Diamond Alkali Natural Resource Damage Assessment

Study Plan for Egg Injection to Evaluate Bird Injury

Draft | April 2025

Prepared by the:

U.S. Department of the Interior
U.S. Fish and Wildlife Service
New Jersey Field Office
4 E. Jimmie Leeds Road, Suite 4
Galloway, New Jersey 08205

With Assistance From:

Dr. Daniel Cristol
College of William and Mary

Dr. Thea Edwards
Dr. Natalie Karouna-Renier
U.S. Geological Survey

Industrial Economics, Incorporated
2067 Massachusetts Avenue
Cambridge, MA 02140

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Executive Summary

As part of the ongoing Natural Resource Damage Assessment and Restoration at the Diamond Alkali Superfund Site (DASS), the U.S. Department of the Interior (DOI), acting by and through the U.S. Fish and Wildlife Service, has prepared this study plan on behalf of the Federal Trustees (DOI and the National Oceanic and Atmospheric Administration) to assess potential injuries to song birds as a result of exposure to DASS-related hazardous substances, including 2,3,7,8, tetrachlorodibenzo-para-dioxin (TCDD) and polychlorinated biphenyls (PCBs).

The DASS provides fragmented, yet important, habitat for a variety of bird species. Birds utilizing portions of the DASS for nesting habitat are exposed to hazardous substances primarily through their diet. This dietary exposure may result in the accumulation of hazardous substances in their tissues, resulting in injury, such as potential adverse effects on reproductive success or altered breeding behavior. The Federal Trustees have developed this study plan which describes field sample collection, egg injection and incubation, and laboratory biological and chemical analyses the Federal Trustees intend to undertake to assess the toxicity and adverse effects of embryonic exposure of barn swallows (*Hirundo rustica*) and gray catbirds (*Dumetella carolinensis*) to dose ranges of TCDD or a mixture of dioxin-like compounds representative of the Lower Passaic River. This study plan is consistent with the Final Natural Resource Damage Assessment Plan for the Diamond Alkali Superfund Site, dated January 2020.

In the future the Federal Trustees may propose additional work to supplement this effort. The Federal Trustees are issuing this Draft Study Plan for a 30-day public review and comment period. Comments should be submitted in writing by May 22, 2025 to: sarah_scheaffer@usfws.gov. Public comments will be included in the Final Report of Assessment.

1 | Introduction

1.1 Background

The U.S. Department of the Interior (DOI), acting by and through the U.S. Fish and Wildlife Service, and the National Oceanic and Atmospheric Administration, (the "Federal Trustees") are conducting a natural resource damage assessment and restoration (NRDAR) to assess and restore the natural resources and associated services injured by hazardous substances released at or from the Diamond Alkali Superfund Site (DASS) (Federal Trustees 2020).

Birds are an integral part of the river ecosystem and provide multiple important ecosystem services such as seed distribution, plant pollination, and insect control. Birds (particularly bird eggs) are also an important source of prey to other species. Birds may be exposed to hazardous substances through direct ingestion of contaminated water, sediment, and soil; and/or through consumption of food items that contain hazardous substances derived from the Passaic River and its floodplain. Contaminated food items linked to the river may include fish, amphibians, benthic invertebrates, adult insects that develop from aquatic larvae, spiders that prey on those insects, plants growing in or near the river, and vertebrates that forage in the floodplain. The dietary exposure of birds to these food items may result in the accumulation and biomagnification of hazardous substances in their tissues, resulting in injury, such as potential adverse effects on reproductive success or altered breeding behavior.

The Final Natural Resource Damage Assessment Plan (DAP) for the Diamond Alkali Superfund Site (Federal Trustees 2020) identified avian health as an area of biological injury investigation. To assess possible injury due to exposure to dioxin and/or dioxin-like contaminants released at or from the DASS. The Federal Trustees prepared this plan to conduct an avian egg injection study.

1.2 Adverse Effects of Contaminants of Concern on Avian Species

Exposure to the types of hazardous substances released at and from the DASS have been shown to negatively impact birds, including increased mortality, reduced fecundity, and reduced growth (e.g., Harris and Elliott 2011). Birds utilizing the DASS for nesting habitat are primarily exposed to hazardous substances through their diet while young are exposed through both diet and maternal transfer to the egg (Custer et al. 2010 and references therein). Songbirds are moderately sensitive to dioxin-like compounds (e.g., coplanar PCBs, TCDD), but a few species have been identified as being highly sensitive, including the gray catbird (Farmahin et al. 2013, Eng et al. 2017).

1.3 Avian Egg Injection Toxicity Studies

Although egg concentrations are reflective of maternal transfer, actual embryonic injury (including hatching success) from the detected concentrations can be best determined under controlled conditions, thereby minimizing environmental factors that may affect embryonic health. Avian egg injection is a well-established technique to assess the effects of contaminants on a developing avian embryo. A robust body of literature on avian egg injection of dioxin-like compounds exists for a range of species (e.g., Allred and Strange 1977, Blankenship et al. 2003, Boily et al. 2003, Bruggeman et al. 2003, Brunström 1986, Brunström 1988, Fox and Grasman 1999, Goff et al. 2005, Grasman and Whitacre 2001, Hoffman et al. 1998, Ivnitski et al. 2001, Janz and Bellward 1996, Jin et al. 2001, Katynski et al. 2004, Lim et al. 2005, Nosek et al. 1993, Powell et al. 1996a, Powell et al. 1996b, Powell et al. 1997, Powell et al. 1998, Stanton et al. 2003, U.S. Environmental Protection Agency 2001, Walker and Catron 2000, Walker et al. 1997). However, only one study injecting dioxin-like

compounds into eggs of the species of particular interest to the Diamond Alkali NRDAR Trustees, barn swallow and gray catbird, has been performed (Eng et al. 2017).

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 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 embryo mortality, 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 dioxin-like compounds (e.g., TCDD, coplanar PCBs) into avian eggs include embryo mortality and malformation. Death, including embryo mortality, for example, and physical deformation, such as external malformation, skeletal deformities, and organ and soft tissue malformation, are injuries pursuant to the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) NRDAR regulations contained in Title 43 of the Code of Federal Regulations Part 11, (the "CERCLA NRDAR regulations"), and would be relevant to determining injury as part of the NRDA.

