

**AVIAN INJURY STUDY
AVIAN EGG INJECTION STUDY PLAN**

AMENDMENT FOR YEAR 2 (2007)

**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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EXECUTIVE SUMMARY

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.

As part of the NRDA, the Trustees have conducted several investigations focused on birds, including studies on Hudson River tree swallows (*Tachycineta bicolor*) in 1994-1995, bird egg preliminary investigations in 2002-2003, and avian injury investigations by the U.S. Geological Survey in 2004-2005. The Trustees also determined that it was appropriate to conduct an avian egg injection study and began such a study in 2006. Year 1 (2006) avian egg injection work focused on injection of test PCBs and development of injection and incubation protocols for eggs from tree swallow, American kestrel and chicken. The 2006 Avian Egg Injection Study Plan noted that these studies were projected to continue into a second year, and that work in Year 2 (2007) would be conducted pursuant to a Study Plan Amendment.

The Trustees performed a peer review of the work proposed for Year 2 and issued a Draft Study Plan Amendment for public review and comment, in accordance with the Hudson River NRDA Plan. All comments received on the Draft Study Plan Amendment, 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 Amendment to produce the Final Study Plan Amendment. In the remaining instances, public comments on the Draft Study Plan Amendment have been addressed by letters to the commentor, acknowledging receipt of comments and providing an initial response and noting that a more detailed Responsiveness Summary will be provided by the Trustees in the near future.

Pursuant to this Final Study Plan Amendment, the Trustees will conduct a study of tree swallows in 2007 to evaluate whether specific avian species in the vicinity of the Hudson River are injured due to exposure to PCBs. The work that will be conducted in 2007 includes: (1) evaluation of the effects of a PCB mixture relevant to tree swallows from the Upper Hudson River in a controlled egg injection study; (2) evaluation of the effects of *in situ* PCB exposure in Upper Hudson River hatchling tree swallows; and (3) a pilot study of injection of a PCB mixture into eggs of Eastern bluebirds (*Sialia sialis*).

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

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).

The Hudson River and surrounding area support more than 150 species of birds, including waterfowl, wading birds, shorebirds, songbirds, and rare species such as the bald eagle, peregrine falcon, and osprey (Andrle and Carroll, 1988). Birds are an integral part of the ecosystem and provide a number of important ecosystem services such as seed distribution, plant pollination, and insect control. Birds are also an important source of prey to other species. Birds may be exposed to PCBs through direct ingestion of contaminated water, sediment, and soil. A more important likely exposure pathway is their consumption of food items that contain PCBs derived from the Hudson River and its floodplain. PCB-contaminated food items linked to the river may include fish, amphibians, benthic invertebrates, adult insects that develop from aquatic larvae, plants growing in or near the river, and mammals that forage in the floodplain.

As part of the NRDA, the Trustees have conducted several investigations focused on birds, including studies on Hudson River tree swallows in 1994-1995 (McCarty and Secord 1999a and 1999b, Secord et al. 1999, Stapleton et al. 2001), bird egg preliminary investigations in 2002-2003 (Hudson River Natural Resource Trustees 2004a, 2005a, 2005b), and avian injury investigations by the U.S. Geological Survey in 2004-2005 (Hudson River Natural Resource Trustees 2004b, 2005c).

The Trustees also determined that it was appropriate to conduct an avian egg injection study and began such a study in 2006 pursuant to study plans (Hudson River Natural Resource Trustees 2006 and 2007a) that were, as appropriate pursuant to the Hudson River NRDA Plan (Hudson River Natural Resource Trustees 2002), subject to peer review and public review and comment.

Year 1 (2006) avian egg injection work focused on injection of test PCBs and development of injection and incubation protocols for eggs from tree swallow, American kestrel and chicken. The 2006 Avian Egg Injection Study Plan noted that these studies were projected to continue into a second year, and that work in Year 2 (2007) would be conducted pursuant to a Study Plan Amendment.

A Draft Study Plan Amendment (Hudson River Natural Resource Trustees 2007b) was developed, and that Draft Study Plan Amendment was peer reviewed and made available to the public for review and comment. All comments received on the Draft Study Plan Amendment, 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 Amendment to produce the Final Study Plan Amendment. In the remaining instances, public comments on the Draft Study Plan Amendment have been addressed by letters to the commentor, acknowledging receipt of comments and providing an initial response and noting that a more detailed Responsiveness Summary will be provided by the Trustees in the near future.

2.0 INTRODUCTION

This Final Study Plan Amendment is for Year 2 (2007) of an avian egg injection study.

The work presented here is a continuation of experiments with tree swallows (*Tachycineta bicolor*) initiated in 2006 (Hudson River Natural Resource Trustees 2006, 2007a). Previously tree swallow eggs were collected from two sites: Patuxent Research Refuge (Patuxent), Maryland and Great Sacandaga Lake, New York. These eggs were injected with PCB 126 in two separate experiments and incubated in the laboratory. Mortality and hatchability of the embryos were monitored and median lethal doses were estimated. The embryos (Patuxent) or hatchlings (Sacandaga) were dissected and tissues are being analyzed for a variety of histological and biochemical endpoints. In addition tree swallow eggs naturally exposed to PCBs in the Upper Hudson River were collected mid-incubation and incubated in the laboratory for the remainder of development. After hatch, chicks were dissected and tissues are being analyzed for a variety of histological and biochemical endpoints.

The next step is to evaluate the effects of a PCB mixture relevant to tree swallows from the Upper Hudson River in a controlled egg injection study.

3.0 PURPOSE AND OBJECTIVE

In 2007, the Trustees will conduct a laboratory and field study of tree swallows and a pilot egg injection study of Eastern bluebirds (*Sialia sialis*) to evaluate whether specific avian species in the vicinity of the Hudson River are injured due to exposure to PCBs.

This study will be used to evaluate whether the viability of avian resources is affected as a result of exposure to PCBs from the Hudson River. The work will inform the Trustees regarding injury to avian resources and guide their future efforts to identify pathway and specific injuries to birds from PCBs, determine causation, and scale restoration, as defined in the DOI NRDA Regulations. The work will be used to identify and evaluate the type(s) of injury(ies), if any, that PCBs are causing to Hudson River birds. This work will also be used to help determine whether future studies will be performed, and if so, to help in their design.

4.0 METHODS

The attached work plan entitled, "Final Work Plan for Tree Swallow Egg Injection Study" (Appendix A) describes the avian investigation that the Trustees will implement to evaluate whether specific avian species in the vicinity of the Hudson River are injured due to exposure to PCBs. The attached work plan includes information regarding the experimental design, Quality Assurance/Quality Control, and Standard Operating Procedures that will be used in the study. The Trustees have developed the design described in Appendix A for work in 2007 to evaluate the effects of exposure of tree swallows to PCBs, through exposure via avian egg injection or through environmental exposure in the field, and to evaluate, in a pilot study, the effects of exposure of Eastern bluebirds to PCBs through exposure via avian egg injection.

There are three components to the work: (1) an egg injection study to be conducted using tree swallow eggs from Patuxent, to be supplemented with additional tree swallow eggs from an upstate New York colony (Cobleskill Reservoir, New York), (2) a study of the effects of *in situ* PCB exposure in Upper Hudson River hatchling tree swallows, and (3) a pilot study of injection of a PCB mixture into eggs of Eastern bluebirds collected from Patuxent.

The PCB congeners to be injected into the tree swallow and Eastern bluebird eggs have been selected by the Trustees based on existing contaminants data from Hudson River biota and other relevant factors. Appendix B provides information on the PCB congener mixture to be used in the avian egg injections in 2007. The doses of the PCB mixture are designed to bracket the exposure of Hudson River birds and allow the determination of a median lethal dose for tree swallows exposed to the PCB mixture.

5.0 QUALITY ASSURANCE/QUALITY CONTROL

This study is being conducted in accordance with the Quality Assurance Management Plan for the Hudson River NRDA (Hudson River Natural Resources Trustees, 2005d).

Strict chain-of-custody procedures will be used throughout the study. All samples collected under this Study Plan will be maintained under chain-of-custody upon collection, and through processing, storage and shipment to the testing laboratory, analytical laboratory or archive facility.

Analysis will be by appropriate methods approved by the Trustees. Analytes may include congener-specific PCBs, including the non-*ortho* congeners, polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated diphenyl ethers (PBDEs), organochlorine pesticides, and metals, as determined appropriate by the Trustees.

In order to minimize analytical costs, and reduce the overall cost associated with the project, the Trustees may conduct the chemical or other analyses in stages, using initial work to inform subsequent decisions regarding which analyses to conduct on which samples.

The laboratories performing analytical work will be contracted to follow the Trustees' Analytical Quality Assurance Plan for the Hudson River NRDA (Hudson River Natural Resource Trustees (2005d). Laboratories will provide fully documented data packages which will enable data validation to be performed based on the criteria provided in the Analytical Quality Assurance Plan for the Hudson River NRDA, applicable laboratory Standard Operating Procedures, and the U.S. Environmental Protection Agency guidelines (1999).

6.0 SPECIAL PROVISIONS

Any necessary collection permits, such as those from New York State or Maryland where eggs will be collected, or from the U.S. Fish and Wildlife Service, will be obtained.

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APPENDIX A

FINAL WORK PLAN FOR TREE SWALLOW EGG INJECTION STUDY

**FINAL WORK PLAN
FOR
TREE SWALLOW EGG INJECTION STUDY**

HUDSON RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

June 1, 2007

Principal Investigator

Co-Principal Investigator

Quality Assurance Coordinator

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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1.0 INTRODUCTION & OBJECTIVES

The work plan presented here is a continuation of experiments with tree swallows (*Tachycineta bicolor*) initiated in 2006. Previously we collected tree swallow eggs from two sites with low polychlorinated biphenyl (PCB) contamination: Patuxent Research Refuge (Patuxent), Maryland and Great Sacandaga Lake, New York. These eggs were injected with PCB 126 in two separate experiments and incubated in the laboratory. Mortality and hatchability of the embryos were monitored and median lethal doses were estimated. The embryos (Patuxent) or hatchlings (Sacandaga) were dissected and tissues are being analyzed for a variety of histological and biochemical endpoints. In addition tree swallow eggs naturally exposed to PCBs in the Upper Hudson River were collected mid-incubation and incubated in the laboratory for the remainder of development. After hatch, chicks were dissected and tissues are being analyzed for a variety of histological and biochemical endpoints. Preliminary results from these experiments suggest potential adverse effects from PCB exposure and responsive end points that are proposed for measure in this study. The next step is to evaluate the effects of a PCB mixture relevant to tree swallows from the Upper Hudson River in a controlled egg injection study. These egg injection studies (2006 & 2007) could provide strong data for determining avian injury in that they encompass both single PCB congener and environmentally relevant PCB mixture exposure for tree swallows. Tree swallows are chosen for study because a suitable number of eggs can be collected, the tree swallows tolerate human disturbance (checking nest boxes and handling eggs) and there is a significant amount of published research on tree swallow natural history and biology.

The objectives of the 2007 work are as follows:

First, determine a median lethal dose for tree swallows exposed to an environmentally relevant PCB mixture. The PCB mixture will mimic the PCB profile found in eggs of tree swallows from the Upper Hudson River. Median lethal dose will be determined through probit analysis of mortality from an egg injection experiment using eggs collected from Patuxent. In addition, biochemical and histological endpoints will be evaluated in tissues from the exposed tree swallows.

