STUDY PLAN
FOR MINK INJURY INVESTIGATIONS
FOR THE HUDSON RIVER

HUDSON RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

HUDSON RIVER NATURAL RESOURCE TRUSTEES

STATE OF NEW YORK

U.S. DEPARTMENT OF COMMERCE

U.S. DEPARTMENT OF THE INTERIOR

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**Names of certain individuals and affiliations have been removed to maintain confidentiality*

EXECUTIVE SUMMARY

Natural resources of the Hudson River have been contaminated through past and ongoing discharges of polychlorinated biphenyls (PCBs). The Hudson River Natural Resource Trustees - New York State, the U.S. Department of Commerce, and the U.S. Department of the Interior - are conducting a natural resource damage assessment (NRDA) to assess and restore those natural resources injured by PCBs.

Many species of mammals rely on the Hudson River, including its floodplain, for habitat, food, and as a breeding ground. Mammals that depend on the river for food and habitat include otter, muskrat, raccoon, beaver, and mink. The Hudson River NRDA Plan identified mink and otter health as an area of biological injury investigation. Mink are the subject of this Final Study Plan for an injury determination effort as part of the Hudson River NRDA.

Based on the results of preliminary investigations conducted by the Trustees, including the mink and otter work conducted in the upper Hudson River drainage during the 1998-1999 and 1999-2000 trapping seasons, input from a panel of mammal experts, review of the existing mink and otter toxicology literature, and considering factors such as the life history of mink and goals of the NRDA, the Trustees have determined that it is appropriate to conduct further investigations focused on mink to be initiated in the year 2006. Pursuant to the Hudson River NRDA Plan, the Trustees have developed a Study Plan for a mink injury determination effort.

A Draft Study Plan for this work was peer reviewed and made available to the public for review and comment. All comments received on the Draft Study Plan, as part of the peer and public review process, have been considered. The Trustees evaluated peer and public comments and, where warranted, incorporated these comments in the Draft Study Plan to produce the Final Study Plan. In the remaining instances, public comments on the Draft Study Plan have been addressed by letter to the commenter, acknowledging receipt of comments and providing an initial response, noting that a more detailed Responsiveness Summary will be provided by the Trustees in the near future.

The Trustees will conduct a laboratory study to evaluate whether mink reproduction and/or development is affected as a result of exposure to PCBs from the Hudson River. In the future the Trustees may propose additional work to supplement this effort.

The purpose of this work is to inform the Trustees regarding injury to mink and guide their future efforts to identify pathways and specific injuries to mink from PCBs, as defined in regulations written by the U.S. Department of the Interior contained in Title 43 of the Code of Federal Regulations Part 11, Natural Resource Damage Assessment. This work will also be used to help determine whether future studies will be performed, and if so, to help in their design.

Pursuant to the Hudson River NRDA Plan, the results of the work conducted pursuant to this Study Plan will be peer reviewed upon completion of the study, and the results then released to the public.

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1.0 BACKGROUND

Past and continuing discharges of polychlorinated biphenyls (PCBs) have contaminated the natural resources of the Hudson River. The Hudson River Natural Resource Trustees - New York State, the U.S. Department of Commerce, and the U.S. Department of the Interior - are conducting a natural resource damage assessment (NRDA) to assess and restore those natural resources injured by PCBs (Hudson River Natural Resource Trustees 2002).

Many species of mammals rely on the Hudson River, including its floodplain, for habitat, food, and as a breeding ground. Mammals that depend on the river for food and habitat include otter, muskrat, raccoon, beaver, and mink. The Hudson River NRDA Plan identified mink health as an area of biological injury investigation. Mink are the subject of this Final Study Plan for an injury determination effort as part of the Hudson River NRDA.

Mink are small carnivorous mammals that are associated with aquatic habitats of all kinds, including rivers, lakes, and wetlands (USEPA 1993). They are opportunistic hunters, feeding on any animal material they can find and kill (Linscombe et al. 1982). Mink appear to select prey primarily based on its availability (Gilbert and Nancekivell 1982) and vulnerability (Eagle and Whitman 1987). The mink diet includes other small mammals such as mice, rats, rabbits and muskrats; aquatic prey including frogs, fish, and crayfish; and terrestrial prey including birds, snakes, insects, and other invertebrates. Mink are exposed to PCBs directly through their diet. Mink are also exposed to PCB-contaminated water and soil or sediments as they build dens and forage for food.

The Trustee agencies have conducted preliminary investigations assessing PCB concentrations in mink from the Hudson River. PCB concentrations in liver (normalized for the amount of fat, or lipids, in each sample) range from 0.13 ppm to 139 ppm in mink (NYSDEC 2001, 2002). PCB concentrations in liver on a wet weight basis range from 0.0082 to 3.34 ppm in Hudson River mink (NYSDEC 2001, 2002).

Those preliminary investigations of mink exposure to PCBs were undertaken to assist the Trustees in determining the extent to which mink in the Hudson River are contaminated with PCBs, to determine if additional pathway and injury assessment studies focused on mink should be conducted as part of the Hudson River NRDA, and for potential use in the design of future studies to assess the health of Hudson River mink.

Several studies have investigated the potential effects of PCB exposure to mammals, including mink. In controlled feeding studies of mink, diets with PCB levels between 0.64 and 5 parts per million (ppm) completely inhibited reproduction (Platonow and Karstad 1973, Bleavins et al. 1980). Moore et al. (1999) predict, based on a dose-response curve, a greater than 99 percent reduction in fecundity (litter size) of ranch mink fed a diet containing 5 ppm PCBs. Bursian et al. (2003), studying the dietary exposure of mink to fish from the Housatonic River, found that a dietary concentration of 3.7 ppm caused a decrease in kit survival and resulted in a maternal hepatic total PCB concentration of 3.1 ppm. Jaw lesions - mandibular and maxillary squamous cell proliferation - were detected in kits fed dietary concentrations as low as 0.96 ppm.

While most of the above-cited studies have focused on adverse effects as a function of contaminant concentrations in the diet, others have evaluated effects as a function of contaminant concentrations in mink tissues. For instance, adverse effects on mink reproduction are expected when PCB concentrations in mink tissues exceed about 0.01 ppm toxic equivalents (TEQs) lipid weight (Leonard et al. 1995, Mason and Wren 2001, Tillitt et al. 1996). In the TEQ approach, the concentration of each dioxin or dioxin-like compound is multiplied by its respective Toxicity Equivalence Factor (TEF), and the products of the concentrations and their respective TEFs are summed in order to obtain a single TCDD TEQ value for the complex mixtures of dioxins or dioxin-like compounds found in the sample (Tillitt 1999, Van den Berg et al. 1998, 2006).

Based on Smit et al. (1996), 21 ppm PCBs (lipid normalized) or more is a critical level for health impairment in mink and otter; this is based on the effects of PCBs on hepatic retinol levels in European otter (Smit et al. 1996). Further, 50 ppm or more PCBs (lipid normalized) is a critical level for reproductive impairment in mink and otters; this is based on reductions in litter size in mink (Leonards et al. 1994, 1995).

In January 2002, the Trustees assembled an expert panel to review the exposure and effects information compiled by the NYSDEC for mink and otter, and to provide guidance to the Trustees on appropriate next steps for determining whether PCBs are causing adverse biological effects in Hudson River mammals, particularly mink and otter. The Hudson River NRDA Plan noted that the Trustees planned to build upon the existing mink and otter studies, potentially conducting further studies to determine PCB effects in mink and otter from the Hudson River.

2.0 INTRODUCTION

Based on the results of preliminary investigations conducted by the Trustees, including the mink and otter work (NYSDEC 2001, 2002), input from a panel of mammal experts, review of the existing mink and otter toxicology literature, and considering factors such as the life history of mink and goals of the NRDA, the Trustees have determined that it is appropriate to conduct further investigations focused on mink to be initiated in the year 2006.

Pursuant to the Hudson River NRDA Plan, the Trustees developed a Draft Study Plan (Hudson River Natural Resource Trustees 2006) for a mink injury determination effort. The Draft Study Plan was peer reviewed and made available to the public for review and comment.

In accordance with the Hudson River NRDA Plan, the Trustees are now issuing this Final Study Plan for a mink injury determination effort. This Final Study Plan describes a laboratory study the Trustees will undertake to evaluate whether mink reproduction and/or development are affected as a result of exposure to PCBs from the Hudson River.

3.0 PURPOSE AND OBJECTIVE

The purpose of this work is to inform the Trustees regarding injury to mink and guide their future efforts to identify pathways and specific injuries to mink from PCBs, as defined in regulations written by the U.S. Department of the Interior contained in Title 43 of the Code of Federal Regulations Part 11, Natural Resource Damage Assessment. This work will also be used to help determine whether future studies will be performed, and if so, to help in their design.

The objective of the laboratory study the Trustees will undertake pursuant to this Study Plan is to evaluate the impacts to mink of dietary exposure to PCB-contaminated fish from the Hudson River. Groups of ranch mink will be fed diets containing different quantities of Hudson River fish and will be assessed to determine reproductive performance, offspring growth and survival, and the development of lesions.

In the future, the Trustees may propose additional work to supplement this effort. Such work may include, but is not limited to: (1) explicit comparisons of the PCBs and other contaminants present in the test diet to contaminants in prey items more likely to be consumed by wild mink (e.g., smaller fish and other prey); making such comparisons may require the collection of additional prey items likely to be part of the wild mink diet, and chemical analyses of those items; and, (2) explicit comparisons of the PCBs and other contaminants in livers of laboratory-fed mink to available information about the contaminants in livers of mink caught in the Hudson River watershed.

4.0 METHODS

4.1 MINK LABORATORY STUDY

4.1 DIETARY EXPOSURE OF MINK TO FISH FROM THE HUDSON RIVER: EFFECTS ON REPRODUCTION AND SURVIVAL

On behalf of the Trustees, beginning in 2006, Principal Investigators (PIs) will conduct a study of the effects on reproduction and survival of mink exposed to PCBs via their diet (fish from the Hudson River). This work will be conducted pursuant to a work plan entitled "Dietary Exposure of Mink to Fish from the Hudson River: Effects on Reproduction and Survival" contained in Appendix A.

The purpose of this investigation is to evaluate if ranch mink fed diets containing PCB-contaminated fish from the Hudson River will exhibit impaired reproductive performance, impaired offspring (kit) growth and survival, and/or development of mandibular/maxillary squamous epithelial proliferation (jaw lesions). Data generated by this investigation can then be compared to existing site-specific field data on concentrations of PCBs in typical prey species and hepatic concentrations of PCBs in wild mink to allow evaluation of risk posed to mink residing in the Hudson River watershed.

The following endpoints will be assessed in this investigation:

- Adult body weights;
- Adult feed consumption;
- Number of females mated;
- Length of gestation;
- Number of females whelping/not whelping;
- Total newborns/female whelped;
- Live newborns/female whelped;
- Average kit birth weight;
- Average litter weight;
- Percent kit survival to three weeks of age;
- Kit body weights at three weeks of age;
- Percent kit survival to six weeks of age;
- Kit body weights at six weeks of age;
- Adult and six-week-old kit organ weights;
- Histopathology of adult and six-week-old kit organs and jaws;
- Total PCB and planar PCB, PCDD, and PCDF analyses of adult and six-week-old kit livers;
- Monthly body weights of seven-month-old juveniles;
- Organ weights of seven-month-old juveniles;
- Histopathology of seven-month-old juvenile organs and jaws; and,
- Total PCB and planar PCB, PCDD, and PCDF analyses of adult and seven-month-old juvenile livers.

This study will enable the Trustees to assess the following injuries to mink: death, disease, cancer, physiological malfunctions (including malfunctions in reproduction), and physical deformations.

As this investigation evaluates injury endpoints, the Trustees have performed a peer review of the proposed study plan. A draft work plan, prepared by the PIs, has been peer reviewed and changes made as a result of the peer review process.

5.0 QUALITY ASSURANCE/QUALITY CONTROL

This study is being conducted in accordance with the Quality Assurance Management Plan for the Trustees' Hudson River NRDA (Hudson River Natural Resources Trustees 2002).

As noted in the Trustees' Responsiveness Summary for the NRDA Plan (Hudson River Natural Resource Trustees 2003), for each data collection effort that is part of the Hudson River NRDA and is identified in the NRDA Plan, the Trustees will develop a project-specific QA Plan that may be an independent document or may be incorporated into the project Study Plan. Such a QA Plan, in combination with the information on QA management described in the NRDA Plan (Hudson River Natural Resource Trustees 2002), will ensure that the requirements listed in the National Contingency Plan and applicable EPA guidance for quality control and quality assurance plans are met.

The work plan for the investigation entitled "Dietary Exposure of Mink to Fish from the Hudson River: Effects on Reproduction and Survival" includes a project-specific QA Plan (Section 6).

Chemical analyses will be conducted in accordance with the requirements of the Hudson River NRDA Analytical QA Plan (Hudson River Natural Resource Trustees 2005).

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Appendix A

Work Plan for Dietary Exposure of Mink to Fish from the Hudson River: Effects on Reproduction and Survival

WORK PLAN

DIETARY EXPOSURE OF MINK TO FISH FROM THE HUDSON RIVER: EFFECTS ON REPRODUCTION AND SURVIVAL

FINAL FOR PUBLIC RELEASE

August 2006

Principal Investigator

Principal Investigator

Quality Assurance Coordinator

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INVESTIGATION TEAM ACKNOWLEDGEMENT OF WORK PLAN REVIEW AND COMPLIANCE

By my signature, I acknowledge that I have read this Work Plan and understand it, and will comply with it in performing this work.

Name (printed): _____ Name (printed): _____

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1. INTRODUCTION

The Hudson River is contaminated with polychlorinated biphenyls (PCBs) from Fort Edward, NY to New York City. General Electric's capacitor manufacturing facilities at Fort Edward and Hudson Falls, NY are considered to be the major source of PCBs in the Upper Hudson River, with discharges beginning in 1947. Between 1966 and 1974, General Electric's Fort Edward and Hudson Falls facilities purchased 35,000 metric tons of PCBs or 15% of domestic sales in the United States. This suggests that General Electric's discharges to the Hudson River Basin could represent approximately 15% of the nationwide total discharges to the environment (Horn et al., 1979).

Foley et al. (1988) reported that mink (*Mustela vison*) collected in the vicinity of the Hudson River contained relatively high concentrations of PCBs in their fat and livers. Comparison of PCB concentrations in the livers of ranch mink fed PCB-contaminated diets and those in wild Hudson River mink suggested that the wild mink could be experiencing similar reproductive impairment with a consequent decrease in abundance (Foley et al., 1988). In a more recent field study, Mayack and Loukmas (2001) reported that there appeared to be no measurable decrease in PCB contamination of mink collected in the vicinity of the Hudson River and that current hepatic PCB concentrations are above the criteria of Leonards et al. (1995) for impairment of mink health and reproduction.

In addition to reproductive impairment, there is concern that mink could develop a squamous epithelial lesion of the mandible and maxilla. Previous studies have indicated that ranch mink exposed to 3,3',4,4',5-pentachlorobiphenyl (PCB 126) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Render et al., 2000a, 2000b, 2001), ranch mink fed diets containing PCB-contaminated fish (Bursian et al., 2006a, 2006b), and wild mink trapped in a PCB-contaminated Superfund site (Beckett et al., 2005) developed a lesion characterized by proliferation of squamous epithelial cells into the periodontal ligament that can cause loose and displaced teeth. The maxilla and mandible become markedly porous because of loss of alveolar bone, with concomitant loss of teeth that leads, in severe cases, to aphagia.

The purpose of the present study is to evaluate if ranch mink fed diets containing PCB-contaminated fish from the Hudson River will exhibit impaired reproductive performance, impaired offspring (kit) growth and survival, and/or development of mandibular/maxillary squamous epithelial proliferation. Data generated by this study can then be compared to existing site-specific field data on concentrations of PCBs in typical prey species and hepatic concentrations of PCBs in wild mink to allow evaluation of risk posed to mink residing in the Hudson River watershed.

The following work plan is based on a similar document prepared for a mink feeding study utilizing contaminated fish collected from the Housatonic River, Berkshire County, Massachusetts (Aulerich et al., 2000). The mink is the species of choice for testing this hypothesis because: (1) they are a semi-aquatic piscivorous species native to the area; (2) they are among the most sensitive species to PCBs (Aulerich and Ringer, 1977) and related polychlorinated dibenzo-*p*-dioxins (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 data base (Shump et al., 1976; Scientifur, 1987, 1992; Sundqvist, 1989; Aulerich et al., 1999).

2. STUDY DESIGN AND METHODS

Table 1 presents an estimated schedule for the mink feeding study. The following paragraphs describe each step in more detail.