1.4 Purpose and Objectives

USFWS will conduct an avian egg injection study with gray catbird and barn swallow eggs to evaluate exposure to dioxin-like compounds representative of the DASS and possible resulting injury. The study may require multiple years to implement based on rates of egg collection.

The objective of the investigation is to evaluate the toxicity and adverse effects of embryonic exposure to dose ranges of TCDD or a mixture of dioxin-like compounds. Eggs will be collected in a similar area of New Jersey where they have previously been screened for low contaminant levels based on field studies conducted in 2020-2021. The eggs will then be injected with a mixture made up of individual PCB, dioxin, and furan congeners and is based on the chemistry that has been observed in eggs collected from the LPR in 2020-2021 as part of the NRDA[1].

This study will be used to evaluate whether avian reproduction and/or development is affected as a result of exposure to dioxin-like compounds in DASS. The purpose of this work is to inform the Federal Trustees' assessment of injury to birds, and to guide future efforts assessing pathways and injuries to birds from hazardous substances, as provided for by the CERCLA NRDAR regulations. This work may also be used to help determine whether additional studies are warranted, and if so, to inform their design.

[1] The study plan for the breeding bird and contaminant survey (U.S. Department of the Interior 2020) is available online at https://www.cerc.usgs.gov/orda_docs/DocHandler.ashx?task=get&ID=6115. Data from the 2020-2021 studies are available through https://ecos.fws.gov/ecdms4/ under catalog numbers 5040085 (2020), 5040086 (2020), 5040088 (2020), 5040089 (2020), 5040090 (2021), 5040091 (2021), and 5040093 (2021).

2 | Methods

2.1 Egg Collection

At the start of the nesting season for each species, field technicians will implement surveys to identify and record gray catbird and barn swallow nest locations. Technicians will search for nests located along the tidal Mullica River in the Great Bay area of southern New Jersey, where eggs have been previously screened for low contaminant levels. Eggs will be assigned a unique identifier that will include the four-letter species alpha code, with GRCA used as a species code for gray catbird and BARS for barn swallow. The identifier will also include a three-digit numerical code for the nest and an alphabetical letter indicating the order of collection of the individual egg, such as "a" for the first egg collected, "b" for the second egg collected. Field collection procedures are provided in the attached Standard Operating Procedure (SOP), "Field Collection of Gray Catbird and Barn Swallow Eggs for Avian Egg Injection Study".

2.2 Egg Incubation and Injection

The egg incubations detailed herein are informed by pilot studies performed by the USFWS in 2023 and 2024 to identify optimal incubation conditions for barn swallow and gray catbird eggs. On the day of collection (Embryonic Day 0; ED0), eggs will be assigned randomly to treatment groups and injected with the calculated volume of dosing solution into the air cell. Eggs will be candled just prior to injection and during incubation at time points approximately equivalent to 15% / 17% (two days post-injection), 46% / 50% (six days post-injection), and 77% / 83% (10 days post-injection) of incubation (BARS / GRCA). At the time of candling, any cracked, dead, or infertile eggs will be removed, and any dead embryos evaluated for stage of development and deformities. During incubation, eggs will be rotated every three hours by incubator rollers and manually turned 180 degrees twice per day. A subset of eggs will be weighed every other day and the humidity adjusted appropriately to ensure correct moisture loss with egg mass loss averaging 14% over the entire incubation period. Egg incubation and injection procedures are detailed in the attached SOP, "Egg Injection and Incubation Procedure for Gray Catbird and Barn Swallow Eggs" (Appendix A.2).

2.3 Dosing Solutions

Certified chemical standards with known weights and purity levels will be purchased as powders from AccuStandard or LGC or a comparable provider. Powders will be dissolved in dimethyl sulfoxide (DMSO). Custom mixes of these DMSO stock standards will be prepared to meet the chemical ratios in the injection solutions representative of the congeners comprising 95% of TEQ concentrations in target species from the Site (BARS/GRCA) (See Appendix A.3). Additionally, chemical concentrations in the DMSO stock standards will be prepared such that the injectable chemical concentrations will match planned dosing concentrations. Dosing solution preparation procedures are detailed in the attached SOP, "Preparation of Dosing Solutions" (Appendix A.3).

2.4 Egg Hatching, Tissue Sampling, and Tissue Analysis

One to two days prior to scheduled hatch (ED11 for BARS; ED10 for GRCA), eggs will be candled and transferred to a separate hatching unit. Presence of pipping will be monitored and noted. Upon hatching, hatchlings will be euthanized by decapitation, necropsied, and sampled for tissue collection. Any eggs that have not hatched within 2 or 3 days of the expected hatching day will be opened and examined for deformities. Deformities will be scored for presence or absence of crossed bill, shortened upper bill, unusually small or large eyes, edema of the neck and head area, incomplete ossification of skull (brain not enclosed in skull), gastroschisis (intestines protrude through hole in abdominal wall), malformed or clubbed feet, and asymmetrical body form. Malposition in the egg and any other abnormal appearances will be noted on the data sheet. Liver

tissue will be collected, frozen, and analyzed for 7-ethoxyresorrufin O-deethylase (EROD) activity as a biomarker of dioxin-like compound exposure. Hatchling carcasses will be frozen at -20°C and stored for potential additional tissue analyses. Tissue sampling and analysis procedures are detailed in the attached SOPs "Necropsy of Hatchling Birds" and "Ethoxyresorufin-o-deethylase (EROD) Assay For CYP450 Activity in Liver Samples" (Appendices A.5 and A.6).

2.5 Tissue Chemistry Analysis

Chemical analysis of a random subset of incubated eggs will be performed to assess the contaminant concentrations to which developing embryos were exposed. The number of eggs analyzed and the timing of the analysis during the incubation period will be determined based on egg availability. Tissue chemistry analysis procedures are detailed in the attached SOP, "Removal of Avian Egg Contents for Contaminants Analysis" (Appendix A.4).

2.6 Statistical Analyses

Data will be analyzed by testing for normality and proceeding with parametric ANOVAs, nonparametric tests, regressions, or other statistical tests as appropriate. Species-specific dose impacts to various endpoints including survival, growth, and deformities will be examined. Analyses verifying the relationship between injection dose and tissue concentration will also be performed.

