1.0 TITLE AND APPROVAL

Quality Assurance Project Plan

For Work Assignment 2-17

Avian Dosing Study

for

EPA CONTRACT NUMBER 68-W-01-023

Project No.: Battelle's Pacific Northwest Division. Project 43496

Work Assignment Leader: Michael Blanton

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**Institution: Battelle's Pacific Northwest Division –
Battelle, Sequim and Battelle, Richland**

SIGNATURE PAGE
Quality Assurance Project Plan for WA 2-17

Avian Dosing Study
EPA Contract Number 68-W-01-023
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4.0 PROJECT/TASK ORGANIZATION

4.1 PROJECT MANAGEMENT

The U.S. Environmental Protection Agency (EPA) is implementing the Endocrine Disruptor Screening Program (EDSP). To support this program, EPA has contracted with Battelle to provide comprehensive toxicological and ecotoxicological testing services, including chemical, analytical, statistical, and quality assurance (QA)/quality control (QC) support to assist the EPA in developing, standardizing, and validating a suite of *in vitro*, mammalian, and ecotoxicological screens and tests for identifying and characterizing endocrine effects through exposure to pesticides, industrial chemicals, and environmental contaminants. The studies conducted will be used to develop, standardize, and validate methods; prepare appropriate guidance documents for peer review of the methods; and develop technical guidance and test guidelines in support of the Office of Prevention, Pesticides and Toxic Substances regulatory programs. The validation studies will be conducted under the EDSP Quality Management Plan (QMP) study protocol, applicable Quality Assurance Project Plans (QAPPs), relevant program and facility standard operating procedures (SOPs), and guidance documents.

The Food Quality Protection Act of 1996 requires the EPA to develop and implement a screening program using valid tests for determining the potential in humans for estrogenic effects from pesticides. EPA proposed a two-tiered screening program in a Federal Register notice in 1998 (63 FR 1542-71568, Dec. 28, 1998) that covered not only pesticides but also commercial chemicals subject to regulation under the Toxic Substances Control Act (TSCA; 15 USC 2601) and environmental and drinking water contaminants. This study plan addresses the need for experimental data regarding:

1) The relative importance of the timing of onset of treatment of the P1 generation (prior to sexual maturation or after proven egg-laying ability) for detecting reproductive and developmental effects over two generations.[1]

2) Whether the F1 generation should receive dietary treatment of the test substance.

A range finding study will be conducted to determine the appropriate dose concentrations for the two generation study.

4.2 GOALS

The completion of Work Assignment (WA) 2-17 will provide data to address the following:

- The overall objective is to determine which dosing scenario for the P1 generation birds (during maturation or after proven breeding ability is established) and F1 birds (exposure from hatch or no additional exposure above *in ovo* exposure) is more biologically

[1] Please note that on page 2 of the Work Assignment Statement of Work the exposure options for the P1 generation are "initiated at sexual maturation or after proven egg-laying ability." Because sexual maturation is often determined by the onset of egg laying, it is assumed that "prior to or during maturation" was intended and conforms to the reference to "pre-breeding" dosing and the exposure scenarios discussed in the Organization for Economic Cooperation and Development (OCED) documents.

sensitive to chemically induced reproductive/endocrine disrupting impacts to species fitness.

- The time series produced by the P1 and F1 generation birds for a given concentration and dosing scenario will allow the evaluation of a possible delay in response time, the form of the time series response (i.e., linear, curvilinear, spline), and the potential carry-over effect of the reproductive response to the F1 generation.

- The dose-response for each generation and dosing scenario will allow the estimation of the EC_{50} and slope of the response. The difference in the slope and EC_{50}s between generations will also allow the evaluation of a potential carry-over effect between generations.

Refer to Section 19.2, Statistical Analysis, of this document for a more detailed discussion.

4.3 ORGANIZATION

Battelle will conduct the assays specified under WA 2-17 as outlined according to the specifications and subsequent modifications by the EPA. A summary of the work assignment organization is shown in Figure 4-1.

The overall work assignment will be managed by **Mr. Michael Blanton,** the Work Assignment Leader (WAL). Michael Blanton is responsible for preparing the technical work assignment, assigning appropriate staff to complete specified tasks within this work assignment, and monitoring the progress of both technical and fiscal milestones, as outlined in the technical work plan. Mr. Blanton will report progress of Battelle on the work assignment to **Dr. David Houchens** at Battelle through a series of planned conference calls and through the use of written monthly reports.

General scientific direction and supervision of the work performed under this work assignment is provided by **Crystal Driver**. Crystal Driver is the Principal Investigator (PI) for the avian dosing assay component of this contract and is responsible for preparing the study protocol for submission and approval by the EPA. Ms. Driver will also prepare and defend the Battelle Pacific Northwest Division Institutional Animal Care and Use Committee (IACUC) study protocol (a document separate and distinct from the study protocol) and ensures that the study is being conducted according to the appropriate study protocol and animal care and use guidelines. **Ms. Teresa Luders**, Animal Care Supervisor, will be responsible for obtaining and housing the 300 Japanese quail (*Coturnix japonica*) eggs and hatching of test birds under the appropriate SOPs. She will work closely with **Ms. Margorie Miller** to care for eggs and birds during the study. Water quality testing is performed under routine animal care facility procedures.