Second, treat eggs from another site with presumed low PCB contamination with selected doses of the PCB mixture. This will serve as a comparison between different populations of swallows and increase the power of the overall study.

Third, obtain tree swallow eggs from the Upper Hudson River, Remnant 3 site, late in incubation. Eggs will complete incubation in the laboratory and hatchlings will be sampled. The additional tree swallows from the Upper Hudson River will be used to expand the sample size from 2006.

Fourth, conduct a pilot study on the effects of the PCB mixture on a limited number of bluebird eggs.

The proposed work for 2007 includes the following:

(1) Tree swallow eggs will be injected *in situ* at Patuxent and these injected eggs will be naturally incubated for the majority of incubation by the parents. This should provide excellent

hatching success when eggs are brought to the laboratory for incubation in the last one third of incubation as was observed with tree swallow eggs managed in this way in 2006. Eggs incubated in the laboratory starting from between day zero and day two of incubation had lower hatching success than those incubated for the first two thirds of incubation in the nest, before incubation in the laboratory. The eggs will be injected with a mixture of PCB congeners that mimics the spectrum of congeners found in tree swallow eggs in the Upper Hudson River. These data will provide a median lethal dose for the PCB and demonstrate effects of *in ovo* PCB exposure in tree swallow hatchlings.

(2) Additional eggs will be injected and collected from a second reference site at Cobleskill Reservoir, NY. These eggs will be used to increase sample sizes but this source is not expected to provide enough eggs to replicate an entire dose-response egg injection experiment.

(3) We will continue studies in tree swallow hatchlings from the Upper Hudson River. This will include collection of three eggs from each active nest at the Remnant 3 site. Three eggs will be collected at approximately day ten of incubation. One egg will be incubated in the laboratory until hatch at which time the chick will be sampled for biochemical and histological endpoints. The other two eggs will be analyzed for PCBs and will be representative of the PCB exposure of the chick that is sampled for biological analyses.

(4) The fourth portion of the work will be a pilot study of egg injection in Eastern bluebirds. The purpose of the Eastern bluebird work is to refine our methods for Eastern Bluebirds and to obtain preliminary data on the response of this species to the PCB mixture as a comparison to tree swallows and other species. Tissues collected from surviving hatchlings will also be used to validate biochemistry and histology assays for this species.

2.0 WORK PLAN

2.1 Study Species And Sites

Tree swallow (*Tachycineta bicolor*)

Eastern bluebird (*Sialia sialis*)

Tree swallow eggs will be collected under appropriate permits from Patuxent, MD, Cobleskill Reservoir, NY and Upper Hudson River, NY. Eastern bluebird eggs will be collected from Patuxent only.

Based upon available information, Patuxent is a historically uncontaminated site. Concentrations of PCBs and other contaminants have been low or non-detectable. Yorks (1999) found an average of 0.7 ± 0.25 SD (N=6) $\mu\text{g/g}$ PCBs in eggs collected in 1995.

2.2 Treatments

Eggs collected from Patuxent and Cobleskill Reservoir will be used in an egg injection study to determine the median lethal dose of a PCB mixture of a profile that is found in tree swallows from the Upper Hudson River. Eggs and embryos collected from the Upper Hudson River will be used to evaluate the PCB exposure and biochemical effects of the PCB exposure in tree

swallows. Tissues from the egg injection study will also be evaluated for PCB-associated effects on biochemistry.

2.3 Endpoints

The literature indicates potentially adverse effects associated with these measures following exposure to PCBs.

1. Embryo mortality

Lethality has the largest impact on fitness. However, incubation in the laboratory is less successful than natural incubation. Therefore, this study will incorporate incubation in the field and movement of the eggs into the laboratory late in incubation.

2. Deformities

Deformities are associated with PCB exposure in birds (Ludwig et al. 1996, Hoffman et al. 1998, Lavoie and Grasman 2007). Photographs will be taken of each embryo or hatchling that is scored for deformities.

3. Bursa Weight and Histology

Decreases in bursa weight and altered cellular morphology are strongly associated with PCB toxicity in chickens (Fox and Grasman 1999; Lavoie and Grasman 2007). Studies in quail have shown similar effects with exposure to other xenobiotics. Impacts on the bursa during B-cell development could result in reduced immunological fitness as nestlings and adults.

4. Liver Histology

Necrotic livers have been observed in chickens exposed to dioxin (Blankenship et al. 2003), suggesting that dioxin-like PCBs may have similar adverse effects. Analysis of liver histology will be conducted under a separate work plan with separate SOPs.

5. Heart histology

Recent literature (Dewitt et al. 2006) demonstrates an association between PCBs and heart deformities in passerine birds. Heart tissues will be collected and preserved as part of this study. Histological and other analyses of heart samples will be conducted under a separate work plan with separate SOPs.

6. Thyroid gland: Thyroxine content

Thyroid hormone balance is impacted by PCB exposure (McNabb and Fox 2003). A decrease in thyroxine reserve as reflected by thyroxine concentration in the gland at time of hatch could be detrimental to growth and survival because thyroid hormone plays a role in thermoregulation and metabolism. The former is especially critical for altricial species, which hatch without thermoregulatory control. Analysis of thyroid gland thyroxine content will be conducted under a separate work plan with separate SOPs.

7. CYP450 enzyme induction (liver)

PCBs have been reported to increase the content or activity of several enzymes in birds, including P450 isozymes (Hoffman et al., 1996a). For example, planar PCBs strongly induce the P450 isozyme CYP1A [measured by increases in aryl hydrocarbon hydroxylase (AHH) or ethoxyresorufin-O-deethylase (EROD) activity]. Analysis of P450 isozyme CYP1A in liver tissue will be conducted under a separate work plan with separate SOPs.

8. Oxidative Stress (liver)

In Hoffman et al. (1996b), for American kestrels there were some associations between oxidative stress (ox-red glutathione ratio) and increasing PCB 126. Liver tissues will be collected and preserved as part of this study. Analysis of oxidative stress markers in liver tissue will be conducted under a separate work plan with separate SOPs.

9. Body and organ (heart, liver and bursa) weights

Organ weights can be affected by PCBs in chickens. Body weight at hatch is generally not affected by *in ovo* PCB exposure (Lavoie & Grasman 2007). However, body weights and organ weights are important cofactors for understanding other endpoints, e.g., body weight may explain unusually small organ weights and organ weights may explain outliers in other analyses. Organ weights will be collected for the liver, heart and bursa.

10. Genetic sex

Blood samples for genetic sexing will be collected for this study, and genotyping will be analyzed by DDC Veterinary, Fairfield, Ohio. Gender is a possible cofactor in statistical analysis; furthermore, genotypic sex will confirm gender that cannot be determined from gonadal morphology if there are morphological changes such as intersex gonads.

3.0 EXPERIMENTAL DESIGN

3.1 Patuxent Egg Injection

Eggs and tissues will be collected, and birds will be handled, under permits from USFWS. Eggs will be assigned to treatment groups: untreated, vehicle injected or PCB injected on the day of injection. Assignment to treatment group will be made under guidance of our statistical consultant. Since we can only estimate the number of eggs available for the study, we will consider sample size, statistical power, sampling day, eggs per breeding pair, and estimated median lethal dose in determining treatment allocation. The goal will be to maximize the number of eggs per independent parent within each treatment group. We will prioritize treatment groups based on data from other avian studies and we will evaluate which treatment groups can be eliminated to maximize sample size and dose response. Approximately 150 eggs are expected to be available for injection (2-4 per nest from ≤ 50 nests).

To conduct the dose response to determine median lethal dose and effects on hatchling tree swallow physiology we plan to incorporate seven groups of the PCB mixture (untreated, vehicle injected (0) and five doses of PCBs). If we assume we can collect 150 eggs: a minimum of eight would be collected for contaminant analysis of background contaminant exposure. This will represent two eggs from each of four nests; eggs from a nest will be collected in one jar for

analysis. This leaves 142 eggs. Since we expect lethality to reach up to 100% mortality in the highest dose groups we will not expect to obtain biochemical data for the most lethal dose groups. However for those in which there is 60% or less lethality we will want to analyze tissues for biochemistry. The sample size distribution in the table below was generated to yield the best design for determining the median lethal dose of the PCB mixture based on predicted lethality.

The table below shows the proposed study design for 142 tree swallow eggs and seven treatment groups:

Treatment µg/g PCBs	# Eggs Injected	Predicted Lethality	Resulting Sample Size
Untreated	10 (no injection)	5%	9
0	27	15%	23
6	21	25%	16
12	21	35%	14
25	21	50%	10
50	21	75%	5
100	21	90%	2
Total	142		

A subset of tree swallow eggs will be assigned to PCB analysis to verify the eggs are suitable as a ‘clean’ source. At a minimum, 2 eggs from each of four nests (one nest per pond site) but potentially more eggs will be selected for PCB analyses. Compositing eggs for PCB analysis will be identified by a combined egg code, for example, 100+101-TRES-2007. Because the number of eggs available from Patuxent may be limited, additional eggs will be assigned to PCB analysis from eggs that fail to develop from the un-injected control group. Eggs containing embryos that die early in development will be archived, along with the eggshells (in separate jars).

Nests will be monitored for initiation of egg laying, clutch completion and initiation of incubation. Tree swallow eggs will be injected at embryonic day (ED) 2.5 and later collected at ~ ED10. We will follow the collection practices of Dr. Chris Custer (USGS), in which the monitored nests will be observed daily for eggs, which are laid at one day intervals. When the fifth egg is laid, then two eggs will be injected at ED2.5 for later collection; if a clutch is six eggs, we will inject and later collect three eggs instead of two. In this way, the female should not abandon the nest because three eggs will remain.

Standard Operating Procedure (SOP): “Egg Injection and Incubation Procedure for Tree Swallow Eggs at Patuxent Research Refuge and Cobleskill Reservoir: Nest Monitoring, Egg Injection, Egg Collection and Egg Incubation”, “Removal of Avian Egg Contents for Contaminants Analysis”.

Egg Incubation and Injection

Eggs will be injected at a time point approximately 18% (ED2.5) of incubation which is specifically defined in the SOP and the same as laboratory work in 2006. A series of numbers 1-199 will be used to identify eggs and embryos from Patuxent Research Refuge. The pointed end of eggs will be numbered gently with soft pencil. Eggs will be candled during incubation at least

once in the field after injection and upon receipt in the laboratory. At time of candling, any dead eggs (first week of development) will be removed and the egg contents will be archived. Any dead embryos (second half of incubation) will be evaluated for stage of development and deformities; abnormal embryos will be photographed, preserved, and archived.

SOP: “Egg Injection and Incubation Procedure for Tree Swallow Eggs at Patuxent Research Refuge and Cobleskill Reservoir: Nest Monitoring, Egg Injection, Egg Collection and Laboratory Egg Incubation”

Dosing Solutions

The PCB mixture solutions prepared in corn oil will be provided by Columbia Environmental Research Center at concentrations designed to deliver 0, 3, 6, 12, 25, 50 and 100 µg/g of egg when 0.4 µL/g of egg are injected.

Egg Hatching and Tissue Sampling

Any eggs that fail to hatch will be opened and condition of the embryo noted. Deformities will be scored for presence or absence of crossed bill, shortened upper bill, missing or deformed eyes, edema of the neck and head area, incomplete ossification of skull (brain not enclosed in skull), gastroschisis in post stage 45 embryos, malformed or clubbed feet, asymmetrical body form, malposition in the egg, and any other abnormal appearances shall be noted on the data sheet. Photographs of deformed and normal embryos and hatchlings will be taken for reference.