2.7 Health and Safety Protocols

Appropriate health and safety procedures will be followed during field activities, egg collection and incubation, and sample processing. When working with potentially hazardous materials, the team will follow USEPA and Occupational Safety and Health Administration (OSHA) standards and corporate health and safety procedures. In order to ensure the safety of team members, all relevant and applicable state, federal, and agency guidance that is available at the time of sampling will be followed, including any guidance on avian influenza.

3 | Quality Assurance and Quality Control

3.1 Overview

Throughout the study, both in the field and analytical laboratory, quality assurance (QA) and quality control (QC) procedures will encompass a range of activities that enable laboratories to achieve and maintain high levels of accuracy and proficiency despite changes in test methods and the volume of specimens collected or tested. Study QC will be designed to detect, reduce, and correct deficiencies in internal analytical processes prior to the release of results; provide a measure of precision, or how well the measurement system reproduces the same result over time and under varying operating conditions. QA/QC objectives will be consistent with the project management, data generation and acquisition, assessment and oversight, and data validation and usability objectives defined in Appendix A of the final DAP (Federal Trustees 2020).

All samples, from the initial eggs through embryos, hatchlings, dead or infertile eggs, necropsy samples, and egg products will be identified and stored following documented procedures to ensure proper identification and handling. All procedures for assessment of biological impacts, including egg injections, observation and measurement of live birds, necropsy, and biological tissue analyses, will be performed following documented procedures to ensure consistent, comparable data.

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. Analytes will include congener-specific PCBs, including the non-ortho congeners, polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). Contract laboratories performing services in support of this study will have a QA/QC system that, among other things:

- establishes standard operating procedures (SOPs) for each step of the laboratory testing process, ranging from specimen handling to instrument performance validation;
- defines administrative requirements, such as mandatory recordkeeping, data evaluation, and internal audits to monitor adherence to SOPs;
- specifies corrective actions, documentation, and the persons responsible for carrying out corrective actions when problems are identified; and
- sustains high-quality employee performance.

The study's QA/QC program will be documented in the final Report of Assessment.

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APPENDIX A | Standard Operating Procedures

A.1 Field Collection of Gray Catbird and Barn Swallow Eggs for Avian Injection Study, Diamond Alkali NRDA

A.1.1 Introduction

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 from tidal river shoreline sites in southern New Jersey will be collected and brought into a laboratory where they are injected with the substance being tested. Proper handling of the eggs during collection and transit to the laboratory is essential to maintain viability in eggs that will subsequently be injected with contaminants and incubated.

A.1.2 Field Materials and Equipment

- Scientific collection permits
- Field notebook, writing instruments (pencils/pens/permanent markers)
- Individual plastic jars padded with three laboratory wipes for transporting eggs
- Labelling pens
- Plastic cooler for keeping eggs from overheating
- Frozen cooler pack(s)
- Avian Egg Collection Data Sheets

A.1.3 Field Procedures

- Collected eggs should be unincubated, whole and not cracked.
- For gray catbirds, the following approach should be used: Incubation of gray catbird eggs starts before the clutch is complete. Eggs are laid at one day intervals, usually between 8:00-10:00 AM. Monitor the construction of nests every 2-3 days until a lining of fine vine tendrils or other fine vegetation is present, and then shift to daily monitoring. Note the initiation of egg laying, marking the egg on the pointed end with one or two small dots indicating if it is the first or second egg, with a fine point pen, and waiting until the third egg is laid. On the day the third egg is laid, collect eggs 2 and 3 from that nest. A fourth egg will likely be laid the following morning.
- For barn swallows, which nest together in colonies, the following approach should be used: Incubation of barn swallow eggs does not start before the clutch is complete, or one day before this. Eggs are laid at one day intervals, often after noon, so nest checks should not be done in the morning, because an egg may be laid after the visit. Monitor the construction of nests every 3 days until a lining of several waterfowl feathers is present, and then shift to daily monitoring. Note the initiation of egg laying, marking the pointed end of each egg with one, two or three small dots, with a fine point pen, and waiting until the fourth egg is laid. On the day the fourth egg is laid collect eggs 2 and 3 from that nest.
- For each egg collected, complete the appropriate information on the Avian Egg Collection Datasheet.
- Fill out chain-of-custody prior to transporting collected eggs.
- Transport collected eggs to lab in hard container with sufficient padding of crumpled tissue around each egg, labelling each container using indelible ink with date, location, nest ID, time of collection, and initials of collector.
- For eggs to be incubated: Follow SOP for Egg Injection and Incubation (Appendix A.2).
- For any eggs that are going to be analyzed for contaminants and not incubated: 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 SOP for Removal of Avian Egg Contents for Contaminants Analysis, Diamond Alkali NRDA (Appendix A.4).

A.2 Egg Injection and Incubation Procedure for Gray Catbird and Barn Swallow Eggs

A.2.1 Introduction

This protocol outlines procedures for incubating eggs and injecting chemicals into the eggs of gray catbirds and barn swallows. The purpose of this is to mimic maternal deposition of chemicals into the egg and determine toxicity toward the embryo. Datasheets are provided in Appendix A.7.

A.2.2`Laboratory Materials and Equipment

- Incubators: Brinsea Ovation 56 EX or 28 Zoological Exotic Egg Incubator
- Egg trays
- Light for candling
- Sponge notched for egg holding
- Ethanol and tissue or alcohol wipes
- Laboratory tissue such as Kimwipes®
- Dremel® and 3/32" diamond tip Dremel bit
- Paraffin or airpore tape
- Heating block
- Laboratory balance (to 0.001 g)
- 2 µL laboratory pipettor and 10 µL laboratory pipettor with extended tips: one tip per egg

A.2.3 Egg Intake Procedures

1. Incubators are equilibrated to 37.5±0.5 °C (99.5°F) and initial humidity of 65% (± 5%) for at least 24 hours prior to setting of the eggs. During incubation, humidity will be adjusted as needed to ensure correct moisture loss. Be sure to measure temperature and humidity at several locations within the incubator (i.e., center versus edges).
2. Egg chiller is set at 13ºC (55.4ºF) and 55-65% relative humidity.
3. Upon receipt of the eggs, check the number assigned to each egg on its transport jar and write the egg number on the egg in fine pen on the pointed end of the egg. It is important to leave the round end of the egg unnumbered for inspecting the air cell. Note on the coding sheet the source, nest number, egg number for the clutch etc.
4. Examine each egg, noting any evidence of damage or embryonic development (by candling). While candling, mark location of air cell on round end.
5. Weigh eggs and enter weight onto data sheet.
6. Eggs will be injected on the day of collection or can be stored temporarily in an egg chiller to synchronize injection and development. Eggs can be stored between 10 to 20°C up to 48 hours, prior to incubation, without significant effects on development and hatchability. Eggs that are to remain in storage are placed round end up on an egg tray placed in a large plastic bag to help retain moisture in the chiller.