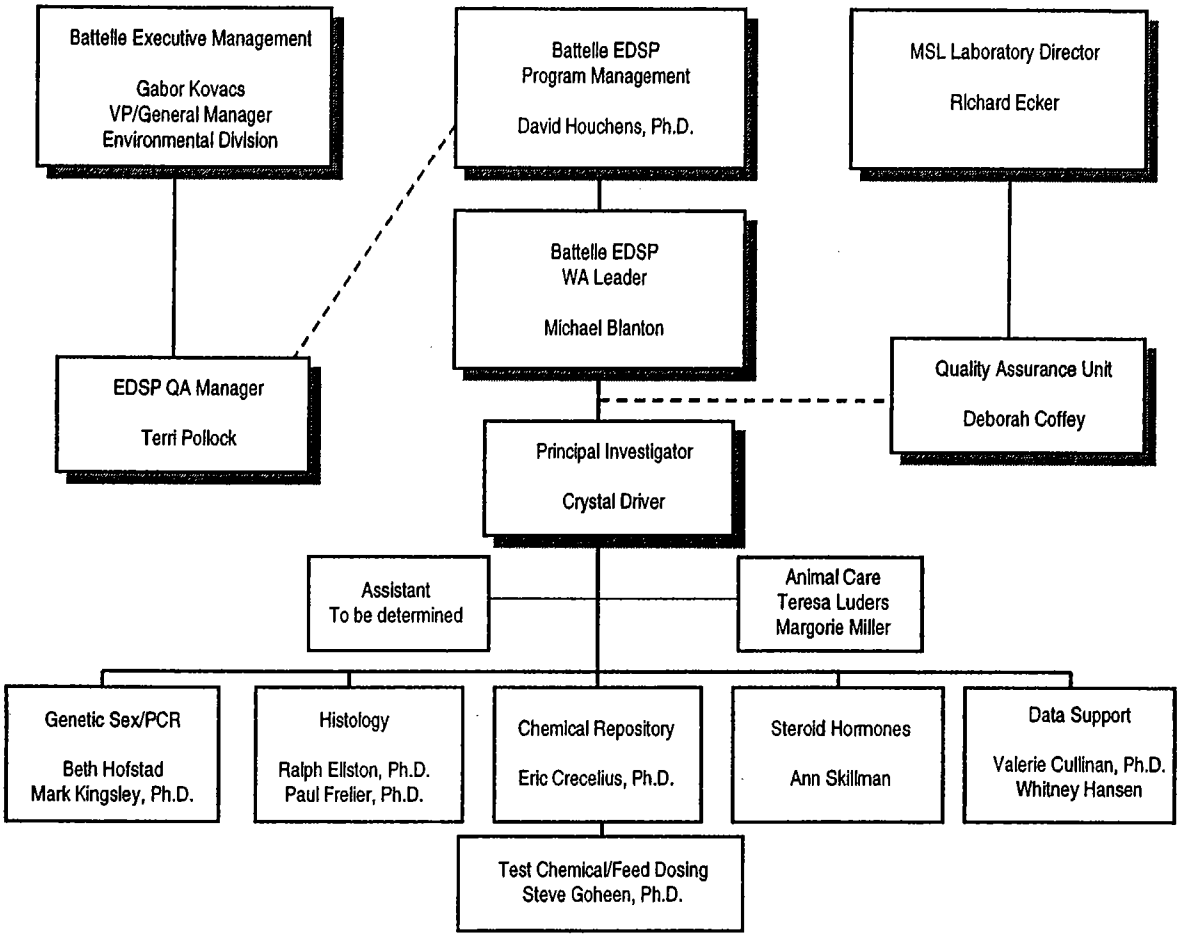

Figure 4-1 WA 2-17 Project Organization Overview

Eggs will be obtained from a local supplier, who regularly supplies quail for toxicological studies. An hourly employee at a Technician 3 level will provide support for egg collection, measures of bird body weight, food consumption, clinical sign observations, eggshell strength and thickness, sample collection and necropsy under the direction of Ms. Driver. Another employee will be hired to assist with daily feeding and cage cleaning activities.

Dr. Ralph Ellston, an independent contractor of his own firm, **AquaTechnics,** located in Sequim, WA, will be responsible for histological analyses of thyroid, adrenal glands, gonad (oviducts, ovaries, testes), liver, and brain tissue of adult birds and oviducts, ovaries, testes and thyroid in F2 chicks. Dr. Ellston will prepare samples and embed organs to create slides for histological analysis. **Dr. Paul Frelier** will perform histological analyses.

Ms. Beth Hofstad who has participated on other EDSP WAs (2-22, 2-30) will support genetic sex determinations. She will work with **Dr. Mark Kingsley** to optimize a PCR method to determine genetic sex in Japanese quail from blood or feather pulp samples. Ms. Hofstad and Dr. Kingsley will report to Ms. Driver.

Ms. Ann Skillman of Battelle, Sequim will provide support by performing steroid analyses of egg content and fecal matter by defining extraction methods and applying test kit ELISA method analyses, similar to analyses performed for EDSP WAs 2-18 and 2-19.

Crystal Driver in conjunction with an additional staff member to be named will function as the day-to-day data coordination supervisor. **Mr. Whitney Hansen**, Battelle, Sequim, will support data delivery and electronic data transfer activities.

Dr. Valerie Cullinan is the lead statistician for the project and is responsible for overseeing the statistical design, analysis, and interpretation of the data. Dr. Cullinan has over 15 years of experience in the design, analysis, and interpretation of multidisciplinary experimental research. Dr. Cullinan will work with Ms. Crystal Driver for statistical analyses.

The Battelle Quality Assurance Unit (QAU) is established as an objective, independent monitor of the work performed at Battelle. Thus, the QA Unit, **Ms. Deborah Coffey,** operationally informs Mr. Michael Blanton, who has overall responsibility for the Battelle ESDP QA program, of QA Program status and implementation. Ms. Deborah Coffey is the designated lead EDSP QA Team Member and is responsible for approving the QAPP and she will also be the designated facility QA specialist for this work assignment. In this capacity, she will administer the QAPP for the EDSP facility QA team members. **Mr. Ron Burkey,** a Battelle Quality Engineer with Good Laboratory Practices (GLP) QA experience, will assist Ms. Coffey, by providing QA support when needed to perform surveillances and assessments, review data, and provide project support during external assessments and facility inspections. Ms. Coffey and Mr. Burkey may assist in facility inspections or assessments at participating laboratories if requested. The specific responsibilities of Ms. Coffey include:

1. Assist the Principal Investigator in preparing the individual QAPP as required for each WA by defining appropriate QA requirements according to EPA/QA-R5, EPA Requirements for Quality Assurance Project Plans for Environmental Data Operations.

2. Interact with the Principal Investigator to ensure that WA personnel understand QA and QC procedures.

3. Conduct technical systems audits (TSAs) and audits of data quality (ADQs) to evaluate the implementation of the program WAs with respect to the EDSP QMP, the WA QAPPs, and applicable program and facility SOPs.

4. Prepare and track reports of deficiencies and submit them to both line and program management.

5. Consult with the Principal Investigator and, as necessary, the EDSP Battelle QA Manager and Program Manager on actions required to correct deficiencies noted during the conduct of the WA.