Embryos from eggs collected in the field and incubated in the lab will be dissected immediately after hatching. Samples from each tree swallow hatchling or tree swallow egg will be identified by a unique code (“sample I.D.”) encompassing the egg code, species, and year, e.g. 1-TRES-2007 for a tree swallow collected in 2007. Each tissue that is collected will be labeled with the complete sample I.D. such as (1-TRES-2007) and the name of the type of tissue: liver, bursa, heart or thyroid. Blood will be collected on sample cards provided by the contracted laboratory, and labeled with the sample ID.

SOP: “Necropsy of Hatchling Tree Swallows”

3.2 Cobleskill Reservoir Embryo Collection

The Cobleskill Reservoir provides another potentially clean site in which the PCB mixture doses will be administered by injection into tree swallow eggs according to the experimental procedures used at the Patuxent site. Nests will be monitored at the Cobleskill Reservoir for initiation and completion of egg clutch. Clutch size and date of initiation of incubation will be noted. Eggs from the Cobleskill Reservoir will be injected per the plan and SOPs for Patuxent. They will be collected at the same time point (~day 10) and stored in a Koolatron brand cooler set at temperature suitable to maintain the eggs for the drive to the laboratory. Contaminant analysis will be conducted on 2 eggs from each of three nest boxes for a total of three samples, with eggs from a nest being collected into one jar for analysis. Compositing eggs for PCB analysis will be identified by a combined egg code, for example, 200+201-TRES-2007.

Eggs and tissues will be collected, and birds will be handled, under permits from USFWS. Eggs will be assigned to the same treatment groups: untreated, vehicle injected or PCB injected on the

day of injection. Assignment to treatment group will be made under guidance of our statistical consultant. Since there will not be as many eggs available at Cobleskill as at Patuxent, we will build upon the experimental design for Patuxent by increasing the sample size in each treatment group. Once the desired sample size for each treatment group is attained, additional eggs may be assigned to another group: 3 µg/g.

All procedures will be the same as for eggs from Patuxent with two exceptions: 1) Eggs will be numbered in a range of 200 to 399 and 2) when eggs are collected they will be transported for an 8-10 hour time period prior to receipt at the laboratory.

3.3 Upper Hudson River Embryo Collection

Nests will be monitored at the Remnant 3 site for initiation and completion of egg clutch. Clutch size and date of initiation of incubation will be noted.

Nests will be observed daily for eggs, which are laid at one-day intervals. At approximately day ten of incubation three eggs will be collected from each nest. Two of these eggs will be preserved for PCB analysis and the third will be transported to the laboratory for incubation and hatch.

In tree swallow eggs, PCB contamination appears not to be affected by egg order (personal communication Drs. Custer). A sample of two eggs per clutch is necessary for determining contaminant levels both in terms of accurate reflection of variation of contaminants within the clutch (Reynolds et al. 2004) and for enough sample volume for all analyses. These eggs will be analyzed for contaminants according to the same methods used for the Patuxent eggs.

Samples from each tree swallow hatchling or tree swallow egg will be identified by a unique code (“sample I.D.”) encompassing the egg code, species and year, e.g. 1-TRES-2007 for a tree swallow collected in 2007. The series of numbers starting at 400 and higher will be used for eggs and embryos from Upper Hudson River, Remnant 3 site. Compositing eggs for PCB analysis will be identified by a combined egg code, for example, 400+401-TRES-2007.

SOP: “Field Collection of Tree Swallow Eggs from Upper Hudson River, New York for Injury Assessment Hudson River NRDA”

Hatchling Sampling

Procedures for sampling hatchlings from Upper Hudson River sites will be consistent with procedures used in 2006.

SOP: “Necropsy of Hatchling Tree Swallows”

3.4 Eastern Bluebird Pilot Egg Injection Study

For Eastern bluebirds, we expect a maximum of 30 eggs. Therefore, we will have only four treatment groups: untreated, zero, medium and high doses of the PCB Mixture.

The table below shows a possible study design for 30 eggs and four treatment groups:

Treatment µg/g PCBs	# Eggs Injected	Predicted Lethality	Resulting Sample Size
Untreated	4 (no injection)	5%	3 or 4
0	8	15%	6
25	9	50%	4 or 5
100	9	75%	2

Because this is a pilot study, no eggs will be collected for contaminants analysis. The sample ID on the data sheets and on the tissues uses EABL to denote eastern bluebirds. The SOPs for the TRES will be used in this pilot study.

Standard Operating Procedure (SOP): “Egg Injection and Incubation Procedure for Tree Swallow Eggs at Patuxent Research Refuge and Cobleskill Reservoir: Nest Monitoring, Egg Injection, Egg Collection and Laboratory Egg Incubation”. “Necropsy of Hatchling Tree Swallows”

3.5 Biological Tissue Analyses

Histological: Bursa, heart and liver tissue will be preserved in appropriate fixatives. Bursa tissues will be embedded, sectioned and stained by standard methods. Slides will be labeled and well organized for retrieval and review. The SOP for the heart and liver histology will be described and conducted under separate Work Plans.

Gender genotyping will be performed on blood collected on cards using polymerase chain reaction (PCR) techniques. SOPs and resulting data will be reviewed for adherence to QA/QC requirements.

Thyroid glands from each hatchling will be collected and stored at -80° C in a microcentrifuge tube. Analysis of thyroid gland thyroxine content will be conducted under a separate work plan with separate SOPs .

Livers will be divided into three (3) parts. One portion will be fixed for histology work, and the two remaining parts will be frozen in separate cryovials. One vial will be used for the measurement of cytochrome P450 activity in liver microsomes by EROD assay. The second vial will be used for measurement of oxidative stress markers. The procedures for these measurements will be described and conducted under a separate Work Plan.

3.6 Statistical Analyses

Data will be analyzed following examination of normality and proceeding with parametric ANOVAs or non-parametric tests, and regressions as appropriate. Mortality data will be analyzed with Fisher Exact Probability test and probit analysis for determining median lethal doses. When necessary, further analyses would be used to understand the significance of dose-responses and non-monotonic trends. If the predictions warrant the use of one-tailed tests, these tests will be used with consultation with our statistician. Additional tests may include bootstrap techniques if data are not normally distributed and sample sizes are low.

The Principal Investigators (PIs) plan to conduct the following comparisons. Null (HO) and alternative (HA) hypotheses are presented below. “PCBs” and “exposed to” refer to the PCB mixture for eggs injected or natural PCB exposure for birds from the Upper Hudson River. “Controls” refers to either uninjected/vehicle injected eggs in the egg injection study or eggs and birds from the reference sites for the field study. “Birds” represents any life stage for which an endpoint is measured.

3.6.1 Compare the embryo survival or hatchability of eggs exposed to PCBs with eggs that are not exposed to PCBs.

- General Hypotheses

HO: Hatchability of eggs injected with the PCBs is equal to the hatchability of control eggs

HA: Hatchability of eggs injected with the PCBs is less than the hatchability of control eggs in a dose response manner

- Statistical tests

Fisher Exact probability tests and probit analysis will be used for determining significant decreases in survival or hatchability and for determining median lethal doses.

3.6.2 Compare occurrence and severity of deformities between PCB exposed embryos and unexposed embryos.

- General Hypotheses

HO: The occurrence and severity of deformities are equal in control and PCB exposed embryos

HA: The occurrence and severity of deformities are increased in PCB exposed embryos compared to controls

- Statistical tests

Fisher Exact probability tests and probit analysis will be used for determining significant increases in deformities and for determining median effect concentrations.

3.6.3 Compare histology of bursa, liver and heart of PCB exposed birds to unexposed birds.

- General Hypotheses

HO: Bursa, liver and heart morphology in PCB exposed birds are not different than controls

HA: Bursa, liver, and heart morphology in PCB exposed birds are different compared to controls and are proportionally related to the dose of treatment

- Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, histological indices of morphology will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test will be used to evaluate dose related effects.

3.6.4 Compare thyroxine (T4) content of thyroid glands from PCB exposed birds to that of unexposed birds.

- General Hypotheses

HO: Thyroid hormone (T4) content of thyroid glands in PCB exposed birds is not different than controls

HA: Thyroid hormone (T4) content of thyroid glands in PCB exposed birds differs from controls and is proportionally related to the dose of treatment

- Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, T4 concentrations will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test shall be used to evaluate dose related effects.

3.6.5 Compare liver EROD activity of PCB exposed birds with unexposed birds.

- General Hypotheses

HO: Liver EROD activity in PCB exposed birds is not different than controls

HA: Liver EROD activity in PCB exposed birds is increased compared to controls and is proportionally related to the dose of treatment

- Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, organ weights will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test shall be used to evaluate dose related effects.

3.6.6 Compare oxidative stress in liver samples from PCB exposed birds to that of unexposed birds.

- General Hypotheses

HO: Oxidative stress level in PCB exposed birds is not different than controls

HA: Oxidative stress level in PCB exposed birds is higher than controls and is proportionally related to the dose of treatment.

- Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, oxidative stress indicators will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test shall be used to evaluate dose related effects.

3.6.7 Compare organ (heart, liver and bursa) weights of PCB exposed birds with unexposed birds.

- General Hypotheses

HO: Organ weights in PCB exposed birds are not different than controls

HA: Heart and liver weights in PCB exposed birds are higher compared to controls and are proportionally related to the dose of treatment

HA: Bursa weight in PCB exposed birds is lower compared to controls and is proportionally related to the dose of treatment

- Statistical tests

Data will be examined for normality and homogeneity of variances as necessary for the tests used. If assumptions of normality and homogeneity of variances are met, organ weights will be compared across treatment groups and sex, and possible interactions examined, using 2-way ANOVA with appropriate post hoc comparisons. Alternately, data transformations will be used as required or appropriate non-parametric tests shall be used. Furthermore, regression analyses with estimates of confidence intervals or dose-response statistics such as the Jonckheere test shall be used to evaluate dose related effects.

These hypotheses and statistical tests may be revised, or not performed by the PIs based on data collected. Further, the PIs may test other hypotheses and conduct additional statistical tests not noted above.

4.0 QUALITY ASSURANCE/QUALITY CONTROL

4.1 Data Quality Objectives, Indicators, and Assessment

4.1.1 Overview

This study is being conducted in accordance with the Quality Assurance Management Plan for the Trustees' Hudson River NRDA. As described in the plan, four general elements of quality assurance/quality control (QA/QC) must be addressed for each data collection effort:

- Project Management
- Data Generation and Acquisition
- Assessment and Oversight
- Data Validation and Usability

This section describes the Quality Assurance Plan (QAP) for the avian egg injection study, based on these four general elements. The objectives of the study are outlined in Section 1 of this Work Plan. To achieve these objectives, the following requirements must be met:

- All samples, from the initial eggs through embryos, hatchlings, dead or infertile eggs, necropsy samples, and egg products must be identified and stored following documented procedures to insure proper identification and handling.
- All procedures for assessment of biological impacts, including egg injections, necropsy, and biological tissue analyses, must be performed following documented procedures to ensure consistent, comparable data.
- PCB mixture preparation and egg contaminant levels: The laboratories performing chemical contaminant testing will follow the requirements of the Hudson River NRDA Analytical QA Plan. This effort is not part of the current work plan and will be funded separately.

