicity were observed; anything unusual about the test; any deviations from the protocol; and other relevant information.

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MAMMALIAN WILDLIFE (MINK AND FERRET) REPRODUCTION TEST[1]

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[1] This work was supported by the U.S. Environmental Protection Agency under contract Number U.S. EPA/NOAA Interagency Agreement #AD 13 F 28800.

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) has been charged, under several laws, with the regulation of many toxic or hazardous substances. The key to regulation of these substances is toxicological testing of the compounds with appropriate test species. The species of choice for testing the toxicity of a compound to man and domestic animals, as specified in federal guidelines (U.S. EPA, 1975, 1978, 1982a,b), have been common laboratory animals such as rats, mice, rabbits, guinea pigs, and hamsters. No representative mammalian wildlife species have been designated in the federal guidelines. Adequacy of risk assessment for wildlife mammals has normally been based on toxicity data for evaluating hazards to humans and domestic animals (U.S. EPA, 1978; Ringer, 1988).

Under special circumstances of hazard assessment, such as secondary toxicity poisoning or evaluation of certain chemicals that bioconcentrate in the food chain, special tests involving wildlife mammals may be required. Carnivorous animals are generally the species of choice in toxicological testing for secondary poisoning (Schitoskey, 1975; Townsend et al., 1984; Aulerich et al., 1986, 1987) and represent the top of the food chain for chemicals that bioconcentrate.

Prompted by these concerns, the mink (Mustela vison) (U.S. EPA, 1975; Aulerich and Bleavins, 1981) and European ferret (Mustela putorious furo) (Thornton et al., 1979; Hoar, 1984) were suggested as representative mammalian carnivores for toxicological testing. The mink may be preferred because it is indigenous to the U.S. and Canada and occurs in the wild throughout much of North America. Its nutritional requirements are well known (National Research Council, 1982), stock of known genetic origin is available, all stages of its life cycle can be successfully perpetuated in the laboratory, and it has a large biological

data base (Shump et al., 1976; Scientifur, 1987; Sundqvist, 1989). The European ferret, although not indigenous to North America, has been examined as a laboratory model for toxicology (Willis and Barrow, 1971; Thornton et al., 1979; Anon., 1981; Hoar, 1984). The mink has been shown to be among the most sensitive, if not the single most sensitive, mammalian species to polychlorinated biphenyl (PCB) toxicity (Aulerich and Ringer, 1977). Research performed with mink was instrumental in setting U.S. water quality standards for PCBs (Aulerich and Bleavins, 1981). Subsequent studies have shown this species to be similarly sensitive to polybrominated biphenyls (Aulerich and Ringer, 1979), hexachlorobenzene (Bleavins et al., 1984), aflatoxins (Chou et al., 1976; Bonna et al., 1991), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Hochstein et al., 1988). Additional toxicological studies with mink and ferrets have been reported in the literature (Frederick and Babish, 1985; Scientifur, 1987; Sundqvist, 1989).

The following protocol for conducting reproduction tests with mink and ferrets was based on procedures used in previous toxicology studies from our laboratory and refined by additional studies on four chemicals (Hornshaw et al., 1986a,b,c, 1987) providing a range of solubilities, volatilities, toxicities, and modes of action.

1. Scope

1.1. This protocol describes a method for determining the reproductive toxicity of a test substance (that can be mixed uniformly into the diet) administered to animals in their daily diet. Reproductive toxicity may be expressed as an adverse effect on: (a) adult survival; (b) oogenesis and/or spermatogenesis; (c) embryo or fetus development; (d) reproductive indices; or (e) offspring growth and survival.

1.2. This protocol is intended for use with carnivorous species, such as the mink (Mustela vison) and European ferret (Mustela putorius furo). Other carnivorous species may be used with appropriate modifications.

2. Summary

2.1. Groups of animals of the same species and age (both sexes) are fed diets containing a test substance in a series of concentrations, plus a control, for 8 weeks prior to breeding, during breeding, gestation, and parturition, and for 3 weeks of lactation (approximately 23 weeks) to measure reproductive toxicity.

2.2. Animals are observed daily and mortalities are reported.

2.3. Data derived from treatment and control groups are compared statistically to detect changes in body weight; feed consumption; length of gestation; percent of females bearing offspring; total offspring born per female (live and dead); average birth weight of offspring; average live litter weight; average weight of offspring at 3 weeks; and percent offspring survival to 3 weeks.

3. Significance

3.1. This protocol provides a means of measuring the reproductive toxicity of a test substance in the daily diet of a carnivore under controlled

conditions. It is recommended that, if possible, tests be conducted indoors. Indoor tests allow greater control of test conditions, and therefore, greater reproducibility. Indoor facilities, if heated, allow more accurate measurements of feed consumption than outdoors, especially during sub-freezing conditions. Indoor facilities also make the possibility of escape of test animals less likely.

3.2. This protocol provides data for assessing the potential adverse effects of chemicals to mammalian carnivores exposed through dietary intake, the normal exposure route in the environment. The mammalian carnivore occupies a position high on the food chain, thus it may be subject to the effects of bioaccumulation of chemicals.

3.3. This protocol permits collection of data on signs of toxicity and mortality over an extended period of dietary exposure, such as may occur in nature.

3.4. This test provides a basis for deciding whether additional toxicity testing should be conducted. Results from a reproduction test may indicate the need for subsequent chronic tests with the test species. These results might also indicate the need for other types of tests, such as aquatic or inhalation, or tests designed for a target organ or organ system. This protocol can provide limited data on the effects of a substance on male reproductive performance. However, if such effects are noted, it would be necessary to conduct further tests employing a different experimental design than the one described in the protocol to quantify male effects.

4. Definitions

4.1. Test substances: The element, chemical compound, formulation, known mixture, or material mixed in diets and fed for the purpose of determining

reproductive toxicity.

4.2. Concentration: The weight of the test substance per unit weight of the diet (expressed as mg/kg of diet).

4.2.1. Theoretical (nominal) concentration: Targeted concentration of the test substance in the diet.

4.2.2. Measured concentration: Concentration of the test substance in the diet as determined by analysis.

4.3. Acclimation period: A period of at least 7 days immediately preceding the exposure period during which the test animals are housed in the test facilities under test conditions and fed the untreated diet and drinking water to be used during the exposure period.

4.4 Definitive test: Test where a substance is fed to a prescribed number of animals at several geometrically-spaced concentrations for a specified period of time during which parameters of toxicity are measured.

4.5. Conventional diet: Feed consisting of both fresh and dried ingredients with water added to provide a semi-solid (hamburger) consistency.

4.6. Dry diet: Feed consisting of only dried ingredients usually fed in pelleted form.

5. Precautions

5.1. Contact with all test substances, solutions, and mixed diets should be minimized with appropriate protective clothing, gloves, eye protection, etc. The use of fume hoods and increased ventilation in test rooms is necessary when handling volatile substances. Information on other mammalian toxicity and special handling procedures should be known before this protocol is used.

5.2. Disposal of excess test substances, solutions, mixed diets, etc., and

treated animals should be done with consideration for health and environmental safety, and in accordance with all federal, state, and local regulations.

5.3. Cleaning and rinsing of glassware, feeders, and other equipment with volatile solvents should be performed only in well-ventilated areas.

5.4. Because this protocol addresses the use of carnivores, appropriate precautions should be used in the handling of test animals, especially mink. Mink are by nature extremely aggressive and may attack a handler if given the opportunity. Appropriate precautions, such as leather gloves and arm-coverings, should be used. Mink and ferrets can transmit certain diseases, most importantly tetanus, and also tuberculosis (TB) and rabies if contacted from outside sources. Researchers should take precautions for these diseases. The possibility of TB infection can be reduced by avoiding the use of pork products in the diet. Proper caging and isolation of test facilities from wild animals can similarly reduce the possibility of rabies infections.