6. Ensure that all data produced as part of the EDSP WAs are maintained in a secure, environmentally protected archive.

7. Ensure, during the conduct of TSAs, that all staff participating on the EDSP are adequately trained.

8. Maintain complete facility-specific QA records related to the program.

9. Submit copies of resolved audits to the EDSP Battelle QA Manager.

10. Submit a QA Statement to the EDSP Battelle QA Manager and Program Manager with each written deliverable that describes the audit and review activities completed and any outstanding issues that could affect data quality or interpretation of the results discussed in the report.

11. Maintain effective communication with the EDSP QA Manager.

12. Act as the facility's EDSP SOP Custodian for all SOPs received from the SOP Administrator.

Ms. Coffey may delegate some of the listed responsibilities to Mr. Burkey when the need arises.

Dr. Eric Crecelius will direct a team of professional chemists and laboratory technicians who will prepare, deliver, and store the chemical test samples to be used by all the participating laboratories on the program. He will have ultimate responsibility for the quality of all chemical test samples used on the program and for their timely delivery to the participating laboratories. Dr. Crecelius is the Manager for the Environmental Chemistry group at the Battelle, Sequim facility. He is responsible for ensuring that appropriate and comparable technical procedures are used for sample analysis, ensuring that performance evaluation samples, standard reference materials, and certified standards are a routine part of the laboratory quality control program, and for providing technical expertise to the analytical laboratories. He is also responsible for ensuring that analyses are scheduled and completed by their due dates, that holding times are met, and that reporting schedules are not compromised. He will also ensure that corrective actions are assigned and completed. Dr. Crecelius reports the status of laboratory analyses and potential problems to the P.I., the MSL Manager and the EDSP Program and QA Managers. Dr. Crecelius reports to the MSL Manager.

Some of the EDSP Chemical Repository support activities for WA 2-17 will be delegated to Dr. Steve Goheen and his staff of the Analytical Chemistry Group, National Security Division, Battelle, Richland. **Dr. Steve Goheen** will be the lead for purity and stability analysis of the test chemical, 17β-estradiol, and testing the game bird feed spiked concentrations, spiking the feed (Startena and Layena) for use in the study, and verifying feed concentrations during the study. Although WA 2-17 is not a GLP-level study, the activities of the EDSP Chemical Repository are conducted at a GLP-level. **Dr. Jim Campbell** is a consultant helping to determine how to solve various analytical problems. **Eric Hoppe,** also a consultant, will provide expertise from work

on previous EPA GLP studies. He will assist with task management. **Ms. Blandina Valenzuela** will provide chemical analyses, and she will be responsible for in-process project records which will be kept locked in a fire-proof cabinet in her office. **Ms.Valenzuela** has prior experience with GLP studies. **Mr. Rich Lucke** is an additional chemical analyst with experience in methods development work. He also has past GLP experience. **Ms. Angie Melville** is a limited-term employee (LTE), providing various support activities, primarily to assist with documentation requirements and assist in the laboratory, supervised by someone with previous analytical experience.

It is important to analyze phytoestrogens in each batch of bird feed. Purina Mills or another laboratory will be identified to perform these analyses.

As EDSP Manager, **Dr. David Houchens** will have ultimate responsibility for quality, timeliness, and budget adherence for all activities on the contract. He will also serve as the principal interface with the EPA's Project Officer on all contract-level administrative and technical issues. Because of the high level of subcontracting and purchases required by the program, such as test laboratory subcontracts and purchases of chemical supplies, Dr. Houchens will be assisted by an administrative deputy manager, **Mr. James Easley**. Mr. Easley will manage the procurement of all subcontracts, consultants, and purchased materials and services, and will facilitate schedule and cost control. He has played a similar role on ten other large, multi-year, level-of-effort task-order contracts for EPA. Thus, he will be able to assure that all purchases are compliant with government regulations and that EPA is provided timely, accurate accounting of these substantial costs in our monthly progress reports.

Ms. Terri Pollock, the EDSP QA Manager at Battelle, will direct a team of QA specialists, including **Ms. Deborah Coffey,** who will monitor the technical activities on the chemical repository program and provide oversight to all associated QA functions. Ms. Pollock will be responsible for reporting her findings and any quality concerns to Dr. Houchens. Ms. Pollock reports, for the purposes of this program, to **Mr. Gabor Kovacs**, Vice President and General Manager of Battelle's Environmental Division. This reporting relationship assures that the QA function is independent of the technical activities on the program.

5.0 PROBLEM DEFINITION/BACKGROUND

The EPA, in collaboration with Organization for Economic Cooperation and Development (OECD), is developing a test guideline to assess the impact of chemicals on the reproduction and development of birds over two generations. The guideline will include both conventional and endocrine endpoints. Several methodological issues that could not be resolved from existing literature were discussed during an OECD Endocrine Disruptor Testing and Assessment Task Force consultation with member country experts (OECD Expert Group on Assessment of Endocrine Disrupting Effects in Birds). One of the key issues needing resolution prior to developing a test guideline is the selection of appropriate exposure scenario(s) during a two-generation test. Some experts argue that dietary treatment of the parental (P1) generation should begin after the onset of egg-laying to 1) allow the option of using pre-treatment measurements as covariates (internal controls), and 2) remove incompatible or infertile pairs

before treatment to reduce non-treatment sources of variation and increase the power to statistically evaluate test parameters (i.e., increase the ability to detect treatment effects if they exist). Other experts believe that exposure should begin prior to sexual maturation to detect effects resulting from delayed or inhibited gonadal development and/or changes in the onset of laying of the P1 generation.

Debate over the exposure regimen also extends to the F1 generation, with some member country experts proposing that the F1 generation also receive dietary treatment of the test substance, while others argue that the F1 generation should not be exposed to the test chemical. Arguments in favor of exposing the F1 generation to the test substance during all critical life stages include the ability to account for endocrine–mediated effects that occur during growth and development of the F1 chicks and to represent a worst-case exposure scenario. Not treating the F1 generation focuses the test on the effects of *in ovo* exposure of the developing embryo (e.g., gonadal abnormalities, altered sex ratio) and the subsequent reproductive success of the F1 generation without the potentially confounding influence of direct toxicity of the test substance to the chicks and the sexually maturing juveniles. However, the response of the F2 generation may provide needed *in ovo* effects data if the F1 exposure regimen is used.