4.1.2 Project Management

The study team is organized based on tasks and levels of responsibility to ensure good communication between all personnel. The Assessment Manager (Kathryn Jahn, USFWS) has overall project oversight responsibility and provides direction to the Quality Assurance Coordinator. The Assessment Manager also provides direction to the Principal Investigator and Co-Principal Investigator, via the Project Coordinator. The Project Coordinator is responsible for ensuring that adequate coordination and communication occurs amongst the Assessment Manager, Quality Assurance Coordinator, Principal Investigator or Co-Principal Investigator. The Principal Investigator and Co-Principal Investigator are responsible for the project's design and implementation and provide guidance and technical expertise as needed to the study team and statistician. They will also work with the Project Coordinator and Quality Assurance Coordinator to ensure that the study is consistent with the overall QA objectives of the NRDA.

The work plan was developed to provide detailed and explicit instructions for the research staff to follow in collecting the study data. The plan has been reviewed, commented on, and approved by key parties to the study. Reliance on a detailed, explicit, and fully reviewed plan ensures that:

- Study objectives, methods, procedures, and details are documented.
- Data are collected in a systematic and consistent way throughout the study.
- Each member of the study team adheres to the requirements of the plan. In particular, the Principal Investigator and Co-Principal Investigator must ensure that their research staff adheres to the plan. Each team member is required to sign a statement that they have read the plan and understand it.

Events may arise during this study that require changes to the procedures documented in the work plan. Deviations from the work plan will be documented in writing, with a detailed explanation of the reasons for these deviations. Predetermined deviations from the plan will be conducted only after the approval of the Principal Investigator or Co-Principal Investigator.

4.2 Data Generation and Acquisition

4.2.1 Data Quality Objectives

Data developed in this study must meet standards of precision, accuracy, completeness, and comparability, and be consistent with sound scientific methodology appropriate to the data quality objectives (DQOs).

Precision is the degree of mutual agreement among individual measurements of the same property under similar prescribed conditions, such as replicate measurements of the same sample. Precision is concerned with the “closeness” of the results. For this study, repeated independent measurements will be performed to assess the precision of several biological assays. Precision will be expressed as the relative standard deviation (RSD) between these replicate measurements on a single sample, and for the hormone assays, will be expressed as Coefficient of Variation.

Accuracy is the degree of agreement of a measurement with an accepted reference value and may be expressed as the difference between the two measured values or as a percentage of the reference value. For this study, evaluation of accuracy will be performed using a positive control sample or reference standard as specified in the SOP for each biological end point.

Completeness is defined for this study as the percentage of the planned data collections compared to data actually collected within the work plan specifications. Because there is uncertainty due to the variables in number and viability of available eggs and hatchlings, the assessment of completeness achieved will be assessed in two ways. First, completeness will be assessed by comparing planned sampling versus samples collected at the end of the study. Secondly, the DQO for completeness of data analysis is 95%, which pertains to no more than 5% of the data points collected are to be rejected as unreliable.

Comparability is defined as the measure of confidence with which results from this study may be compared to another similar data set. For this study, evaluation of comparability will be performed using external reference standards or an internal standard prepared from a serum pool

extract or a standard prepared within our laboratory, aliquoted and frozen into individual units for utilization within each assay as an internal quality control measure. These comparisons will also take into consideration inter-assay variability due to reagent differences. For example, antibodies used in hormone assays may differ in the forms of their cross reactivity with closely related hormones thereby providing differing absolute concentrations.

4.2.2 Study Documentation

All study procedures and results will be documented on data sheets, which will be placed in binders and retained for review. To the extent possible, information will be recorded on pre-formatted data sheets. The use of pre-formatted data sheets is a QA/QC measure designed to:

- ensure that all necessary and relevant information is recorded for each sample and each sampling activity
- serve as checklists for the Principal Investigator, Co-Principal Investigator and their staff to help ensure completeness of the data collection effort
- assist the research staff by making data recording more efficient
- minimize the problem of illegible or hard-to-follow notebook entries

The researcher performing each procedure will be responsible for recording information on data forms.

Data entries will be made in waterproof ink, and corrections will be made with a single line through the error accompanied by the correction date and corrector's initials. Each completed data sheet will be reviewed, corrected (if necessary), and initialed by the Principal Investigator, Co-Principal Investigator, or their designee. Following completion of the study, data sheet originals will be retained by the laboratory.

4.2.3 Sample Identification Procedures

Strict sample identification procedures will be used throughout the study. The sample identification procedure will begin when an egg is collected. Each egg will be identified by a unique egg code.

The four-letter code of TRES will be used for tree swallow samples. Each egg will be assigned a unique egg code as follows: Series of numbers 1-199 for Patuxent Research Refuge, 200-399 for Cobleskill Reservoir, NY and 400 and higher for Hudson River. Samples from each egg/embryo will be identified by a sample ID encompassing the egg code, species, and year, e.g. 1-TRES-2007. Sampling of embryos and hatchlings will include body weight, organ weights, and collection of tissue.

The sample identification described above will be recorded on all data sheets used to document all procedures. This identification along with tissue type will be transferred to all other sample types originating from the egg, including hatchlings (live and sacrificed), and necropsy samples.

The sample ID will be used to uniquely identify all samples, either on a label or written directly on the container. The code will be recorded using a waterproof marker. If applicable, the label should also include the type of sample and date of collection and researcher's initials.

4.3 Assessment and Oversight

The QA management plan specifies that studies that generate data will be audited to ensure that the project-specific plans are being properly implemented. Several mechanisms for internal audits of the data generation process will be used for the avian egg injection study. These mechanisms include:

- A project management structure that defines clear lines of responsibility and ensures communication between researchers and trustees. Clear responsibilities and communication can serve as a means of providing internal audits of the study as it proceeds.
- A requirement that laboratory notebooks and data forms be completed daily and be reviewed weekly by the Principal Investigator or Co-Principal Investigator.
- The use of pre-formatted data sheets that serve as a checklist for study procedures and assay results.
- The Quality Assurance Coordinator will conduct an audit of the procedures and documentation of the study.

4.4 Data Validation and Usability

This study employs documented, repeatable procedures to perform the experiments and assays required to generate the data for this study. The work plan has been reviewed for the adequacy of the design and proposed methodology. The original data sheets and other study records will be maintained by the laboratory and archived for a minimum of eight years. Disposal of these records will require the approval of the Assessment Manager. Findings from this study can be reviewed against the data sheets to ensure that the data presented in the reports represent complete and accurate information. Chemistry contaminant data will be validated as specified in the Analytical QA Plan.

The Principal Investigator or Co-Principal Investigator will perform oversight of all egg injections and data collection for measurement endpoints. They will validate that Project Scientists and Technicians are correctly following the standard operating procedures and correctly documenting the results.

Data analysis will be performed using JMP IN version 5, release 5.1, SAS Institute Inc and SAS programming but not be limited to these statistical programs. All numeric data presented in reports will contain basic statistical properties and uncertainty. The robustness of each parameter studied will be presented.

4.5 Chain of Custody Procedures

Chain of Custody (COC) procedures will be used during the field sample collection and transfer to the laboratories for incubation or analysis. The purpose of COC is to assure the integrity of each sample and be able to clearly identify who was responsible for the sample at each step. The COC procedure will begin when an egg is collected from the nest. That collection is documented on field data forms (Avian Egg Collection Data Sheets), which clearly identify the team member(s) responsible, as well as the date and time. The egg collection forms will clearly identify to whom the sample was delivered for further processing, and will also include the date and time.

The immediate team members are personally responsible for the care and custody of the samples that are in their possession. A sample is in custody of the immediate team member if any of the following occur:

- The sample is in the individual's physical possession;
- The sample is within view after being in possession;
- The sample is in a locked or sealed container that prevents tampering after being in possession; or,
- The sample is in a designated secure area.

When the samples are packed in coolers or other containers for shipment to the laboratory or storage facility, completed COC records will accompany the samples. The COC form will contain the following information:

- Project name;
- Sample identification (unique for each sample);
- Sample matrix (e.g., egg contents, liver) which may be part of the sample ID;
- Name and signature of individual relinquishing custody;
- Name and signature of individual accepting custody;
- Sample shipping date and mode.

Other information such as date of sample collection, collection location, and jar sizes may be on the COC form or on accompanying documentation.

An original COC record for the samples in that cooler will accompany each shipping container. All sections of the COC form will be completed. Indication of the number of coolers per shipment (e.g., 1 of 3) will be listed on the form if more than 1 container is shipped. Once the form is completely filled out, it will be placed securely inside the cooler (in a plastic sealable bag to keep it dry). Field personnel will maintain a copy of the COC to keep with the air bill. The

cooler will be sealed with custody seals or the containers inside the cooler may be sealed with custody seals. Custody seals are used to detect unauthorized tampering with samples after sample collection until the time of use or analysis. Signed and dated gummed paper seals may be used for this purpose. The seals will be attached so that they must be broken to open the shipping container. Each cooler will be sturdy and well sealed with strapping or other tape. All samples will be kept in locked locations or with custody seals at all times until shipped.

An air bill, Federal Express shipping label, etc. can be used to document the transfer of a sample from the field team to an intermediate storage location, the analytical laboratory, or archive freezer.

Coolers or other containers containing samples will be opened at the analytical laboratories or archiving facility only by a person authorized to receive the samples. The containers will first be inspected for integrity of the chain of custody seals or other signs of tampering. The receipt of each sample in the coolers or containers will be verified on the COC forms. The signed COC forms will be photocopied, and the photocopy will be mailed to the sending party. Samples will be stored in a secure area according to procedures documented for each analytical facility.

5.0 PRINCIPAL INVESTIGATORS

Principal Investigator

The Principal Investigator is a neuroendocrinologist with twenty-five years of experience studying avian neuroendocrinology and reproduction. The Principal Investigator will oversee all aspects of the studies.

Co-Principal Investigator

The Co-Principal Investigator is an avian toxicologist with experience in egg injection studies and immune and endocrine disruption studies in birds. The Co-Principal Investigator will plan the logistics of all aspects of the study and participate in assays, data collection and data analysis.

Research Technicians with expertise in endpoints required as part of the study will conduct assays and analyze data as needed.

Graduate and Undergraduate Level Students will assist with animal care, sampling and assays as needed.

The full names, contact information, written signature and written initials of all individuals working on this project shall be maintained in the project file.

6.0 LITERATURE CONSULTED

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7.0 STANDARD OPERATING PROCEDURES

7.1 Recording and Handling Data for Avian Egg Injection Study

This protocol describes procedures for recording and handling data in this laboratory.

Procedure

- 1) Data sheets are in the study binder.
- 2) Data entry:
 - Entries will be made in ink.
 - All blank cells in the sheets should be filled with data, or marked with "NA". Large areas left blank (such as the bottom part of a partially-filled sheet) should be crossed out.
 - Any changes will be made by crossing through the error with a single line, and initialing and dating the change.
 - Data recorder will date and initial each sheet; the sheets will contain documentation such that each individual performing the injections/measurements can be identified.
- 3) After hard copies of data sheets are filled out they must be reviewed by the Principal Investigator or Co-Principal Investigator then stored in the project notebook in the Co-Principal Investigator's office.
- 4) Data should be input as soon as possible, after collection, into electronic files (Excel or JMP) and files stored on the Principal Investigator's or Co-Principal Investigator's computers. Data entry must be 100% verified against the hard copy by someone other than the person who performed the initial data entry.
- 5) Back-up copies should be made to a CD after any additions or changes to files are made. A back-up copy of data on CD will be made weekly and will be stored at the homes of the Principal Investigator or Co-Principal Investigator.
- 6) Any deviations from the protocols will be written out in detail by the Principal Investigator and added to the project notebook.