Table 1
Study Schedule

Task	Estimated Start Date	Estimated End Date
Collect fish for use in mink feed	6/15/06	6/30/06
Ship to mink study facility and homogenize	6/30/06	8/31/06
PCB analyses of Hudson River fish homogenate	9/1/06	10/15/06
Mix diets	10/15/06	10/31/06
Animal acclimatization	12/15/06	12/31/06
Feeding study implementation	1/1/07	1/1/08
Test diet feeding (adults)	1/1/07	7/1/07
Breeding	3/1/07	3/21/07
Gestation and parturition	3/21/07	5/15/07
Weaning/analysis of six-week kits, adults	7/1/07	12/31/07
Analysis of seven-month kits	11/15/07	6/30/08
PCB analysis of tissues	1/1/07	6/30/08
Data analysis and report generation	6/30/08	12/31/08

2.1 COLLECTION OF FISH AND FEED PREPARATION

Fish were collected from the Northumberland Pool, from the Lock 2 vicinity, and from the first 2000 feet of Moses Kill. Collection and transport of fish was handled by New York Department of Environmental Conservation and/or U.S. Fish and Wildlife personnel and in general followed fish handling and shipping procedures presented in Appendix 1.

When fish arrive at the mink study facility, they will be identified, sorted, and weighed by collection site. All fish will be ground and then placed in a 500 kg capacity mixer for 30 minutes to ensure equal distribution of contaminants. The total amount of Hudson River fish collected is approximately 1500 kg. Because the capacity of the mixer that will be used to blend the ground fish is 500 kg, the fish will have to be ground and mixed in three loads (Loads 1 – 3). For Load 1, one third of the fish from each of the three collection sites will be ground and mixed together such that the product is representative of the entire study area rather than a specific collection site. The ground, blended fish will be expelled from the mixer into approximately 20 plastic pans (capacity approximates 30 kg) for freezing. Pans will be numbered consecutively from 1 to 20. One sample (of approximately 30 grams) associated with each pan will be collected and

placed in a chemically clean glass container. There will be one glass container per set of four pans. Thus, there will be five samples of blended fish collected over 20 pans. The glass containers will be labeled, reflecting the pan numbers associated with each, and frozen for subsequent analysis for total PCBs (tPCBs) according to procedures outlined in the Hudson River Natural Resource Damage Assessment Analytical Quality Assurance Plan (AQAP; Hudson River Natural Resource Trustees, 2005). The pans of blended fish will be placed in the freezer for approximately 48 hours. After the fish is frozen, it will be removed from the pans in individual blocks that will be placed in individually numbered plastic bags (bags will have the same number as the pan from which the block was taken) and stored on a pallet in the freezer. The procedures for Loads 2 and 3 will be the same except that the resulting blocks of fish will be numbered 21 through 40 and 41 through 60, respectively). It is anticipated that grinding and mixing the Hudson River fish will be completed in one day.

"Clean" ocean fish will be purchased from a supplier that routinely services the fur industry and will be shipped frozen to mink study facility. This fish will be processed, sampled, and analyzed in the same manner as the Hudson River fish except that samples from approximately 10 pans will be composited, such that there will be two sample containers for each grind of the ocean fish. Four loads of ocean fish are anticipated altogether.

2.2 DIETARY TREATMENTS

The diets will be conventional mink diets formulated to meet the nutritional requirements of mink (National Research Council, 1982) as described in Ringer et al. (1991; Appendix 2). There will be six dietary treatments, each containing the same percentage of fish (for example, 40%). The control diet will contain 40% "clean" ocean fish. The remaining five diets will contain a mixture of ocean fish and the homogenized fish from the test site(s). Based on past fish sampling efforts, Hudson River carp are anticipated to contain average PCB concentrations in approximately the 10 to 15 mg/kg (ppm) range. The targeted PCB concentrations for use in the mink dietary treatments will depend on the PCB concentrations actually present in the Hudson River fish. For instance, assuming a concentration of 15 ppm in these fish, the highest dose would be 6.0 mg/kg feed (40% * 15 ppm). Sequentially lower doses are designed to be 0.75x, 0.5x, 0.25x and 0.125x, which would result in targeted doses of 4.5, 3.0, 1.5 and 0.75 mg/kg feed. A concentration of 10 ppm in Hudson River fish would, correspondingly, result in targeted PCB concentrations of 4.0, 3.0, 2.0, 1.0 and 0.5 mg/kg feed. Reproductive impairment has been reported in mink fed diets containing PCB concentrations lower than 5.0 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 Hudson River. Table 2 presents the estimated quantities of Hudson River and ocean fish required for each dietary treatment.

Table 2 Approximate Quantity of Fish Required[a]					
Dietary PCB Concentration, assuming 15 ppm in Hudson fish (ppm)	**Dietary PCB Concentration, assuming 10 ppm in Hudson fish (ppm)**	**Hudson River Fish (kg)**	**Hudson River Fish (% of Diet)**	**Ocean Fish (kg)**	**Ocean Fish (% of Diet)**
0	0	0	0%	576	40%
0.75	0.5	52	5%	364	35%
1.5	1.0	104	10%	312	30%
3.0	2.0	208	20%	208	20%
4.5	3.0	432	30%	144	10%
6.0	4.0	576	40%	0	0%
Total		**1,372**		**1,604**	

Notes:
a. These figures assume a mink diet containing 40% fish.

2.3 PREPARATION OF DIETS

It is anticipated that the six 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, with the exception of the number of samples analyzed.

For the initial batch of feed, after thorough mixing of the dietary ingredients for 30 minutes, feed will be expelled into storage pails. As feed is being expelled into a storage pail, a sample of approximately 30 grams will be taken from the stream and placed into one of three chemically clean glass containers. The first grab will be placed into the first jar; the second into the second jar; the third into the third jar; and the fourth grab will again be placed into the first jar. This procedure will be continued during feed expulsion such that a sample from each bucket will be included in one of the three sample jars. These samples will be frozen for subsequent chemical contaminant analysis (organochlorine pesticides [OCs], tPCBs, non-*ortho* PCB congeners, mono-*ortho* PCB congeners, polychlorinated dibenzo-*p*-dioxin [PCDD] isomers, polychlorinated dibenzofuran [PCDF] isomers, polybrominated diphenyl ether [PBDE] isomers and potentially toxic and bioaccumulative metals). Congener-specific analyses will allow calculation of TCDD toxic equivalents (TEQs) in feed samples using mammalian toxic equivalency factors (TEFs) presented in Van den Berg et al. (2006). An additional sample from each dietary treatment will be collected for nutrient (proximate) analysis (moisture, dry matter, fat, crude protein, crude fiber, ash, total digestible nutrients, Ca, K, Mn, Mg, Fe, Na, Cu, Zn and P).

During preparation of subsequent batches of feed, three composite samples from each of the dietary treatments will be collected as described above. One sample will be archived and two will be submitted for PCB analysis by high resolution mass spectrometry (Hudson River Natural Resource Trustees, 2005). An additional grab sample will be collected for nutrient analysis. Chemical analyses of grab samples will be completed prior to providing feed from the associated batch to the mink.

In addition to the sampling described above, for the first dietary batch, five grab samples of approximately 30 grams from each of three dietary treatments (1x, 0.5x, and control) will be collected. These samples will be collected at regular intervals as the feed is extruded from the mixer and will be analyzed for PCBs using the low resolution mass spectrometry method. These examples are intended to provide information about the variability of PCB exposure within a dietary group.

Feed will be placed in appropriately labeled, sealed plastic containers and stored frozen in a walk-in freezer at -7ºC as described by Ringer et al. (1991). A sufficient quantity of feed for one day will be removed from the freezer in the morning and thawed slowly over the next 24 hours at room temperature, or if conditions require, under a minimal heat source suspended above the material to be thawed. Thawed feed that remains after animals have been fed for the day will be placed in the walk-in cooler for feeding the next day. Thawed feed is kept no longer than 48 hours.

Because the fish species used in the diets are known to contain thiaminase, supplemental thiamine will be provided to the animals on a daily basis to prevent Chastek's paralysis (National Research Council, 1982). Twenty-five mg thiamine hydrochloride (USB, Cleveland, OH) will be dissolved in 50 ml water and then mixed into 950 g of ranch feed. Each mink will be fed approximately 10 g of the thiamine-containing feed, which provides 0.25 mg thiamine hydrochloride/day, at least two hours before feeding of the treatment diets.

2.4 ANIMALS

There will be 15 uniquely identified, first-year (virgin), natural dark, female mink (*Mustela vison*) and five uniquely identified, first-year, natural dark, male mink from the mink study facility herd randomly assigned to the 1x, 0.75x and control groups and 10 females and five males assigned to each of the 0.5x, 0.25x and 0.125x groups. 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. 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 at least seven 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 feed consumption as described by Ringer et al. (1991), if weather permits.

2.7 DEFINITIVE TRIAL

Three unexposed females and males from the breeding stock will be euthanized and their livers analyzed for OCs, PCBs (HRMS), PCDDs, PCDFs, PBDEs, and potentially toxic and bioaccumulative metals. After the acclimation period, the definitive test will begin on or around 1 January 2007, which is eight weeks prior to the initiation of breeding. Test diets will be fed daily to both females and males for approximately 150 days through 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 all the adult females, adult males and 15 kits (approximately evenly split between males and females) randomly selected from each treatment will be euthanized by asphyxiation (CO_2) and necropsied for analysis. Fifteen kits from each treatment group will be maintained on their respective diets through November to assess possible effects of PCBs on developmental parameters. To the degree possible, the sets of 15 kits will include one kit randomly selected from each female within the treatment group. For treatment groups of 10 females, one kit will be randomly selected from each female, with the remaining kits being randomly selected from the treatment group as a whole.

Although Aleutian disease has not been observed in the mink study facility 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 of 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 two weeks and feed consumption weekly. Feed consumption will be assessed on a weekly basis by measuring food consumption for two days during this period. Breeding of treated females and males within the same group will begin on or around 1 March 2007 and will follow procedures outlined in Ringer et al. (1991). A ratio of approximately one male for every three females will be used. Attempts will be made to ensure that females will have two or more 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 three and six weeks of age. Their dams will be weighed at the same times.

When the last litter whelped is weaned at six weeks of age, the adult females, males, and associated kits from each treatment group will be euthanized with 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 placed in a 10% formalin-saline solution for subsequent histological examination. Additional liver samples will be frozen for subsequent contaminant analysis (tPCBs, non-*ortho* PCB congeners, mono-*ortho* PCB congeners, PCDD isomers, and PCDF isomers). Congener specific analyses will allow calculation of TEQs in liver samples using mammalian TEFs presented in Van den Berg et al. (2006). The remaining portion of each liver will be archived in the event that additional analyses (such as retinoid analyses) are desired at a later date. Heads will also be collected and placed in 10% formalin-saline for subsequent examination of mandibular and maxillary squamous epithelial proliferation. All collected materials will be appropriately labeled (type of tissue, identification of the individual animal that the tissue came from, date of collection, and project identification).

Fifteen kits from each treatment group will be maintained on their respective diets through November 2007. 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 four weeks. At the end of the growth period in November, these juveniles will be euthanized by CO_2 and necropsied with tissues being handled as described above. In addition to the organs collected from the six-week-old kits, the reproductive tracts of all male and female juveniles will be removed and processed for subsequent histological examination. Any mink (except unweaned kits) that die during the trial period will be evaluated by a board certified veterinary pathologist.

Scat samples will be collected from each adult female and each seven-month-old juvenile just prior to necropsy. These samples will be archived in the event that contaminant analysis of these samples is deemed desirable.

3. CHEMICAL ANALYSIS

Chemical analyses will be conducted in accordance with the Hudson River AQAP (Hudson River Natural Resource Trustees, 2005). Table 3 indicates the types and numbers of samples to be taken for each analysis.

Table 3

Anticipated Sample Analyses

	Sample	No. Samples	OCs	PCBs		PCDDs/ PCDFs	PBDEs	Metals	Lipids	Necropsy/ Histopathology	Nutrient Analysis (feed)
				LRMS	HRMS						
Feed Preparation											
	HR fish	15	0	15	0	0	0	0	15	N/A	0
	Ocean fish	8	0	8	0	0	0	0	8	N/A	0
	Dietary mix - first batch (6 treatments * 3 samples)❖	18	18	15	18	18	18	18	18	N/A	6
	Dietary mix - second batch (6 treatments * 2 samples)❖	12	0	0	12	0	0	0	12	N/A	6
	Dietary mix - third batch (6 treatments * 2 samples)❖	12	0	0	12	0	0	0	12	N/A	6
Experimental Results											
Pre-Trial	Adult livers, individual	6	6	0	6	6	6	6	6	N/A	N/A
Weaning	Adult individuals (3 treatments of 15F and 5M, plus 3 of 10F and 5M)	105	N/A	N/A	N/A	N/A	N/A	N/A	N/A	105	N/A
	Adult livers, individual (3 treatments of 15F and 5M, plus 3 of 10F and 5M)	105	0	0	105	105	0	0	105	N/A	N/A
	Kits @ weaning (15 kits * 6 treatments)	90	N/A	N/A	N/A	N/A	N/A	N/A	N/A	90	N/A
	Kit livers @ weaning, individual (15 kits * 6 treatments)	90	0	0	90	90	0	0	90	N/A	N/A
7 mos.	Kits @ 7 mos. (15 kits * 6 treatments)	90	N/A	N/A	N/A	N/A	N/A	N/A	N/A	90	N/A
	Kits livers @ 7 mos., individual (15 kits * 6 treatments)	90	0	0	90	90	0	0	90	N/A	N/A

Note: The adult individuals evaluated at the pre-trial stage include three males and three females. The adult individuals evaluated at weaning include both females (10-15 per treatment) and males (5 per treatment). All kit evaluations include approximately equal numbers of males and females. As indicated in Hudson River Natural Resource Trustees (2005), organochlorine (OC) pesticides include: aldrin, α-BHC, ß-BHC, γ-BHC, α-chlordane, γ-chlordane, chlordane, 2,4'-DDD, 2,4'-DDE, 2,4'-DDT, 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrine ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, methoxychlor, cis-nonachlor, trans-nonachlor, oxychlordane, and toxaphene. Congeners measured using LRMS include: 8, 18, 28, 31, 44, 45, 47, 49, 52, 56, 66, 70, 74, 77, 81, 87, 95, 99, 101, 105, 110, 114, 118, 123, 126, 128, 138, 146, 149, 151, 153, 156, 157, 158, 167, 169, 170, 174, 177, 180, 183, 187, 189, 194, 195, 201, 206, 206, plus homologues and tPCBs. HRMS measurements include all LRMS values plus: 105, 114, 118, 123, 156, 157, 167, and 189. Metals include aluminum, antimony, arsenic, barium, beryllium, cadmium, calcium, chromium, cobalt, copper, iron, lead, magnesium, manganese, mercury, nickel, potassium, selenium, silver, sodium, thallium, vanadium, and zinc.

❖ = This calculation reflects the number of analyses for most analytes. As described in the text, the number of samples to be analyzed for some analytes differs.

4. 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 time when kits are three weeks old; at time when kits are six weeks old; at necropsy (Ringer et al., 1991)
Adult feed consumption:	During the acclimation period; weekly (two consecutive days/week) during the definitive trial (if the temperature is above 0ºC) 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/ not whelping:	(Ringer et al., 1991)
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 three weeks of age:	(Ringer et al., 1991)
Kit body weights at three weeks of age:	(Ringer et al., 1991)
Percent kit survival to six weeks of age:	(Ringer et al., 1991)
Kit body weights at six weeks of age	(Ringer et al., 1991)
Adult and six-week-old kit organ weights:	(Heaton et al., 1995a)
Histopathology of adult and	

six-week-old kit organs and jaws:	(Heaton et al., 1995b; Bursian et al., 2006a,b)
Total PCB and planar PCB, PCDD and PCDF analyses of adult and six-week-old kit livers:	(Hudson River Natural Resource Trustees, 2005)
Monthly body weights of seven-month-old juveniles:	(Heaton et al., 1995a)
Organ weights of seven-month-old juveniles:	(Heaton et al., 1995a)
Histopathology of seven-month-old juvenile organs and jaws:	(Heaton et al., 1995b; Bursian et al., 2006a,b)
Total PCB and planar PCB, PCDD and PCDF analyses of seven-month-old juvenile livers:	(Hudson River Natural Resource Trustees, 2005)

5. STATISTICAL ANALYSIS

5.1 STATISTICAL METHODS

Twenty measurement endpoints of interest are identified in Section 4. These endpoints can be classified into three data types: continuous measurements such as total PCB concentrations in livers; counts, such as the number of mandibular lesions per mink; or binary outcomes such as whether or not an individual kit survived to three weeks. Statistical analyses will be conducted using a generalized linear model framework (McCullagh and Nelder, 1989), where each data type and specific distributional characteristics will be used to select the most appropriate class of linear model. In general, continuous endpoints will be analyzed using normal-theory linear models (Neter et al., 1996) such as analysis of variance or repeated measures analysis of variance (Miliken and Johnson, 1984). Count variables will be analyzed using Poisson or overdispersed Poisson regression models (McCullagh and Nelder, 1989), and binary variables will be analyzed using logistic regression models for clustered sampling designs (McCullagh and Nelder, 1989). Each of the endpoints is classified by data-type and anticipated analysis method in Table 4. For endpoints measured at three or more points in time, repeated measures analyses will be used to test for differences in growth profiles *(i.e.,* profile analysis, Seber 1984).