5.5. Mink and ferrets are known to be sensitive to handling and other disturbances during the first 2 weeks post-partum; contact and outside disturbances should be minimized during this period.

6. Test Animals

6.1. This protocol is intended for use with carnivorous species, such as the mink (Mustela vison) and European ferret (Mustela putorius furo). Other carnivorous species may be used with appropriate modifications, such as diet, cages, etc.

6.2. All animals for a given test must come from one source and strain and be of approximately the same age to minimize variability. Test animals may

be obtained from commercial sources or may be reared in laboratory colonies, but they must not have been used in a previous test. It is recommended that test animals be immunized against diseases commonly afflicting the test species. Immunization for mink should include canine distemper, virus enteritis, infectious pneumonia, and botulism (Joergensen, 1985). It is recommended that ferrets be vaccinated against canine distemper and botulism (Fox, 1988). Mink and ferrets are also susceptible to Aleutian Disease (AD). Since no vaccine exists for this viral disease, testing and certification of the animals as AD-free is recommended. Animals that are deformed, injured, emaciated, or phenotypically different from normal animals must not be used as test subjects. The population of animals from which the test subjects (treated and control) are selected should be considered unsuitable for testing if mortality exceeds 5% during the acclimation period.

6.3. It is preferable to use animals that are proven breeders. However, availability and cost may dictate that animals in their first breeding season be used.

7. Facilities

7.1. Space requirements for most carnivores have not been standardized. However, adherence to the guidelines of the Fur Farm Animal Welfare Coalition (1988) should provide a basis for adequate space and husbandry requirements. Individual cages measuring 61 x 76 x 46 cm (24 x 30 x 18 in) and nest boxes measuring 38.1 x 27.9 x 26.7 cm (15 x 11 x 10.5 in) have proven adequate for tests performed in conjunction with the development of this protocol. Mink and ferrets must be caged individually. In designing a caging system for carnivores, it is important to prevent both cross-

contamination of treatment groups and contact between individual animals. To prevent aggressive animals from attacking neighboring animals, use solid dividers between adjoining cages or provide adequate space between adjoining cages if wire mesh cage material is used.

7.2. Construction materials in contact with animals should not be toxic, nor be capable of excessively adsorbing or absorbing test substances. Stainless steel, galvanized steel, or materials coated with perfluorocarbon plastics are acceptable materials, but other construction materials may also be useful.

7.3. A nest area or nest box containing nesting material (such as straw, shredded wood, or marsh hay) must be provided for all females prior to the parturition period. It is very important to ensure that newborn are protected from toxic compounds. A particular area of concern is wood by-products which may be contaminated with compounds to which mink are sensitive.

7.4. Adequate ventilation should be provided at all times. The Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals, 1985) recommends 10 to 15 room air changes/hour for animal facilities.

7.5. This protocol addresses the use of mink and ferrets during their natural breeding seasons. Photoperiodic manipulations may permit the use of this protocol at other seasons. If the animals were raised outdoors and the test is conducted indoors, the photoperiod should simulate ambient conditions appropriate to maintain the normal reproductive status throughout the acclimation period and definitive test. In order to bring mink and ferrets into breeding condition indoors, it is necessary to gradually

increase the length of photoperiod during the test. If the animals are held indoors for an extended period of time prior to the test, it is also necessary to gradually decrease the photoperiod prior to the acclimation period to provide a necessary quiescent period of sexual development for the animals. Since very low intensities of light may alter the reproductive cycle, care must be taken to ensure that total darkness is maintained during the appropriate periods. If the test is conducted outdoors, care must be taken to ensure that the photoperiod is not altered by extraneous light sources.

8. Diets

8.1. Diets must be formulated in accordance with the nutrient requirements of the test species (National Research Council, 1982). Suggested ranges of composition of conventional mink diets are shown in Table 1. Any unmedicated commercial diet that meets the minimum nutritional standards of the test species is acceptable.

8.2. Fresh diets and water must be provided daily and fed ad libitum.

9. Diet Preparation

9.1. Knowledge of the physical, chemical, and biological properties of the test substance is important in test diet preparation.

9.2. Test diets can be prepared by mixing the test substance directly into the feed or by dissolving or suspending the test substance in a solvent or carrier prior to mixing with the feed. The use of solvents or carriers may be necessary to achieve uniformity. If solvents or carriers are used, they must also be added to the control diet in an equivalent volume. It is very important to assure uniform distribution of a test substance in the diet. In many instances, this will be more easily accomplished using the

conventional diet, because many substances can be mixed into a diet more uniformly if the diet is semi-solid and capable of being machine-mixed. For some test substances, especially water soluble ones, this may be the only method of assuring uniform distribution, since pelleted diets are not conducive to being coated with aqueous solutions, but rather tend to become a mash. No matter which type of diet is used, it is recommended that innocuous solvents or carriers, such as distilled water or corn oil, be used if possible. (It is recommended that, unless the amount of test substance to be added is large, solvents or carriers be used to introduce the substance to the diet to ensure uniform distribution). If an innocuous solvent or carrier cannot be found, it is recommended that a volatile solvent, such as acetone or hexane, be used to dissolve the test substance, if possible, and the resultant solution be added to a small amount of either the dry diet or a dry ingredient (e.g., cereal) of the conventional diet. After the solvent is evaporated, the pre-mix can then be mixed with the rest of the diet uniformly. (If this procedure is used, it must likewise be used on the control diet).

9.3. If the researcher chooses to use the conventional diet, it is important not to freeze the diets in containers too large, because the diets will not remain fresh under refrigeration for more than 2-3 days. When conventional diets are used, sufficient diet should be mixed to provide adequate feed for approximately 4 weeks. The amount of feed to be mixed may be based upon the feed consumption data obtained during the acclimation period. When mixed, the diet should be stored frozen in containers large enough to hold 1-2 day's feed. In testing volatile substances, sealable containers must be used and stored upside down, one day's feed per container. The feed should

fill the container, allowing no headspace. When dry diets are used, they should be stored so as to maintain the stability of the test substance in the diet. The frequency with which the feed is mixed is dependent upon the physical/chemical properties of the test substance.

9.4. It is imperative that all diets be analyzed for the concentration of the test substance in the diet and the measured concentration reported with the test results. A significant number of samples should be analyzed to accurately determine both the concentration of test substance in each diet as well as the homogeneity of the mixture.

10. Procedure

10.1 Dietary concentrations of test substance.

10.1.1. Establishing the dietary concentrations of a test substance for a reproductive study is a difficult but essential first step in determining an environmental effect of a chemical substance upon reproduction. A number of procedures exist for establishing the dietary concentrations to be used. Three are presented in this protocol.

10.1.1.1. If a dietary LC_{50} test has been conducted with the species under consideration, the highest dietary concentration at which no signs of toxicity were observed should approximate the highest of a series of geometrically-spaced dietary concentrations, plus a control.

10.1.1.2. Another method of establishing dietary concentrations utilizes known or expected environmental concentrations of the test substance. Two or more dietary concentrations, plus a control, should be used. Examples of series of concentrations that may be used include 1X, 3X, and 5X or 1X, 3X, and 10X, where X equals the

measured environmental concentration.