5.1 PROBLEM DEFINITION

WA 2-17 addresses the need for experimental data regarding:

1) The relative importance of the timing of onset of treatment of the P1 generation (prior to sexual maturation or after proven egg-laying ability) for detecting reproductive and developmental effects over two generations.[2]

2) Whether the F1 generation should receive dietary treatment of the test substance.

The range finding study will consist of three treatment levels with no controls. Each treatment level will expose three reproducing pairs of Japanese quail to dosed feed (six birds * three treatment levels or 18 birds total). Doses for the treatment levels will be 1 ppm, 10 ppm and 100 ppm.

The test duration will evaluate adult birds through 14 days of egg-laying, to incubating eggs and hatching offspring. Hatchlings will be evaluated at 3 days for normality. Endpoints will be the survival of adult birds, egg production, embryo viability, and production of normal hatchlings.

For the definitive test, 300 eggs will be purchased, incubated and hatched. Eggs hatch at about 18 days of age. Birds will initially be fed Purina Startena game bird feed. Birds in the P1A population will start on dietary treatment when the gender of the birds can be determined by a visual examination of the plumage (about 3 weeks of age, Day 0). At this time, the birds will

[2] Please note that on page 2 of the Work Assignment Statement of Work the exposure options for the P1 generation are "initiated at sexual maturation or after proven egg-laying ability." Because sexual maturation is often determined by the onset of egg laying, it is assumed that "prior to or during maturation" was intended and conforms to the reference to "pre-breeding" dosing and the exposure scenarios discussed in the OCED documents.

be separated by gender and assigned to treatment groups. Birds can be considered as adults about 6 weeks of age. The P1A birds will continue on treatment through maturation and approximately 8 weeks of egg laying (approximately Day 84). Older birds will be fed Purina Layena game bird feed. Males and females will be housed separately until the fifth week of egg production (about Day 56) at which time they will be paired together. Eggs collected after pairing at the beginning of the fifth week post-onset of laying will be counted, candled for cracks or other abnormalities, sub-sampled for eggshell quality measurements and for steroid content (1 egg per pen per week of weeks 5 and 8). All remaining eggs will be incubated and candled on Day 8 of incubation to determine embryo viability. Except for a subset of eggs (4 per pen) from the eggs produced in week 8 of egg laying, all eggs will be discarded after the Day 8 candling.

The remaining subset of eggs will be incubated to hatch to produce the F1 breeding population (F1, Day 0). Hatchlings from this final egg batch will be assigned to one of two cohorts as they are removed from the hatcher. The F1a cohort will receive no dietary treatment. Hatchlings assigned to the F1b cohort will receive dietary treatment from hatch through egg laying (approximately Day 98). Both cohorts will be brought into egg production, the eggs will be collected for 5 weeks after the onset of laying to measure test endpoints.

P1B birds will be raised as described for the P1A birds, but without dietary treatment until they are paired. Females laying at least 3 eggs per week by the fifth week post onset of egg production will be considered proven breeders at which time they will be paired with males and the breeding pairs will start on dietary treatment. Treatment will last 4 weeks such that treatment ends at the same time as for the P1A birds. Endpoint measurements will be collected, the F1 breeding populations established and the F1 cohorts treated as described for the P1A birds.

5.2 BACKGROUND

The process of selection of the test chemical is discussed in great detail in the Study Plan (Battelle, 2002). The following criteria for selecting a suitable test substance for comparing exposure regimens were applied to the candidate compounds:

1) The test substance should have the potential to affect the maturation of parents in such a way to determine what endocrine-mediated effects may not be observed by starting treatment during the egg-laying period (P1), or by not treating the F1 chicks.

2) The test substance should give rise to inter-generational effects so that the impact on reproductive/endocrine endpoints in the F1 generations of the two P1 exposure regimes can be compared. This also provides for a comparison of the reproductive performance of untreated F1 and treated F1 birds and the survivability of their offspring.

3) The test compound must clearly act on a hormone system (not simply alter a process that is under normal endocrine control).

4) There should be sufficient knowledge of the effects and/or mode of action of the test substance that appropriate, sensitive endpoints can be selected.

A compound such as 17ß-estradiol satisfies the general selection criteria (obvious action on a hormonal system, ability to affect maturation, documented transfer from hen to egg, induction of intergenerational effects) for the dosing study and is applicable for evaluating the F1 exposure regimen in the absence of confounding toxicity. Therefore, 17ß-estradiol was selected for use in the dosing study, specifically to evaluate the relative importance of the timing of onset of treatment of the P1 generation (prior to sexual maturation or after proven egg-laying ability) for detecting reproductive and developmental effects.

An additional advantage of using 17β-estradiol for this dosing study, is the availability of data on the effects of dietary estradiol on the reproductive performance of the northern bobwhite (*Colinus virginianus*). Studies by Lien et al. (1985 and 1987) provide information on potential dose levels, endpoint sensitivity and range testing. In addition, using 17β-estradiol in this study with Japanese quail will allow a comparison of the relative sensitivity of this more domesticated species with the bobwhite, a species that has experienced less inbreeding pressure and presumably is more representative of wildlife responses. Although estradiol is not volatile (1.26E-008 mm Hg vapor pressure), care must be taken to avoid exposure of staff and cross-contamination of controls to this potent estrogen. These issues are addressed in greater detail in the Study Protocol (Battelle, 2002).

6.0 PROJECT/TASK DESCRIPTION

6.1 PROJECT OVERVIEW

6.1.1 General Japanese Quail Life Cycle

Eggs will be purchased from a commercial supplier. Eggs will be kept in incubators until hatching. The incubation period is around 18 days, ranging from 16 to 19 days. The duration of incubation varies depending upon temperature, humidity and genetic variability. The genetic variability of these eggs is initially an unknown factor. On Day 15, the eggs will be transferred to another incubator and placed in pedigree baskets where they will be allowed to hatch. Those chicks that have not hatched within about 24 hours of the majority of chicks, will be considered unhatched. The newly hatched quail chicks are transferred directly to the brooder from the incubator. Hatchlings will be housed in stainless steel or galvanized brooding pens by treatment group. Hatchlings will be housed in brooding pens until 21 days of age. Sexing is possible by three weeks of age by the cinnamon-colored feathers on the breast of the male bird, but there are some birds that defy definite sexing by this method, even when adults. Egg laying begins at approximately 56 days of age and males are capable of fertilizing eggs by 42 days of age. The expected duration of the in-life portion of the test will be approximately 40 to 44 weeks.