Table 4
Summary of Data Types and Anticipated Statistical Analyses

Endpoint	Data Type	Statistical Methods
Number of females mated	Binary	Logistic Regression; Spearman Karber LCp
Number of females whelping	Binary	Logistic Regression; Spearman Karber LCp
Kit survival at three and six weeks	Binary	Logistic Regression; Spearman-Karber LCp
Adult body weight	Continuous	ANOVA / Regression
Adult feed consumption	Continuous	ANOVA / Regression
Length of gestation	Continuous	ANOVA / Regression
Kit weight at birth, three and six weeks	Continuous	Repeated Measures ANOVA / Regression (Profile Analysis)
Average litter weight	Continuous	ANOVA / Regression
Adult and six-week-old kit organ weights	Continuous	ANOVA / Regression
Total PCB and planar PCB, PCDD and PCDF analyses of adult and six-week-old kit livers	Continuous	ANOVA / Regression
Monthly body weights of seven-month-old juveniles	Continuous	Repeated Measures ANOVA / Regression (Profile Analysis)
Organ weights of seven-month-old juveniles	Continuous	ANOVA / Regression
Total PCB and planar PCB , PCDD and PCDF analyses of seven-month-old juveniles livers	Continuous	ANOVA / Regression
Number whelped per female	Count	Poisson Regression (log transform instead of logit)
Number whelped live per female	Count	Poisson Regression
Histopathology of adult and six-week-old kit organs and jaws	Count/Binary	Poisson/Logistic Regression
Histopathology of seven-month-old juveniles	Count/Binary	Poisson/Logistic Regression

The minimum dose necessary to induce a specified proportion (p) of kit mortality (LCp) will be estimated based on the maximum likelihood estimates provided by the generalized linear model analysis (i.e., logit or probit analysis), as well as using the nonparametric Spearman-Kärber method (Spearman 1908, USEPA 1993). Estimated LCp from both methods will be compared, although based on simulation studies conducted by Miller and Ulrich (2001), it is anticipated that the Spearman-Kärber method will provide the most robust estimates. Dose response relationships will be estimated for total PCB concentrations as well as TEQs. Statistical analyses will include both hypothesis testing and estimation of confidence intervals for parameter estimates and effect sizes.

In addition to estimating the dose response relationships, differences in endpoints among dosing groups will also be estimated. The precision of estimates will be quantified using confidence limits for differences. Point estimates combined with

confidence limits express both the magnitude of effects as well as the precision with which they are estimated (Cherry, 1998 and Johnson, 1999). Additionally, lower confidence limits for differences can be interpreted as tests for no difference among treatments, while upper confidence limits can be interpreted as tests against a pre-specified minimal difference of interest. For example, when an upper confidence limit for the difference is less than a pre-specified effect size of interest, this is equivalent to rejecting a test of bioequivalence (e.g., the reverse null hypothesis) (McDonald and Erickson, 1994). Additional statistical evaluations may also be employed.

5.2 SAMPLE SIZE CONSIDERATIONS

The number of mink to be placed on trial will balance a reasonable expectation of detecting biologically meaningful effects subject to the limitations of available time and resources to conduct the study. One of the objectives of this study is to identify relationships between dietary PCB doses in adult females and kit survival rates. Survival rates are estimated from binary data summarizing kit survival. Effects are indicated by differences in control and treatment survival rates. In this section, a power analysis is conducted to provide estimates of the probability of detecting differences in survival rates among control and treatment mink. Conducting a power analysis with respect to this particular endpoint (i.e., kit survival) is reasonable not only because of the importance of the endpoint from a biological perspective but also because, assuming similar effect sizes, detecting differences amongst groups requires the largest sample sizes when the measurement metric is binary in nature. As a result, the power associated with the other endpoints proposed in the study will be higher given the same sample size.

In general, to conduct a prospective power analysis one requires estimates of the nature of the anticipated data and the effect sizes (differences in survival rates) of interest. In this study, one null hypothesis (H_0) is that the survival rates are equal among control and treatment groups. The alternative hypothesis (H_a) is that treatment survival rates are lower than the control rate. For this power analysis, we used the results of a similar study conducted by Bursian et al. (2003) as a source of data to estimate expected control and treatment survival rates and variability. Bursian et al. (2003) report control survival rates of 96% at birth and 85% at three and six months. They also reported that each female whelped approximately 4 to 6 kits and that survival of kits whelped from PCB dosed females ranged from 46% to 99% depending on the dose.

Based on these results we developed four scenarios to calculate the power to test H_0. The first scenario represents the comparison of survival rates at birth for which the control survival rate was assumed to be 96% and the dosed survival rate was assumed to be approximately 90%. The additional three scenarios represent comparison of control survival rate (85%) with dosed survival rates assumed to be approximately 46%, 60% and 70%. These are representative of the range of reduced survival rates observed by Bursian et al. (2003) in kits whelped from dosed adult females. For each of these four scenarios, power was estimated for samples of 10, 12, 15, 18 and 20 adult females. It was assumed that on average five kits would result from each female in the test. A group of 15 females, for example, would therefore contribute approximately 75 (5x15) kits to be

monitored for survival and other endpoints. For each combination of the four scenarios and five sample sizes, we calculated the power of a one sided test of the null hypothesis of equal survival rates (Fleiss, 1981). Calculations were conducted using an internet based Java Applet developed by Lenth (2005). The results of these calculations are summarized in Figure 1 and Table 5.

Figure 1

Power of the Test of the Null Hypothesis to Detect Differences in Survival Rates

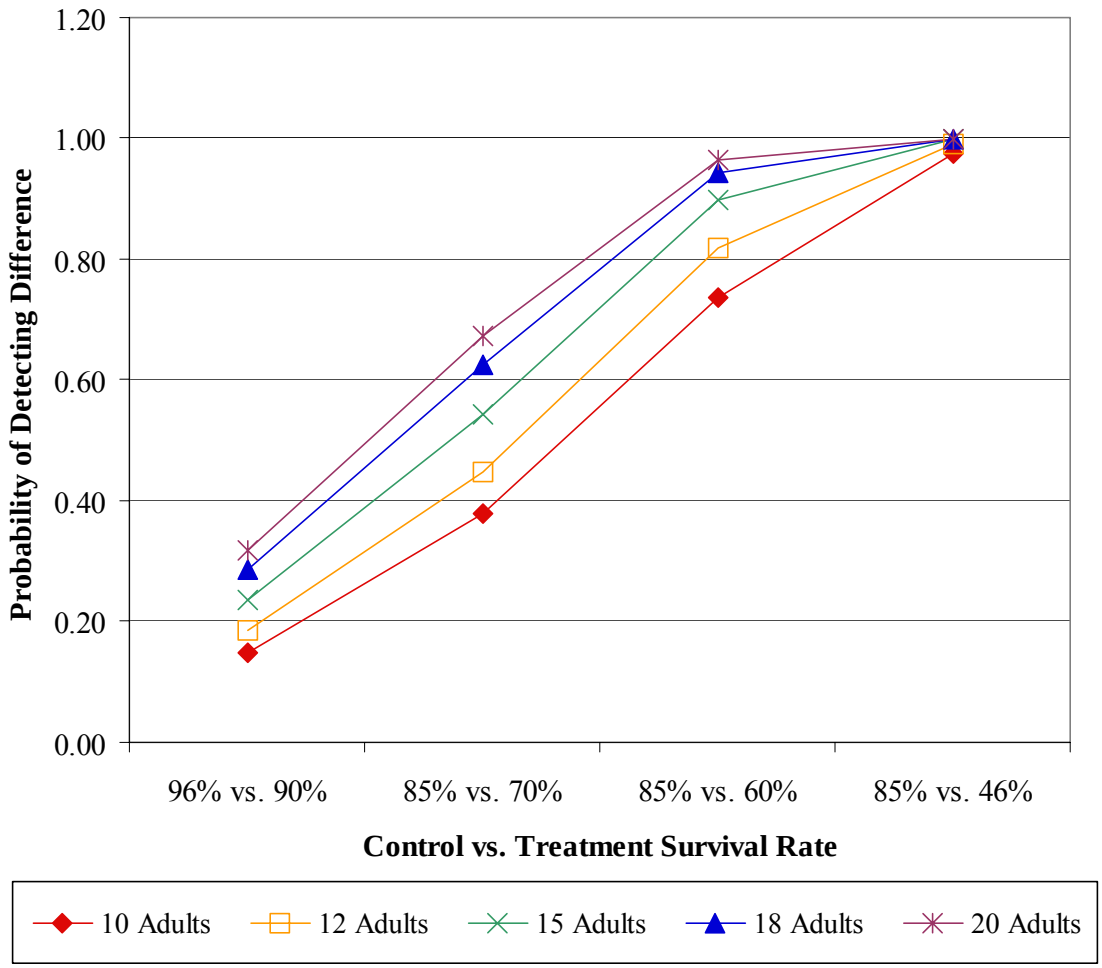

Table 5 Power to Detect Differences in Proportions					
	Number of Adult Females				
Control vs. Treatment Survival	**10**	**12**	**15**	**18**	**20**
96% vs. 90%	0.1495	0.1843	0.2359	0.2862	0.3189
85% vs. 70%	0.3778	0.4476	0.5421	0.6240	0.6718
85% vs. 60%	0.7370	0.8174	0.8976	0.9443	0.9633
85% vs. 46%	0.9751	0.9911	0.9982	0.9997	0.9999

It is anticipated that kits from the most heavily dosed females will have survival rates ranging from 60% to 70%. Assuming these survival rates, the number of females required per treatment to maximize the probability of detecting differences between the control and treatment groups is in the range of 15 to 20. However, the mink study facility does not have capacity for more than the proposed number of adult females per treatment (*i.e.,* 10 or 15), for the contemplated number of treatments.

It should be noted that the power analysis we conducted is approximate and not completely aligned with the analyses that are anticipated. These power estimates are based on standard statistical methods for comparing proportions (Fleiss, 1981) from independent trials, while it is anticipated that litter mates may not be statistically independent. Therefore, these power estimates may overestimate the actual power that will be realized.

6. QUALITY ASSURANCE/QUALITY CONTROL

The objectives of the quality assurance (QA) plan for the proposed study are: 1) to ensure that the mink reproductive toxicity tests are conducted and properly documented according to protocols and the standard operating procedures (SOP) of the mink study facility (Appendix 3), and in accordance with all applicable animal use and care requirements of the facility, and 2) to ensure that the analytical measurements and biological/toxicological assays are accurate and precise. 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).

6.1 DATA QUALITY OBJECTIVES

The data quality objectives for the mink dietary exposure study are directly linked to endpoints presented in Section 4 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. To achieve these objectives, the following types of data will be required:

Reproduction, growth and survival data for control and treatment groups
Dietary exposure chemistry
Mink liver chemistry
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 work plan is to further document the measures being taken to ensure that these standards are met.

6.2 DATA QUALITY INDICATORS

Data developed in the mink dietary study must meet acceptable standards of precision, accuracy, completeness, representativeness, comparability and sensitivity. Each of these data quality indicators, some of which are not readily quantifiable, is 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 characteristics. Precision for this study is assessed by the performance of several replicates (up to 15) per treatment. For the measurements that are not unique to the mink dietary study, such as diet and tissue chemistries, precision is evaluated as described in the Hudson River AQAP (Hudson River Natural Resource Trustees, 2005).

Accuracy is defined as the agreement of a measure with its true value. For the parameters unique to this study (tissue weights, reproductive effects and pathology), accuracy is defined as meaning that tissues are correctly weighed, and reproductive effects and tissue pathology are 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 diet and tissue chemistry and dietary nutrient content, accuracy is defined as the degree of agreement of an analytical measurement with the true or expected concentration.

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 researchers at the selected mink study facility for over 35 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., 10 or 15 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 sampling processing design 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 parameters are specified in Hudson River Natural Resource Trustees (2005). These, in conjunction with reproductive and pathological effects, will provide sufficient sensitivity for the purpose of providing insight into the potential for the measured contaminants to impact resident mink populations.

6.3 SAMPLE HANDLING, TRANSPORTATION AND ANALYTICAL PROCEDURES

Samples of fish, diets, and livers will be collected at the mink study facility and sent to Alpha Woods Hole Lab (AWHL) and/or Axys Analytical Services, Limited (Axys), as appropriate, for chemical analyses. Table 6 sets forth which laboratories will conduct which chemical analyses. The laboratory project managers are:

Gerard Zschau
Alpha Woods Hole Lab
375 Paramount Drive, Suite 2
Raynham, MA 02767-5154
(508) 822-9300; FAX (508) 822-3288
gschau@alphalab.com

Pam Riley
Axys Analytical Services, Limited
2045 Mills Road West
Sidney, British Columbia, Canada V8L358
(250) 655-5800; FAX (250) 655-5811
priley@axys.com

Table 6
Anticipated Laboratory Roles for Chemical Analyses

Sample	Alpha Woods Hole Lab	Axys Analytical Services
Ocean fish blend	PCBs, lipids, moisture	--
Hudson River fish blend	PCBs, lipids, moisture	--
Dietary mix - initial batch	PCBs, lipids, metals, moisture	PCBs, OCs, PCDDs/PCDFs, PBDEs, lipids, moisture
Dietary mix - subsequent batches	--	PCBs, lipids, moisture
Pre-trial adult livers	Metals, moisture	PCBs, OCs, PCDDs/PCDFs, PBDEs, lipids
Adult and kit livers at weaning	--	PCBs, PCDDs/PCDFs, lipids
Kit livers at 7 months	--	PCBs, PCDDs/PCDFs, lipids

Fish, diet and tissue samples for chemical and nutritional analyses will be stored in I-Chem jars at -80ºC prior to shipment. Fish, diet and tissue samples for chemical analysis will be shipped by overnight courier frozen on dry ice. Diet samples for nutritional analysis will be shipped by overnight courier to Litchfield Analytical Services[1] frozen on dry ice. Chain of custody documentation (Appendix 4) will accompany all shipped samples.

Chemical analyses of fish, diet, and tissue samples will be performed in conformance with the Hudson River Natural Resource Damage Assessment AQAP (Hudson River Natural Resource Trustees, 2005).

Tissue samples preserved in formalin for histopathological analysis will be transported under Chain of Custody by the Principal Investigator from the mink study facility at the end of each necropsy session (at weaning [adult females and males and six-week-old kits] and when juveniles are seven months old) to a board certified veterinary pathologist where they will be processed. All tissues are assigned a unique number upon receipt by the pathology lab, which follows the tissue through processing and reading of the slides. Tissue blocks are returned to the Principal Investigator when the pathology report is submitted. A subset of slides will also be reviewed by a second pathologist to confirm interpretations.

6.4 DATA REDUCTION VALIDATION AND REPORTING

All experimental information is recorded in bound notebooks or on forms kept in loose leaf notebooks and will be signed and dated. 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 checked internally by the Principal Investigator on a weekly basis. Copies of the computerized data files are maintained in a project notebook and on CD in the project file. During the course of the experiment, an external audit will be conducted by the Hudson River Quality Assurance Coordinator to evaluate adherence to relevant protocols and ensure that procedures are in place for proper sample handling, processing, and documentation of results. Prior to use by the Principal Investigator, analytical data will be validated as described in the Hudson River AQAP (Hudson River Natural Resource Trustees, 2005).

6.5 SAMPLING METHODOLOGY

Fish sampling in the Hudson River will be conducted according to procedures outlined in Appendix 1. Carp (*Cyprinus carpio*) is the primary target of this sampling

[1] Contact information for Litchfield is as follows: Stan W. Force, President, Litchfield Analytical Services. P.O. Box 457, 535 Marshall Street, Litchfield, MI 49252. Telephone: 517-542-2915.

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.

6.6 EQUIPMENT

All equipment used in these studies (grinder, feed mixer, freezers, cooler and balances) is routinely inspected, calibrated, and preventive maintenance is performed. A logbook is kept for each instrument to document its use, performance, calibration, and maintenance.

6.7 STATISTICAL ANALYSIS OF DATA AND SAMPLING DESIGN

The statistical treatment of the data is described in Section 5 of the work plan. Sampling design in general follows procedures described by Ringer et al. (1991) (Appendix 2).

6.8 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 and reported to the Principal Investigator.

6.9 TRAINING

All sampling and analyses will be directed by the Principal Investigator or by the appropriate supervisor, depending upon the task, who have experience in the collection and shipping of samples, the analyses of tissue and diet chemistry, and the evaluation of mink reproductive endpoints and pathology. Supporting staff will receive training from the Principal Investigator in overall goals of the study and in techniques to be followed to ensure collection of quality data.

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APPENDIX 1

Fish Collection Standard Operating Procedure

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FISH COLLECTION

STANDARD OPERATING PROCEDURE

Hudson River Natural Resource Damage Assessment

HUDSON RIVER NATURAL RESOURCE TRUSTEES

State of New York
U.S. Department of Commerce
U.S. Department of the Interior

June 2006

Project Manager Name (printed): _____

Signature: _____

Field Crew Leader Name (printed):_____

Signature: _____

Quality Assurance Coordinator Name (printed): _____

Signature: _____

FIELD TEAM ACKNOWLEDGEMENT OF SOP REVIEW

Name (printed): _____ Name (printed): _____

Signature: _____ Signature: _____

Date: _____ Date: _____

Title: _____ Title: _____

Name (printed): _____ Name (printed): _____

Signature: _____ Signature: _____

Date: _____ Date: _____

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Signature: _____ Signature: _____

Date: _____ Date: _____

Title: _____ Title: _____

Introduction

This standard operating procedure (SOP) was developed by the New York State Department of Environmental Conservation (NYSDEC) to guide fish collection efforts undertaken in anticipation of the Hudson River natural resource damage Trustees' mink feeding study. This SOP focuses on the mechanics to be followed with respect to fish collection, temporary storage, shipment, record-keeping, and chain of custody and is based in part on the NYSDEC's general fish collection procedures.