10.1.1.3. If LC_{50} data are lacking, it is useful to conduct a preliminary study with several widely spaced dietary concentrations of the test substance. The dietary concentrations for the definitive test may be established from these preliminary studies. It is recommended that 3 or more dietary concentrations plus a control be tested in the definitive test if this procedure is followed. If an estimate of a dietary concentration at which signs of toxicity are not observed is lacking, it is recommended that a preliminary study be conducted to aid in establishing dietary concentrations for the definitive test. This study may be patterned after the protocol for mammalian dietary LC_{50} tests, using several widely-spaced concentrations over a short period (e.g., 7-14 days) to determine an approximate no effect concentration. Because the data from a study such as this would be expected to be fragmentary, it is suggested that at least 3 dietary concentrations be tested in the definitive test in order to maximize the possibility of meeting the criteria for an acceptable test while minimizing the possibility of wasting time, money, and animals.

10.2. Experimental design.

10.2.1. This protocol is intended for use with individually caged animals only. Males and females will be paired only during breeding attempts, and one male will be assigned to a treatment group for each 3 or 4 females. Thus, this protocol is primarily designed to test female reproductive effects, and provides only limited data on male reproductive effects. If data on male reproductive effects are desired, a

different experimental design will be necessary.

10.2.2. Relatively few background data are available to aid in determining the proper number of male and female mink and ferrets to use to detect a significant difference for a given reproductive parameter. Due to considerations of cost and availability of proven breeders, it may be necessary to use animals which have not had breeding experience. If this is the case, based on reproduction tests performed in conjunction with the development of this protocol and on other reproduction tests with mink and ferrets, it is recommended that a minimum of 12 females per treatment group be used to provide a margin of safety against females which will not accept males, are barren, or do not have proper maternal instincts (each of these reproductive anomalies will be exhibited by a small percentage of first year females within a cohort). Because the male's only function in reproduction is the mating act, it is not necessary to house equal numbers of males and females, unless male reproductive effects are expected. Thus, it is only necessary to house one male for every three or four females per dietary concentration. Again, if first year animals are used, it is suggested that the male:female ratio be 1:3, to provide a margin of safety against males which will not attempt to mate or which produce no viable spermatozoa. If proven breeders are used, it may be possible to meet the criteria for an acceptable test with as few as eight females and two males per dietary concentration. It is recommended that breeding attempts be made only between males and females within the same treatment group.

10.2.3. If this experimental design is selected, one of the following

criteria must be met:

A. One dietary concentration must produce an effect.

B. The highest dietary concentration must contain at least 1000 mg/kg.

C. The highest dietary concentration must be at least 100 times the highest known or expected environmental concentration.

If the researcher selects an experimental design based on considerations of Type I and Type II error, the number of females per treatment group may be specified by the researcher's levels of power, significance, and difference between means to be detected.

10.2.4. Each test animal should be randomly assigned to a specific test diet concentration and be uniquely identified.

10.3. Acclimation period.

10.3.1. All animals should be conditioned to the test facilities including: photoperiod, temperature, and caging for a minimum of 7 days. A longer acclimation period may be desirable especially if the animals to be used in a test are changed from outdoor to indoor housing (or vice versa) or if the diet or water to be used in a test is different from what the animals are accustomed to consuming. During this period all animals should be given the untreated (control) diet and drinking water, as used during the definitive test. Test animals should be weighed at the start of the acclimation period. It is recommended that feed consumption be measured during the latter part of the acclimation period as these measurements can provide an indication of how much feed the animals should consume once the test starts, how much diet to mix for the test, and can also serve as a control value for each group.

10.4. Definitive test.

10.4.1. The test diets must be fed daily throughout the pre-breeding, breeding, gestation, parturition, and lactation periods, a duration of approximately 20-23 weeks. The suggested length of the mammalian reproduction test of approximately 20-23 weeks is designed to conform to the normal reproductive seasons of mink (March through June) and European ferrets (April through July), with an 8 week exposure period prior to the reproductive season. The total time of the mink exposure period can be expected to be somewhat longer than the ferret exposure period because mink exhibit a variable delay in implantation of fertilized ova, while ferrets do not. Thus, the gestation period for mink can range naturally from approximately 42 to 60 days, whereas for ferrets the gestation period will normally be approximately 42 days. The length of this test allows ample time for absorption, distribution, metabolism, enzyme induction, re-distribution, bioconcentration, and elimination to occur, and for tolerance to be acquired, similar to that which might occur to animals chronically exposed to a substance in the environment.

10.4.1.1. Pre-breeding period: Individual body weights must be recorded at the initiation of the definitive test and bi-weekly (once every other week) thereafter for the 8 weeks of the pre-breeding period. Feed consumption must also be measured weekly during the pre-breeding period, and should be based on a minimum of two consecutive day's consumption because feed consumption can be adversely affected on a short-term basis by temperature, weather, and other factors. Feed consumption should be measured on days when

the animals are not handled (e.g., during weighing, moving, etc.), because handling can produce a temporary reduction in feed consumption.

10.4.1.2. Breeding period: Under natural conditions, mating attempts begin at the first of March for mink and at the end of April for ferrets. In breeding mink, a female is presented to a male and, if receptive, is allowed to mate. If not receptive, the female is removed and presented to a male approximately 4 days later. Once a successful mating occurs (as verified by the presence of viable spermatozoa in a vaginal aspiration taken just after copulation), the female is given the opportunity to mate a second time (with the same male or a different male), either 8 days after the initial mating or the next day (if the first mating occurs late in the breeding season). In breeding ferrets, females are presented to males when they are judged to be in estrus (determined by the extent of vulvar swelling) and left overnight. They are not normally given the opportunity for additional matings. If the researcher has reason to suspect male reproductive effects, vaginal aspirations may be taken for examination of spermatozoa. Generally, it is advisable to discontinue recording body weights and measuring feed consumption once the breeding attempts begin. The increased handling of the animals during the breeding period causes perturbations in the animals' daily routines, resulting in decreased feed consumption by some animals. In addition, some animals respond to increased handling by becoming excitable. Repeated breeding attempts, coupled with routine weighings, may produce some females

that are so excitable that breeding them becomes extremely difficult. Once the breeding period is over, it is best that the animals are left undisturbed as much as possible, especially during the first 2 weeks post-partum.

10.4.1.3. Gestation period: This period lasts approximately 6 weeks for ferrets and 6-8 weeks for mink. During this period, the animals should not be weighed, handled, or unduly disturbed.

10.4.1.4. Parturition period: This period lasts up to 3 weeks, depending on species. During this period females are checked daily for newborn. All newborns are counted, weighed, sexed, and recorded within 24 hours post-partum. It is suggested that in checking for newborn, care is taken not to disturb the females more than necessary. If a nest box is not employed, visual inspection often is sufficient to determine whether a litter has been born. If a nest box is employed, it may be necessary to exclude the female from the nest box while checking the nest for newborn. When the female refuses to leave the nest box, it is often an indication that parturition has occurred.

10.4.1.5. Lactation period: Individual body weights of all surviving newborn are recorded at the end of this 3 or more week period. This period should not extend beyond 6 weeks, the normal weaning time for mink and ferret offspring. During this period, offspring may come in contact with or eat (after 3 weeks) the maternal diet.

10.4.1.6. Termination: At the termination of the test, all males and at least an equal number of females chosen at random from each dietary group should be killed by accepted humane procedures (AVMA

Panel on Euthanasia, 1986) and necropsies performed. Valuable information on the mode of action of the test substance and its effect on target organs or organ systems can sometimes be gained from gross observation of the test animals at necropsy. Histopathological examination of tissues and tissue residue analyses can often provide more information. Weights of internal organs and blood parameters of controls and treated animals can also be compared statistically to determine effects of the substance.

10.4.2. General considerations.

10.4.2.1. All animals must be observed daily. All overt clinical signs and any abnormal behavior must be recorded. If mortality occurs, the date and body weight must be recorded and necropsy performed.