6.1.2 Exposure Regime

The exposure options will be evaluated in an experimental design that compares two P1 exposure scenarios (P1A receiving treated diet prior to sexual maturation and P1B receiving treatment after proven egg-laying ability has been established) and F1 exposure options (F1a receiving no dietary treatment and F1b receiving treated diet from hatch through egg laying) using two cohorts of F1 chicks that survived *in ovo* exposure from the P1 parents (Figure 6-1).

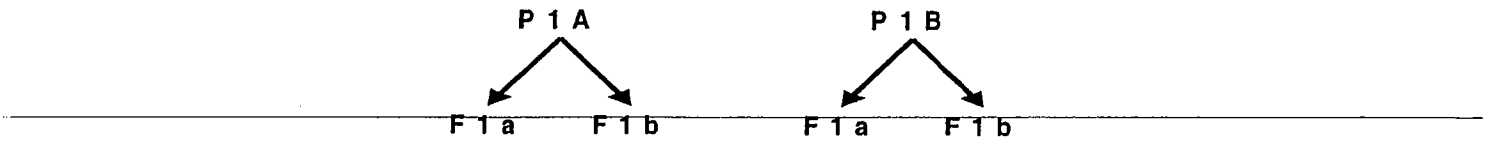

Figure 6-1. Exposure Design. The F1 Populations are Obtained from the Last Week of Eggs Collected from the P1 Birds.

Both P1 populations will be assigned randomly to a control group or one of a geometrically spaced series of four dietary concentrations (1x, 0.33x, 0.11 x and 0.033x where x = 33 ug/g.) The dietary concentrations will be determined from the results of a range-finding trial. Dietary concentrations for the study will define the dose-response relationships for the more sensitive test endpoints. The control group will be fed diet containing any solvents or carriers in amounts equivalent to those used in treated diets. The specific solvent/carrier used in this study for 17β-estradiol will be determined at the conclusion of the solubility trial in which cottonseed oil, commercial Mazola oil, DMSO, acetone and ethanol are being tested. The dietary route of administration was selected because it represents the most likely route of exposure to avian species in the environment. The diet will be analyzed for caloric content and the presence of natural endocrine-active compounds.

Birds in the P1A population will start on dietary treatment when the gender of the birds can be determined by a visual examination of the plumage (about 3 weeks of age). At this time the birds will be separated by gender and assigned to treatment groups. The P1A birds will continue on treatment through maturation and approximately 8 weeks of egg laying. The date of onset of laying will be recorded. Males and females will be housed separately until the fifth week of egg production at which time they will be paired together. All eggs produced prior to pairing will be collected, counted, candled for cracks and other abnormalities, sub-sampled (1 egg per pen per week) for shell quality measurements (thickness and strength) and discarded. Eggs collected after pairing at the beginning of the fifth week post onset of laying will be counted, candled for cracks or other abnormalities, sub-sampled for eggshell quality measurements and for steroid content (1 egg per pen per week of weeks 5 and 8). All remaining eggs will be incubated and candled on Day 8 of incubation to determine embryo viability. Except for a subset of eggs (4 per pen) from the eggs produced in week 8 of egg laying, all eggs will be discarded after the Day 8 candling. The remaining subset of eggs will be incubated to hatch to produce the F1 breeding population. Hatchlings from this final egg batch will be assigned to one of two cohorts as they are removed from the hatcher. The F1a cohort will receive no dietary treatment. Hatchlings assigned to the F1b cohort will receive dietary treatment from hatch through egg laying. Once chicks have reached the age where gender can be determined, the F1 breeding population will be selected as described in the study protocol (Appendix A of the Study Plan). Both cohorts will be brought into egg production, and the eggs will be collected for

5 weeks after the onset of laying to measure test endpoints. All intact eggs, except those removed for eggshell quality measurements (1 egg per pen per week) and steroid analysis (1 egg per pen of week 5), will be artificially incubated and hatched on a weekly basis. Embryo viability will be determined on Day 8 of incubation and F2 hatchling survival will be monitored for 14 days. Gross necropsy, body weight, and histological examination of excised tissues will be performed on all adult birds when terminated. Terminal body weight will be obtained for all F2 chicks. Necropsies will be conducted on a subset of the F2 chicks (33% of each pen's chicks) and histological examination of the reproductive system will be performed on a total of one third of the F2 chicks produced from eggs collected during week 5. Genetic sex determination will be performed on about one half of the week 5 F2 chicks. The cloacal gland will be measured in all male birds.

P1B birds will be raised as described for the P1A birds, but without dietary treatment until they are paired. Females laying at least 3 eggs per week by the fifth week post onset of egg production will be considered proven breeders at which time they will be paired with males and the breeding pairs will start on dietary treatment. Treatment will last 4 weeks such that treatment ends at the same time as for the P1A birds. Endpoint measurements will be collected, the F1 breeding populations established and the F1 cohorts treated as described for the P1A birds.

The experimental treatments are shown in Table 6-1. The actual test concentrations can not be selected until the range-finding test is completed. Figure 6-2 shows the number of adult birds used, number of eggs collected and produced in the different phases of the study. Figure 6-2 shows that 558 adult birds, 6832 eggs and 2325 hatchlings are anticipated to be required to conduct this study.

6.2 SPECIFIC STUDY OBJECTIVES

The specific objectives of the study are to:

1) Compare dose-response relationships of endocrine and fitness endpoints between the two exposure scenarios to define the most appropriate exposure regimen for detecting and quantifying a range of endocrine-mediated effects. Emphasis will be placed on comparing the relationships on the basis of slope, relative sensitivity and relative variability of the endpoints, and determining endocrine-mediated effects that may not be observed by initiating treatment after the onset of egg laying.

2) Compare dose-response relationships of endocrine and fitness endpoints between the two exposure scenarios for the F1 generation of each P1 exposure scenario and between all F1 exposure groups to define the most appropriate exposure regimen for detecting and quantifying a range of endocrine-mediated effects. Time series data will be used to assess the daily/weekly/etc. within-class variation in response, time lag between exposure and response, and appropriateness of the exposure duration.