Target Species and Site

Common carp (*Cyprinus carpio*) is the target species. These fish will be caught in the Upper Hudson River between Fort Edward and Lock C-1 at Waterford.

Personnel and Oversight

The Project Manager (Mr. Larry Gumaer of NYSDEC) will have overall responsibility for managing the implementation of this fish collection effort. It is anticipated that NYSDEC personnel experienced in fish collection efforts will undertake this task. NYSDEC personnel may be supported by appropriately trained staff from the U.S. Fish and Wildlife Service and/or the National Oceanic and Atmospheric Administration. The Project Manager will be responsible for ensuring that all personnel involved in this fish collection effort are adequately trained in the fish collection, handling, labeling, and transportation procedures described in this SOP, as well as in NYSDEC health and safety protocols. The designated Field Crew Leader (Mr. Chris Balk of NYSDEC) will be responsible for implementation of this SOP during the field collection effort and also will be responsible for health and safety requirements in the field.

Collection Procedures

The goal of the anticipated collection effort is to catch and rapidly preserve approximately 1,400 kg of carp from the Upper Hudson River. The focus of collection efforts is on the bulk acquisition of the required quantity of fish from the correct stretch of the river and of the correct species. Information on location and collection date is important for record-keeping purposes. Bulk weight information is also important. Accordingly, the following procedures are to be followed:

1. The boat proceeds to the sampling location. Available carp are caught at that location by electrofishing. Electrofishing will continue at a particular location until, in the professional opinion of the electrofishing boat's captain and crew, further fishing at the site is unlikely to be sufficiently productive to warrant further effort. At that point, the boat will proceed to a different location and attempt to take carp there. Attachment 1 depicts anticipated sampling locations. In general, it is anticipated that sampling will occur starting at the most upstream locations and proceeding downstream. Carp will be kept in the boat's live-well until return to shore.

2. When fishing is complete at a particular location or when the boat's live well nears capacity, the boat shall return to shore. Immediately upon return, fish will be sacrificed by placement on dry ice. Fins, gills and tails will be removed from the fish, and groups

of carp from the same location shall be wrapped in a plastic bag or bags. Each bag shall be tagged and labeled in indelible ink with the following information:

1. Bag ID number. This number shall have the format "MFS001", short for "mink feeding study, bag 1."

2. Date collected, in the format YYYYMMDD (*e.g.*, 20060630 for June 30, 2006).

3. Collection location, as indicated by the river mile or river mile range from which the fish were collected.

4. Weight (to the nearest 100g).

3. The above information shall also be recorded on the Fish Collection Log form (Attachment 2). New Fish Collection Log forms shall be used for each collection date and shall be signed as indicated. If multiple Fish Collection Log forms are needed for a single day, they shall be sequentially numbered in the appropriate space on the form.

4. The sampling location, as recorded on the Bag ID tags, shall be recorded on a photocopy of a topographic map or navigation chart of good quality. This map shall be attached to and kept with the Fish Collection Log forms used that day.

5. After tagging, bagged fish shall be frozen on dry ice as soon as possible. Bagged fish and dry ice will be carefully proportioned into coolers to allow for rapid and thorough freezing of the fish. Personnel handling dry ice shall wear gloves and suitable eye protection. In no event will any fish remain unfrozen for more than 12 hours after collection. Fish will be kept on dry ice in the coolers until they are transported to a temporary storage freezer maintained by the New York Department of Environmental Conservation.

6. Once at the temporary storage freezer, the bags of fish will be re-packed into rigid containers (coolers, cardboard boxes with Styrofoam inserts, or equivalent). (Dry ice shall not be kept in the temporary storage freezer.) Each container will be sealed with Chain of Custody tape to the extent that opening the box would require breaking of the tape. Each container shall be labeled with a unique identification number in the form of "MFSB001," short for "mink feeding study, box 1." Box labels shall also indicate the Bag ID numbers packed inside. As boxes are packed and sealed, appropriate entries will be made into the Chain-of-Custody and Field Collection Log forms.

7. Prior to transport, the fish shall be stored in the temporary storage freezer. This freezer is located at:

Delmar Wildlife Resources Center
56 Game Farm Road
Delmar, NY 12054
(518) 439-8082

The freezer is capable of maintaining a temperature of -20ºC and shall be locked, save for those times when fish are actively being placed in it, or removed from it, or when other NYSDEC staff require access to the freezer for other projects.

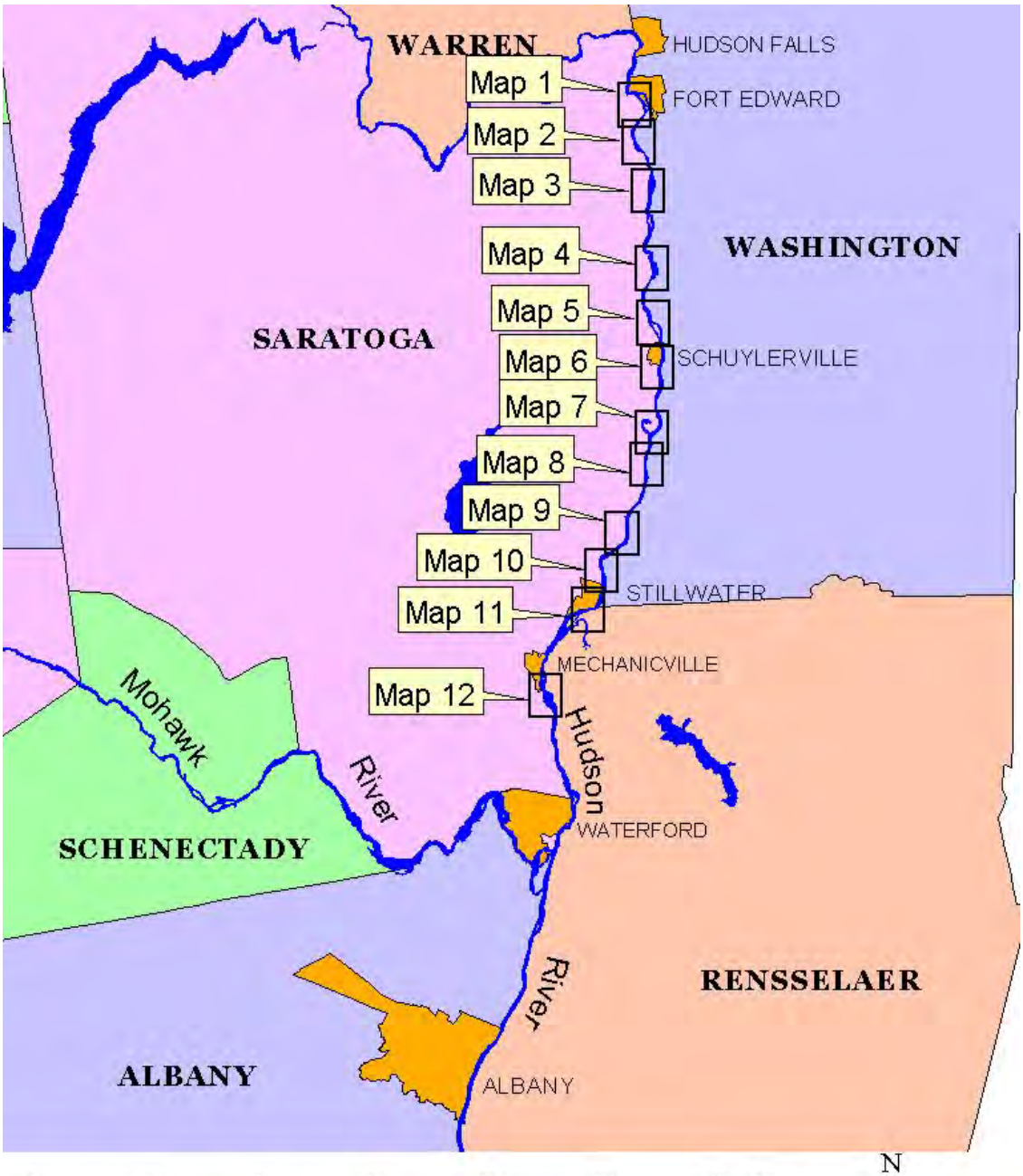
8. Transportation from the temporary storage freezer to the mink study facility will occur by freezer truck, using a temperature-validated service that will provide a record of temperatures at intervals no less than one hour. The truck will be capable of maintaining the frozen fish in a frozen state during transport and until delivery and is expected to have temperatures no higher than -4ºC. After loading, the truck will remain locked until delivery.

9. Containers will be shipped to the mink study facility, care of the mink feeding study Principal Investigator.

10. The Chain of Custody forms (Attachment 3) and their associated Field Collection Log shall accompany the fish during delivery to the Principal Investigator. The forms will be placed in a clear plastic shipping window and securely attached to the inside of one of the shipping containers. A copy of all Chain of Custody and Field Collection Log forms shall be maintained by NYSDEC. Additional copies shall be sent to the mink feeding study Principal Investigator and to the Quality Assurance Coordinator.

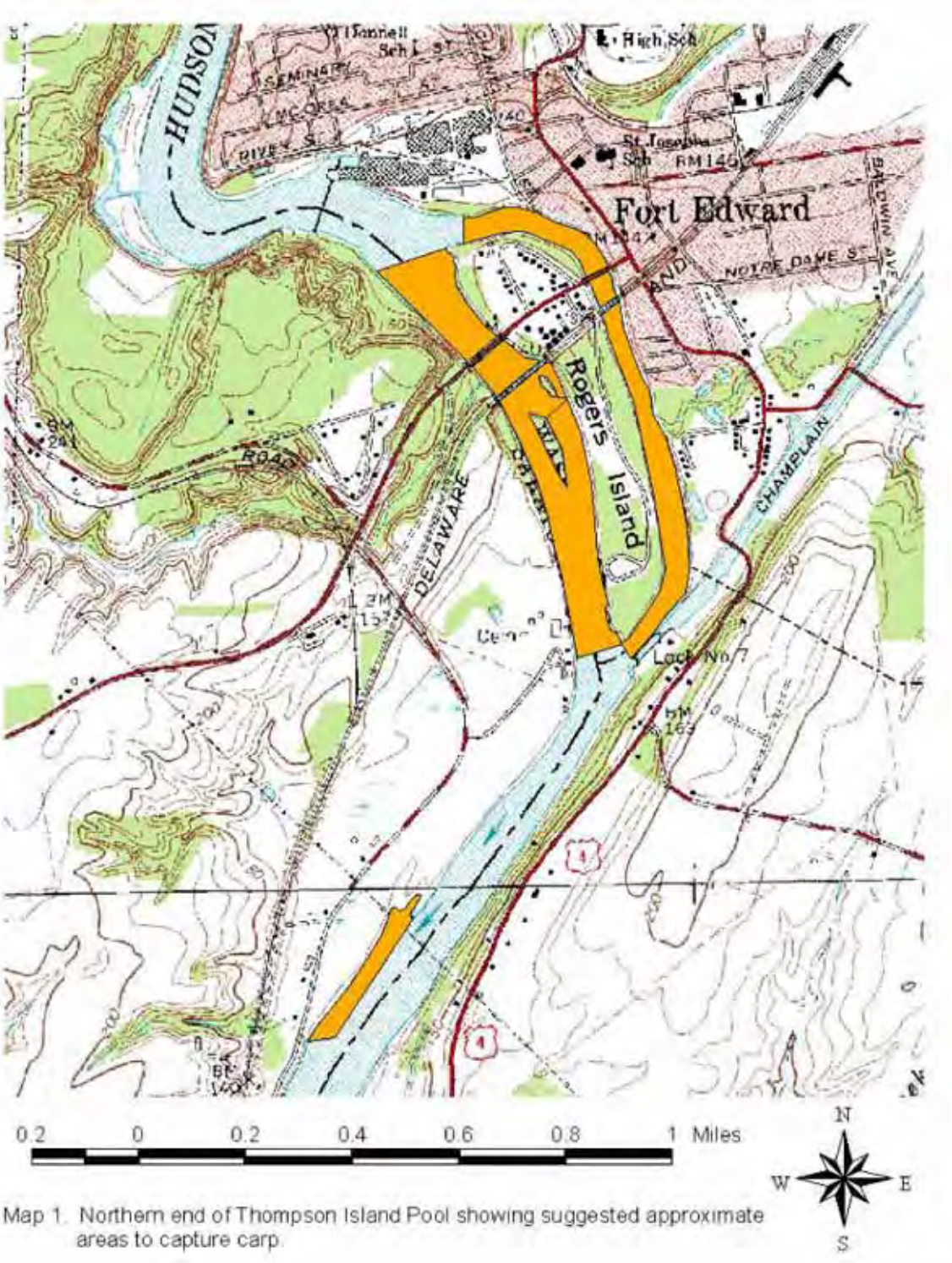
11. Upon delivery to the mink feeding study facility, the fish containers will be inspected for contents against the Chain of Custody forms. Temperature of the freezer and general condition of the fish will be noted on the forms. Forms will be signed when receipt is verified of all bags. The fish will be placed into a -7°C freezer, where they will be stored until they are ground and mixed into a homogeneous blend for use in feed preparation.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



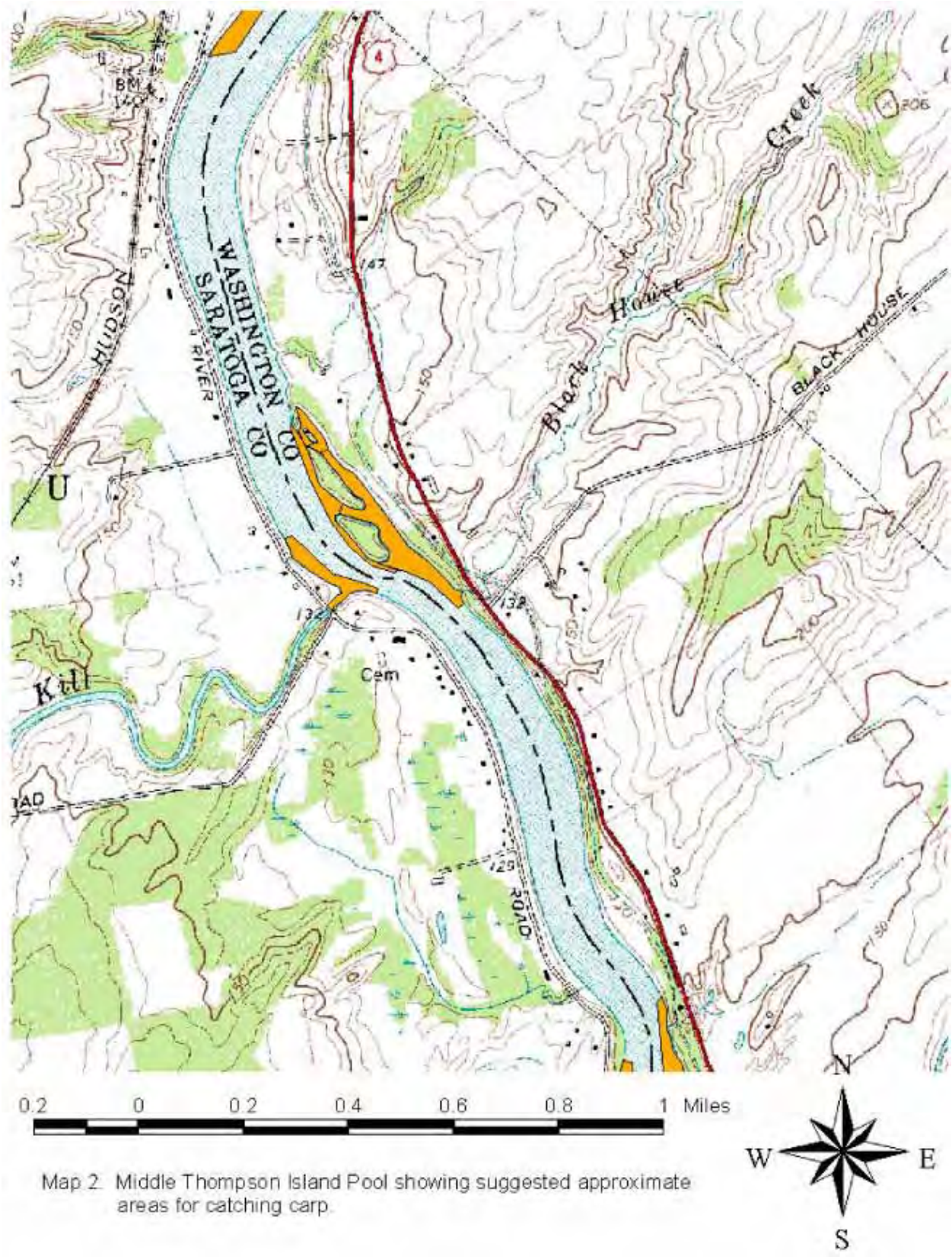
Overview map of central eastern New York State showing the upper Hudson River and the approximate coverages of the 12 maps that indicate suggested approximate carp catching locations.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