10.4.2.2. A test must be considered invalid if more than 20% of the control animals die during the definitive test. It is highly unlikely, based on the results of tests conducted in conjunction with the development of this protocol and on general mortality patterns observed in the fur industry, that more than 20% of a population of healthy mink or ferrets would die over the course of a 23 week reproduction test (Joergensen, 1985). If a researcher suffers the loss of greater than 20% of control animals in a test, it is possible that problems may exist in the diet or husbandry practices, or that disease has affected the stock.

10.5. Reproductive indices.

10.5.1. The reproductive indices required in this protocol were selected based on features of the reproductive performance of mink and ferrets.

Weights of all offspring (live and dead) are not required to be tested and reported because mink and ferrets are known to consume dead or stillborn young, thus, testing this reproductive index may produce incorrect or misleading results. Percent survival and weights of offspring are required at 3 weeks to allow minimal disturbance of dams and offspring during the critical period after birth and to ensure that nourishment received by offspring is almost totally of maternal origin. Percent survival and weights of offspring at 6 weeks is not required because the young usually begin consuming at least some solid feed by 4 weeks of age. As mentioned previously, mink are known to exhibit a variable delay in implantation of fertilized ova, thus the length of gestation may not be useful in assessing effects of a substance on gestation in mink. It may, however, by very useful in assessing these effects in ferrets.

10.5.2. The following reproductive indices must be calculated:

A. Length of gestation: The time, in days from the last confirmed mating until parturition.

B. Number whelped, not whelped: The number of females giving birth and not giving birth in a treatment group. Number whelped includes females that die during the process of whelping from problems associated with parturition. This value is expressed as the number of females whelped or not whelped per the number of females with confirmed matings in a treatment group.

C. Live newborn/female whelped: The average number of live newborn produced by all females that give birth in a treatment group. This value does not include females that die during the process

of whelping from problems associated with parturition.

D. Average birth weight: The average weight of all live newborn born in a treatment group, weighed to the nearest tenth of a gram within 24 hours post-partum.

E. Average litter weight: The average weight of all litters (live newborn only) born in a treatment group, weighed to the nearest tenth of a gram within 24 hours post-partum.

F. Percent newborn survival to 3 weeks: The number of live newborn in a treatment group surviving to 21 days of age, expressed as a percentage of all live newborn born in a treatment group.

G. Average 3 week body weight: The average weight of all live newborn in a treatment group, weighed to the nearest gram on the 21st day after birth.

10.5.3. The following reproductive indices may also be useful:

A. Total newborn/female whelped: The average number of all newborn (alive and dead) produced by all females that give birth in a treatment group. This value includes females that die during the process of whelping from problems associated with parturition.

B. Percent newborn survival to 6 weeks: Identical to 21 day survival, but extended to 42 days.

C. Average 6 week body weight: Identical to 21 day weights, but measured at 42 days of age.

10.6. Statistical analysis.

10.6.1. The following variables may be analyzed by analysis of variance (Sokal and Rohlf, 1969) and significant differences may be tested by

Dunnett's method for comparison with control (Dunnett, 1964):

A. Body weight changes

B. Feed consumption

C. Length of gestation

D. Live offspring/female whelped

E. Total offspring/female whelped

F. Average birth weight

G. Average litter weight

H. Average 3 week body weight

I. Average 6 week body weight

10.6.2. The following variables may be analyzed by contingency tables (Zar, 1974) and significant differences may be tested by Bonferroni's Chi-square test (Gill, 1978):

A. Number whelped, not whelped

B. Percent newborn survival to 3 weeks

C. Percent newborn survival to 6 weeks

10.6.3. The statistical procedures suggested are only a few of the valid statistical methods which may be used. Use of other methods may prove more appropriate in detecting significant differences. Certain procedures may permit testing two or more combined reproductive indices to assess the true effect of a substance on reproductive performance, even though none of the indices by themselves are statistically significant (Brown, 1975).

11. Quality Assurance

11.1. In order to assure the quality and reliability of data developed using this protocol, testing facilities should have a quality assurance unit that

is responsible for monitoring the test along with the investigator to assure that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with good laboratory practice standards (U.S. EPA, 1989) and are legally defensible and appropriate for the intended use.

12. Reporting Requirements

12.1. All data and information required by this protocol should be recorded in black ink on appropriate forms and/or bound laboratory notebooks. A duplicate set of records should be kept at a location other than at the test site. The following information must be recorded.

12.1.1. Name of the investigator(s), laboratory, laboratory address, location of raw data, and date of initiation and termination of test.

12.1.2. Name of species tested, including scientific name, source, and age of the animals at the beginning of the test.

12.1.3. A detailed description of the test substance including its chemical name, synonyms, structure, formulations, purity, source, batch, lot number, and physical/chemical properties and name and source of solvent or carrier, if used.

12.1.4. Description of the test facilities and housing conditions, including test cages, temperature, and photoperiod. If conducted outdoors, adverse weather conditions may alter test results, especially during the parturition period, and should be reported.

12.1.5. Name and source of feed, including description and proximate analysis of diet.

12.1.6. The dietary concentration; number of males and females per concentration; body weights; feed consumption; signs of toxicity; abnormal behavior; mortality; reproductive indices; statistical methods

employed; significant necropsy findings (including organ weights, if recorded); anything unusual about the test; any deviations from the protocol; and other relevant information.

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MAMMALIAN WILDLIFE (MINK AND FERRET) DIETARY LC_{00} TESTS TO ASSESS PRIMARY AND SECONDARY TOXICITY[1]

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[1] This work was supported by the U.S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, OR, under Cooperative Agreement CR810785.

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) has been charged, under several laws, with the regulation of many toxic or hazardous substances. The key to regulation of these substances is toxicological testing of the compounds with appropriate test species. The species of choice for testing the toxicity of a compound to man and domestic animals, as specified in federal guidelines (U.S. EPA, 1975, 1978, 1982a,b), have been common laboratory animals such as rats, mice, rabbits, guinea pigs, and hamsters. No representative mammalian wildlife species have been designated in the federal guidelines. Adequacy of risk assessment for wildlife mammals has normally been based on toxicity data for evaluating hazards to humans and domestic animals (U.S. EPA, 1978; Ringer, 1988).

Prompted by these concerns, the mink (Mustela vison) (U.S. EPA, 1975; Aulerich and Bleavins, 1981) and European ferret (Mustela putorius furo) (Thornton et al., 1979; Hoar, 1984) were suggested as representative mammalian carnivores for toxicological testing. The mink may be preferred because it is indigenous to the U.S. and Canada and occurs in the wild throughout much of North America. Its nutritional requirements are well known (National Research Council, 1982), stock of known genetic origin is available, all stages of its life cycle can be successfully perpetuated in the laboratory, and it has a large biological data base (Shump et al., 1976; Scientifur, 1987; Sundqvist, 1989). The European ferret, although not indigenous to North America, has been examined as a laboratory model for toxicology (Willis and Barrow, 1971; Thornton et al., 1979; Anon., 1981; Hoar, 1984). The mink has been shown to be among the most sensitive, if not the single most sensitive, mammalian species to polychlorinated biphenyl (PCB) toxicity (Aulerich and Ringer, 1977). Research performed with mink was instrumental in setting U.S. water quality standards for PCBs (Aulerich

and Bleavins, 1981). Subsequent studies have shown this species to be similarly sensitive to polybrominated biphenyls (Aulerich and Ringer, 1979), hexachlorobenzene (Bleavins et al., 1984), aflatoxins (Chou et al., 1976; Bonna et al., 1991), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Hochstein et al., 1988). Additional toxicological studies with mink and ferrets have been reported in the literature (Frederick and Babish, 1984; Scientifur, 1987; Sundqvist, 1989).