A.2.4 Egg Injection and Incubation

1. If working with stored eggs, when ready for incubation, remove eggs from chiller and set trays on table in incubation room to allow eggs to come to room temp (≈ 1 hour).

2. Assign eggs to treatment groups with consideration of number of eggs available and number of eggs from the same clutch. Ideally, eggs from the same clutch/nest should be dispersed among treatments.
3. Calculate and record the volume of dosing solution to be added to each egg (0.5 µL/g egg). Round the volume to the nearest 0.01 µL.
4. Make injections into the egg as follows:
    a. Place the egg to be dosed on sponge that has a carved-out indent to hold the egg.
    b. Hold the egg with one hand and using a Dremel®, drill a <1.5 mm hole into the center of the air cell using a 3/32" diamond tip Dremel bit.
        i. The shell and outer membrane are pierced slowly (i.e. with a firm but controlled pressure) to ensure the egg contents are not disrupted.
        ii. The hole must be made wide enough to loosely fit the pipette tip but not too tight, so that air exchange is maintained, and dosing solution is not expelled.
    c. Vortex the dosing solutions thoroughly prior to injection.
    d. Slowly inject dosing solution into the air cell using a micro-pipettor and extended tip.
    e. Seal the hole with paraffin or other sealant such as airpore tape and place the egg round-end up into a rack.
    f. Allow the egg to sit at room temperature for 30 minutes.
5. Mark the eggs with an X and an O on opposite sides to confirm eggs are turned properly throughout incubation.
6. Place eggs into the incubator on their sides in an egg rack of suitable size. Randomly place treatment groups across the egg racks.
7. Eggs will be partially turned every 3 hours by the incubator rollers. In addition, turn eggs 180º by hand twice per day (before 10 am and after 4 pm), using a quick, firm motion.
8. On days 2, 6 and 10 of incubation, weigh eggs and check viability by candling. Remove infertile and dead eggs from the incubator and retain for contaminants analysis; follow SOP "Removal of Avian Egg Contents for Contaminants Analysis, Diamond Alkali NRDA".
9. Check moisture loss by weighing a subset of eggs every other day and adjust the humidity appropriately to ensure correct moisture loss with egg mass loss averaging 14% over entire incubation period. For an average 1.9 g egg, for example, over a 15-day incubation, moisture loss should average approximately 1% or 0.02 g per day.
10. One or two days before the estimated hatch date (ED10 for GRCA and ED11 for BARS), a subset of eggs will be collected for analysis of contaminant levels in the embryos.
11. One to two days prior to scheduled hatch, eggs will be candled and transferred to a separate hatching unit set at 37.2ºC and relative humidity of 70%.
12. Any eggs that have not hatched within 2 or 3 days of the expected hatching day will be opened and examined for deformities. Presence of pipping will be noted.
13. Hatchlings will be euthanized by decapitation and necropsied.

A.2.5 Literature Consulted

Fasenko GM. 2007. Egg Storage and the Embryo. Poultry Science 86, 1020-1024.

Heinz GH, Hoffman DJ, Klimstra JD, Stebbins KR, Kondrad SL, Erwin CA. 2009. Species Differences in the Sensitivity of Avian Embryos to Methylmercury. Arch Environ Con Tox 56, 129-138.

A.3 Preparation of Dosing Solutions

A.3.1 Introduction

Dioxins, furans, and PCBs are readily soluble in dimethyl sulfoxide (DMSO, CAS# 67-68-5). For example, PCB-77 is soluble to at least 14.6 mg/ml (50 mM). Once dissolved in DMSO and stored in a sealed amber vial, these chemicals are stable at room temperature or in a refrigerator for months to years.

DMSO is an optimal solvent because it effectively moves chemicals through the egg membrane; it can be accurately pipetted, being non-viscous and non-greasy; and it is an analytical solvent with a known chemical composition.

Potential dosing chemicals are listed in Table A-1. Table A-2 details parameters that affect dosing calculations. To ensure chemical solubility in DMSO, we considered likely maximum doses to the eggs. Based on LPR egg data from 2020-2021 (see footnote 1), the maximum total TEQ was under 1,200 pg/g. For this maximum estimate to ensure solubility, and assuming a maximal egg mass of 5 g, we would need to apply 1,200 pg/g x 5 g = 6,000 pg per egg = 6 ng per egg. If the dosing volume is 1 µl of water with 0.1% DMSO, we would need to be able to dissolve 6 ng TEQ/0.001 µl DMSO = 6 mg TEQ/ml DMSO. This maximal dose is within the DMSO solubility limits for the chemicals in Table A-1 and ensures stock solutions will be fully dissolved. Additional higher doses may be evaluated, dependent on the number of eggs available.