Table 6-1. Proposed Treatment Groups for the Exposure Comparison Study

Onset of Exposure	Pens per P1 or F1 Group (1 cock and 1 hen per pen)	17ß-Estradiol Exposure Concentration (ppm)			
		adults	F1a	F1b	F2
P1A (pre-breeding; 2-3 wks old)	8	0	0	-----[2]	0
	8	1x	0	1x	0
	8	0.33x	0	0.33x	0
	8	0.11x	0	0.11x	0
	8	0.033x	0	0.033x	0
P1B (adult; proven layers)	8[1]	0	0	-----	0
	8	1x	0	1x	0
	8	0.33x	0	0.33x	0
	8	0.11x	0	0.11x	0
	8	0.033x	0	0.033x	0

[1] 10 pairs will be established in each group initially to provide for at least 8 pairs of breeding pairs during treatment. (Both birds of a pair will be removed if one of the pair dies or is injured).

[2] No additional control group is used. The F1a control groups serve as controls for both F1a and F1b populations.

6.3 SUMMARY

The general consensus of the expert group was to include in the pre-validation most of the "fitness" endpoints (Table 6-2) and to apply a subset of "physiological" or "endocrine" endpoints that identify endocrine-mediated effects during sexual maturation and egg production. Because the proposed test substance is 17ß-estradiol, the selected endpoints emphasize measures with underlying estrogenic mechanisms and measures for feminization of males (Table 6-3). Feminization of the P1 and F1 offspring will be measured by determining genetic sex in hatchlings (Table 6-2) and the hormonal status and internal sexual characteristics of a subset of genetic male and female chicks at 14-days of age (Table 6-3). Because of the apparent sensitivity of the oviduct to endocrine changes (Lien et al, 1985), the oviduct will be weighed and the degree of differentiation in the organ will also be evaluated. Some measures also are relevant for general toxicity assessment, especially in embryonic stages. Also, because there are a number of interactions among various endocrine axes and estrogen is known to depress production of thyroid hormone, Dr. Anne Fairbrother (EPA) suggests the addition of thyroidogenic endpoint measures to monitor this interaction and begin to develop baseline information for the Japanese quail.

Table 6-2. Fitness Endpoints for Exposure Comparison Study

Endpoint	Endocrine Activity	
	Estrogenic	Thyroidogenic
For Breeding birds (P1 and F1)		
Body weight at start and end of treatment		X
Food consumption weekly during treatment		X
Survival	toxicity	
Signs of toxicity	toxicity	
Number of eggs laid per pair	X	
Number of fertile eggs per eggs laid	X	
Number of cracked eggs (at set)	potential	potential
Number of eggs hatched per eggs set[1]	X	
Eggshell strength and thickness	potential	
Early and late viability per eggs set[2]		
F1 and F2 Chicks		
Sex ratio of chicks	X	
Number of chicks surviving to 7 and 14 days per eggs set and per eggs hatched[3]		
Growth rate of chicks (weight at days 1, 14)[3]	X	X

[1] Only F1 eggs from the last week of egg-laying (week 8) will be hatched. F2 eggs from all collection periods will be hatched.
[2] Late viability will be determined on all F2 eggs and F1 eggs from last week of egg-laying.
[3] F2 chicks and those F1 chicks hatched from the last week of eggs.