Under special circumstances of hazard assessment, such as secondary toxicity poisoning, special tests involving wildlife mammals may be required. Carnivorous animals are generally the species of choice in toxicological testing for secondary poisoning (Schitoskey, 1975; Townsend et al., 1984; Aulerich et al., 1986, 1987) and represent the top of the food chain for chemicals that bioconcentrate. However, protocols have not been developed for mammalian wildlife species to compare the toxicity of metabolized forms of a xenobiotic (as contaminated prey substituted for similar uncontaminated animal products in the diet) with comparable concentrations of an unmetabolized form of the chemical added to the feed of test species. Standardized test protocols are needed to provide experimental techniques suitable for routine assessment of secondary toxicity.

The results of a secondary toxicity test may be difficult to interpret. For example, if the resulting primary and secondary LC_{50} test results were different, it could be because of differences in the bioavailability of the test chemical due to biological incorporation in the tissues of the prey species, or it could be due to formation of toxic metabolites. If the latter case is suspected, additional analytical chemistry would be required to identify the metabolites. If the metabolites are known, then the total toxicity of the parent compound plus the metabolites could, in some cases, be compared to the parent compound alone.

The following protocol was developed to provide a means of assessing primary versus secondary toxicity of chemicals to mammalian wildlife (mink and ferrets). The protocol is based on procedures used in previous toxicology studies from our laboratory (Aulerich et al., 1986; 1987).

1. Scope

1.1. This protocol describes a method for determining the subacute dietary toxicity of a test substance as used in field applications administered to animals in their daily diet (primary toxicity) and for comparing the primary toxicity of the compound to the toxicity of the same test substance contained within contaminated prey animal tissues (secondary toxicity). Toxicity is expressed as the median lethal concentration of the test substance (LC_{50}) and the slope of the dose-response curve.

1.2. This protocol is intended for use with carnivorous species, such as the mink (Mustela vison) and European ferret (Mustela putorius furo). Other carnivorous species may be used with appropriate modifications.

2. Summary

2.1. Groups of animals of the same species and age (both sexes) are fed diets containing a substance in a geometric series of concentrations for 28 days to measure lethality. This exposure period may be followed by a withdrawal period during which lethality is also measured. At the same time, an equal number of groups of animals of the same species and age (both sexes) are fed the identical concentrations contained in animal tissues contaminated by previous exposure to the same test substance. Data derived from the two tests are compared.

2.2. Daily observations for signs of toxicity and mortality are reported.

2.3. Data derived from treatment and control groups are compared statisti-

cally within and between groups to detect changes in body weight, feed consumption, and an index of toxicity.

3. Significance

3.1. This protocol provides a means of measuring and comparing the toxicity of a test substance in the daily diet of a carnivore, as the result of primary and secondary exposure, under controlled conditions. The use of a 28-day dietary exposure period allows metabolic transformations of the test substance to occur. It is recommended that, when possible, tests be conducted indoors. Indoor tests allow greater control of environmental test conditions and, therefore, greater reproducibility. Indoor facilities, if heated, allow conducting tests at any time of the year. Indoor facilities also make the possibility of escape of test animals less likely.

3.2. This protocol permits collection of data on signs of toxicity in addition to mortality.

3.3. The dose-response curves provide a basis for comparison of primary and secondary toxicity of a test substance to an animal.

4. Definitions

4.1. LC_{50}: The calculated concentration of a test substance which causes 50% lethality of a test animal population under the conditions of the test.

4.2. Primary toxicity: Poisoning or intoxication of an animal due to the consumption of a technical grade or formulated chemical.

4.3. Secondary toxicity: Poisoning or intoxication of an animal due to consumption of contaminated prey tissues.

4.4. Test substance: The element, chemical compound, formulation, known mixture, or material mixed in diets and fed for the purpose of determining an LC_{50}.

4.5. Concentration: The weight of the test substance per unit weight of the diet (expressed as mg/kg of diet).

4.5.1. Theoretical (nominal) concentration: The targeted concentration of test substance mixed into the diet.

4.5.2. Measured concentration: The concentration of test substance in the diet determined by analysis.

4.6. Acclimation period: A period of at least 7 days immediately preceding the exposure period when the test animals are housed in the test facilities under test conditions and fed the untreated diet and drinking water to be used during the exposure period.

4.7. Range-finding test: Test conducted to determine the concentration of the test substance to be used in the definitive test.

4.8. Palatability test: Test where the highest proposed dietary concentration of a test substance for use in a definitive test is fed to a few animals to determine if they will consume the diet containing this concentration of the test substance.

4.9. Definitive test: Test where a substance is fed to a prescribed number of animals at several geometrically-spaced concentrations for a specified period of time (exposure period may be followed by a withdrawal period) when parameters of toxicity, including lethality, are measured.

4.10. Exposure period: The 28-day period when the test animals are fed diets containing the test substance.

4.11. Withdrawal period: The period following an exposure period when all animals are fed an untreated diet to allow for observation of delayed mortality.

4.12. Diet: Feed consisting of both fresh and dried ingredients with water

added to provide a semi-solid (hamburger-like) consistency.

5. Precautions

5.1. Contact with all test substances, solutions, and mixed diets should be minimized with appropriate protective clothing, gloves, eye protection, etc. The use of fume hoods and increased ventilation in test rooms is necessary when handling volatile substances. Information on other mammalian toxicity and special handling procedures should be known before this protocol is used.

5.2. Disposal of excess test substances, solutions, mixed diets, etc., and treated animals should be done with consideration for health and environmental safety, and in accordance with all federal, state, and local regulations.

5.3. Cleaning and rinsing of glassware, feeders, and other equipment with volatile solvents should be performed only in well-ventilated areas.

5.4. Because this protocol addresses the use of carnivores, appropriate precautions should be used in the handling of test animals. Mink are by nature extremely aggressive and may attack a handler if given the opportunity. Appropriate precautions, such as leather gloves and arm-coverings, should be used. Ferrets are normally much less aggressive than mink but precautions should also be taken when they are handled. Mink and ferrets can transmit certain diseases, most importantly tetanus, and also tuberculosis (TB) and rabies if contacted from outside sources, so researchers should take precautions for these diseases. The possibility of TB infection can be reduced by avoiding the use of pork products in the diet. Proper caging and isolation of test facilities from wild animals can similarly reduce the possibility of rabies infections.

6. Test Animals

6.1. This protocol is intended for use with carnivorous species, such as the mink (Mustela vison) and European ferret (Mustela putorius furo). Other carnivorous species may be used with appropriate modifications, such as housing and diet.

6.2. All animals for a given test must come form one source and strain and be of approximately the same age to minimize variability. Test animals may be obtained from commercial sources or may be reared in laboratory colonies, but they must not have been used in a previous test. It is recommended that test animals be immunized against diseases commonly afflicting the test species. Immunization for mink should include canine distemper, virus enteritis, infectious pneumonia, and botulism (Joergensen, 1985). It is recommended that ferrets be vaccinated against canine distemper and botulism (Fox, 1988). Mink and ferrets are also susceptible to Aleutian Disease (AD). Since no vaccine exists for this viral disease, testing and certification of animals as AD-free is recommended. Animals that are deformed, injured, emaciated, or phenotypically different from normal animals must not be used as test subjects. The population of animals from which the test subjects (treated and control) are selected should be considered unsuitable for testing if mortality exceeds 5% during the acclimation period.

6.3. It is preferable to use animals that have approached their mature body size (for mink and ferrets this is about 18-20 weeks of age). Older animals can also be used to determine the LC_{50}. The use of young, rapidly growing animals may yield a distorted LC_{50} value because the change in body weight far exceeds the change in feed consumption resulting in a decreased amount

of test substance consumed per unit of body weight over the 28-day period. Because of the sex difference in size of mink and ferrets, the two sexes should be treated as separate sub-groups.

