Study Plan
for
Avian Egg Injection Study

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

DRAFT
for
PUBLIC REVIEW AND COMMENT

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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.

In 2002, the Trustees conducted an avian egg exposure preliminary investigation for the Hudson River. That preliminary investigation revealed that of the eleven avian species tested, the highest PCB levels were found in belted kingfisher and spotted sandpiper.

Based on the results of avian investigations conducted by the Trustees, and considering factors such as the life histories of various Hudson River avian species, avian toxicology, and the goals of the NRDA, the Trustees determined that it was appropriate to conduct further investigations focused on avian species. Pursuant to that determination and to the Hudson River NRDA Plan, the Trustees conducted a study of belted kingfisher, spotted sandpiper and tree swallow in 2004 and 2005. The Trustees further proposed conducting an avian egg injection study.

Accordingly, the Trustees have developed this Draft Study Plan for an avian egg injection experiment focused on Hudson River bird species. As this study will entail injury endpoints, the Trustees will perform a peer review of the proposed study and are issuing this draft Study Plan for public review and comment, in accordance with the Hudson River NRDA Plan.

The Trustees are interested in receiving feedback on this Draft Study Plan. To facilitate this process, the Trustees are asking the public and the party or parties responsible for the contamination to review this Draft Study Plan and provide feedback on the proposed approach. Comments should be submitted by March 23, 2006. These comments will help the Trustees plan and conduct an assessment that is scientifically valid and cost effective and that incorporates a broad array of perspectives. To that end, the Trustees request that you carefully consider this Draft Study Plan and provide any comments you may have to:

CONTACT FOR PUBLIC COMMENTS

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1.0 Background

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

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.

In 2002, the Trustees conducted an avian egg exposure preliminary investigation for the Hudson River. The investigation entailed collection of eggs, and subsequent analysis for PCBs, from six primary species (belted kingfisher (*Ceryle alcyon*), American robin (*Turdus migratorius*), Eastern phoebe (*Sayornis phoebe*), spotted sandpiper (*Actitis macularia*), red-winged blackbird (*Agelaius phoenicius*), and American woodcock (*Scolopax minor*)) and from five additional species (Eastern screech owl (*Otus asio*), common grackle (*Quiscalus quiscula*), northern rough-winged swallow (*Stelgidopteryx serripennis*), barn swallow (*Hirundo rustica*), and Eastern bluebird (*Sialia sialis*)) based on the opportunities for survey team members to locate the nests of these species. The geographic scope of the 2002 avian egg investigation was the Hudson River and its floodplains, from Hudson Falls to Lower Schodack Island, New York.

That preliminary investigation was undertaken by the Trustees to assist the Trustees in determining the extent to which avian species in the Hudson River are contaminated with PCBs, to determine if additional pathway and injury assessment studies focused on avian species should be conducted as part of the Hudson River NRDA, and for potential use in the design of future studies to assess the health of Hudson River birds. The Trustees noted in the Hudson River NRDA Plan that, based on the results of the bird egg study, the Trustees would determine whether injury determination and quantification studies were warranted.

That preliminary investigation revealed that of the eleven avian species tested, the highest PCB levels were found in belted kingfisher and spotted sandpiper (Hudson River Natural Resource Trustees, 2003). Spotted sandpiper eggs contained a mean of 15 ppm PCBs (as total homologues, fresh weight basis). Of the eleven species tested, spotted sandpiper eggs exhibited the highest individual egg concentration of PCBs (56 ppm) as well as the highest average PCB concentration (15 ppm). Of the eleven species tested, belted kingfisher eggs exhibited the second highest individual egg concentration of PCBs (43 ppm).

Based on the results of avian investigations conducted by the Trustees, including the tree swallow (*Tachycineta bicolor*) work (McCarty and Secord 1999a, 1999b, Secord *et al.* 1999) and the 2002 avian egg preliminary investigation (Hudson River Natural Resource Trustees 2003), and input from a panel of avian experts, and considering factors such as the life histories of various Hudson River avian species, avian toxicology, and the goals of the NRDA, the Trustees determined that it was appropriate to conduct further investigations focused on avian species, and initially on belted kingfisher, spotted sandpiper, and tree swallow, to be started in the year 2004.

Pursuant to that determination and to the Hudson River NRDA Plan, the Trustees released in 2004 a "Study Plan for Year 2004 Avian Investigations for the Hudson River – Final, Public Release Version," dated June 15, 2004 (Hudson River Natural Resource Trustees, 2004). That Avian Injury Study Plan described the activities that constituted the Trustees' planned approach to conducting investigations of injury to avian species as part of the Hudson River NRDA.

As noted in the Avian Injury Study Plan, the Trustees planned to assess the following potential injuries to birds: reduced avian reproduction and overt external malformations. The Trustees planned to: (1) assess the relationship between contaminant concentrations in nest sample eggs and parameters of nest reproduction by application of appropriate statistical analysis of data to determine whether reproductive success of spotted sandpipers, tree swallows and belted kingfishers nesting on the Hudson River is negatively affected by PCB exposure; (2) assess the incidence of gross deformities in embryos or hatchlings; (3) assess organic contaminant accumulation rates in belted kingfisher chicks on the Hudson River; and (4) initiate an avian egg injection pilot study in 2004.