7.2 Removal of Avian Egg Contents for Contaminants Analysis

Introduction

Avian eggs are a common sample for contaminants analysis. An accurate analysis depends upon getting the egg contents from the shell to a clean sample jar without introducing other sources of contamination. This protocol, which has been developed and refined by many researchers over the decades, was written for those who have minimal experience. Your first egg should be a practice egg. *It is suggested that all personnel practice on several quail eggs to improve technique. Chicken eggs may be used if quail eggs are not available.*

Laboratory Materials And Equipment

- ❑ Avian Egg Processing Data Sheets
- ❑ paper or other towels
- ❑ green scrubby or sponge
- ❑ Acculab V-200 balance, weighs to nearest 0.01 gm
- ❑ calipers
- ❑ Chemically-clean jars, 1 per sample
 - ✓ Make sure they are cleaned for the contaminants you are sampling, e.g., I-Chem pesticide/PCBs Series 200 or 300.
 - ✓ Size: 4 oz.
- ❑ chemically-clean stainless steel scalpel blades (No. 21 or No. 22 with No. 4 handles work well)
- ❑ chemically-clean forceps
- ❑ aluminum foil sheets (approximately 30 x 30 cm square), 1 per egg
- ❑ sharps container for used blades or disposable scalpels

Laboratory Procedures

- ❑ Fill out the Avian Egg Processing Data Sheet; use one data sheet per egg.
- ❑ If debris is present, rinse egg in cool water while gently scrubbing with green scrubby or sponge. Do not soak the egg.
- ❑ Dry and weigh whole egg to the nearest .01 g
- ❑ Transfer egg contents to chemically-clean jar using the following procedure:
 1. Use nitrile gloves for this part of the procedure. Avoid letting contents run over your hands into the sample jar. Note that the two eggs collected from a nest will both be placed in one jar for analysis.
 2. Create a catch basin out of the aluminum foil by turning edges up and securing the corners. This will catch egg contents in case they spill over the edge of the jar. Use a separate piece of foil for each sample. The foil also is a clean place to place your instruments when they are not in use.

3. Weigh the clean empty jar with lid on, and note this tare weight on data sheet.
4. Place jar in center of aluminum foil, and loosen the lid.
5. Score equator with serrated blade or scalpel blade. Use a new, chemically-clean scalpel blade for each egg. This part takes practice. Cradle the egg in one hand (don't squeeze too tightly!) and gently score while rotating the egg. Many light strokes are preferable to a fewer deeper strokes, increasing the evenness of the score and decreasing the possibility of eggshells not separating cleanly or of punching through the shell. Continue to work on your score until you see the membrane, which usually appears gray underneath the white of the eggshell. When you see the first bit of membrane, remove the lid from the jar so that it will be ready as soon as you need it. Avoid getting shell dust, or anything else besides the egg contents, in the jar. Try to expose the membrane evenly around the entire egg. Often the score line can be used to help pick the egg shell apart using forceps.
6. Place the egg over the jar and cut through membranes with the scalpel. For large eggs a new scalpel blade may be used at this point to reduce the potential for cross contamination and since the blade may become dull during the cutting process. The scalpel can also be used to finish scoring down to the membranes. Pour contents into jar, or use the scalpel to gently scrape if that is necessary. Small stainless steel scoops may also be used to help remove the contents. Use forceps to remove any shell fragments from the jar. Cover the jar.
7. For swallows, hold the egg vertically with air cell end up. Using scissors cut the top of the eggshell just below the air cell. Pour contents into the jar, and use a pipet to gently collect egg contents that don't freely flow out. Use forceps to remove any shell fragments from the jar. Avoid getting shell dust, or anything else besides the egg contents, in the jar.
8. Save the egg shell and associated membranes separately in a second I-chem jar labeled with the same sample ID as for the egg contents and note 'egg shell' on the jar.
9. The target for the minimum weight of egg tissue is 4 grams for analysis. It may be possible to analyze smaller samples ranging from 1 – 2 grams. Analysis of these samples may result in a lower ability to detect contaminants due to the lack of mass. An effort must be made to maximize the amount of each sample that is usable. The weight of each sample should be made in the laboratory during egg processing using the following procedure:
 - a. Place a small jar on a balance that reads to at least 1 milligram and that has been appropriately calibrated.
 - b. Tare the jar or record the jar weight if the balance cannot be tared.
 - c. Open the egg, according to the procedures referenced above and empty the contents into the jar.
 - d. Record the weight, to the nearest .01g, of the egg contents if the balance was tared. If the balance was not tared, then record the weight for the egg contents and the jar, then subtract the previously

recorded weight of the jar. Record the weight of the egg contents in the field notebook and on the jar label.

- e. If egg is developed, estimate age of embryo. Documentation of embryo development is very limited (Powell *et al.* 1998; Bird *et al.* 1984), therefore, documenting this phase of the egg processing is important. Note amount of decay or anything else pertinent to your study, and examine for deformities, particularly bill deformities such as crossed bills or lack of jaws, but also lack of skull bones, club feet, rotated ankles, or dwarfed appendages (Gilbertson *et al.* 1991).
 - f. Repeat these procedures for any other eggs that need to be added to the sample jar.
- ✓ Do not touch or move the jar between steps b. and d. above. It is preferable to add the egg contents to the jar while the jar is still on the balance, immediately after taring the jar.
 - Place label on jar. Place clear tape over the label to keep it from getting wet.
 - Prepare Chain of Custody records and maintain egg samples under chain of custody.
 - Freeze samples. Ship under Chain of Custody (see attached COC form) overnight on dry ice to the sample archive or analytical laboratory.

Literature Consulted

Bird, D.M, J. Gautier, and V. Montpetit. 1984. *Embryonic growth of American kestrels*. Auk 101:392-396.

Gilbertson, M., T. Kubiak, J. Ludwig, G. Fox. 1991. *Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease*. J. Toxicol. Environ. Health 33:455-520.

Powell, D.C., R.J. Aulerich, R.J. Balandier, K.L. Stromborg, and S.J. Bursian. 1998. *A photographic guide to the development of double-crested cormorant embryos*. Colonial Waterbirds 21(3):348-355.

These egg-processing guidelines were developed by the U.S. Fish and Wildlife Service and modified for the project based on consultation with the author of these guidelines and on conversations with the Quality Assurance Coordinator for this project.

AVIAN EGG PROCESSING DATA SHEET

Processor(s): Name _____

Name _____

Signature _____

Signature _____

Date Processed: _____

Egg Code: _____

Sample ID: _____

Jar lot number _____ Balance within limits? Yes OR No

Whole Egg Weight (g): _____

Contents weight:

Weight of jar (g) : _____

Weight of jar + contents (g): _____

Weight of contents (g): _____

Membrane location: ___ with embryo OR ___ with eggshell

Contents condition (embryo development ¹, state of decay, etc.) and other comments:

Other comments: _____

Contaminants disposition (catalog number and date submitted, etc):

¹ None, 1/4, 1/2, 3/4 , full term

Data Sheet checked by: _____ Date: _____

Name/Initials

PROCEDURES FOR NRDA SAMPLE ARCHIVE SHIPMENT

Chain-of-Custody (COC)/Transfer Record

1. You may use the original Chain-of-Custody if has at least one set of “Relinquished By” and “Received By” lines remaining.
2. If the original COC is not available, a new COC or Transfer Record (form is attached to these instructions) shall be prepared. List each sample or inventoried container (e.g., rack of vials, etc.) on a separate line, identifying with original field ID, laboratory ID and description or other identifier. (A Transfer Record is attached to this procedure).
3. Record on the COC, as appropriate:
 - Volume or quantity of sample, if available
 - Comments – apparent preservation problems or custody concerns
4. Cross check all sample identifiers from container to COC before packaging the samples.
5. Sign and date the “Relinquished By Signature” block on the COC. Make a copy for your records. Place the COC in a ziplock bag and tape it to the inside lid of the appropriate cooler.

Packaging/Shipping

1. Sample shipments are best made early in the week. Do not ship samples on Friday unless specific arrangements have been made with the courier and Receiver for Saturday delivery.
2. Wrap or package each item, as appropriate and place in cooler/package. Bubble wrap is a good cushion. **Dry ice or other coolants are not cushioning material, because the jars will become loose as the ice melts or evaporates.**
3. Place coolant, e.g., dry ice, wet ice or frozen gel packs (see below for dry ice info) in the cooler *so that the contents will remain at temperature for a minimum of 48 hours.*

For Dry Ice:

- **You must use < 5 kg/package**
 - **The drain plug on the cooler must be taped open for ventilation of carbon dioxide (CO₂) gas that occurs when the dry ice vaporizes**
 - **Indicate that dry ice has been used as a coolant on the shipping documents, however, when less than 5 kg is used as a coolant, dry ice is not considered a “Dangerous Good”.**
4. Seal the lid shut. Wrap duct/shipping tape around either end of the cooler (three times) to ensure a tight seal.

5. Place a minimum of two COC seals on the cooler in such a manner that if the container was opened, the seals would have to be broken.
6. Sign and date the COC seals, which are placed on the outside of the cooler. The same person who signed the COC record should do this.
7. Place clear shipping tape over the COC seals.
8. Adhere the appropriate address label on the top, outside surface of the cooler with clear shipping tape.
9. Fill out appropriate shipping documents:
 - Coolers are to be sent by Federal Express Priority One-Day service or a comparable, traceable service.
 - The cooler/package should be sent to:
"Receiving Contact Name" at:
NOAA Building 32
7600 Sand Point Way NE
Seattle, WA USA 98115-0070
 - The contact phone number on the airbill should be Receiving Contact's number
10. Fax or email a Notification of Shipment Form (attached to this procedure) to Receiver. Receiver will:
 - Coordinate receipt with NOAA Shipping and Receiving Department, on the day of arrival
 - Sign the COC in the "Received By Signature" block
 - Make sure that the cooler/package(s) are placed into the archive freezer at NOAA ARD West (Bldg. 32).

APPENDIX A: CHEMICALLY-CLEAN INSTRUMENTS FOR COLLECTING CONTAMINANTS SAMPLES

To minimize cross-contamination when collecting biological samples for contaminants analysis, a primary requirement is use of chemically-clean instruments. These are made of appropriate materials (stainless steel or teflon) and rinsed with alcohol and solvents to remove contamination and organics. Once rinsed, the instruments should be treated as sterile instruments, e.g. not placed on unclean surfaces.

Because every laboratory situation is different, this document tells you what to do, but not how to do it. The chemicals used for rinsing are hazardous, so you should follow proper safety and laboratory protocols when using them. This includes proper personal protective equipment (lab coats, gloves specific to the chemical, eye protection), proper laboratory equipment and procedures (use of hood, proper storage and disposal methods), and knowledge of chemical hazards such as flammability, reactivity, and toxicity (MSDS required). If this is all new to you, enlist the help of a chemist to help you make the proper decisions and reduce your risks of exposure and accident.

For organics, rinse with a reagent grade isopropyl alcohol, air-dry, rinse with reagent-grade hexanes, and air-dry.