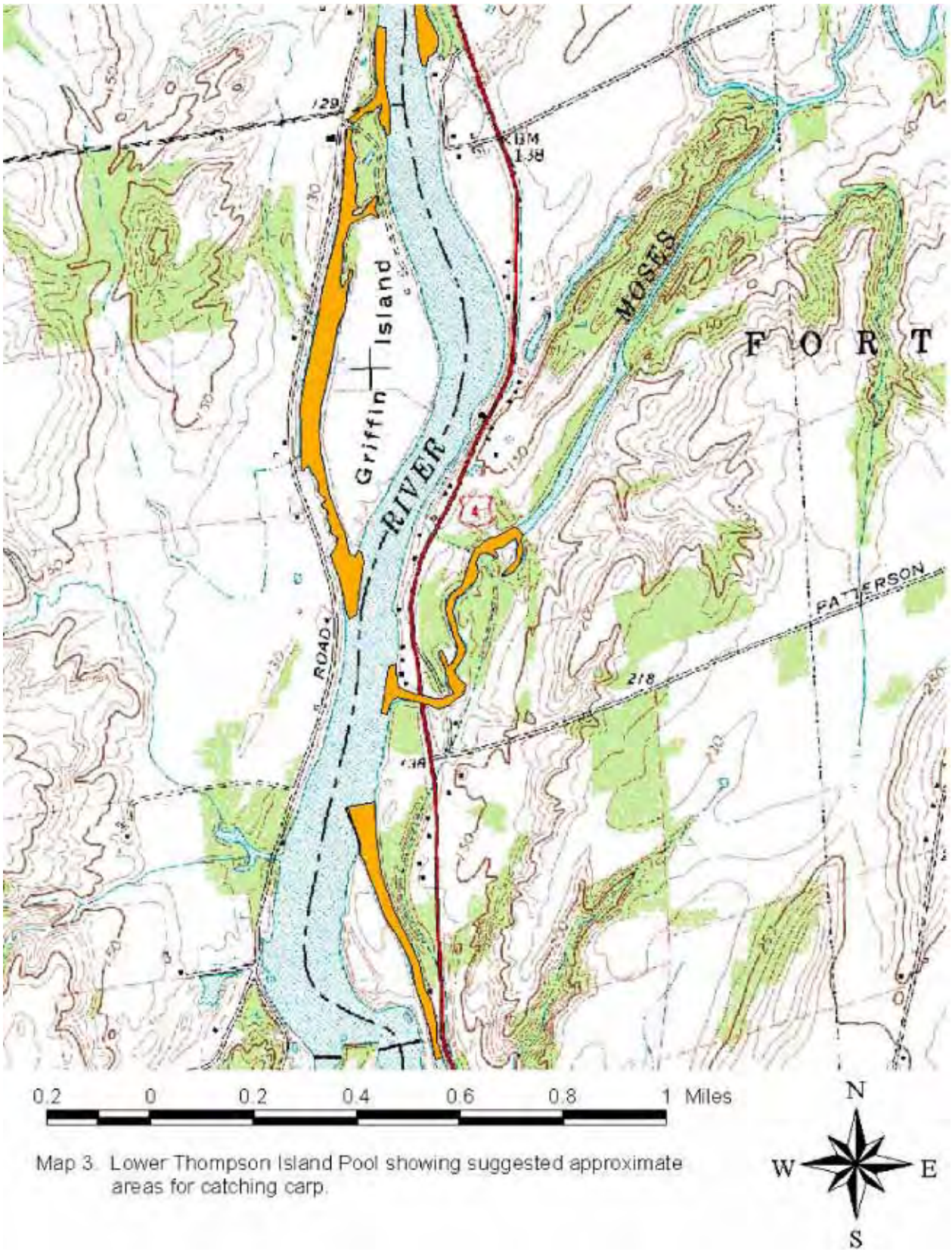
Map 1. Northern end of Thompson Island Pool showing suggested approximate areas to capture carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



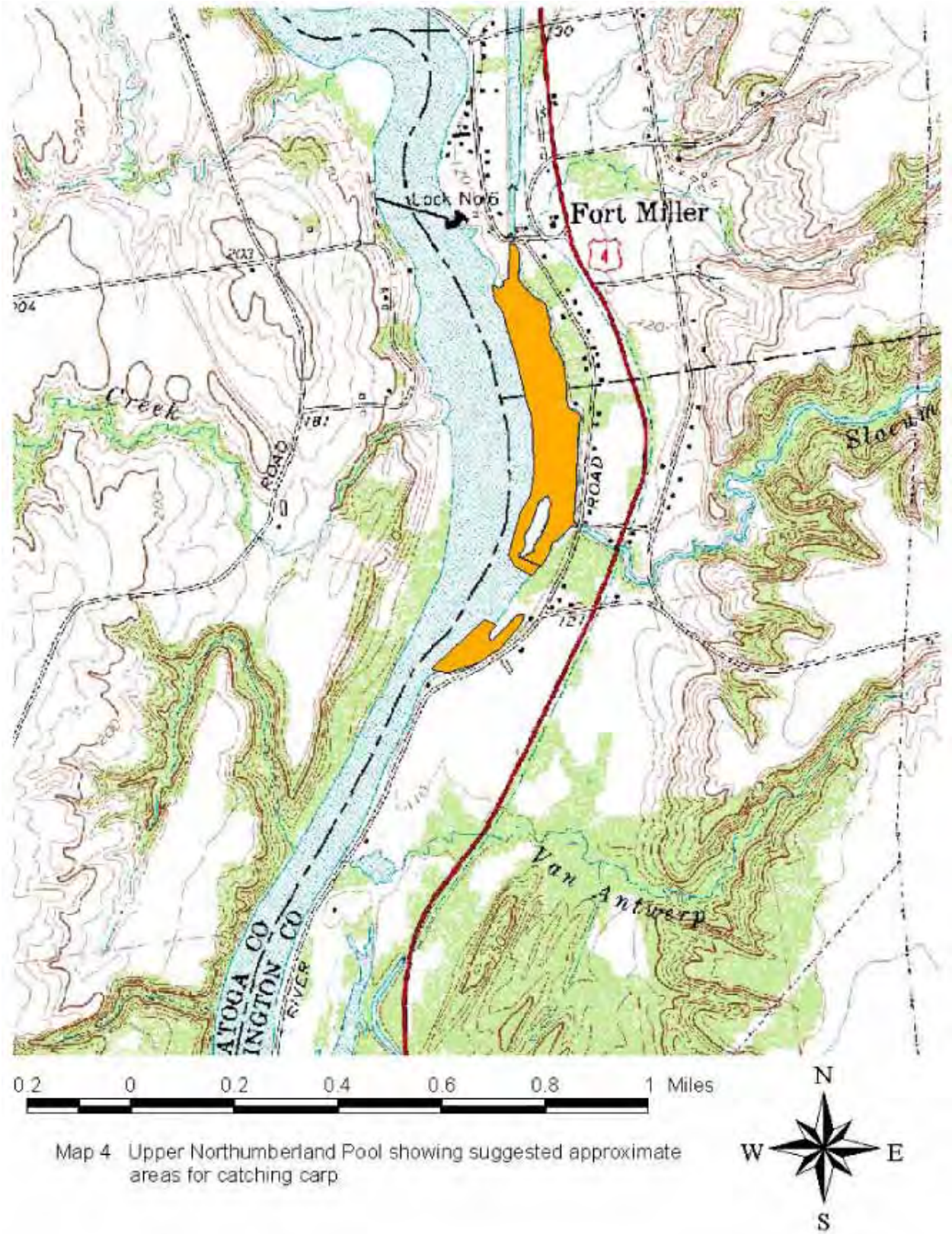
Map 2. Middle Thompson Island Pool showing suggested approximate areas for catching carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



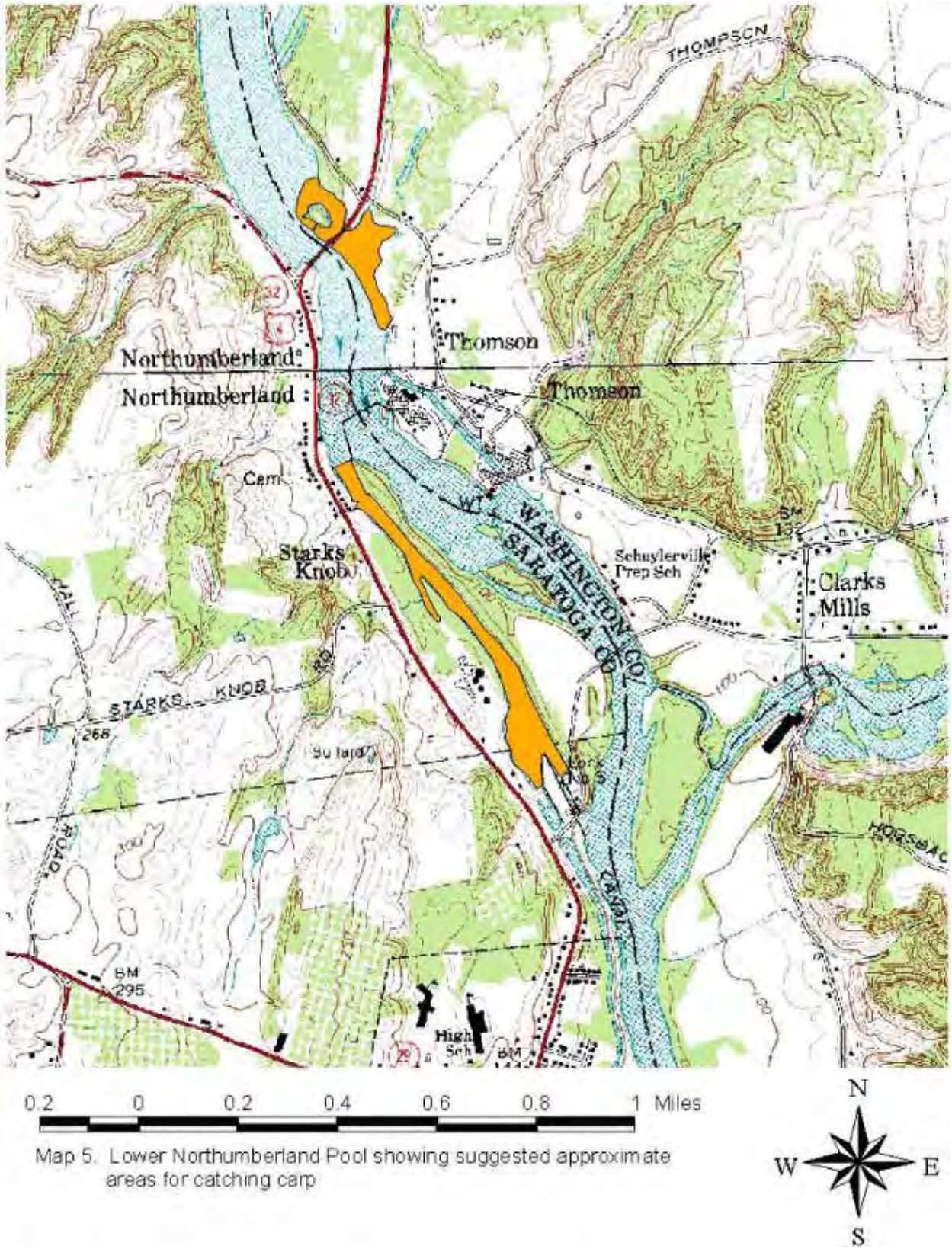
Map 3. Lower Thompson Island Pool showing suggested approximate areas for catching carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



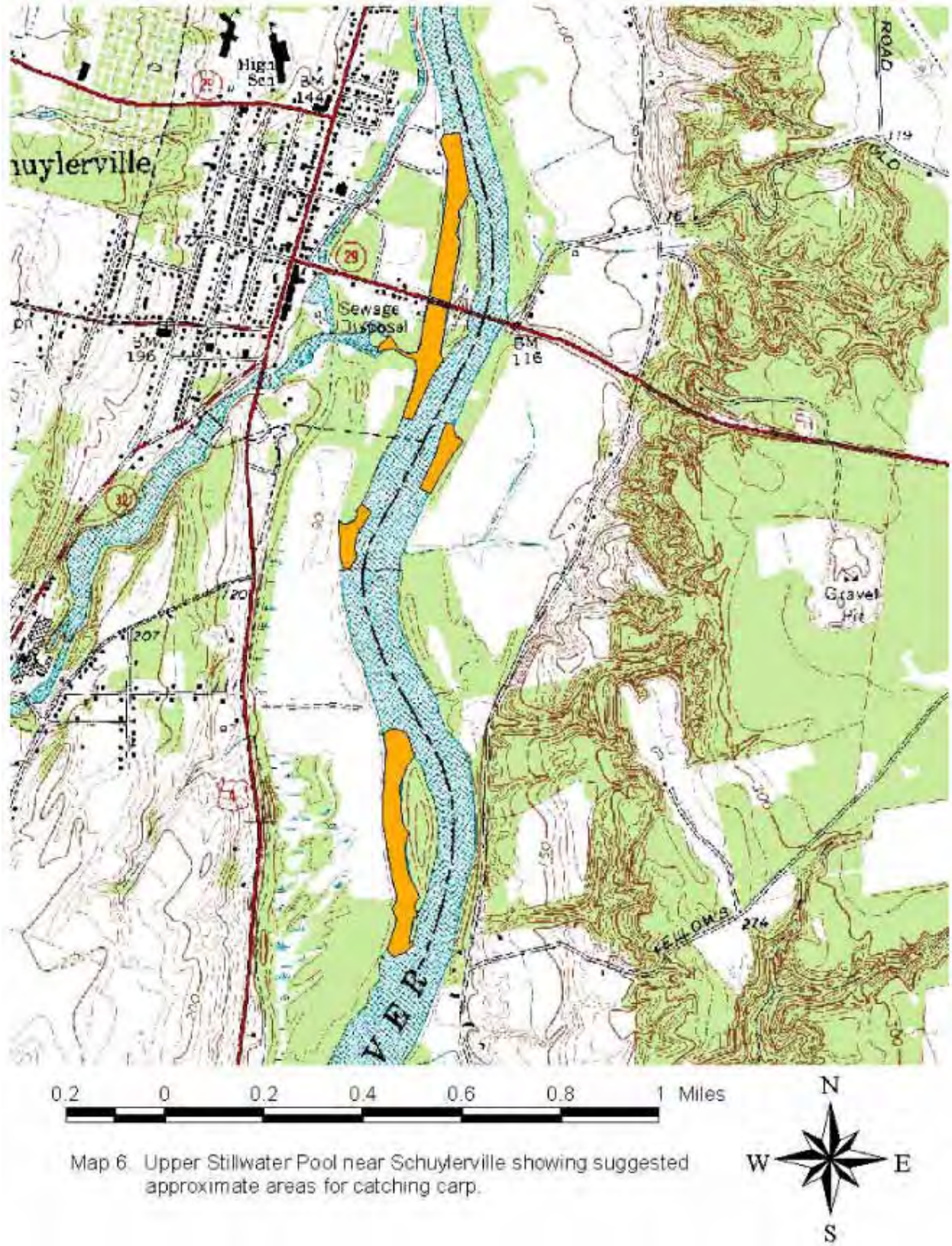
Map 4 Upper Northumberland Pool showing suggested approximate areas for catching carp