Table A-1. Characteristics of Dosing Chemicals

Chemical	Short Name	CAS Number	Molecular Weight (g/mol)	Chemical Formula
2,3,7,8-Tetrachlorodibenzo-p-dioxin	2,3,7,8-TCDD	1746-01-6	321.96	$C_{12}H_4Cl_4O_2$
3,3',4,4'-Tetrachlorinated biphenyl	CL4-PCB-77	32598-13-3	291.99	$C_{12}H_6Cl_4$
3,4,4',5-Tetrachloro-1,1'-biphenyl	CL4-PCB-81	70362-50-4	291.99	$C_{12}H_6Cl_4$
2,3,7,8-Tetrachlorodibenzofuran	2,3,7,8-TCDF	51207-31-9	305.97	$C_{12}H_4Cl_4O$
2,3,4,7,8-Pentachlorodibenzofuran	2,3,4,7,8-PECDF	57117-31-4	340.4	$C_{12}H_3Cl_5O$
3,3',4,4',5-Pentachlorobiphenyl	CL5-PCB-126	57465-28-8	326.43	$C_{12}H_5Cl_5$
1,2,3,7,8-Pentachlorodibenzo-p-dioxin	1,2,3,7,8-PECDD	40321-76-4	356.4	$C_{12}H_3Cl_5O_2$
2,3,3',4,4'-Pentachlorobiphenyl	CL5-PCB-105	32598-14-4	326.43	$C_{12}H_5Cl_5$
1,1'-Biphenyl, 2,3,4,4',5-Pentachloro-	CL5-PCB-114	74472-37-0	326.43	$C_{12}H_5Cl_5$
1,1'-Biphenyl, 2,3',4,4',5-pentachloro-	CL5-PCB-118	31508-00-6	326.43	$C_{12}H_5Cl_5$
1,2,3,4,7,8-Hexachlorodibenzofuran	1,2,3,4,7,8-HXCDF	70648-26-9	374.86	$C_{12}H_2Cl_6O$
1,2,3,6,7,8-Hexachlorodibenzofuran	1,2,3,6,7,8-HXCDF	57117-44-9	374.86	$C_{12}H_2Cl_6O$
2,3,3',4,4',5-Hexachlorobiphenyl	CL6-PCB-156	38380-08-4	360.88	$C_{12}H_4Cl_6$
2,3,3',4,4',5'-Hexachloro-1,1'-biphenyl	CL6-PCB-157	69782-90-7	360.88	$C_{12}H_4Cl_6$

Table A-2. Parameters That Affect Dosing

Parameter	Grey Catbird Value(s)	Barn Swallow Value(s)
Egg mass range (grams)	3.8 to 4.2	1.6 to 2.2
Volume of egg injection (µl) (0.5 µl/g egg mass)	1.9 to 2.1	0.8 to 1.1
Final percent DMSO in water-based dosing solution	0.1%	0.1%

Table A-3. Proposed Parameter Ratios, based on overall contribution to Total TEQs in 2020-2021 eggs from LPR River Miles 1-8

Parameter	Grey Catbird[1]	Barn Swallow
CL4-PCB-77	39%	61%
2,3,7,8-TCDD	25%	20%
2,3,7,8-TCDF	3%	6%
2,3,4,7,8-PECDF	6%	--
CL4-PCB-81	--	5%
CL5-PCB-126	19%	8%
1,2,3,7,8-PECDD	3%	--
CL5-PCB-105	4%	--

[1]Percentages may not sum to 100% due to rounding.

A.3.2 Preparing DMSO Stock Standards and Injection Solutions

Certified chemical standards with known weights and purity levels will be purchased as powders from AccuStandard (New Haven, CT) or LGC (https://www.lgcgroup.com/). Powders will be dissolved in a known volume of DMSO, added to the original vials and sonicated for 20 minutes in a warm water bath, heated to 37 °C. DMSO (100%) is antimicrobial, so DMSO stocks do not need further treatment for sterility (Ansel et al. 1969)

Custom mixes of these DMSO stock standards will be prepared to meet the planned chemical ratios in the injection solutions (Table A-3). Control solutions with 2,3,7,8-TCDD and with plain DMSO will also be prepared. Additionally, chemical concentrations in the DMSO stock standards will be prepared such that, when diluted with sterile ultrapure water to create injection solutions containing 0.1% DMSO, the injectable chemical concentrations will match planned dosing concentrations (Table A-4). Custom mixtures in DMSO, aqueous dilutions (injection solutions), and aliquots of the injection solutions will be prepared and stored in sterile glass culture tubes with Teflon-faced rubber lined screw caps (similar to Fisher # 149 3010G, 20 x 125 mm). DMSO solutions will be stored at 4°C and aqueous dilutions will be stored at -80 °C. Aliquots from the dilutions will be sent for analytical chemistry to verify the concentrations of the components. All pipette tips, tubes, and other supplies that contact the injection solutions will be autoclaved or purchased as pre-sterilized supplies.

Table A-4. Example Calculations for Preparing DMSO Stock Solutions and Diluted Injection Solutions

Preparatory Calculations			Make Stock Concentration in 1ml DMSO	Dilute Stock in Water (10µg stock + 9.99 ml water)	Final Injection Solution	
Intended Dose to Egg (pg/g)	Egg Mass (g)	Mass of Chemical Applied to Egg (pg)	Target DMSO Stock Concentration (mg/ml)	Injection Concentration (µg/ml)	Mass of Chemical Dosed to Egg in 1 µl injection	Percent DMSO In Injected Solution
3	2	6	0.006	0.006	6	1%
30	2	60	0.06	0.06	60	1%
200	2	600	0.6	0.6	600	1%

A.3.3 Shipping Vials

Aliquots of aqueous injection solutions will be individually bagged in a 4" x 4" sealable plastic bag, wrapped in bubble-wrap and placed in a cardboard shipping box inside a styrofoam shipping box with approximately 10 lbs. dry ice for overnight shipping to the injection site. An inventory of the vials will be included with the shipment and emailed separately to the injection site. Solutions will be stored at -80 °C until use.

A.3.4 Literature Consulted

Ansel HC, Norred WP, and Roth IL. 1969. Antimicrobial activity of dimethyl sulfoxide against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*. Journal of Pharmaceutical Sciences 58(7): 836-839.

A.4 Removal of Avian Egg Contents for Contaminants Analysis

A.4.1 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, was written for those who have minimal experience with egg injection. 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.*

A.4.2 Laboratory Materials and Equipment

- Avian Egg Processing Data Sheets
- Paper or other towels
- Laboratory wipes such as Kimwipe brand
- Laboratory-grade 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. or smaller depending on product availability, lab recommendation, and storage space
- 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

A.4.3 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 laboratory wipe. Do not soak the egg. This step is rarely necessary.
- Dry and weigh whole egg to the nearest 0.01 g
- Take three measurements each of egg length and maximum egg width with calipers. Compute average of three measurements for final width and length measurements.
- 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.
  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. 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 of the egg contents to the nearest 0.01g, 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. Wet weight conversion will be made based on the weight and egg measurements. 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. Using these procedures, the weight of each egg's contents will be measured, even for eggs whose contents are combined into a single 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 the label on the jar. Place parafilm over the label and lid to keep it from getting wet and to seal the lid onto the jar.
- Prepare Chain of Custody records and maintain egg samples under chain of custody.
- Freeze samples. Ship under Chain of Custody overnight on dry ice to the sample archive or analytical laboratory.