7. Facilities

7.1. Space requirements for mink and ferrets have not been determined. However, adherence to the guidelines of the Fur Farm Animal Welfare Coalition (1988) should provide a basis for adequate space and husbandry requirements. This space requirement is currently 32,774 cu cm or 2000 cu in. Individual cages measuring 61 (L) x 76 (W) x 46 (H) cm (24 x 30 x 18 in) have proven adequate for tests performed in conjunction with the development of this and other protocols. Cages must be constructed to prevent cross contamination of treatment groups and contact between individual animals. To prevent aggressive mink from attacking neighboring animals, use solid dividers between adjoining cages, or provide adequate space between adjoining cages if wire mesh cage material is used. Species not conducive to colony rearing, such as mink, must be caged individually.

7.2. Construction materials in contact with animals should not be toxic, nor be capable of excessively adsorbing or absorbing test substances. Stainless steel, galvanized steel, or materials coated with perfluorocarbon plastics are acceptable materials, but other construction materials may also be useful.

7.3. Adequate ventilation should be provided at all times. The Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals, 1985) recommends 10 to 15 room air changes/hour for animal facilities.

7.4. If the animals have been reared outdoors and the test is conducted

indoors, the photoperiod should simulate ambient daylight conditions because altered photoperiods may subject mink and ferrets to slight adjustments in normal body rhythms with respect to feeding, sleeping, fat mobilization or deposition, coat development, and hormonal activity. It should be noted that the effects of the length of the light/dark cycle on toxicity have not been investigated in these species. If the animals are reared indoors, the photoperiod should not be altered.

8. Prey (Contaminated) Animals

8.1. Prey animals for carnivores include any species that may be contaminated with the test substance and consumed by the test animals. Laboratory studies using contaminated prey animals in dietary, secondary toxicity trials have utilized fish (salmon, perch, alewife, sucker, carp, and bloater chubs), birds (chickens), and mammals (cattle, nutria, rabbits, prairie voles, pocket gophers, rats, and mice).

8.2. Contamination of prey animals may be via dietary, inhalation, or dermal routes. The prey animals should be exposed to the same test substance (same source and lot number) as fed in the definitive, primary toxicity test.

8.3. Before prey are contaminated, it may be necessary to conduct a range-finding trial using several widely-spaced concentrations to determine that concentration necessary to cause approximately 50% lethality in the test animals. Then it must be determined, through analytical procedures, whether sufficient body burdens can be achieved in the prey species. This body burden should allow for dilution of the tissues by the remainder of the dietary ingredients as per the nutrient requirements of the test animal (e.g., given that 10 mg/kg causes a 50% lethality in range-finding tests and

40% prey tissue is desired in the diet, then a prey body burden of 25 mg/kg is needed to yield a final dietary concentration of 10 mg/kg). LC_{50} estimates for other species may be helpful in setting dietary concentrations, although in general mink and ferrets are more sensitive to toxic compounds than laboratory animals. Palatability tests may also be employed to save time and animals. It is recommended that the highest proposed dietary concentration be fed for several days to 2-4 animals to determine if they will eat the diet at this concentration of the test substance. If not, it may be necessary to reduce the highest concentration to a level at which the diet will be eaten.

8.4. Prey animals that do not succumb to the test substance should be killed rapidly at the desired time by accepted humane methods (AVMA Panel on Euthanasia, 1986) that will not interfere with the test results. Prey animals that die should be frozen and stored to be used as part of the diet.

8.5. Depending on the nature of the test substance and the purpose of the test, it may be desirable to remove the contents from the alimentary tract before chemical analyses and/or incorporation into the diet. The removal of the alimentary tract contents eliminates the possibility of the primary chemical that has not been digested from being incorporated into the final diet. However, it should be noted that with certain test substances, the removal of the digestive tract contents may yield little or no body burden due to rapid metabolism and elimination by the prey species, although, considerable test substance may be present if the entire carcass including the alimentary tract content is utilized. Compounds that bioaccumulate, such as halogenated hydrocarbon compounds and certain metals, result in secondary poisoning with or without inclusion of digestive tract contents;

however, chemicals such as organophosphates, carbamates, and many rodenticides are metabolized rapidly and removal of contents from the digestive tract often renders the prey tissues non-toxic. With test substances such as organophosphates, it may be necessary to gavage the prey animals with high concentrations that may cause rapid death in order to secure tissue concentrations sufficient to be lethal to the test animals.

8.6. Carcasses of all clean prey animals should be ground and blended thoroughly to yield a homogeneous mixture. Samples should be taken for chemical analyses of test substance. The contaminated prey animals should be processed in the same manner and a sufficient number of samples to determine the concentration and homogeneity of the test substance taken for analyses. All ground carcasses should be stored in a frozen condition in tightly sealed containers until incorporated into the final diet.

9. Diets

9.1. Diets may be formulated in accordance with the nutrient requirements of the test species (Table 1 and National Research Council, 1982). Any unmedicated commercial diet that meets the minimum nutritional standards of the test species is acceptable. Diets must be formulated so that the same percentage of prey animal tissue is incorporated into each primary and secondary diet. The prey animals should be of the same species and source in all diets.

9.2. Fresh diets and water must be provided daily and fed ad libitum.

10. Diet Preparation

10.1. Knowledge of the physical, chemical, and biological properties of the test substance is important in test diet preparation.

10.2. Diets for the primary toxicity test can be prepared by mixing the

test substance directly into the feed or by dissolving or suspending the test substance in a solvent or carrier prior to mixing with the feed. The use of solvents or carriers may be necessary to achieve uniformity. If solvents or carriers are used, they must also be added to the control diet and the secondary toxicity test diets. It is very important to assure uniform distribution of a test substance in the diet. It is recommended that innocuous solvents or carriers, such as distilled water or corn oil, be used if possible. (It is recommended that, unless the amount of test substance to be added is large, solvents or carriers be used to introduce the substance to the diet to ensure uniform distribution). If an innocuous solvent or carrier cannot be found, it is recommended that a volatile solvent, such as acetone or hexane, be used to dissolve the test substance, if possible, and the resultant solution be added to a small amount of a dry ingredient (e.g., cereal). After the solvent is evaporated, the pre-mix can then be mixed with the rest of the diet uniformly.

10.3. To yield geometrically-spaced concentrations of the test substance in prey animals for the secondary toxicity test, appropriate quantities of contaminated and clean animal tissue, based on chemical analyses, should be thoroughly blended together and then mixed with the other dietary ingredients.

10.4. Sufficient diet should be prepared to provide adequate feed for the 28-day exposure period. The amount of feed to be mixed may be based upon the feed consumption data obtained during the acclimation period. When mixed, the diet should be stored frozen in containers large enough to hold 1-2 day's feed. It is important not to freeze diets in containers too large, because they will not remain fresh under refrigeration for more than

2-3 days. When testing volatile substances, sealable containers must be used. One day's volume of feed should be stored in each container and the feed should fill the container allowing no headspace. The containers should be stored upside down.