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



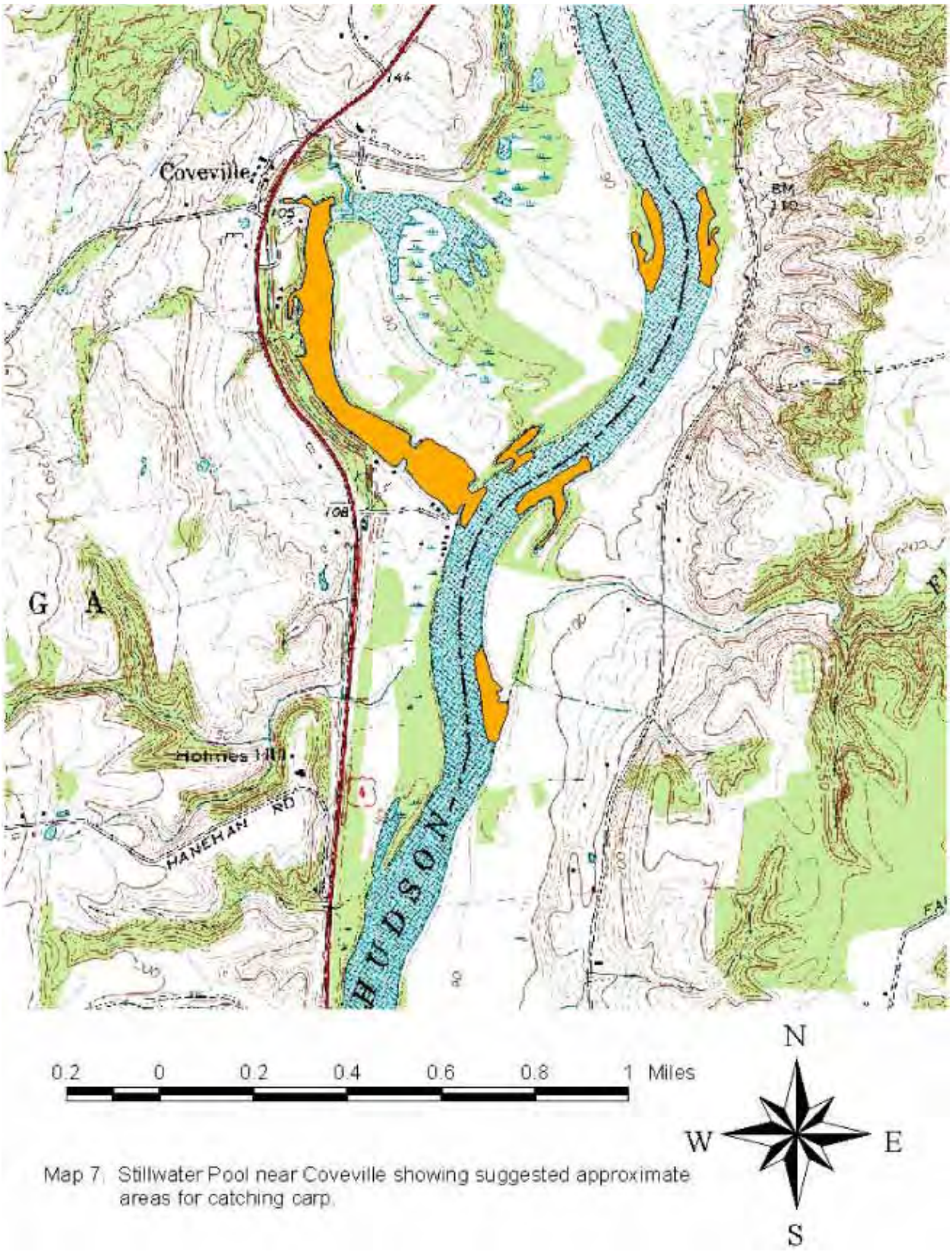
Map 5. Lower Northumberland Pool showing suggested approximate areas for catching carp

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



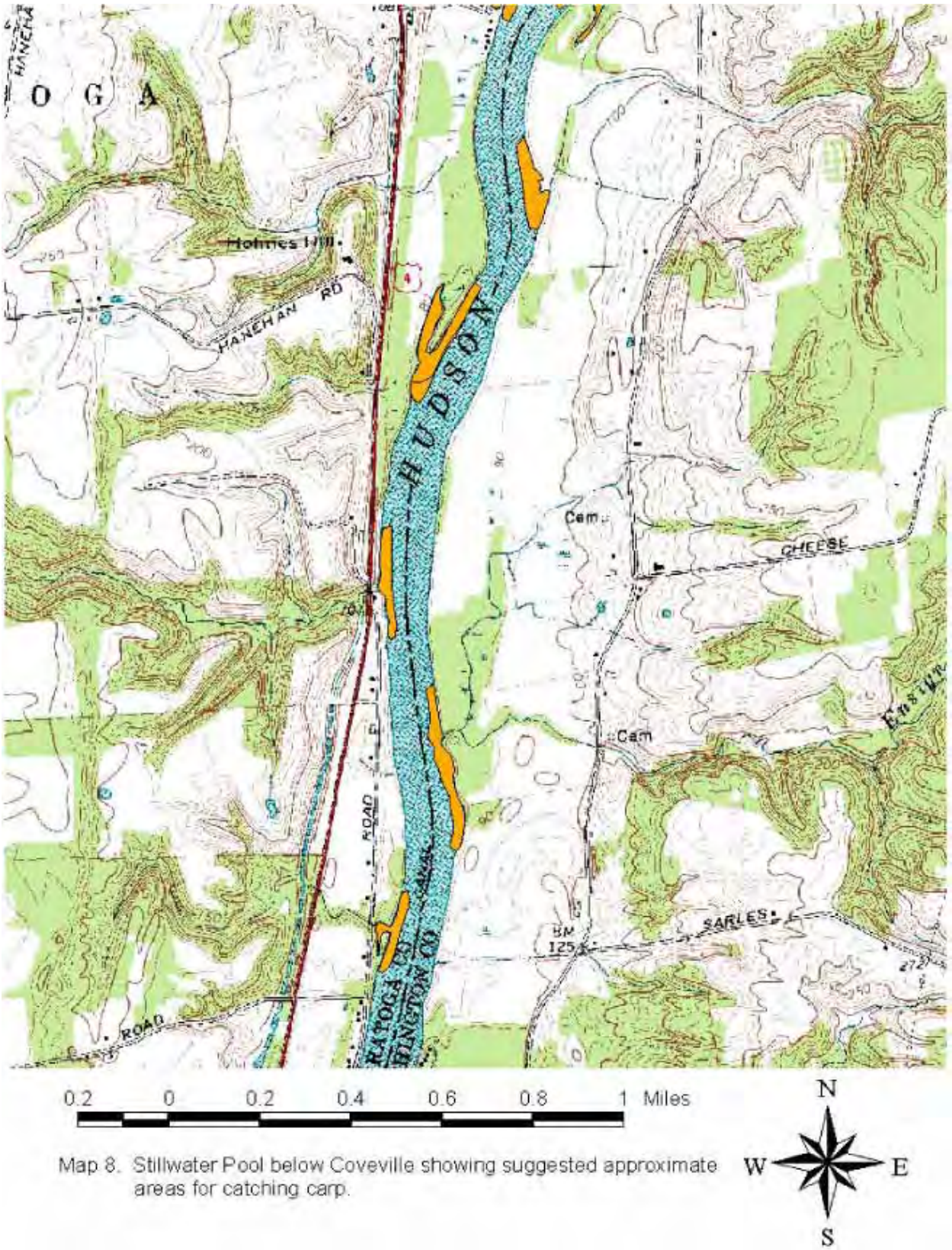
Map 6. Upper Stillwater Pool near Schuylerville showing suggested approximate areas for catching carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



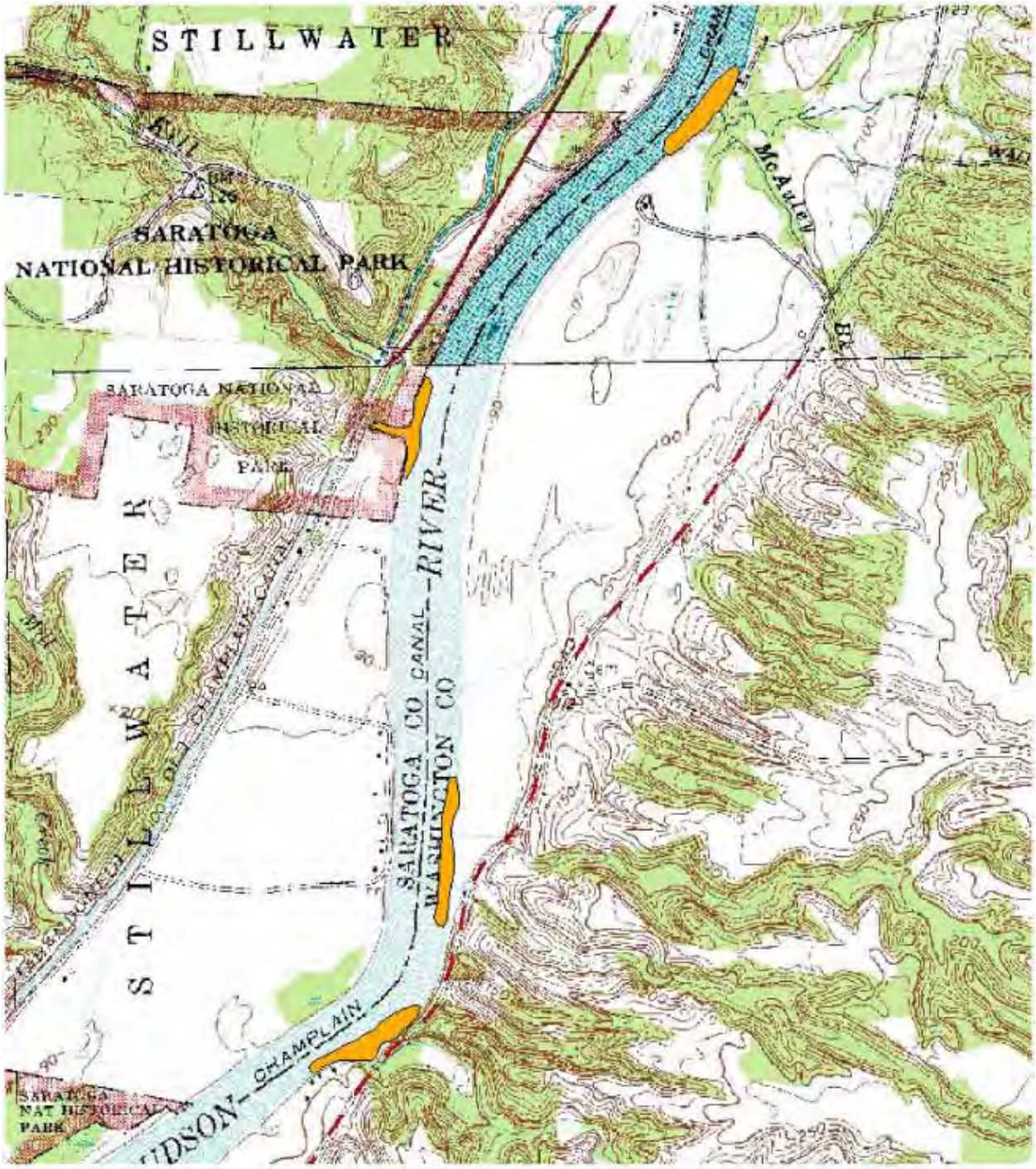
Map 7. Stillwater Pool near Coveville showing suggested approximate areas for catching carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



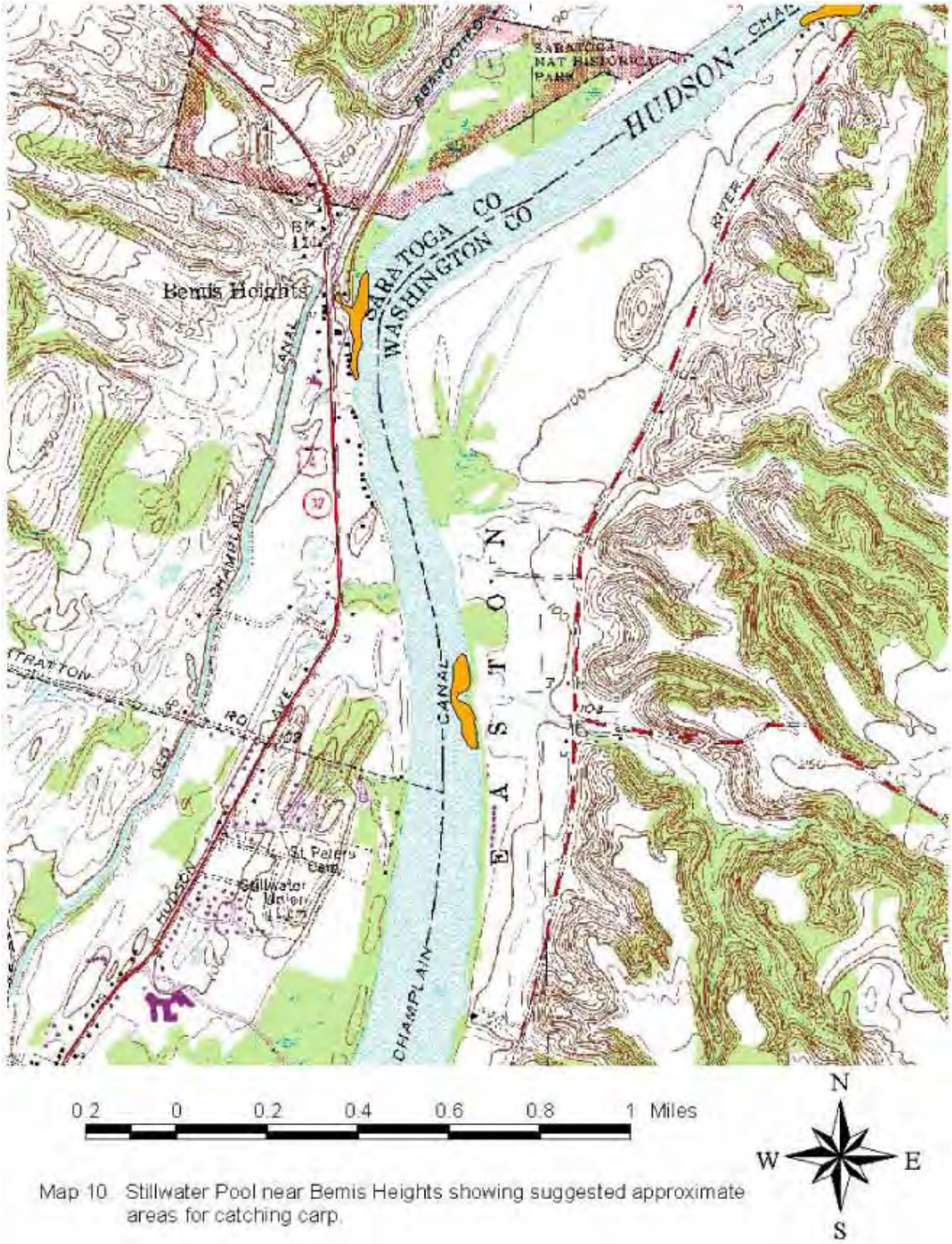
Map 8. Stillwater Pool below Coveville showing suggested approximate areas for catching carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



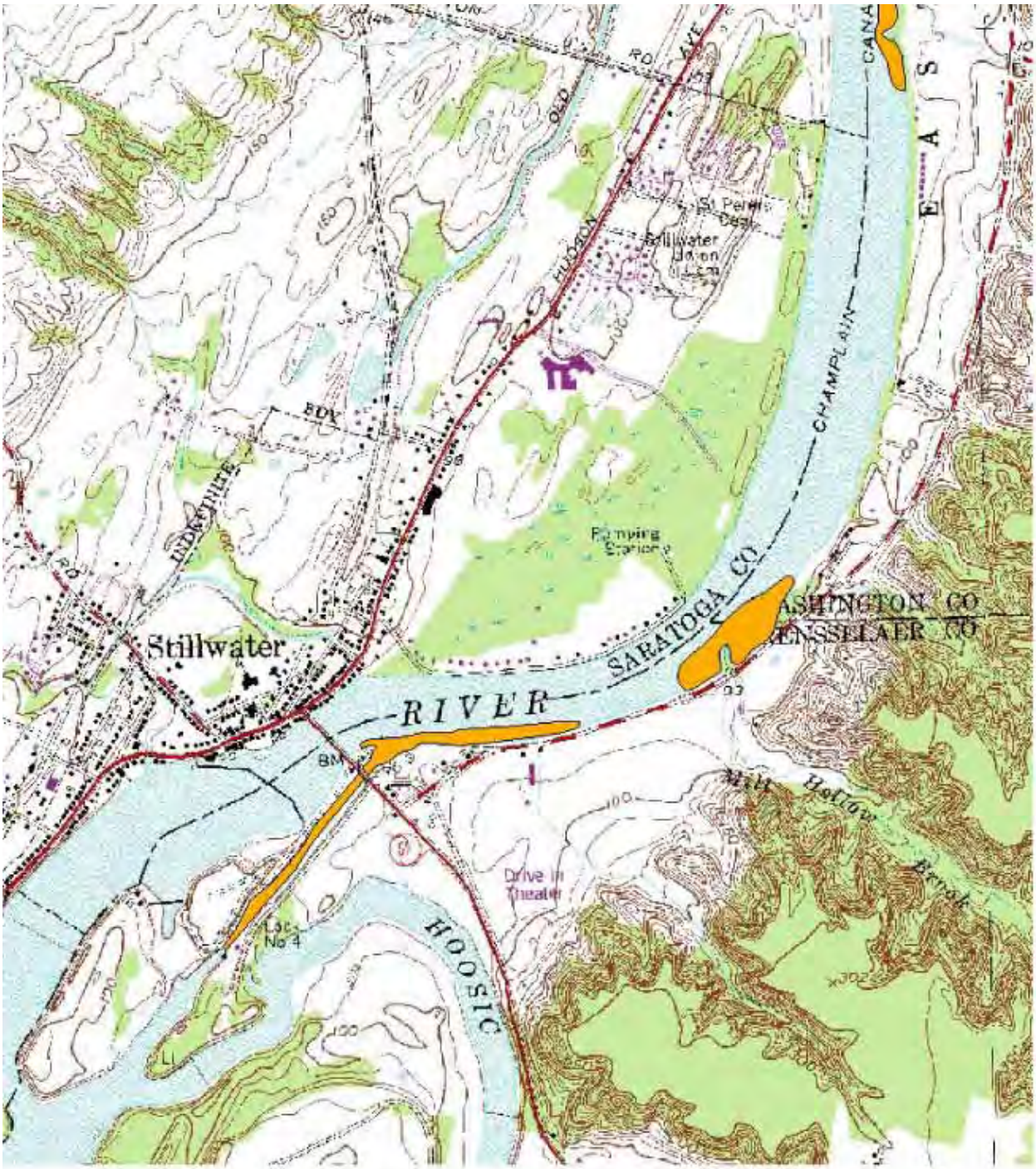
Map 9. Stillwater Pool near Saratoga National Historical Park showing suggested approximate areas for catching carp