A.4.4 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 HDPE) 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 to enable each person to comply with laboratory-specific protocols. 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.

A.4.5 Literature Consulted

Bird DM, Gautier J, and Montpetit V. 1984. Embryonic growth of American kestrels. Ornithology 101:392-396.

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

Powell DC, Aulerich RJ, Balander RJ, Stromborg KL, and Bursian SJ. 1998. A photographic guide to the development of double-crested cormorant embryos. Colonial Waterbirds 21(3):348-355.

A.5 Necropsy of Hatchling Birds

A.5.1 Introduction

Hatchling birds are sacrificed by decapitation once dry, usually within 2-6 hours of hatching.

A.5.2 Laboratory Materials and Equipment

- Scales sensitive to 0.00001 grams
- Scales (510 - 0.001 g)
- Scissors for decapitation
- Dissecting scissors and forceps
- Cryovials – two per bird, one for EROD and the second for remaining liver (if any) for residue analysis.
- Small weigh boats
- Liquid nitrogen
- Small glass jars
- Hatchling Sampling Data Sheet

A.5.3 Laboratory Procedures

1. Bring one hatchling at a time to the necropsy bench.
2. Record time necropsy is initiated and completed. Record all data in Hatchling Sampling Data Sheet.
3. Weigh the hatchling.
4. Euthanize the hatchling by decapitation with scissors.
5. Excise the liver while carefully removing the gall bladder. Place the liver on a pre-tared weigh boat and record weight.
6. Cut a ~ 50 – 100 mg piece of liver from the right lobe and place in a labeled cryovial for EROD analysis. Place in liquid nitrogen immediately. Samples are to be stored long-term in an ultra-low (-70^{o}C) freezer.
7. Any remaining liver should be placed in a second cryovial for residue analysis.
8. Place the remaining carcass in a labeled sample jar by treatment and place in the freezer.

A.6 Ethoxyresorufin-o-deethylase (EROD) Assay For CYP450 Activity in Liver Samples

A.6.1 Introduction

This protocol outlines a method for preparing microsomes from hatchling avian liver tissue by differential centrifugation and measuring enzyme activity of cytochrome P450-1A (a monooxygenase or mixed function oxidase) in those microsomes. The expression/activity of CYP450 is elevated by exposure to xenobiotics. Halogenated aromatic hydrocarbons (HAHs), especially planar dioxins, furans and polychlorinated biphenyls increase CYP450 expression via interaction with the aryl hydrocarbon receptor.

Expression of CYP450 is a biomarker of exposure to HAHs. CYP450 is a phase I metabolic enzyme that has dealklyase activity. The enzyme activity is measured by assaying EROD (ethoxyresorufin-o-deethylase) activity. 7-ethoxyresorufin serves as a substrate for this enzyme which yields resorufin as a fluorescent product. NADPH is the cofactor which donates electrons. The reaction is $NADPH_2$ + substrate + O_2 → substrate-O + H_2O + NADP. Enzyme activity is expressed as nmol or pmol of resorufin per mg protein per minute. This protocol is adapted from Melancon (1997) and Brunstrom and Halldin (1998).

A.6.2 Laboratory Materials and Equipment

- Tissue homogenizer (for example Bullet Blender®, Next Advance, BBX24B)
- 0.9-2.0 mm blend Stainless Steel Beads
- Centrifuge (9,000 xg)
- Ultra centrifuge (100,000 xg)
- Black 96-well plates
- Clear 96-well plates
- BCA Protein Assay kit (Pierce BCA assay BCA kit cat. # 23225)
- Temperature controlled plate shaker
- Fluorescence microplate reader, excitation 544 nm, emission 590 nm

A.6.3 Preparation of Reagents

Homogenization Buffer

Na/K Phosphate pH 7.4

The homogenizing buffer, at pH 7.4, is prepared by mixing approximately 1 part stock A to 4 parts stock B until a pH 7.4 is reached. Stock A will decrease pH while stock B will increase it.

- Stock A: 0.2M KH2PO4 = 27.22 g/L Potassium Phosphate-Monobasic, using distilled water
- Stock B: 0.2 M Na2PO4 = 28.40 g/L Sodium Phosphate-Dibasic, using distilled water

Microsomal Resuspension Buffer

0.05M Na/K Phosphate containing 10-3M Disodium Ethylenediamine Tetraacetate (EDTA), pH 7.6

The resuspension buffer is prepared by starting with Stock C and raising pH to 7.6 using Stock D - approximately 6:1 (D:C).

- Stock C: 6.80g KH2PO4 + 0.372g EDTA/L using distilled water
- Stock D: 7.10g Na2PO4 + 0.372g EDTA/L using distilled water

66 mM Tris-HCl, pH 7.4

- Solution A: 4.0 g Trizma Base (Tris(hydroxymethyl) aminomethane/ 500 ml distilled water)

- Solution B: 20.8 g Trizma HCl / 2 L distilled water)

Mix solutions A and B as needed to get a pH of 7.4 (~6 parts B:1 part A). Make in advance and store at 4°C.