Pursuant to the Final Avian Injury Study Plan and a May 4, 2005 Modification to that Study Plan (Hudson River Natural Resource Trustees, 2005a), the U.S. Geological Survey (USGS) conducted a study of belted kingfisher, spotted sandpiper and tree swallow in 2004 and 2005. The USGS study was directed at items (1), (2) and (3) above; review by the Trustees of data and results from the USGS study are ongoing.

Regarding item (4) above, the Trustees' Final Avian Injury Study Plan proposed a "pilot" study, a preliminary investigation focused on incubation of eggs of Hudson River avian species in 2004 with injection of PCBs into eggs of avian species of interest in a subsequent year. The Trustees subsequently determined that, considering preliminary work done by the Trustees and the literature on avian egg injection studies, it was not necessary to conduct a separate incubation-focused pilot study prior to initiating an avian egg injection study.

As this study will entail injury endpoints, the Trustees will perform a peer review of the proposed study and are issuing this Draft Study Plan for public review and comment, in accordance with the Hudson River NRDA Plan.

The Trustees are interested in receiving feedback on this Draft Study Plan. To facilitate this process, the Trustees are asking the public and the party or parties responsible for the contamination to review this Draft Study Plan and provide feedback on the proposed approach. Comments should be submitted by March 23, 2006. These comments will help the Trustees plan and conduct an assessment that is scientifically valid and cost effective and that incorporates a

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2.0 Introduction

Egg injection assesses the effects of contaminants on a developing avian embryo. Avian egg injection is a well-established technique to assess the effects of contaminants on a developing avian embryo.

To conduct an avian egg injection experiment, eggs are collected and brought into a laboratory where they are injected with the substance being tested. In avian egg injection experiments various doses of a contaminant of concern (for example, PCBs in a vehicle or carrier solution) are typically injected into the yolk sac, air cell, or albumen of eggs. The eggs are then incubated in a laboratory and their development monitored. Measurement endpoints may include embryomortality, malformations, and hatching success. Measurement endpoints may also extend to hatchlings, for which chick growth and development, for example, may be measured.

Results reported in the literature of injecting contaminants, such as PCBs, into avian eggs include embryomortality and malformation. Death, including embryomortality, for example, and physical deformation, such as external malformation, skeletal deformities, and organ and soft tissue malformation, are injuries pursuant to the regulations written by the U.S. Department of the Interior contained in Title 43 of the Code of Federal Regulations Part 11, Natural Resource Damage Assessment (the "DOI Regulations"), and would be relevant to determining injury as part of the NRDA.

3.0 Purpose and Objective

The purpose of this work is to inform the Trustees regarding injury to avian resources and guide their future efforts to identify pathway and specific injuries to birds from PCBs, as defined in the DOI Regulations. This work will also be used to help determine whether future studies will be performed, and if so, to help in their design.

The objective of this study is to evaluate whether avian reproduction and/or development is affected as a result of exposure to PCBs from the Hudson River.

4.0 Methods

The Trustees have developed the preliminary design described below for an avian egg injection experiment focused on Hudson River bird species.

These studies are projected over two years to allow development of injection and incubation protocols for eggs from wild species and to produce statistically useful sample sizes.

The Trustees are proposing to conduct these studies in at least two avian species from, but not limited to the following list: tree swallow, Eastern screech owl, American kestrel, Eastern bluebird, great blue heron, barn swallow, American robin, Eastern phoebe, red-winged blackbird, spotted sandpiper, and belted kingfisher.

These species have been preliminarily selected for additional consideration because they represent a range of positions in the ecosystem, breed in the Hudson River basin, and may be sensitive to PCB exposure. It will be possible to obtain eggs more easily from some species (such as swallows) than others (such as kingfishers or sandpipers). Additionally, egg injection experiments may be conducted using chicken (*Gallus gallus*) eggs to provide a point of reference for impacts observed in other species and in relation to effects levels identified in the toxicology literature for PCBs, dioxins and other chemicals.

The measurement endpoints that are proposed are those which, from the literature and our preliminary investigations, are expected to be important and useful across the species to be assessed. These measurement endpoints are:

- Viability – hatching/pipping success, embryomortality;
- Gross abnormalities;
- Gonadal and thyroid gland morphology;
- Endocrine measures – including hormone levels;
- Status of neural systems important in metabolic and reproductive axes function – hypothalamic neurotransmitters, aromatase enzyme, and gonadotropin-releasing hormone (GnRH-I);
- Cytochrome P450 enzyme activity; and,
- Endocrine-disrupting compound-induced abnormalities, such as heart or gonadal morphology, biochemical and hormonal endpoints evident in the hatchling.