10.5. It is critical to analyze all diets for the concentration of the test substance in the diet.

11. Procedure

11.1. Both the primary and secondary portions of the test should be conducted simultaneously.

11.2. Range-finding test: In most cases, LD_{50} estimates for mink or ferrets will not be available to aid in setting dietary concentrations for the LC_{50} test. Therefore, range-finding procedures can be used to save both time and animals by reducing errors or miscalculations in setting these concentrations. LD_{50} estimates for other species may be helpful in setting dietary concentrations, although, in general, mink and ferrets are more sensitive to toxic compounds than other animals. For this reason, if LD_{50} estimates are available for other species, these values can be used as the upper limit of doses in the range-finding procedure. This procedure can be a geometrically-spaced series of doses (e.g., in multiples of 2 or ½) administered by gavage to 2 animals per dose, in which case the approximate LD_{50} is the dose at which 1 or 2 animals die after an appropriate period of observation (often one week). It is suggested that, when administering an oral dose to mink or ferrets by gavage, a piece of plastic large enough to force the animal's mouth open, with a small hole in the center, be used. The tube can then be inserted through the opening without the animal biting it (Figure 1). A three inch, 14 gauge, curved, stainless steel animal feeding needle

can also be used to administer the test substance. If LD_{50} estimates are not available for other species, widely-spaced doses (e.g., 1, 10, 100, and 1000 mg/kg) can be administered to one animal per dose to find a lethal dose. The range-finding procedure described above can then be employed, centering on the lethal dose. If range-finding procedures yield an approximate LD_{50} value, the highest dietary concentration should then be set to ensure that an animal will consume the equivalent of an LD_{50} dose in one day's feed. If a lethal dose is not found, the highest dietary concentration should be set at 5000 mg/kg because concentrations above this value are assumed to be nontoxic. Palatability tests should also be employed to save time and animals. It is recommended that the highest proposed dietary concentration be fed for several days to 2-4 animals to determine if they will eat the diet at this concentration of the test substance. If not, it may be necessary to reduce the highest concentration in the diet to a level that will be eaten.

11.3. Acclimation period: All animals should be conditioned to the test facilities, including: photoperiod, temperature, and caging for a minimum of 7 days. A minimum of 7 days is required, but a longer period may be necessary, especially if the animals to be used in a test are changed from outdoor to indoor housing (or vice-versa) or if the diet or water to be used in a test is different from that which the animals are accustomed to consuming. During this period all animals should be given the untreated (control) diet and drinking water, as used during the definitive test. It is important to measure feed consumption during this period as these measurements can provide an indication of how much feed the animals should consume once the test starts, how much diet to mix for the test, and can

also serve as a control value of each group. Test animals should be weighed at the start of the acclimation period.

11.4. Definitive test:

11.4.1. Each test animal should be randomly assigned according to weight class to a specific test diet concentration, individually caged, and be uniquely identified.

11.4.2. The test diets must be fed for 28 days. In some instances, it may be possible to achieve satisfactory results with a test of shorter duration using higher concentrations of the test substance, but the possibility of feed rejection or avoidance becomes greater with increasing concentrations. Also, certain substances cause delayed mortality, whether administered as a single dose or multiple dosages. Increasing the concentration of the substance does not necessarily shorten the time to death. A 28-day test allows time for absorption, distribution, metabolism, enzyme induction, redistribution, bioconcentration, and elimination to occur, similar to what might occur in animals subacutely exposed to a substance via diet in the environment. A 28-day test also allows testing of slow-acting or bioaccumulating substances. Tests of shorter duration could yield negative or misleading results. For some test substances, it may be necessary to include a withdrawal period, when the test diets are replaced with untreated feed, in order to observe prolonged or delayed toxicity. A withdrawal period is recommended when animals are still exhibiting signs of toxicity at the end of the exposure period. This period provides a more accurate estimation of the true toxicity of a test substance, especially if the substance causes delayed or cumulative injury. By

observing the animals and measuring feed consumption during this period, the permanence of the injury can also be estimated. It is recommended that a withdrawal period not exceed 14 days.

11.4.3. Individual body weights must be recorded at the initiation of the definitive test and at weekly intervals thereafter, and on the day of death. Feed consumption must be measured weekly for the exposure and withdrawal periods, and should be based on a minimum of two consecutive days' feed consumption. In estimating feed consumption by mink or ferrets, several precautions are necessary. Because feed consumption can be adversely affected on a short-term basis by temperature, weather, and other factors, estimates should be based on at least two consecutive days' consumption. These days should also be days when the animals are not handled (e.g., during weighing, moving, etc.), because handling can produce a temporary reduction in feed consumption.

11.4.4. Mortality, behavioral abnormalities, and other signs of toxicity should be recorded daily during the test.

11.4.5. For tests conducted indoors, the photoperiod should be maintained at the same schedule in effect at the conclusion of the acclimation period because a changing photoperiod may subject mink or ferrets to slight adjustments in normal body rhythms with respect to feeding, sleeping, fat mobilization or deposition, coat development, and hormonal activity. It should be noted that the effects of the length of the light/dark cycle on toxicity have not been investigated in these species.

11.4.6. A minimum of eight animals for each test concentration should be used. The test concentrations should be geometrically spaced so as to

result in at least 2 dietary concentrations yielding 10-90% mortality. These results usually can be obtained with 4-6 dietary concentrations including a control. It is possible to conduct an LC_{50} test with as few as 3 dietary concentrations and 8 animals per concentration if a good estimate of the LC_{50} for the test species is available. In many instances, however, accurate results can be achieved with 5 dietary concentrations and 10 animals per concentration if a good estimate of the LD_{50} is available from range-finding procedures.

11.4.7. A test should be considered invalid if more than 12.5% of the control animals die during the definitive test.

11.4.8. It is strongly recommended that a dietary concentration group should be removed from testing when food consumption measurements indicate that 10% or less feed, compared to controls and/or acclimation period values, is consumed daily for the first two weeks' feed consumption measurements or the animals lose 30% of their original body weight.

11.4.9. Necropsies should be performed on all mortalities. At the termination of the test, all surviving test animals should be killed by accepted humane methods (AVMA Panel on Euthanasia, 1986) and necropsies performed. It is suggested that necropsies be performed on all test animals, either the day of death or at the termination of the test. Valuable information on the mode of action of the test substance and its effect on target organs or organ systems can sometimes be gained from gross observation of the test animals at necropsy. Histopathological examination of organs and tissues can often provide more information. Weights of internal organs of control and treated animals can be

compared statistically to determine effects of the substance, although the effects of starvation can sometimes be confounded with effects of the substance.

11.4.10. Body weight changes and feed consumption may be analyzed by analysis of variance (Sokal and Rohlf, 1969) and significant differences may be located by Dunnett's method for comparison with the control (Dunnett, 1964). An LC_{50} value, including confidence limits and slope of the dose-response curve may be calculated for the primary and secondary test by the method of Litchfield and Wilcoxon (1949). Other valid statistical procedures may also be used to analyze the data.

12. Quality Assurance

12.1. In order to assure the quality and reliability of data developed using this protocol, testing facilities should have a quality assurance unit that is responsible for monitoring the test along with the investigator to assure that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with good laboratory practice standards (U.S. EPA, 1989) and are legally defensible and appropriate for the intended use.

13. Reporting Requirements

13.1. All data and information required by this protocol should be recorded in black ink on appropriate forms and/or bound laboratory notebooks. A duplicate set of records should be kept at a location other than the test site. All data should be maintained in a secure location to prevent tempering or destruction of the records. The following information should be reported.

13.1.1. Name of the investigator(s), laboratory, laboratory address,

location of raw data, and date of initiation and termination of test.

13.1.2. Name of species tested, including scientific name, source, history, and age of the animals at the beginning of the test.

13.1.3. A detailed description of the test substance including its chemical name, synonyms, structure, formulations, purity, source, batch, lot number, and physical/chemical properties and name and source of solvent or carrier, if used.