Stock Solutions

- Substrate Stock: 1.0 mM ethoxyresorufin (Fisher/MP Biomedicals ICN15796405; MW 240.18).
  - 2.41 mg/10 ml DMSO. Aliquot 95 ul into labeled tubes. Indefinite shelf life at -20°C.
- Standard Stock: 100 uM Sodium Resorufin (Sigma Aldrich R3257; MW 235.17).
  - 2.35 mg resorufin salt / 100 ml methanol. Aliquot ~1 ml each in to 1.5 ml centrifuge tubes and store in -20°C freezer. Protect from light. *Approximately 50 ul is required per assay. Resorufin in methanol will not freeze but it may precipitate leaving fine particles on the side of the tube. Therefore, bringing the resorufin aliquot to room temperature and vortexing vigorously improves the assay.*

Working Solutions

- Substrate Solution: 1.25 µM 7-ethoxyresorufin in Tris buffer - Dilute 47.85 µl of 1.0 mM substrate stock in 22 mL cold 66 mM Tris buffer.
- Standard Solution: 1 x 10^{-6} M resorufin in Tris buffer - Add 50 ul of 100 uM standard stock to 450 ul 66 mM Tris buffer = 10 µM.
- NADPH (ACROS Organics AC328740010; MW =905.4) – 3.25 mM solution in 66 mM Tris buffer
  - Make fresh daily. To 6.0 mg of NADPH, add 2.0 mL cold 66 mM Tris buffer. This is more than enough for 2 plates. Protect from light.

A.6.4 Preparation of Microsomes

Keep all samples in a benchtop cooler that was frozen at -70°C. Pre-cool all centrifuges and rotors.

1. Place labeled 1.5 ml centrifuge tubes on frozen thermal beads or ice.
2. Remove sample from benchtop cooler and quickly weigh excised liver on a tared weigh boat. Optimally, the assay requires 50 – 100 mg of tissue. If necessary, cut liver fragment to this range. Record the weight and transfer to a cooled labeled tube from step 1. Return unused tissue to storage at -70°C.
3. Add 1.3 times the sample weight of 0.9-2.0 mm blend of rinsed and dried stainless-steel beads to each sample, e.g. for 100 mg of tissue, weigh 130 mg of beads.
4. Add 1.0 ml of homogenization buffer to each sample tube. Vortex to dislodge tissue from the sides.
5. Homogenize samples for 1 minute, then place samples briefly on ice to cool. Homogenize an additional 30 seconds.
6. Centrifuge homogenate for 20 minutes at 9,000xg in a pre-cooled (4°C) centrifuge.
7. Transfer the supernatant to a labeled and chilled ultracentrifuge tube. Avoid transferring the floating layer of fat commonly found in homogenized embryonic or hatchling livers.
8. Centrifuge in pre-cooled Sorvall WX80 at 100,000g for 60 min at 4°C. Use pre-cooled TFT-45.6 rotor at 40,000 RPM.
9. The resulting pellet contains the microsomal fraction. Pour off the supernatant, blot and remove any remaining moisture by using a cotton swab.

10. Resuspend microsomes in resuspension buffer at 1:10 w/v - e.g. for 100 mg of tissue, resuspend in 1.0 ml of resuspension buffer (final concentration 100 mg/ml). For small livers, add no less than 300 ul of buffer.

11. Using a glass rod cooled in ice cold DI water, gently crush the microsomal pellet into the resuspension buffer. Gently pipette up and down with a 1.0 ml pipette for a foamless, homogeneous suspension. Transfer to a pre-labeled 1.5 ml "stock" tube on ice.
12. Aliquot microsomes from each sample into 2 or more tubes to minimize freeze-thatw in case sample must be rerun.
13. Store the microsomes in aliquots at -70ºC. Multiple freeze/thaw cycles can affect activity.
14. Reference samples: prepare reference samples from 2 different sample pools – reference 1 is typically prepared on the first day of microsome preparation and reference 2 is prepared on the last. For reference samples, transfer 100 ul from samples with > 0.7 ml of 100 ug/ul homogenate. If homogenate contains > 1.0 ml, transfer 200 -300 ul to reference pool. Both reference 1 and reference 2 are required for each EROD plate.

A.6.5 EROD Assay

The quantity of microsomes used should be that which gives a linear response over the time of the assay, within the range of the standard curve. If enzyme activity is highly induced, one may need to re-run the assay with a smaller number of microsomes.

1. Turn off lights in lab room. Assay is run in subdued light.
2. Preheat plate shaker and microplate reader to 37°C.
3. Remove Standard solution (resorufin in Tris buffer), Substrate solution (7-ethoxyresorufin in Tris buffer), and NADPH (in Tris buffer) from freezer and bring to room temperature. Resorufin is light sensitive and should be kept in foil.
4. Prepare a resorufin standard curve (0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 uM) by serially diluting in 66 mM Tris buffer. Standards are assayed in triplicate.
5. Prepare working solutions of NADPH and substrate as described above in section 2.3.
6. Thaw 22 sample microsomes and 2 reference microsomes on ice or frozen thermal beads and vortex before proceeding with the dilutions for the assay.
7. Dilute microsomes with cold resuspension buffer. Starting diluting is 1:5 or 1:6 of a 100 ug/uL liver suspension. This may need to be adjusted based on the results of the first plate.
8. Transfer 50 uL of each microsome dilution to a new labeled tube for protein analysis. Store on ice until use.
9. Vortex diluted microsomes, microfuge briefly and place on ice.
10. Make a total of 1.5 mL of pooled microsomes in a 15 mL tube on ice using either diluted reference microsomes or diluted sample microsomes or both, depending on available volume of each. The amount of microsomes to take from each sample (including the reference microsome sample) can vary depending on availability. These are the "pooled microsomes" used in the standard wells to account for protein background that may affect fluorescence.
11. Make a STANDARD Master Mix solution of substrate, buffer and microsomes by combining the following:
    a. 1.5 ml pooled microsomes.
    b. 4.5 ml substrate solution
    c. 300 ul cold 66 mM Tris buffer
12. Make a SAMPLE Master Mix solution by combining:
    a. 5.0 ml Tris buffer;
    b. 15.0 ml substrate solution
13. Add the following to the appropriate wells of a black round bottom, 96 well plate:
    a. 50 ul standard in triplicate (use Tris buffer for Blank)
    b. 50 ul reference or sample microsomes in triplicate.
    c. 210 µl of STANDARD Master Mix solution using a multi-channel pipette to the wells containing the standards

d. 200 ul SAMPLE Master Mix solution using a multi-channel pipette to the wells containing the sample microsomes and reference microsomes

14. Cover plate with foil to protect from light and place in preheated plate shaker and mix at ~800 rpm for thirty seconds. Incubate for 10 minutes at 37°C.
15. Remove plate. Quickly add 10 ul NADPH to wells containing sample and reference microsomes. **Do not add NADPH to blank or standard wells.**
16. Immediately place plate in microplate reader and read at 544 excitation/590 emission. Fluorescence readings are taken in Relative Fluorescence Units (RFU) every 1.5 minutes over twelve readings for a total of 18 minutes.
17. Proceed to protein assay.