Rinsing should be done using glass pipettes or wash bottles (made of appropriate material for the rinsing agent). Glass funnels, wide enough to accommodate your instruments and foil sheets, are invaluable in directing the flow of used chemicals into disposal containers or waste jars. Use disposal containers that are the same as your source chemical containers (e.g. brown glass). Never rinse into or pour unused chemicals back into your source chemical bottle.

7.3 Egg Injection And Incubation Procedure For Tree Swallow Eggs At Patuxent Research Refuge And Cobleskill Reservoir: Nest Monitoring, Egg Injection, Egg Collection And Laboratory Egg Incubation

Nest Checking Procedures:

- 1) In March, repair nest boxes and remove old nesting material.
- 2) Beginning in late April, check nest boxes for signs of nesting and egg laying.
- 3) Upon initiation of egg laying, record date of clutch initiation on data sheet and visit nestbox(es) at least every other day to determine completion of clutch and initiation of incubation (note accordingly on data sheet).
 - Patuxent: During egg laying randomly select one nest at each of the four pond sites and collect two eggs per nest for PCB analysis: Refrigerate eggs until opened, no longer than 48 hrs. Processing of eggs for contaminants analysis will be completed on a daily basis as much as practical. Follow Standard Operating Procedure for Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA.
 - Cobleskill Reservoir: During egg laying randomly select three nests and collect two eggs per nest for PCB analysis: Refrigerate eggs until opened, no longer than 48 hrs. Processing of eggs for contaminants analysis will be completed on a daily basis as much as practical. Follow Standard Operating Procedure for Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA.
- 4) In nests containing at least five eggs: On day two of incubation (initiation of incubation = day zero), in the afternoon, select 3 eggs from the nest. Candle these eggs and determine the two (if any) that fit stage 1-2 of development as described in the table below, this will determine approximately a 2.5 day old embryo (~18% of incubation):

Developmental Stage	Appearance of Vascularization & Embryo
0	Nothing visible
1	Pale, faint vascularization, faint embryo (red spot)
2	Obvious embryonic spot and vascularization
3	Classic embryonic shape apparent, vascularization around ~ 1/3 diameter of egg
3+	Significant vascularization and defined embryo

If available eggs in the stage 1-2 range are limited, assign any stage 3 or 3+ eggs to the untreated group as appropriate.

- 5) If eggs meet stage 1-2 appearance, assign each of the eggs from a clutch to a treatment group with no assignment of multiple eggs in a clutch to the same treatment. The egg will be labeled gently with soft pencil with an egg I.D. number on the pointed end of the egg and an 'X' and 'O' marked on opposite sides of the egg. Inject the egg using the following procedure being sure to limit the time the egg is out of the nest:

Injection Procedures

- 1) Assign the eggs to treatment groups and weigh each egg to the nearest one hundredth of a gram. Calculate and record the volume of dosing solution to be added to each egg (egg weight multiplied by 0.4). The volume will be rounded to the nearest 0.01 μL .
- 2) Make injections into the egg as follows, allowing the eggs to be outside the nest for not more than 30 minutes:
 - a. Wipe the blunt (air cell) end of the egg with 70% ethanol.
 - b. Gently make a hole in the egg with Dremel drill with a fine drill bit.
 - c. Inject the dosing solution, 0.4 $\mu\text{L/g}$ egg into the air cell, with a micro-pipettor and extended tip or Hamilton syringe.
 - d. Seal the hole with a piece of fabric band-aid or paraffin wax.
 - e. Allow the egg to sit pointed end down for at least 10 minutes.
- 3) Place eggs back into the nest.
- 4) Monitor the nests containing injected eggs while visiting adjacent nests during the nest monitoring period. Candle injected eggs at least once after injection and before collection to confirm they are developing post-injection.
- 5) On day ten of incubation collect the injected eggs from the nest and transport them to the laboratory for completion of incubation in an incubator; transport the eggs in individual compartments surrounded in saw dust and in a Koolatron incubator to maintain warmth.

Laboratory Incubation Procedures

- 1) Upon receipt of the eggs at the laboratory, examine them noting any evidence of damage or embryonic development (by candling).
- 2) Weigh eggs and note weight. Determine total weight (moisture) loss based on egg weight at injection.
- 3) Place eggs in an incubator in an egg rack adapted for tree swallow eggs, on their sides. Incubate at 99.5°F and humidity adjusted as needed to ensure correct moisture loss. Eggs will be turned hourly by automatic rotation in the incubator for a total of 60° every two hours. In addition turn eggs 180° by hand or using a scoopula twice per day (before 10 am and after 4 pm). Confirm turning of eggs by assigning the O to day-time orientation and the X to night-time orientation.
- 4) On day 12, candle the eggs and transfer each egg to its own hatching 'nest' (a weigh boat with a piece of fabric in the bottom such as quilt batting) and place in incubator without egg turner, 99.5°F and 70-80% humidity.
- 5) Upon hatching, sample tissues per necropsy protocol.

Deformity Scoring Procedures

Any eggs that fail to hatch should be opened and condition of the embryo noted on the Deformity Score Sheet. Deformities should be scored for presence or absence of crossed bill, shortened upper bill, missing or deformed eyes, edema of the neck and head area, incomplete ossification of skull (brain not enclosed in skull), gastroschisis in post stage 45 embryos, malformed or clubbed feet, asymmetrical body form, malposition in the egg, and any other abnormal appearances shall be noted on the data sheet. Photographs of deformed and normal embryos will be taken for reference. Deformed embryos will be preserved in a liquid fixative such as 70% ethanol or 10% neutral buffered formalin. Original memory cards from the digital camera should be kept under Chain-of-Custody (attached).

Equipment Needed

- Scientific collecting permits
- Incubators: Natureform NMC2000 and GQF Sportsman 1502
- Egg trays
- Light for candling
- Ethanol and tissue or alcohol wipes
- Dremel drill
- Hamilton syringes: one per treatment
- Paraffin and tool to apply it to eggs
- Heating block
- Scales (510 - 0.001 g) Mettler Toledo PG503-S
- Rainin Pipettman with extended tips: one tip per egg

Data Sheets

“Nest Checking”, “Egg Collection” (Versions 1 and 2), “Egg Incubation” (Versions 1 and 2), “Incubator Record”, “Egg Treatment and Incubation Log” (Versions 1 and 2), “Deformity Score Sheet”, and Chain of Custody for camera memory cards.

(Note: Use latest version of Data Sheet provided. Former versions are included because they were used for some initial data collection.)

Literature Consulted

Robertson, R. J., Stutchbury, B. J., and R. R. Cohen. 1992. Tree Swallow. *In The Birds of North America, No. 11* (A. Poole, P. Stettenheim, and F. Gill, Eds.). Philadelphia: The Academy of Natural Sciences; Washington, DC: The American Ornithologists' Union.

Robertson et al. (1992) in *Birds of North America* describe the nests, eggs, incubation and the hatching of tree swallow chicks and summarized below:

Tree swallow nests are constructed before the laying period. The nest cup is built primarily of grasses, especially when located near fields. It can also be composed of mosses, small roots, sticks, aquatic vegetation, and other plant materials. Feathers are a distinguishing characteristic, usually contour feathers of other fowl, are added after the formation of the nest cup. They are oriented so that the quill is buried in the nest, and the ends of the feathers cover the eggs when the female is not incubating the eggs. Bluebird nests are similar in appearance to tree swallow nests when in a nest box but do not have feathers lining the nest. Eggs are 18.7 x 13.2 mm and 1.4 to 2.6 g in size with an average weight of 1.9 g. Approximately 14% of mass is lost between laying and the end of incubation. Egg color translucent and rosy pink at time of laying turning to pure white (without any markings) around the fourth day of incubation. Eggs become glossier during incubation. Incubation length for tree swallow eggs averages 14-15 days but ranges from 11 to 19 days. Female incubation rhythms have been reported as 11 minutes on the nest and 9 minutes off the nest. An embryo takes one to two hours to hatch from start of pipping and clutches hatch over a one to two day period, occasionally over three days. Hatchlings weigh 1.5 to 1.7 g, eyes are closed, skin is uniform pink and the gape is yellow. Hatchling is able to raise head to beg and position itself with the dorsal side up.

7.4 Necropsy of Hatchling Tree Swallows

This protocol outlines appropriate dissection techniques and sample storage conditions for several tissues including:

Blood for genotyping

Liver for biochemistry and histology

Liver, Heart and Bursa for weights and histology

Thyroid for thyroxine analysis

- 1) Record time necropsy is initiated and completed. Record all data on appropriate data sheet.
- 2) Weigh the hatchling to the nearest 0.01 g on the Mettler PG503-S scale.
- 3) Kill the hatchling by cervical dislocation and decapitate with scissors. Collect blood onto a clean piece of card, gauze or cotton swab labeled with the sample ID. Store each sample individually in a sealed paper envelope.
- 4) Quickly dissect out the heart (trying to remove it before it has stopped beating), weigh it and preserve in appropriate fixative following these procedures: trim the heart of blood vessels in a standard manner from sample to sample, being careful not to remove any heart muscle. Rapidly immerse the heart in potassium chloride (25 mM) until it stops beating (at least one minute). Dab excess liquid from the heart, weigh it (to the nearest 0.00001 g using the Mettler MT5 balance) and then preserve it in cold 4% paraformaldehyde for 24-72 hours. For storage longer than 48 hours, replace the paraformaldehyde with phosphate buffered saline.
- 5) Dissect the liver, remove the gall bladder and weigh the liver (to the nearest 0.00001 g using the Mettler MT5 balance). Cut the liver into three approximately equal parts, place two pieces each in a cryovial, and flash freeze them in liquid nitrogen for biochemical analysis. Fix the third piece of the liver in 4% paraformaldehyde with a ratio of fifteen parts fixative volume to one part tissue volume. After 24-72 hours of fixation replace the fixative with phosphate buffered saline.
- 6) Remove both right and left thyroid at the same time. The thyroid is located at the caudal point of the thymus just anterior to the heart. Thyroid is within the thorax, ventral to and bound by fascia to the carotid artery. Place thyroids together in a 1.5 mL micro-centrifuge tube, and freeze thyroids on dry ice (thyroids are too often too small to get an accurate weight in hatchling tree swallows).
- 7) Remove the bursa, weigh it (to the nearest 0.00001 g using the Mettler MT5 balance) and fix it in Bouin's fixative, fifteen parts fixative to one part tissue volume. After 24 hours of fixation, replace the Bouin's fixative with phosphate buffered saline. Make a second saline replacement in another 24 hours.

8) Identify the gonads to determine gender. Males have two circular shaped testes. Females have one left ovary.

9) Discard remainder of carcass appropriately.

Long term storage:

Store frozen tissues at -80°C. Store fixed tissue at room temperature.

Equipment Needed:

Scales sensitive to 0.00001 grams

(Mettler MT5)

Scales (510 - 0.001 g) Mettler Toledo PG503-S

Dissecting scissors and forceps

Swabs and envelopes for blood collection

Dry Ice

Cryovials

1.5 mL microcentrifuge tubes

25 mM potassium chloride

Bouin's Fixative

4% paraformaldehyde

Liquid nitrogen

Labor: ideally a minimum of four people participate to ensure rapid dissection and storage of tissues

Data Sheets

“Hatchling Sampling Data Sheet”

7.5 Histological Analysis Of Avian Embryo Tissue: Bursa of Fabricius

This protocol describes the process of dehydrating, embedding, sectioning and staining tissue for histological study under light microscopy.