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



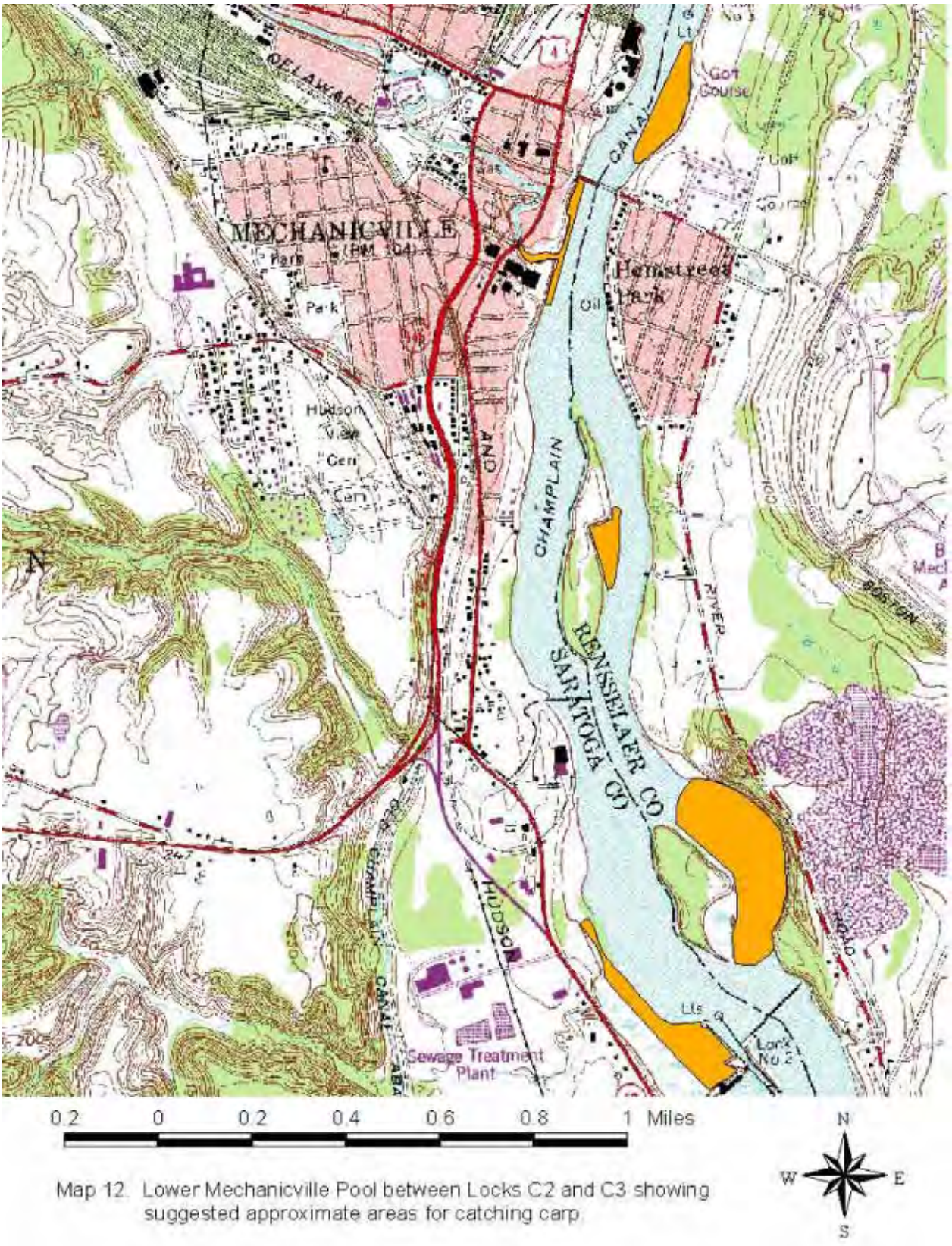
Map 10 Stillwater Pool near Bemis Heights showing suggested approximate areas for catching carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



Map 11. Lower Stillwater Pool showing suggested approximate areas for catching carp.

ATTACHMENT 1 – POTENTIAL HUDSON RIVER CARP FIELD COLLECTION LOCATIONS



Map 12. Lower Mechanicville Pool between Locks C2 and C3 showing suggested approximate areas for catching carp

ATTACHMENT 2 – HUDSON RIVER CARP FIELD COLLECTION LOG

Date: _____ **Page _____of _____**

Bag ID	Collection Location	Number of Fish	Weight (g)	Box ID	Notes

PRINT name of field crew leader: _____ Field crew leader signature: _____

PRINT name of data recorder: _____ Data recorder signature: _____

ATTACHMENT 3 – CHAIN OF CUSTODY RECORD – COLLECTED FISH

I, _____, of _____, collected the items noted below on _____.
[Print Name] [Print Affiliation Name] [Date]

Said sample(s) were in my possession and handled according to standard procedures provided to me prior to collection.
The sample(s) were placed in the custody of a representative of the New York State Department of Environmental Conservation on _____.
[Date]

I, _____, have received the above mentioned sample(s) on the date specified. I have overseen the packaging of the samples into boxes as indicated below. The boxes remained in my custody until subsequently transferred, prepared, or shipped at times and dates as attested to below.

_____ _____
[Signature] [Date]

Box ID	Box Contents (Bag IDs)	Field Collection Log (date and page no.)	Remarks
Second Recipient: (print name) SIGNATURE:		Time & Date:	Purpose of Transfer:
Third Recipient: (print name) SIGNATURE:		Time & Date:	Purpose of Transfer:

APPENDIX 2

Ringer et al. 1991

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PB91-216507

EPA/600/3-91/043
July 1991

MAMMALIAN WILDLIFE (MINK AND FERRET) TOXICITY TEST PROTOCOLS (LC50, Reproduction, and Secondary Toxicity)

by

R.K. Ringer
T.C. Hornshaw
R.J. Aulerich
Department of Animal Science
Michigan State University
East Lansing, MI 48824-1225

USEPA/NOAA IAG #13 F 28800

Project Officer

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CORVALLIS ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CORVALLIS, OREGON 97333

TECHNICAL REPORT DATA
(Please read instructions on the reverse before completing)

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

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16. ABSTRACT

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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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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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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_{50} TESTS TO ASSESS PRIMARY AND SECONDARY TOXICITY[1]

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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

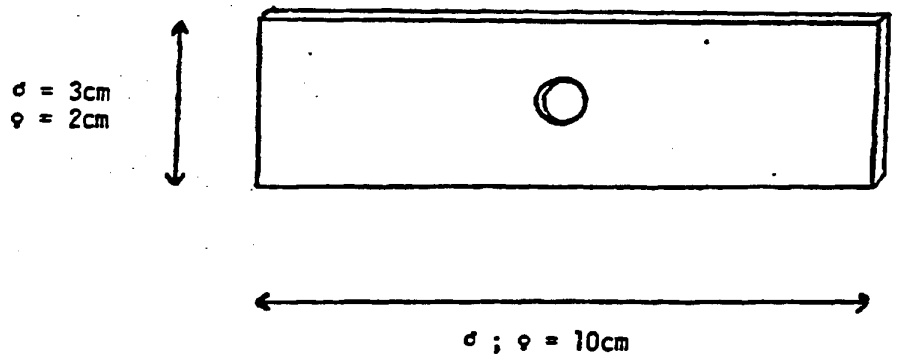

Figure 1. Plastic device for gavage.

APPENDIX 3

Mink Facility Standard Operating Procedures

June 2005
(Revised July 1, 2005)

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A. Institutional Policies and Responsibilities

1. Monitoring the Care and Use of Animals

a. Animal Care and Use Protocol Review and Approval

1) All research projects and teaching activities at the farm must have an approval form from the relevant department and the Committee on Animal Use & Care (CAUC) on file with the manager before initiation of the project or use of the animals for teaching purposes.

b. Physical Restraint

1) Restraint of animals beyond that necessary for routine husbandry and clinical procedures is not a feature of operations at the farm.

c. Multiple Major Surgical Procedures

1) Multiple major surgical procedures are not a feature of mink research at the farm.

2. Personnel Qualifications and Training

a. Animal Resource Professional/Management/Supervisory Personnel

1) Principal Investigator: Ph.D. in Physiology; M.S. in Ecology and Behavioral Biology, B.S. in Experimental Biology; Experience with fur bearing animals since 1979.
2) Farm Manager (Poultry/Fur): M.S. in Animal Science, B.S. in Animal Science; Experience with fur bearing animals since 1979
3) Consulting Veterinarian: DVM, Ph.D. in Pathology.
4) Consulting Pathologist: D.V.M., Ph.D. in Pathology.

b. Animal Care Personnel

1) Herder I: Experience with fur bearing animals since 1979.

c. Research Staff

1) All researchers are required to have either attended the mandatory general seminar on animal use and care or taken the CAUC on-line training. Records are on file with the CAUC Training Coordinator.

d. Personnel Training for Specific Procedures

1) **Staff involved with the use of hazardous agents in animals**
   a) The Principal Investigator or Farm Manager instructs the staff in the use of hazardous or biological chemicals in animals and the importance of following protocol or label directions.
2) **Educational program(s)**
   a) All personnel involved in fur animal use and/or care are instructed by the professional staff in the standard operating procedures of the mink facility that include proper procedures for handling and restraining the animals (use of heavy leather gloves, capture nets, transfer cages), and the use of specialized equipment (feed preparation, maintenance, and pelting equipment).
   b) The Principal Investigator is responsible for informing personnel about zoonoses, personal hygiene, hazardous agents, and other considerations regarding occupational health and safety.
   c) Training records pertaining to the mink facility are on file with the Farm Manager, AUC, the Radiological, Chemical and Biological Safety Office (RCBSO) , or Laboratory Animal Resources (LAR) depending on the nature of the training.

3. Occupational Health and Safety of Personnel

a. Hazard Identification and Risk Assessment

1) RCBSO, the Public Safety Department (PSD), and the Occupational Health Service (OHS) assist in the identification and evaluation of potential hazards via safety and fire

inspections, consultation with the Principal Investigator or the Farm Manager, evaluation of projects submitted to CAUC or health issues, and direct assistance when requested.

2) The Principal Investigator is responsible for informing the Farm Manager of hazardous agents involving research projects. Additionally, the Principal Investigator may schedule meetings with RCBSO and/or other appropriate parties as needed or as directed by CAUC to address health and safety issues that may impact worker or public safety at the facilities.
3) The Farm Manager is responsible for informing personnel about hazardous agents and other considerations regarding occupational health and safety.

b. Medical Evaluation and Preventive Medicine for Personnel

1) **Occupational health and safety program**
   a) All full time employees working at the farm must complete the Occupational Health and Safety Program for Animal Use and Care.

c. Personal Hygiene and Protection

1) **Personal Protective Equipment/Work Clothing Provided**
   a) Coveralls, shirts, pants, gloves, and rubber boots are provided.
   b) Any personnel associated with the use of hazardous or biological chemicals are provided with appropriate protective garments (masks, gloves, lab coats, coveralls, etc.).
   c) Laundry is done by the facility's laundry service.
2) **Provisions for washing hands, showering, and changing clothes / Are work clothes worn outside the animal facility?**
   a) Locker room and bathrooms are located in the Service Center.
   b) A shower is available in the men's locker room. The shower is available and used by both men and women. There is a room with lockers for women.
   c) Persons in contact with fur animals are encouraged to wear protective clothing and boots. Soiled footwear and clothing are to be cleaned or changed before leaving the farm.
3) **Procedures**
   a) Any personnel associated with the use of hazardous chemicals or biologicals are instructed on the potential hazards and proper storage, use and disposal procedures in consultation with RCBSO.
4) **Policies regarding eating, drinking, and smoking in animal facilities**
   a) Eating, drinking, and smoking are prohibited in animal housing, feed storage and feed preparation areas. Eating and drinking are done in the break room of the Service Center.

d. Procedures Involving Hazards

1) **Institutional policies**
   a) The CAUC and the RCBSO evaluate all teaching and research projects to determine potential use of hazardous materials.
   b) Any hazardous agents used must have a use form that is kept in the manager's office in the Right to Know file.
   c) Material Safety Data Sheets for hazardous materials can be accessed via the RCBSO website, fax or phone call to RCBSO.
2) **Apply to personnel potentially exposed to hazardous agents**
   a) There are procedures for reporting, diagnosis, treatment, and care when an injury occurs to farm employees, staff, or others. This is posted at the farm. For a life threatening incident call 911. For non-life threatening incidents employees should report the incident to their supervisor immediately and then go to the designated Primary Care Facility with an authorization to treat form.

B. Animal Environment, Housing, and Management

1. Physical Environment

a. Housing

1) Primary Enclosures

a) Mink are housed in cages in open-sided buildings similar to those used commercially.
b) Adult animals are housed individually in cages of different sizes depending upon the situation.
c) Caging
   (i) Cages are built-in, suspended from the ceiling or walls, or on racks in open-sided sheds.
   (ii) Cages are constructed of galvanized wire mesh, 1" x 1" on the side and top, and 1.5" x 1" mesh on the bottom.
   (iii) During whelping and the first 4 weeks of life, the cages are equipped with false floors and a 6" high extra side wall of 0.5" x 0.5" mesh to prevent the kits from falling through the wire.
d) Types of Cages
   (i) Breeder cages are 24" x 30" x 18" = 12,690 cubic inches
   (ii) Individual cages are 12" x 30" x 18" = 6,480 cubic inches
   (iii) Cages in the Research House are 18" x 30" x 15" = 8,100 cubic inches
   (iv) Grower cages are 12" x 24" x 15" = 4,320 cubic inches

2) Behavioral Management

a) Caging is such that there is sufficient room for exercise and animals are in visual contact with one another.
b) Bred females are allocated to breeder cages with attached nest boxes prior to whelping.
c) After whelping, litters are kept with their mother until weaning at 6-7 weeks of age.
d) Upon weaning, kits are initially housed in groups of 3-4, then as pairs until 10-12 weeks of age at which time animals are housed individually.

2. Husbandry

a. Feed

1) Type and Source

a) Fur animals are fed a commercial pelleted feed or a wet food diet made of ocean fish or fishmeal and slaughter house by-products, together with a proprietary brand of cereal or wheat mids. (Occasionally, other appropriate feed ingredients such as cheese are incorporated into the diet if available). Farm Manager has recipes of diets on file.
b) Cereal, wheat mids, fishmeal, and spray-dried liver and eggs are supplied in sealed bags on pallets wrapped in plastic that are stored in an enclosure in Mink-House 11.
c) Other feed components are purchased frozen from suppliers and stored in one of the two walk-in freezers at <10°F.
d) Fresh chicken carcasses may be flash frozen individually on the floor of the freezer for a day and then placed in barrels with lids.
e) If raw eggs are part of the diet, biotin is added at 25 mg/1000 lbs.
f) In research projects where thiaminase-containing fish may be fed, supplemental thiamine is provided.
g) Extra salt is added (2 lbs/1000 lbs) during lactation and summer.
h) Corn oil and/or wheat germ may be added at breeding, gestation, and lactation.
i) An oral larvicide is incorporated into the diet from mid-April to late fall.