A.6.6 Protein Assay

The protein assay is used to determine the amount of protein in the sample in order to express the activity of enzyme per milligrams of protein. (Pierce BCA assay BCA kit catalog number 23225)

1. Add 10 ul of bovine serum albumin (BSA) standards (0.125 to 2.0 mg/ml), blank (resuspension buffer), sample microsomes and reference microsomes in triplicate to a clear 96 well plate.
2. To make the working reagent, combine 50 parts of BCA reagent A with 1 part of BCA reagent B. For one full plate, 22 mL of reagent A and 0.44 mL reagent B are required.
3. Add 200 ul of working reagent to each well and mix plate thoroughly on a plate shaker for 30 seconds.
4. Incubate at 37ºC for 30 minutes and cool at room temperature for 15 minutes.
5. Read the plate at 562 nm on the microplate reader.

A.6.7 Data Analysis

1. The standard curve for the EROD and the protein assay is produced using 4- parameter fit.
2. The first reading of the EROD assay generally has the highest %CV. Therefore, the difference between the last reading and the second reading is used to calculate the amount of resorufin. The replicate readings per sample are averaged to determine picomoles of resorufin using the standard curve. This value is then divided by the total number of minutes to obtain picomoles of resorufin per minute.
3. The value obtained from the EROD assay for "picomoles of resorufin per minute" is divided by the value for the protein assay to obtain the final enzyme activity for each sample (as picomoles of resorufin per milligram of protein per minute).

A.6.8 Literature Consulted

Melancon MJ. 1997. Development of Cytochromes P450 in Avian Species as a Biomarker for Environmental Contaminant Exposure and Effect: Procedures and Baseline Values. Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment (5th. Volume). David A. Bengston and Diane S. Henshel, Eds. American Society for Testing and Materials, Philadelphia.

Brunstrom B and Halldin K. 1998. EROD induction by environmental contaminants in avian embryo livers. Comparative Biochemistry and Physiology Part C 121: 213-219.

A.7 Representative Data Sheets

Avian Egg Collection Data Sheet – Passaic River Avian Egg Injection Study

Page __ of ___

Collector _____

Data Recorder _____
Name Signature

Egg Code[1]	Location[2]	Date Collected[3]	Time Collected[4]	Clutch Size	Eggs Warm Yes or No	Comments

[1] Egg Code: Numeric code beginning at 1; [2] Site Name and Nest number or description; [3] In MM/DD/YEAR format, such as 04/30/2006 for April 30, 2006; [4] In 24-hour format, such as 1300 for 1PM

Custody of samples listed above transferred from field collection crew to laboratory crew as follows:

Relinquished by: _____
Signature Print Name

_____
Company/Title Date Time

Received by: _____
Signature Print Name

_____
Company/Title Date Time

Data Sheet checked by: _____ Date: _____
Name/Initials

Incubator Record – Passaic River Avian Egg Injection Study

Incubator	Date	Time	Temperature	% Humidity or Wet Bulb Temp	Eggs Turned (O/X)	List any adjustments	Initials

Make any additional notes on reverse. Reviewed by: _____ Date: _____

Egg Treatment and Incubation Log – Passaic River Avian Egg Injection Study

Species: _____ Injection Mixture: _____

Treatment (ug/g)	Egg ID	Egg Mass (g)	Dosing Concentration (ug/ul)	uL injected	Date of Injection	Date Death Detected	Stage at death	Initials

Make any additional notes on reverse. Reviewed by: _____ Date: _____

Egg Monitoring Log – Passaic River Avian Egg Injection Study

Species: _____ Date & Time Eggs Set: _____ Date Injected: _____

Date	Time	Investigator	Embryonic Day	# infertile	# dead	# remaining	Initials

Make any additional notes on reverse.

Reviewed by: _____ Date: _____

Moisture Loss Data Sheet – Passaic River Avian Egg Injection Study

Species: _____

Date	Time	Egg ID	Embryonic Day	Weight (g)	Comments	Initials

Make any additional notes on reverse. Reviewed by: _____ Date: _____

Deformity Score Sheet – Passaic River Avian Egg Injection Study

Note "Y" (yes) or "N" (no) to note presence or absence of the deformity. If embryo is not old enough to detect a structure, or is too decomposed, note "NS" (not scored) under the deformity type.

Date	Species	Egg ID	Date Death Detected	Stage	Cross Bill	Short Upper Bill	Abnormal Eye Size	Neck/head Edema	Incomplete Skull	Clubbed Feet	Mal-position	Gastroschisis (post stage 45)	Other	Initials

Make any additional notes on reverse. Reviewed by: _____ Date: _____

Avian Egg Processing Data Sheet – Passaic River Avian Egg Injection Study

Egg sample ID_____

Site_____Lat_____Long_____

Original Clutch Size_____#eggs sampled_____

Collection: Date_____Time_____Collector initials_____

Distance from nearest water_____

Nest Details_____

Processing: Date_____Time_____Processor's initials_____ Processing Location_____

Jar mass without lid_____

Displacement Volume of egg A _____

Length of egg A _____ _____ _____ Width of egg A _____ _____ _____

Whole egg mass A_____ Jar plus egg A contents_____

Displacement Volume of egg B _____

Length of egg B _____ _____ _____ Width of egg B _____ _____ _____

Whole egg mass B_____ Jar plus eggs A+B contents ____

Displacement Volume of egg C _____

Length of egg C ____ ____ ____ Width of egg C ____ ____ ____

Whole egg mass C_____ Jar plus eggs A+B+C contents _____

Final sample mass (all eggs – jar) _____-_____=_____

Into freezer: Location_____Time_____

Hatchling Sampling Data Sheet

Date: _____ **Investigators:** _____

Egg ID	Hatchling Appearance	Time Start	Body Mass (g)	Liver (mg)	Time Finish	Initials

Make any additional notes on reverse. Reviewed by: _____ Date _____