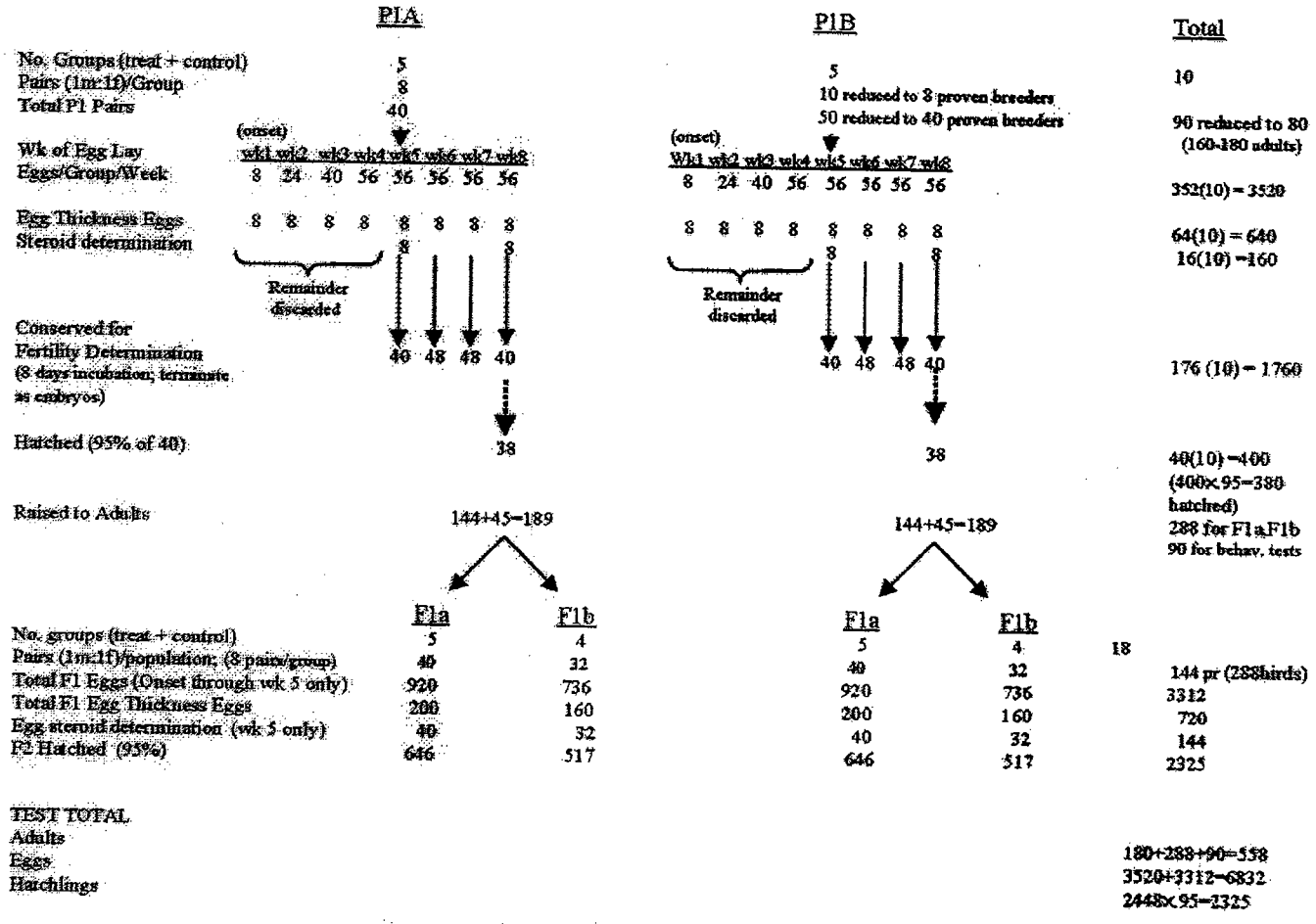

Figure 6-2. Number of Adults, Eggs, and Hatchlings Used During Different Phases of the Dosing Study

Table 6-3. Endocrine or Physiological Endpoints for Exposure Comparison Study

Endpoints	Estrogenic	Thyroidogenic	Androgenic
For Breeding Birds (P1 andF1)			
Gross morphology & histology			
weight of testes, ovaries, thyroid, adrenals, oviduct, cloacal gland, liver	X	X	X
histology of thyroid, adrenals, gonads, brain	X	X	X
testicular spermatid counts and morphology	feminization	X	
gross anomalies of the genital tract	X		X
Developmental Landmarks			
feather dimorphism	X	X	
cloacal gland size, 1st appearance of foam			X
1st egg laid	X		
sexual behavior[1]	feminization		
Fecal/urate hormones			
steroid hormones (estradiol, testosterone)[2]	X		X
For F2 Chicks			
steroid hormones (estradiol, testosterone)	X		X
Gross morphology & histology			
size and dimorphism of gonads	X		X
histology of gonads (relative amount of cortex and oocytes), thyroid, oviduct	X		X
presence, weight and differentiation of oviduct	X		X
thyroid, cloacal gland, liver, brain, pancreas	X	X	X
wing and bone length		X	

[1] F1 males only
[2] P1 only

7.0 QUALITY OBJECTIVES AND CRITERIA FOR MEASUREMENT DATA

A number of the specified tasks incorporate quality objectives relative to the accuracy of the measures to be made (refer to Table 7-1). The large number of birds to be used in the test are needed to provide a statistically-valid sample to detect effects from the test chemical. Fitness and endocrine or physiological endpoints are listed in tables 6-2 and 6-3 above. Standard applications of data quality objectives do not readily apply to this work. Measurement systems and equipment will be calibrated before use. Much of the data to be collected are counts, observations, weights, and measures, which are discussed in detail in Section 10.4. Gross morphological assessments depend on the level of training of staff. A detailed procedure, MSL-T-043, Avian Necropsy and Gross Organ/Tissue Morphology will provide a strong reference for staff training and completing this task. Histology will be performed by experts. Genetic sex of hatchlings will be made by a gel electrophoresis method polymerase chain reaction (PCR). Steroid hormone determinations are made using commercially available kits. Eggshell thickness is made using micrometers and eggshell strength is made using a instrument modified for this purpose.

Table 7-1. Quality Objectives and Criteria for Measurement Data

Parameter	Units	Comments
Eggs		
Egg production: Eggs will be collected and counted daily.	Counts	Eggs will be counted on a per pen basis
Eggshell quality: At the end of the weekly interval, all eggs will be removed from the cold room, counted, candled, and selected eggs taken for egg quality tests and analysis of test substance content.	Counts	Cracked or abnormal eggs will be recorded and discarded
Cracks or abnormal egg: Remaining eggs will be candled weekly with an egg-candling lamp to detect eggshell cracks or abnormal eggs.	Counts and Observations	Cracked or abnormal eggs will be recorded and discarded
Early embryo viability: Eggs will be candled on Day 8 of incubation to determine early embryonation (embryo viability)	Number with a viable embryo will be recorded	Cracked or abnormal eggs will be recorded and discarded
Hatching success: On Day 15 viable eggs will be will be transferred to another incubator and placed in pedigree baskets where they will be allowed to hatch.	The number hatched and unhatched will be recorded.	Those chicks that have not hatched within about 24 hours of the majority of chicks will be considered unhatched.
Eggshell Quality		
Egg shell thickness	mm	An average of five measures around the circumference of the egg will be reported.
Egg shell strength	The load (±1%) will be recorded in Newtons	The egg will be placed on its side on the test stand so that the compression head will contact the egg at the equator between two parallel stainless steel surfaces advancing at a constant rate
Steroid Hormone Determination		
Egg content: collection and analysis	pg/mL Estradiol, Testosterone	Commercial enzyme immunoassay (EIA) kits
Fecal matter	pg/mL estradiol and Testosterone	Commercial enzyme immunoassay (EIA) kits
Observations		
Clinical observations: All adults and offspring will be observed daily throughout the test for overt signs of toxicity or abnormal clinical observations.	Daily Counts and Observations	A record will be maintained of all mortalities and observations.
Survivability of hatchlings: F1 offspring (F2) will be observed over a 14-day period beginning when birds are first removed from the incubator.	The number surviving to 14 days will be recorded. Survivability of chicks hatched for F1a and F1b populations and male mating behavior trials will also be recorded.	The observation period will be extended if late mortality occurs, which appears to be treatment related.
WEIGHTS		
Adult body weight: Individual body weights of the adults will be measured at test initiation (Day 0) and at adult termination.	Individual body weights (g)	Body weights will not measured during egg laying because of the possible adverse effects that handling may have on egg production.
Offspring body weight: Individual body weights of offspring by parental pen will be recorded at hatching and on Day 14 post hatch.	Group body weights (g) of offspring by parental pen	