13.1.4. Description of the test facilities and housing conditions, including test cages, temperature, and photoperiod.

13.1.5. Analyses of contaminated prey carcasses and details of contamination methodology.

13.1.6. Name and source of feed and/or ingredients, including description and proximate analysis of diets.

13.1.7. The theoretical and measured dietary concentrations; number of animals per concentration; body weights; feed consumption; signs of toxicity; behavioral changes; % mortality for each concentration; significant necropsy findings; calculated LC_{50} values and 95% confidence limits, slope of the dose-response curves and 95% confidence limits, and the name and reference of the statistical method used; highest dietary concentration at which no signs of toxicity were observed; anything unusual about the test; any deviations from the protocol; and other relevant information.

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Table 1. Suggested ranges of composition of conventional diets for mink[1].

Ingredients	Percent
Fortified cereal[2]	15-30
Liver	0-10[3]
Quality protein feedstuffs (cooked eggs, whole poultry, whole fish, horsemeat, rabbits, nutria, etc.)	0-30[4]
Beef by-products (tripe, lungs, lips, udders, spleen, etc.)	10-30
Poultry by-products (heads, entrails, feet)	10-70
Fish scrap	10-50
Fat supplementation (rendered animal fat or vegetable oils)	0- 6[5]
Proximate analysis[6] of diet	
Protein	25-40
Fat	18-30
Carbohydrate	20-50
Ash	6-12

[1] From: National Research Council, 1982.

[2] May consist of single-cooked grains such as oat groats or wheat in combination with vitamin and trace mineral supplementation or commercially prepared fortified cereal mixtures.

[3] Reproduction-lactation diets (March-May) often contain 5-10% beef liver, although necessity for this has not been universally accepted.

[4] Level of quality-protein feedstuffs is often increased during the critical fur development and reproduction-lactation phases - a practice consistent with the higher protein requirements of the mink during these critical periods.

[5] That level of fat supplementation that provides proper protein/energy balance for each phase of the life cycle.

[6] That proximate analysis consistent with the optimum nutritional balance for each phase of the life cycle.

Table 2. Mortality pattern of mink fed sodium monofluoroacetate (Compound 1080) during a 28 day LC_{50} test.[1]

Concentration (mg/kg)	No. of animals dying/day of test																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
0																												
0.05																												
0.90																												
1.62																												
2.90																							1	2			1	
5.25					1			1			1				1				1	1	1					1	1	

[1] Hornshaw et al., 1986b

Table 3. Mortality pattern of ferrets fed sodium monofluoroacetate (Compound 1980) during a 28 day LC_{50} test.[1]

Concentration (mg/kg)	No. of animals dying/day of test																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
0																												
4.76										1																		
8.56														1														
15.40										2											1	1	1			1	1	

[1] Hornshaw et al., 1986b

Table 4. Mortality pattern of mink fed Aroclor 1254 during a 28-day LC_{50} test.[1]

Concentration (mg/kg)	No of animals dying/day of test																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
0																																			
10.0																														1					
18.0																																			
32.4																																1			
58.3																				1	1				1		1	1				1			
105.0																				1	1				3	1		2				1			

1 Hornshaw et al., 1986c

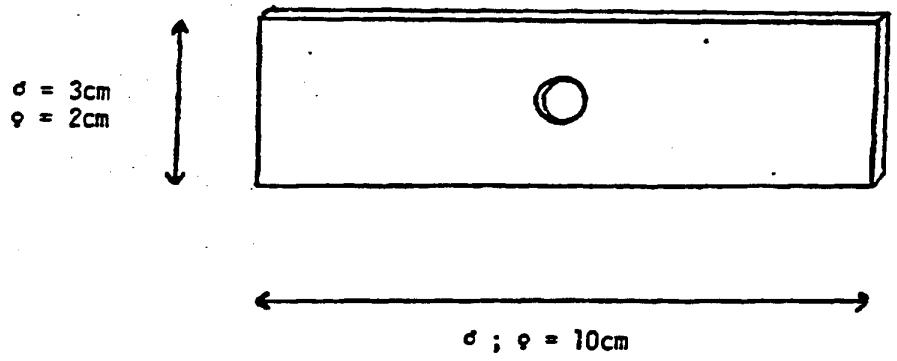

Figure 1. Plastic device for gavage.

ATTACHMENT 2

SAMPLE TRANSMITTAL, RECEIPT, AND INVENTORY (SOP P.200 CF.162)

SAMPLE TRANSMITTAL, RECEIPT, AND INVENTORY

The Environmental Chemistry Branch of the Columbia Environmental Research Center (CERC) has certain procedures which must be followed in the transmittal and receipt of samples that are to be processed by the Branch. Adherence to these instructions will facilitate incorporation of samples into the Branch's sample management system.

Sample Transmittal

1. **The individual responsible for transmission of samples (from outside CERC or internally)** must contact Jesse Arms of CERC via phone (573-876-1856) PRIOR TO any sample transmission[1]. He will send you the necessary forms to fill out and provide additional instructions. Information on methods of preservation used during transmission can be provided at this time. **NOTE: PARTICULARLY SPECIFY ANY SAMPLES REQUIRING -80°C STORAGE REQUIREMENTS; CONTACT PERSONALLY - DO NOT LEAVE VOICE MAIL.**

2. The person responsible for the samples, or designated individuals, must fill out the attached "Batch History" form in detail. Failure to complete this form in its entirety will delay and may jeopardize sample processing.

3. The form entitled "Chain of Custody Record" must be completed. Each sample is to be listed on an individual line along with other requested information. The header and appropriate footer information must be filled out for each sheet that is used.

4. **For samples being transmitted to CERC from outside:** place all completed forms in protective covering and put in packaging with samples. Seal all boxes, coolers, or other packaging so that it can be indicated if tampering has occurred. Once transmission (shipment) has taken place, immediately contact Jesse Arms (573 876-1856) and provide shipment and expected date of arrival information. **Do not leave a voice mail; a direct contact with specified CERC personnel prior to shipment must be established.** Following this phone call, specified CERC personnel must complete the "Pre-Shipment" section of the form entitled "Sample Shipment Record."

5. **For samples being transmitted from one group to another within CERC:** steps 1-4 above must be completed. Following notification of J. Arms or designate, manual transmission (hand-carrying) of samples to the Analytical Building is appropriate. However, contact J. Arms beforehand to assure completion of the transmission process. Completion of "Pre-Shipment" section of "Sample Shipment Record" is still required by specified CERC personnel (J. Arms).

[1]If Jesse Arms is unavailable, contact Tom May at 573-876-1858.

6. **For samples being transmitted from CERC Chemistry to a second party outside CERC:** A yellow Batch History form (samples out) must be completed along with "Chain of Custody Record" for all samples, following instructions in steps 2 and 3. This documentation is packaged and shipped with samples to the outside party. A copy of documentation is placed in a green pocket file and filed in specified Analytical Building Rm. 5 file drawer. At time of shipment, CERC personnel (J.Arms or alternate) will call the outside party and inform them of shipment details. Once the shipping details have been verified, fill out the "Pre-Shipment" section of the "Sample Shipment Record." When the outside party notifies CERC of sample receipt, J. Arms or alternate will obtain information to fill out "Receipt" portion of "Sample Shipment Record." This completed "Sample Shipment Record" is filed with other documentation in the appropriate green file pocket.

Sample Receipt (receipt at CERC)

1. J.Arms or alternate CERC personnel must immediately notify responsible party at CERC when samples are received. Also J. Arms or alternate CERC personnel must complete a sample check-in procedure following guidelines depicted in "Receipt" section of form entitled "Sample Shipment Record."

Sample Inventory (at CERC)

1. Samples are logged-in or inventoried into the Chemistry Division sample management system by J.Arms or alternate CERC personnel following guidelines depicted on a form entitled "Sample Log-in."

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