2) **Storage in Animal Facilities**
   a) The ready-to-feed mix is stored frozen in buckets or metal pans covered with plastic wrap.
3) **How Feed is Provided**
   a) Thawed feed is provided to the animals.
   b) Adult and growing animals are fed on top of the cages, fresh feed being supplied daily after orts from the previous day's feeding have been removed.
   c) During freezing temperatures, it may not be possible to remove orts daily.
   d) Beginning at 3 weeks of age, kits are fed adult feed thinned with water by placing it on a metal plate incorporated into the finer mesh 'kit-floor'.
   e) From 4-7 weeks of age, the feed given to kits is increasingly thicker in consistency, after which kits are fed the same feed as adults.
   f) During studies involving determination of feed consumption, feed is provided to the fur animals in porcelain crocks placed on the cage floor.
4) **Special Food Quality Control Procedures**
   a) Proximate analysis of ranch diets is conducted periodically to insure appropriate percentages of fat and protein. Samples of every experimental diet are submitted for proximate analysis.
   b) The frozen feed is thawed in the feed mixing area with accessory heat in the winter.
      (i) Thawing experiments were performed at the request of the USDA inspector to indicate that feed is still cold (39°F) and wholesome at the time of feeding.
      (ii) Thawing of feed in the refrigerator is not feasible.

b. Water

1) **Source, Treatment or Purification Process and How Provided**
   a) Water comes from 17 wells that are interconnected and balanced in operation across all wells. The water in use is not treated with any chemicals.
   b) Water is provided to the animals by nipple waterers or by water cups. Water cups are filled twice daily from an automatic water line, or more often in hot weather. In the winter, hot water is poured into the cups twice daily.
2) **Quality Control Methods**
   a) The well is tested according to the potable water requirements of the Water Quality Division of the state Department of Environmental Quality. The water meets or surpasses all federal and state drinking water standards (Information provided by Power and Water Division, Physical Plant).

c. Bedding

1) Poplar or aspen wood chips are used for bedding in the nest boxes when kits are present. Wood chips are received bundled and are stored in Mink-House 11.
2) Pine shavings are used in the nest boxes at other times. Pine shavings are also used under the suspended cages. Shavings are received bundled and are stored in Mink-House 10.
3) Wood Wool® (excelsior) is provided during whelping and lactation and to all animals during the fall and winter. Excelsior is received bundled and is stored in Mink-House 11.

d. Miscellaneous Animal Care and Use Equipment

1) **Motorized Vehicles**
   a) 2-Pickup trucks
   b) 1-Tractor
   c) 1-Skid Loader
   d) 1-Lawn Mower
2) **Other Animal Care Related Equipment**
   a) High pressure sprayer, Table top and Platform Scales, Feed mixer, Meat grinder, Meat cutter

e. Sanitation

1) **Bedding Change**
   a) Nest box litter is changed if soiled.
2) **Cleaning of Primary Enclosures**
   a) Waste food is scraped off the top of the cage before new food is provided during the summer while in the winter, food may be left on top of the cage, especially if it freezes.
   b) The cage top is brushed monthly (weekly for Mink-Shed 2) with a wire brush to remove small pieces of food stuck to the wire.
   c) Manure under the cages is removed quarterly, weather permitting, and monthly in Mink-Shed 2.
3) **Support Areas**
   a) The outdoor sheds used for sheltering fur animals throughout the year are power-washed as needed.
   b) The feed mixing floor is cleaned daily after each use.
   c) Corridors and feed storage areas are swept as needed.
   d) The grass around the sheds is cut during the summer, except from mid-April until mid-June (time of gestation and parturition) in efforts to not distress females and encourage cannibalistic behavior.
4) **Sanitation of Equipment**
   a) **Implements**
      (i) Feed Mixer
         1. Feed residue is rinsed from the equipment with hot water (the highest temperature possible, about 156°F).
         2. The mixer is disinfected with a commercial disinfectant (diluted according to the manufacturer's recommendations) using a brush.
         3. The mixer is rinsed again with hot water after the disinfection procedure.
         4. If toxic substances are incorporated in mink diets, the feed preparation equipment may be washed with appropriate solvents, prior to disinfection and rinsing.
   b) **Feeders**
      (i) When feed consumption is being measured, the individual porcelain jars are washed every 2 days.
   c) **Watering Devices**
      (i) Water cups are dumped when soiled and washed at least monthly (in winter, when weather permits) with sulfuric acid and hot water (300 mls concentrated sulfuric acid in 44 gallons of water).
   d) **Enrichment Devices**
      (i) Nest boxes are cleaned and disinfected with a commercial disinfectant (O-SYL 128) prior to whelping.
   e) **Transport Cages, Equipment**
      (i) Transport cages (used for moving individual animals from one cage to another) are washed and disinfected with a commercial disinfectant (O-SYL 128) if soiled.
5) **Assessing the Effectiveness of Sanitation**
   a) Visual inspection

f. Waste Disposal Methods

1) **Soiled Bedding and Refuse**
   a) Soiled bedding from the nest boxes, and manure and wood chips from under the cages are stored in an enclosed room and spread on the fields twice a year. Composting is done in the white shed on the farm designed for that purpose. It

contains 4 bins. Temperature of the pile is monitored and turned with a skid loader when the material has cooled to the appropriate temperature.

2) **Animal Carcasses**
   a) Animal carcasses are incinerated at the Diagnostic Center.
3) **Hazardous Wastes - infectious, toxic, radioactive**
   a) Hazardous waste is disposed of in accordance with relevant guidelines via RCBSO. Medical/Biohazardous waste is disposed of in accordance with relevant guidelines via RCBSO.

g. Pest Control

1) **Program**
   a) The PSD pest control officer attends the monthly Farm Managers meeting to stay abreast of any current or future problems and responds to pest reports from the Farm Manager. PSD oversees the agents used and application methods depending on the type of pest problem. The Farm Manager purchases pest bait directly from retail sources. A rotational program using Rampage, Jaguar and Hawk rodenticides is utilized at the farm.
   b) Fly control
      (i) Larvadex 2 SL® is incorporated into the diet of ranch mink at 65 mls/1000 lbs feed from mid-April until fall.
      (ii) Manure underneath the cages of experimental animals is sprayed with Demon® (cypermethrin) whenever larvae as seen in the manure.
      (iii) An electric fly killer is used in Mink-House 11.
   c) Chemicals and traps used for vermin control are not accessible by the mink.

h. Provisions for Emergency, Weekend, and Holiday Care

1) **Procedures for Providing Weekend/Holiday Care**
   a) The Farm Manager develops employee work schedules for personnel during these time periods. Animal feeding, care and health assessment are completed daily.
2) **Procedures for Contacting Responsible Animal Care and/or Veterinary Personnel**
   a) If the Farm Manager is not working, the Principal Investigator or Consulting Veterinarian can be contacted by phone in emergency situations.
3) **Brief Description of Disaster Plan**
   a) The Farm Manager has a notebook with an Emergency Farm Disaster Plan. The plan contains emergency contact names and phone numbers, risk assessment facilities and sites, feed storage sites, maps, available animal transportation equipment and contacts, emergency feed resources, mass animal mortality procedures, electrical load shedding schedule, and veterinary care contacts.

i. Routine Management Practices

1) Sperm checking
   a) To determine if a female has been successfully bred, she is transported to Mink-House 11 and restrained while a glass medicine dropper containing 0.1 ml 0.9% sodium chloride is inserted into the vagina.
   b) The vaginal contents are aspirated and examined under a microscope for viable sperm.
2) Toe-nail clipping
   a) Occasionally a drop of blood is required for diagnostic purposes such as Aleutian disease testing.
   b) The animal is placed on a table and manually restrained by one person.
   c) A second person clips a nail on a hind claw just below the quick for collection of blood.
   d) Once blood has been collected, pressure is applied to the clipped nail until bleeding stops.

e) The animal is observed periodically through the next 24 hours to ensure that bleeding has ceased.

3) Collection of blood via the jugular vein
   a) The animal is anesthetized with the appropriate dose of Ketamine HCl (0.3 or 0.4 ml, 100 mg/ml, depending upon sex).
   b) The ventral region of the neck is shaved with an electric razor to allow visualization of the jugular vein.
   c) Blood (no more than 5 ml) is collected with a syringe and 22 gauge needle.

j. Marketing

1) Animals are pelted the first week of December and processed pelts are auctioned.

3. Population Management

a. Identification and Records

1) **Methods for Animal Identification**
   a) Individual cage cards contain information in code for year of birth, color, sex, origin, individual identification number, experimental group, and experimental treatment.
2) **Procedure(s) for Maintaining Individual Records**
   a) Individual records are on file in the office in Mink-House 11.

b. Genetics and Nomenclature

1) **Animal Inventory**
   a) Animal inventory consists of all animals that will be used on approved trials for the upcoming year plus a breeding herd of approximately 50 females and 20 males.
2) **Breeding program**
   a) Breeding begins on or around March 1.
   b) A female is placed in a male's cage and observed for 5-10 minutes.
   c) If fighting occurs or if the animals do not interact, the female is removed.
   d) After mating, females may be removed and transported to Mink-House 11 where a vaginal sperm check is performed.
   e) Each female is provided an opportunity to mate at least once every 4 days until mating is obtained.
   f) At the beginning of the season, bred females will be rebred 8 days later whereas mink bred later in the season will be rebred the following day.
   g) Most mink are bred by March 21 and all breeding is terminated by March 28.

C. Veterinary Medical Care

1. Animal Procurement and Transportation

a. Sources of animals are suggested by the Principal Investigator.
b. Any required permits are acquired by the Principal Investigator.
c. Transportation of animals is arranged by the Principal Investigator.

2. Preventive Medicine

a. Quarantine, Stabilization, and Separation

1) Upon delivery, all animals are inspected by the Farm Manager and/or Principal Investigator.
2) Any animals that appear to be ill or injured are referred to the Consulting Veterinarian.
3) All new animals brought onto the fur farm are quarantined for 3 weeks away from other animals.
4) These animals are routinely cared for after the other animals on the farm have been cared for.

b. Program for Separation of Animals by Species, Source, and Health Status

1) All animals are routinely housed in individual cages (except nursing females with their litters, newly weaned litters, or as specified in approved research protocols).
2) Both mink and ferrets may be housed within the same building or room on occasion.

c. Surveillance, Diagnosis, Treatment, and Control of Disease

1) **Program**

a) **Procedure for Daily Observation of Animals**

(i) All animals are observed at least once per day, by the animal care staff, 365 days per year.

(ii) Any animal not eating will receive a gross physical exam by the animal care staff to check for obvious items such as a bone chip stuck in the mouth, abscesses or bite wound from mating or sibling rivalry.

1. Bone chips stuck in the dental arcade can be removed by the animal care staff, whereas any other lesion will be reported to the Consulting Veterinarian via the Farm Manager or Principal Investigator.
2. Mink are occasional intermittent feeders, especially in the spring or fall.
3. On the first day that an animal does not eat a normal amount of feed, the cage card is turned 90° to ensure that the animal is closely looked at the next day and if the animal does not eat a normal amount of feed on the following day, it will be offered supplemental feed such as liver or egg yolk.

b) **Procedure for Providing Veterinary Medical Care**

(i) The Consulting Veterinarian observes the animals on a monthly basis.

(ii) Minor injuries to animals will be treated by the Farm Manager or Principal Investigator while serious injuries will be reported to the Consulting Veterinarian.

(iii) If a mink is observed with roughened or missing hair, it will be examined to determine if there is an open wound or scab.

1. Cage hardware will be examined if a wound is discovered to ensure that a neighboring mink or the cage or cage hardware can no longer cause injury to the affected animal.
2. Female mink may have rough fur or superficial bite marks on the back of the neck after breeding.
   a. If bite wounds are noted, this will be recorded and topical treatment with approved mediations will be carried out by the animal care staff.
   b. Minor scratches or superficial abrasions that are not bleeding will be considered as normal occurrences that do not warrant treatment or recording.
3. Mink with clipped, chewed, or missing fur other than for obvious reasons will be considered as having behavioral anomalies.
   a. Historical review of records has shown that this occurs more often in summer.
   b. Animals with this condition will be removed from the herd at pelting.

(iv) Occasionally, the tongue or gums may bleed from abrasion on frozen drinking water and/or water cup during cold temperatures.

1. When blood is observed in a water cup, the animal's mouth will be examined.
2. The water cup will be examined for sharp edges and replaced if necessary or ice will be removed from the cup and replaced with fresh water.
3. If the bleeding has stopped and the animal is capable of drinking water, no action will be taken.
4. If bleeding continues, the animal will be housed inside until the tongue or gum lesion has healed.

(v) Policy is designed for prevention, rather than therapy, since stress imposed on the animal by repeated handling may not be warranted.
(vi) A herd health approach is taken with attention to mortality rates, necropsy findings, and preventative programs rather than individual treatment.
(vii) Euthanasia is generally preferable to medical treatment.

c) **Procedure for Maintaining Medical Records**
(i) Records are maintained on all animals that are brought inside including those that do not eat for more than 72 hours.
(ii) Entries are made in the daily log book by the staff and the Consulting Veterinarian maintains contact with the animal care staff and investigators.

d) **Preventative Medicine Programs**
(i) Vaccination
1. Mink and ferret kits are vaccinated at approximately 10 weeks of age against canine distemper, botulism, virus enteritis, and *Pseudomonas pneumonia*.
2. The vaccination is administered subcutaneously in the inguinal region using a 20 gauge needle. The Farm Manager and/or Farm Coordinator administer the vaccinations.
3. Research animals are exempt from the state requirement for rabies vaccination.

(ii) The herd is Aleutian virus-free and serological tests are run approximately every 5 years.

2) **Diagnostic Resources**
a) The Diagnostic Center provides diagnostic, necropsy, and histopathology support when needed.
b) Veterinary pathologists serve as consultants on postmortem interpretation.

3. Surgery

a. No major surgery is conducted at the farm.
b. The Principal Investigator, other researchers, or the Consulting Veterinarian occasionally performs non-invasive procedures at the facility's laboratory.

4. Pain, Distress, Analgesia, and Anesthesia

a. Agents Used
1) Ketamine HCl
a) Ketamine HCL is commonly used as an anesthetic for mink and ferrets.
b) The drug is administered under direct supervision of the Principal Investigator, other researchers or the Consulting Veterinarian.
c) The Principal Investigator, Farm Manager, Consulting Veterinarian, and full-time animal caretakers have experience in the use of this anesthetic.
d) The dose rate for Ketamine HCl (100 mg/ml) is 0.3 ml for adult females and 0.4 ml for adult males.
e) Ketamine HCl is commonly used for blood sampling from the jugular vein.

b. How Veterinarian Provides Input to Choice and Use of Drugs
1) Analgesics are administered according to an approved animal use form or as designated by the Consulting Veterinarian.

5. Euthanasia

a. Euthanasia is usually by carbon dioxide in a pre-charged chamber unless research protocols require other means of euthanasia (such as cervical dislocation) that are performed with approval of CAUC according to the Report of the American Veterinary Medical Association (AVMA) Panel on Euthanasia.

6. Drug Storage and Control

a. General Storage Arrangements for both Controlled and Noncontrolled Substances

1) Pharmaceuticals and antibiotics are stored in a locked room.
2) Inventory of therapeutic drugs is monitored routinely to eliminate expired products.
3) Because Ketamine HCl is a controlled substance, it is kept in a locked drawer of the Farm Coordinator's desk.

b. Record Keeping Procedures for Controlled Substances

1) Use of Ketamine HCl is recorded in a log book.

D. Physical Plant

1. Location and Construction

a. Location and Size of Animal Facility

1) The mink facility is located on 22 acres.
2) The facility is designed to conform to the standards of the commercial mink industry.
   a) There are 3 outside sheds and a lean-to that have pole barn roofs with screened-in walls to prevent entry of animals larger than rodents.
   b) The cages are suspended over manure pits bedded with pine shavings.
   c) There is a perimeter fence with sheet metal at the top that is designed to keep wild animals out and the resident animals in.

b. Functional Space for Animal Care

1) Mink-Shed 2 is used to house research animals when the research may have human health implications and thus is managed more intensely than other farm facilities.
2) Other farm facilities
   a) House 5 contains 2 17' x 30' rooms with heat and air conditioning.
   b) House 10 contains 2 22' x 38' rooms with heat.
   c) House 2 contains 4 10' x 15' rooms with controlled lighting and ventilation only.
   d) There are 2 60' x 19' open-air sheds that can hold 236 cages in Mink Sheds 1 and 3.
   e) There is 1 90' x 12' lean-to that can hold 92 cages (attached to Mink-House 11).
   f) The 3 sheds and lean-to are on a one-acre plot of land that is enclosed by a fence.

c. Support Areas

1) Quarantined animals will be housed in whichever facility will allow complete isolation of the animals.
2) Sick animals are kept in individual cages in isolation in Mink-House 11.
3) Major surgery would be conducted in the Veterinary Center.
4) Necropsies are to be done either in the laboratory in Mink-House 11 or in the necropsy facility of the Diagnostic Center depending on the circumstances.
5) Some experimental procedures such as perfusions prior to removal of organs would be done in a small room off of the toxicology laboratory.
6) Radiography would be done in the Veterinary Center.
7) Feed is prepared in Mink-House 11 in an 18' x 35' area.
8) Feed can be stored in 2 walk-in freezers (16' x 16' and 22' x 10') and 1 walk-in cooler (12' x 12') located in Mink-House 11.
9) Feed ingredients such as cheese and chickens are stored in the outside walk-in freezer.
10) Cereal storage is in a 12' x 33' area within Mink-House 11.
11) Bedding is stored in a 22' x 22' area of Mink-House 11.
12) Sinks, lockers, and toilets are in the Service Center.
13) The break area is in the Service Center.
14) Administrative space (11' x 10') is located in Mink-House 11.
15) Feed and animal (tissue) samples are stored frozen in 2 chest freezers in Mink-House 11.

d. Physical Relationship to Research Labs

1) Research and administrative areas are not located at the mink facility.

2. Procedures for Maintaining Security in Animal Housing Area

a. Building doors are locked after normal working hours. The Department of Public Safety regularly patrols the area.

NOTE: Any exceptions to the procedures listed above will be detailed in the appropriate animal use form.

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APPENDIX 4

Chain of Custody Form

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Final for Public Release

CHAIN OF CUSTODY RECORD – SAMPLES FOR ANALYSIS

Cooler # _____

Sampler(s): Printed Name and Signature		
Sample ID	Sampling Date	Remarks
Special Instructions/Comments:		

	Signature	Print Name	Company/Title	Date	Time
Relinquished by:					
Received by:					
Relinquished by:					
Received by:					
Relinquished by:					
Received by:					

CHAIN OF CUSTODY FORM


U.S. DEPARTMENT OF THE INTERIOR
MARCH 3, 1849


NOAA
NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
U.S. DEPARTMENT OF COMMERCE