Organ specific details:

Bursa: Section transversely.

Procedure

1. Fix tissue with appropriate fixative per necropsy protocol.
2. Dehydration:
 - a. 70% EtOH 1 hour
 - b. 85% EtOH 1 hour
 - c. 95% EtOH 40 minutes
 - d. 95% EtOH 40 minutes
 - e. 100% EtOH 40 minutes
 - f. 100% EtOH 1 hour
 - g. 50% EtOH:50% xylene 1 hour
 - h. 100% xylene 1 hour
 - i. 100% xylene 1 hour
 - j. 100% xylene 1 hour
 - k. Paraffin 40 minutes @ 56°C
 - l. Paraffin 1 hour @ 56°C
 - m. Paraffin 1 hour @ 56°C
3. Embed tissue in paraffin with desired orientation (longitudinal orientation for bursas; ****this is very important****). This step must happen quickly in order for the paraffin to solidify in one block.
4. Section tissue embedded in paraffin into 5-10 μ m sections. Make three slides. Place 3-5 sections on one slide. Use plus-treated slides, as the tissue will stay on the slides while they are taken through the washing and staining process.
5. Place slides in an oven or on a hotplate at 60°C for 30-60 minutes. This helps with keeping the sections bonded to the slide.
6. Stain the tissue by washing the slides as follows:
 - a. Xylenes 2 minutes
 - b. Xylenes 2 minutes
 - c. 100% EtOH 1 minute

d. 100% EtOH	1 minute
e. 95% EtOH	1 minute
f. 95% EtOH	1 minute
g. Tap water (non-running)	10 minute
h. Mayer's hematoxylin	15 minutes
i. Lukewarm running tap water	20 minutes
j. Eosin	2 minutes
k. Non-running tap water	1 minute
l. 95% EtOH	2 minutes
m. 95% EtOH	2 minutes
n. 100% EtOH	2 minutes
o. 100% EtOH	2 minutes
p. 100% EtOH	2 minutes
q. Xylenes	2 minutes
r. Xylenes	2 minutes
s. Xylenes	2 minutes

7. Set slides out to dry.

8. Mount cover slip with mounting medium after the slides are dry. Try not to use too much mounting medium.

Endpoints:

Bursa: For bursa analysis, measure number of follicles per section, number of vacuoles, thickness of epithelial layer, and follicle size. Other qualitative aspects to also consider with each section are arrangement of bursal buds and arrangement of epithelial layers.

Record a digital image of each section used.

Equipment Needed:

Tissue Tek(VIP1000) for tissue dehydration and embedding

Microtome for tissue sectioning

Warm water bath for mounting tissue onto slides

Hot plate for warming slides prior to washing

Additional Supplies:

Paraplast

10-20 baths for washing fluids

Plus-treated slides

Cover slips

Mounting medium

Hematoxylin

Eosin

Ethanol

Xylenes

Quality Assurance Parameters and Procedures:

Sample Analysis: Multiple tissue sections per slide. Slides coded to obscure identifying marks and presented to reader in random order.

Duplicate (repeat) count of 10% of slides.

Criterion: reject results and repeat counts if the difference between counts from the replicate slides exceeds $2x \sqrt{(\text{highest count})}$.

Performance Evaluation: Independent recount of a previously counted set of slides.

Frequency: Once for every tissue type or every 10 sets of slides.

Criterion: Repeat counts of previous sets if the difference between repeated counts exceeds $3x \sqrt{(\text{highest count})}$.

7.6 Field Collection Of Tree Swallow Eggs From Upper Hudson River, New York For Injury Assessment Hudson River NRDA

INTRODUCTION

Tree swallow eggs from a PCB-contaminated location will be collected late in incubation and incubated to hatching. A subsample of eggs from the PCB-contaminated location will be selected for contaminants analysis.

MATERIALS AND EQUIPMENT

FIELD:

- ❑ Scientific collecting permits
- ❑ Field notebook, writing instruments (pencils/pens/permanent markers)
- ❑ Padded egg collection boxes (hard-sided container, e.g., Tupperware or tackle box, with padding such as sawdust or holofill)
- ❑ Avian Egg Collection Data Sheets

PROCEDURES

FIELD:

- ❑ Collected eggs should be whole and not cracked.
- ❑ For tree swallows, the following approach should be used: Incubation of tree swallow eggs doesn't start until the clutch is complete. Monitor nests every two to three days. Tree swallows generally lay eggs at one day intervals with a maximum clutch size of about 5-7 eggs. When a nest is 2-5 days pre-hatch (based on when the clutch was completed and incubation began), collect three eggs from that nest -- one egg will be incubated at the laboratory and the other 2 sibling eggs will be subject to contents collection for PCB analysis. In order to facilitate transport of eggs to the laboratory, eggs for PCB analysis can be collected independently of those to be transported to the laboratory. Eggs should be collected from all active nests at Remnant 3.
- ❑ For each egg collected, complete the appropriate information on the Avian Egg Collection Datasheet. Maintain separate Avian Egg Collection Datasheets for eggs to be transported to the laboratory and for eggs to be analyzed for contaminants.
- ❑ Place eggs in individually numbered compartments (one for each egg or eggs from each clutch). A list of the egg codes associated with each compartment will be placed inside the container. A fishing tackle box with compartments lined with sawdust or holofill is ideal – all eggs should be treated the same. Place this box in a hard-sided container with sufficient padding. Transport to the processing laboratory in a hard container avoiding temperature extremes and jostling.
- ❑ For eggs that are going to be analyzed for contaminants and not incubated: Refrigerate eggs until opened, no longer than 48 hours. Processing of eggs for contaminants analysis

will be completed on a daily basis as much as practical. Follow Standard Operating Procedure for Removal of Avian Egg Contents for Contaminants Analysis, Hudson River NRDA, compositing the 2 eggs from each nest in one jar. Archive samples at NYSDEC laboratory within two weeks of collection.

- For eggs that are going to be incubated: Transport promptly to the laboratory. Prompt transport under appropriate conditions is essential. Use of a “Koolatron” to maintain a proper temperature of eggs during transport is recommended. A hot water bottle can be substituted if a Koolatron is not practical or malfunctions. Maintain a temperature of about 90 to 95 degrees F, unless the transport time is going to be 8 hours or more, in which case a temperature as close as possible to 99.5 degrees should be maintained. Complete chain of custody transfer of samples from field collection crew to laboratory crew on Egg Collection Data Sheet.

APPENDIX B

EGG INJECTION DOSING MIXTURE FOR TREE SWALLOWS (2007)

Table 1. PCB congeners in the certified solution received from Accustandard in April 2007. This solution was designed to represent the PCB congener profile observed in Hudson River tree swallow eggs and was used to develop dosing solutions for egg injection studies.

PCB Congener	Cert. Conc. received (ug/mL)	Cert. total cong. Mass (ug)	Cert. mass (% of nominal)	% of total
28	680.100	170025	100	7.42
52	596.800	149200	100	6.51
66	576.300	144075	100	6.29
138	560.700	140175	100	6.12
163	432.400	108100	100	4.72
164	424.500	106125	101	4.63
118	420.400	105100	101	4.59
153	380.400	95100	100	4.15
74	378.000	94500	100	4.12
47	363.700	90925	101	3.97
48	340.100	85025	101	3.71
75	272.000	68000	101	2.97
43	256.200	64050	101	2.80
49	255.200	63800	100	2.78
70	240.100	60025	100	2.62
101	208.100	52025	102	2.27
31	156.100	39025	100	1.70
41	148.300	37075	101	1.62
71	138.900	34725	101	1.52
64	123.700	30925	100	1.35
110	119.900	29975	100	1.31
105	119.000	29750	99	1.30
99	118.900	29725	101	1.30
56	119.100	29775	101	1.30
60	107.600	26900	101	1.17
149	100.300	25075	104	1.09
139	87.900	21975	101	0.96
95	76.090	19023	101	0.83
37	75.550	18888	104	0.82
85	71.930	17983	105	0.78
87	72.200	18050	105	0.79
115	68.080	17020	103	0.74
180	64.320	16080	103	0.70
128	63.640	15910	102	0.69
170	59.860	14965	105	0.65
190	55.960	13990	105	0.61
92	55.560	13890	106	0.61
146	51.480	12870	101	0.56
187	51.520	12880	103	0.56
63	48.240	12060	104	0.53
158	48.280	12070	107	0.53
44	48.240	12060	107	0.53
156	44.190	11048	100	0.48
117	44.200	11050	102	0.48
91	43.910	10978	104	0.48
97	39.680	9920	100	0.43
42	39.320	9830	101	0.43
59	38.080	9520	101	0.42
109	34.400	8600	100	0.38
77	33.900	8475	102	0.37
132	30.890	7723	99	0.34
141	26.800	6700	100	0.29
15	25.500	6375	100	0.28
151	22.340	5585	100	0.24
130	21.000	5250	101	0.23
72	14.030	3508	102	0.15
137	13.870	3468	102	0.15
183	13.180	3295	101	0.14
167	11.950	2988	100	0.13
114	11.440	2860	101	0.12
157	10.760	2690	101	0.12
123	2.951	738	100	0.03
189	2.647	662	101	0.03
81	2.405	601	101	0.03
126	1.081	270	101	0.01
169	0.028	7	102	0.0003
Total PCBs	9164	2291051		

Table 2. Target and nominal concentrations of total PCBs of Hudson River (HR) tree swallow mixture in each dosing solution in corn oil for 2007 egg injection studies.

Vial & Cap #	Solution Description	Target Concentration ($\mu\text{g}/\mu\text{L}$) ¹	Nominal Concentration ($\mu\text{g}/\mu\text{L}$) ²
X-10	Stock Solution of HR tree swallow PCB mixture	250	250
X-11	2-fold dilution	125	124
X-12	4-fold dilution	62.5	62.0
X-13	8-fold dilution	31.3	31.2
X-14	16-fold dilution	15.6	15.5
X-15	32-fold dilution	7.8	7.8
X-9	Isooctane blank	0	0

¹ Target concentrations for the dosing solutions of the custom Hudson River tree swallow PCB mixture.

² Nominal total PCB concentrations are based on the sum of the certified analyte concentrations from AccuStandard (Attachment 1), a volume of 27.3 mL of the 250-mL of original custom Hudson River tree swallow PCB mixture, a one-mL stock solution volume, and the measured weights of transferred solutions for each dose.



AccuStandard, Inc.



CERTIFICATE OF ANALYSIS

CATALOG NO: S-15880-250ML
DESCRIPTION: Custom PCB Congener Standard
LOT #: B7040178
SOLVENT: Isooctane

EXPIRATION: Apr 23, 2017

See reverse for additional certification information.

This product is guaranteed accurate to $\pm 0.5\%$ of the Certified Analyte concentration through the Expiration Date on the Label.