Trials will be conducted using select PCBs or other relevant compounds administered early in embryonic development. The PCBs or other compounds to be injected into the eggs will be determined by the Trustees based on existing contaminants data from Hudson River biota and other relevant factors. Samples will be taken at hatch for measurement of selected endpoints known to be steroid-responsive and sexually dimorphic. It is expected that endocrine-active substances will impact these endpoints, thereby making treated individuals less sexually dimorphic.

In Year 1, the Trustees propose to focus on injection of test PCBs and incubation methods for eggs from chosen species. If injection and incubation methods are successful, tissues will be

collected for analysis, providing initial datasets for these species. That will position the Trustees to have some completed data sets (if sufficient power has been attained with the numbers that hatch) as well as replicate the exposure in eggs from chosen species of interest in the following year. The Trustees anticipate that Year 1 (2006) work will focus on those species with eggs that are more easily obtainable than others, with potential assessment of additional species in Year 2 (2007).

It will be necessary to obtain eggs from areas which are not contaminated with contaminants of concern on the Hudson River. A random subsample of eggs will be analyzed for chemical residues. Eggs may be obtained from the wild or from commercial sources. For box-nesting species, such as tree swallow, Eastern screech owl, or Eastern bluebird, the Trustees may place nest boxes in appropriate locations to provide a source of eggs. For other species, such as great blue heron, American robin, or Eastern phoebe, it will be necessary to collect eggs from existing nests found in the field. There are captive breeding colonies of some bird species, such as American kestrel, as well as Eastern screech owl, that may be used as sources of eggs for the egg injection experiments.

There will be a separate experiment conducted for each species. The timing of each experiment will depend on the availability of eggs for that species. In the case of chickens, for example, eggs will be available over much of the year, so those experiments will be scheduled around the work with the other species.

Treatment will consist of injection into the egg of the vehicle control or chemical treatment at the appropriate dose (with a total of 6 different doses). Eggs will be randomly assigned to the treatment groups. There will also be an "untreated" group. Treatments will be made at or before embryonic day 3 (in part in order to study gonadal effects and subsequent impact on sexual differentiation of neural systems). Sampling will occur at hatch to detect effects on gonads, plasma hormones, reproductive tract, liver, bursa, and hypothalamic systems.

The following endpoints are proposed to be assessed:

- Hatching/pipping success/embryo mortality;
- Gross morphology – including abnormalities;
- Organ weights for liver, heart, brain, thyroid, thymus, bursa, spleen, and gonads;
- Gonad histology and morphology of the reproductive tract;
- Bursal morphology;
- Cardiac morpohology and histology; and,
- Biochemical analyses
    - Liver CYP4501A;
    - Brain monoamines/GnRH-I (gonadotropin releasing hormone-I);
    - Thyroid hormones: T3 and T4 in thyroid gland and T3 in serum; and,
    - Estradiol and androgen concentrations in fecal matter.

As noted above, eggs will be injected with PCBs (individual congeners and/or a mixture reflective of chemical exposures in the Hudson River region) or other relevant compounds. A vehicle control and an untreated group of eggs will be included. The table below shows the ideal design which may be modified based on egg availability.

Table 1. Numbers of eggs to inject (if available) and number of eggs to be sampled in study per chemical. This assumes obtaining a minimum of 8 male and 8 female hatchlings per dose and reflects consideration of the potential lethality of the chemical and injection procedure

Treatment	Sample/hatch
Untreated	20/16
Vehicle only	28/16
Dose 1	32/16
Dose 2	45/16
Dose 3	45/16
Dose 4	60/16
Dose 5	60/16
Dose 6	60/16
Total	350/128

The following methods are proposed:

- Gonadal histology and gross morphology of the reproductive tract: These analyses require observations and weights at sacrifice, if possible, and histology of the gonads. Histology will require sectioning and staining with Berg's stain to differentiate spermatozoa and mature sperm.
- Hypothalamic monoamines and GnRH-I: These will be measured from the posterior hypothalamus, which is the area of storage and release of GnRH-I. Monoamines are measured by HPLC with electrochemical detection. GnRH-I is measured by an EIA (enzyme immuno assay), using an antibody specific for GnRH-I.
- Plasma steroids and thyroid hormones: These will be measured by RIA (radioimmunoassay) or ELISA (enzyme-linked immunosorbent assay), as appropriate for the chemical under investigation and according to techniques validated by the Trustees' laboratory. Thyroid hormone stores will be measured in an extract from the thyroid gland in order to assess the thyroid system status.
- Liver CYP 450 enzymes: These will be measured in microsomal preparations of the liver according to appropriate methods published for birds.
- Bursas: Bursal weight will be recorded and morphology will be examined histologically. A subset of bursas representing each treatment group will be analyzed histologically to determine if the immunocompetence is likely to be impaired in PCB-treated birds.

Statistical analyses will be conducted using ANOVA (analysis of variance) and dose-response statistical analyses.

5.0 Quality Assurance/Quality Control

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

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 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), and polybrominated diphenyl ethers (PBDEs), and organochlorine pesticides, as determined appropriate by the Trustees.

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 2005b). 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 other states where eggs will be collected, or from the U.S. Fish and Wildlife Service, will be obtained.

7.0 Literature Cited

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