Component	CAS #	Purity % (GC/MS)	Prepared Concentration ¹ ($\mu\text{g/mL}$)	Certified Analyte Concentration ² ($\mu\text{g/mL}$)
4,4'-Dichlorobiphenyl	2050-68-2	100	39.32	± 1.5728 39.32
2,4,4'-Trichlorobiphenyl	7012-37-5	100	680.1	± 27.204 680.1
1,6,6'-Trichlorobiphenyl	16606-02-3	100	340.1	± 13.604 340.1
3,4,4'-Trichlorobiphenyl	38444-90-5	99.7	124.1	± 4.964 123.7
2,2',3,4'-Tetrachlorobiphenyl	52663-59-9	99.3	60.28	± 2.4112 59.86
2,2',3,4'-Tetrachlorobiphenyl	36559-22-5	100	38.08	± 1.5232 38.08
2,2',3,5'-Tetrachlorobiphenyl	70362-46-8	99.9	364.1	± 14.564 363.7
2,2',3,5'-Tetrachlorobiphenyl	41464-39-5	99.0	64.28	± 2.5712 63.64
2,2',4,4'-Tetrachlorobiphenyl	2437-79-8	100	240.1	± 9.604 240.1
2,2',4,5'-Tetrachlorobiphenyl	70362-47-9	99.9	156.3	± 6.252 156.1
2,2',4,5'-Tetrachlorobiphenyl	41464-40-8	100	44.20	± 1.768 44.20
2,2',5,5'-Tetrachlorobiphenyl	35693-99-3	99.3	601.0	± 24.04 596.8
2,3,3',4'-Tetrachlorobiphenyl	41464-43-1	99.6	120.4	± 4.816 119.9
2,3,3',6'-Tetrachlorobiphenyl	74472-33-6	98.5	11.61	± 4.644 11.44
2,3,4,4'-Tetrachlorobiphenyl	33025-41-1	99.0	120.2	± 4.808 119.0
2,3,4,4'-Tetrachlorobiphenyl	74472-34-7	100	72.20	± 2.888 72.20
2,3,4,6'-Tetrachlorobiphenyl	52663-58-8	100	208.1	± 8.324 208.1
2,3',4,4'-Tetrachlorobiphenyl	32598-10-0	100	576.3	± 23.052 576.3
2,3',4',5'-Tetrachlorobiphenyl	32598-11-1	99.0	384.2	± 15.368 380.4
2,3',4',6'-Tetrachlorobiphenyl	41464-46-4	100	48.24	± 1.9296 48.24
2,3',5,5'-Tetrachlorobiphenyl	41464-42-0	99.0	31.20	± 1.248 30.89
2,4,4',5'-Tetrachlorobiphenyl	32690-93-0	100	420.4	± 16.816 420.4
2,4,4',6'-Tetrachlorobiphenyl	32598-12-2	99.5	12.01	± 4.804 11.95
3,3',4,4'-Tetrachlorobiphenyl	32598-13-3	100	48.28	± 1.9312 48.28
3,4,4',5'-Tetrachlorobiphenyl	70362-50-4	100	2.405	± 0.0962 2.405
2,2',3,4,4'-Pentachlorobiphenyl	65510-45-4	99.0	120.1	± 4.804 118.9
2,2',3,4,5'-Pentachlorobiphenyl	38380-02-8	99.5	108.1	± 4.324 107.6
2,2',3,4,5'-Pentachlorobiphenyl	68194-05-8	99.0	52.00	± 2.08 51.48
2,2',3,5,5'-Pentachlorobiphenyl	52663-61-3	99.7	76.32	± 3.0528 76.09
2,2',3,5,5'-Pentachlorobiphenyl	38379-99-6	99.1	140.2	± 5.608 138.9
2,2',3',4,5'-Pentachlorobiphenyl	41464-51-1	99.0	52.04	± 2.0816 51.52
2,2',4,4',5'-Pentachlorobiphenyl	38380-01-7	99.6	256.2	± 10.248 256.2
2,2',4,5,5'-Pentachlorobiphenyl	37680-73-2	99.4	380.3	± 15.212 378.0
2,3,3',4,4'-Pentachlorobiphenyl	32598-14-4	100	256.2	± 10.248 256.2
2,3,3',4,6'-Pentachlorobiphenyl	74472-35-8	100	48.24	± 1.9296 48.24
2,3,3',4',6'-Pentachlorobiphenyl	38380-03-9	100	272.0	± 10.88 272.0
2,3,4,4',5'-Pentachlorobiphenyl	74472-37-0	100	21.00	± 0.84 21.00
2,3,4,4',6'-Pentachlorobiphenyl	74472-38-1	99.5	10.81	± 4.324 10.76
2,3,4',5,6'-Pentachlorobiphenyl	68194-11-6	99.0	56.12	± 2.2448 55.56
2,3',4,4',5'-Pentachlorobiphenyl	31508-00-6	100	560.7	± 22.428 560.7
2',3,4,4',5'-Pentachlorobiphenyl	65510-44-3	99.8	13.21	± 5.284 13.18
3,3',4,4',5'-Pentachlorobiphenyl	57465-28-8	100	1.081	± 0.4324 1.081
2,2',3,3',4,4'-Hexachlorobiphenyl	38380-07-3	99.7	88.16	± 3.5264 87.90
2,2',3,3',4,5'-Hexachlorobiphenyl	52663-66-8	99.3	33.32	± 1.3328 33.09
2,2',3,3',4,6'-Hexachlorobiphenyl	38380-05-1	99.8	44.28	± 1.7712 44.19
2,2',3,4,4',5'-Hexachlorobiphenyl	35694-06-5	99.7	26.88	± 1.0752 26.80
2,2',3,4,4',5'-Hexachlorobiphenyl	35065-28-2	100	424.5	± 16.98 424.5
2,2',3,4,4',6'-Hexachlorobiphenyl	56030-56-9	99.4	2.969	± 1.1876 2.951
2,2',3,4,5,5'-Hexachlorobiphenyl	52712-04-6	99.0	40.08	± 1.6032 39.88
2,2',3,4',5,5'-Hexachlorobiphenyl	51908-16-8	99.3	76.08	± 3.0432 75.55
2,2',3,4',5,6'-Hexachlorobiphenyl	38380-04-0	99.9	148.4	± 5.936 148.3
2,2',3,5,5',6'-Hexachlorobiphenyl	52663-63-5	100	34.40	± 1.376 34.40
2,2',4,4',5,5'-Hexachlorobiphenyl	35065-27-1	100	432.4	± 17.296 432.4
2,3,3',4,4',5'-Hexachlorobiphenyl	38380-08-4	99.5	56.24	± 2.2496 55.96
2,3,3',4,4',5'-Hexachlorobiphenyl	69782-90-7	99.0	14.01	± 5.604 13.87
2,3,3',4,4',6'-Hexachlorobiphenyl	74472-42-7	100	68.08	± 2.7232 68.08
2,3,3',4,5,6'-Hexachlorobiphenyl	74472-44-9	100	100.3	± 4.012 100.3
2,3,3',4',5,6'-Hexachlorobiphenyl	74472-45-0	99.7	44.04	± 1.7616 43.91
2,3',4,4',5,5'-Hexachlorobiphenyl	52663-72-6	99.2	22.52	± 9.008 22.34
3,3',4,4',5,5'-Hexachlorobiphenyl	32774-16-6	100	0.028	± 0.0112 0.028
2,2',3,3',4,4',5'-Heptachlorobiphenyl	35065-30-6	100	64.32	± 2.5728 64.32
2,2',3,4,4',5,5'-Heptachlorobiphenyl	35065-29-3	99.0	120.3	± 4.812 119.1
2,2',3,4,4',5',6'-Heptachlorobiphenyl	52663-69-1	99.0	25.76	± 1.0304 25.50
2,2',3,4',5,5',6'-Heptachlorobiphenyl	52663-68-0	99.4	72.36	± 2.8944 71.93
2,3,3',4,4',5,5'-Heptachlorobiphenyl	39635-31-9	99.9	2.650	± 1.06 2.647
2,3,3',4,4',5,6'-Heptachlorobiphenyl	41411-64-7	100	14.03	± 0.5612 14.03

received 4/26/07 883

1. All weights are traceable through NIST, Test No. 822/272103-05
 2. Certified Analyte Concentration = Purity x Prepared Concentration. The Uncertainty calculated for this product is the Combined Uncertainty $u_c(y)$. It represents an estimated standard deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U which is $U_c(y) * K$ where K is the coverage factor at the 95% confidence level ($K=2$). Values reported above are Expanded Combined Uncertainty.
 3. A product with a suffix (-1A, -2B, etc.) on its lot# has had its expiration date extended and is identical to the same lot# without the suffix.

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OR-ORG/INO-001 Rev. 10/06

CERTIFICATION REPORT

1. **Intended Use:** The product covered by this Certificate is designed for Calibration or for use in Quality Control procedures for the specified chemical compounds listed on the reverse side. This product can be used for Identification and/or Quantification. This product can also be used as a Reference Material to validate analytical procedures, subject to the conditions under Section 8.
2. **Raw Materials:** Reference Standards are prepared from the highest quality starting materials with defined purities. All analytes and solvents are obtained from pre-qualified vendors and then analyzed or evaluated according to ISO 9001 requirements prior to use.
3. **Manufacturing:** AccuStandard, Inc. manufactures its products under an ISO 9001 certified quality system. Balances used in the manufacturing process are calibrated regularly. All weights are traceable through the National Institute of Standards and Technology (NIST).
4. **Homogeneity Assessment:** Homogeneity of the finished product is assessed by analyzing sample batches or by other methods consistent with the intended use of the product and by procedures that comply with the ISO 9001 Quality System.
5. **Stability Assessment:** AccuStandard, Inc. guarantees the stability of this solution through the expiration date stated on the label, when handled and stored according to the conditions stated on the label. To ensure a uniform solution, mix the contents of the sealed container thoroughly prior to use. Care should be taken not to contaminate the contents of the original container.
6. **Analytical Quality Control:** Products are tested by validated analytical methods covered under the company's ISO 9001 Quality System.
7. **Uncertainty Statistics and Confidence Limits:** The maximum Uncertainty stated on the face of this certificate has been calculated in accordance with the EURACHEM/CITAC Guide – Quantifying Uncertainty in Analytical Measurement - Second Edition. The Uncertainty given is the Expanded Combined Uncertainty and represents an estimated Standard Deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U which is $U_c(y) \cdot K$, where K is the coverage factor at the 95% confidence level ($K=2$). The Expanded Uncertainty is based on the combination of uncertainties associated with each individual operation involved in the preparation of the product.
8. **Legal Notice and Limit of Liability:** This product is for research use only. No warranty for any particular application is expressed or implied. Due to their hazardous nature, they should be handled by trained personnel. The company's liability will be limited to replacement of product or refund of purchase price. Notice of claims must be made within thirty (30) days from date of delivery.



AccuStandard, Inc.



CERTIFICATE OF ANALYSIS

CATALOG NO: S-15880-250ML

EXPIRATION: Apr 23, 2017

DESCRIPTION: Custom PCB Congener Standard

LOT #: B7040178

See reverse for additional certification information.

SOLVENT: Isooctane

This product is guaranteed accurate to $\pm 0.5\%$ of the Certified Analyte concentration through the Expiration Date on the Label.

Component

CAS #

Purity %
(GC/MS)

Prepared
Concentration¹
($\mu\text{g/mL}$)

Certified Analyte
Concentration²
($\mu\text{g/mL}$)

1. All weights are traceable through NIST, Test No. 822/272103-05
2. Certified Analyte Concentration = Purity x Prepared Concentration. The Uncertainty calculated for this product is the Combined Uncertainty $u_c(y)$. It represents an estimated standard deviation equal to the positive square root of the total variance of the uncertainty of components. The Expanded Uncertainty is U_c which is $U_c(y) * K$ where K is the coverage factor at the 95% confidence level ($K=2$). Values reported above are Expanded Combined Uncertainty.
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