Parameter	Units	Comments
Feed consumption: Feed consumption for each pen will be measured at least weekly and at test termination. More frequent measures will be made if feed must be changed more often than weekly to maintain 80% of original concentration under test conditions.	Feed consumption is determined by weighing the freshly filled feeder (g) on Day 0, recording the amount of any additional diet added during the week and weighing the feeder and remaining feed (g) at the end of the seven-day feeding period. Estimated weight (g)	Since wasted feed normally is scattered and mixed with water and excreta, no attempt will be made to quantify the amount of feed wasted by the birds. Therefore, feed consumption will be presented as an estimate.
Reproductive Parameters		
The following reproductive parameters will be measured and recorded by pen: # eggs laid # eggs cracked # eggs broken	Counts	
# 14-day old survivors	Counts	
Body weight of 14-Day-old survivors	Weight (g)	
# eggs incubated (set) # Infertile or clear eggs # dead embryos (Day 8, Day 15) # viable embryos (Day 8, Day 15) # unhatched eggs # hatchlings	Counts	
Genetic Sex of hatchlings		
Polymerase chain reaction (PCR) – genetic amplification at hatch (toe clip, or feather pulp), subset of F2 and subset at necropsy	Genetic male or female determination from blood or feather pulp	Gel electrophoresis
Steroid Hormones		
Fecal matter	pg/mL estradiol and testosterone	Commercial enzyme immunoassay (EIA) kits
Maturation Endpoints		
Feather dimorphism	Visual assessment, feather spot (mm)	Calipers
Bone length and morphology	Tibia (necropsy), mm Wing (aging) mm	Micrometer
Cloacal gland measures	mm	Calipers
Oviduct weight	mg	
Oviduct differentiation	Visual and histological assessment	
Fixed organ weights	Thyroid, adrenal (mg)	Weights will be determined post-preservation to limit damage to tissue
Organ Weight at Necropsy/Histology	Oviducts, ovaries, testes, liver, and brain tissue	
Organ weight at Necropsy/Histology F2	Oviducts, ovaries, testes (thyroid as fixed)	
Copulatory Behavior	E.g., average number of mountings per pen, and cloacal contact movement	

8.0 SPECIAL TRAINING/CERTIFICATION

Although the 17β-estradiol is minimally volatile (1.26 E-008 mm Hg), there may be potential for dermal and respiratory exposure of staff through handling the diet and/or from

airborne fines from the feed. Therefore, staff handling the preparation and distribution of the treated diets and accessing the animal rooms will be trained relative to the hazards and safe handling of this potent estrogen and will wear appropriate personal protective equipment as determined by an Environmental Safety and Health subject area expert.

There are no obvious requirements for specialized training for the exposures to be performed under this WA. Battelle will demonstrate to EPA's satisfaction its ability to do any of the tasks listed in the protocols by offering to have each technician who will be doing these tasks demonstrate proficiency (i.e., during an audit).

9.0 DOCUMENTS AND RECORDS

9.1 RETENTION OF SPECIMENS AND RECORDS

All specimens and records that remain the responsibility of Battelle will be retained in the project data files for the length of time stipulated in the contract. The files will remain with the Principal Investigator until the study is complete and then be placed in the archived files for Battelle in Richland, WA. Records are maintained according to a policy of limited access. The archivist is responsible for archiving and retrieving WA materials. An archive inventory will be maintained and storage capability will provide for the expedient retrieval of materials. Specimens and samples will be disposed of only after assessing that they no longer afford evaluation.

9.2 QUALITY ASSURANCE PROJECT PLAN

This QAPP will be distributed to Battelle project participants, initially, and whenever revised. Previous versions either will be marked as "obsolete" when newer versions are distributed, or will be collected and destroyed so that there is no confusion regarding the version in effect. The right-justified document control header example shown here

Version 1
Month Year
Page 1 of 1

is used to ensure that revision numbers and dates are obvious to document users. The QAPP will be reviewed annually if the project extends beyond one year, and a determination made to either modify the document, based on new or modified project requirements, or leave as is. Controlled copies of the QAPP are maintained, tracked, and managed by the Battelle QA Unit Manager through the use of a master distribution list.

9.3 STANDARD OPERATING PROCEDURES

Applicable procedures for conducting work exist within the MSL QA Program. The following existing SOPs will be followed:

ADMINISTRATION

MSL-A-001 Sample Log-In Procedure
MSL-A-002 Sample Chain of Custody
MSL-A-003 Guidelines for SOP Format and Control
MSL-A-005 Deviations From Established Requirements
MSL-A-006 Marine Sciences Laboratory Training
MSL-A-008 Control of Reagents/Solutions, Test/Control Articles and Specimens
MSL-A-010 Document Control

DOCUMENTATION, RECORDS, REPORTS

MSL-D-001 Recording Data on Data Sheets and Laboratory Notebooks
MSL-D-004 Data Reporting, Reduction, Back Up, and Archiving

INORGANIC CHEMISTRY

MSL-I-026 Use of Laboratory Refrigerators and Freezers

CONVENTIONAL/GENERAL CHEMISTRY

MSL-C-004 pH in Water
MSL-C-009 Use and Performance Checks of Balances
MSL-C-010 Calibration and Use of Pipettes

TOXICOLOGICAL TESTING

MSL-T-041 Removal of Animals for Terminal or Interim Weighing and Sacrifice
MSL-T-043 Avian Necropsy and Gross Organ/Tissue Morphology
MSL-T-044 Avian Identification with Wing Clips and Leg Bands
MSL-T-045 Clinical Observations for Acute and Repeated Dose Studies
MSL-T-047 Avian Indications for Euthanasia
MSL-T-048 Daily Care of Bioassay Animals and Cleaning of Exposure Rooms
MSL-T-049 Receiving Eggs and Birds, and Quarantine of Birds
MSL-T-050 Collecting, Incubating , and Hatching Eggs
MSL-T-051 Brooders, Juvenile Bird Husbandry, and Data Collection
MSL-T-052 Maturation Endpoint Measures
MSL-T-053 Copulatory Behavior Study
MSL-T-054 Diet Sampling
MSL-T-055 Steroid Content of Eggs and Fecal/Urate Samples
MSL-T-056 Eggshell Strength and Thickness
MSL-T-057 Food Consumption
MSL-T-058 Determining Genetic Sex
MSL-T-059 Avian Study Range Finding Test

This project requires requesting test chemicals from the EDSP Chemical Repository following specifications found in:

EDSP.C-001 The EDSP Chemical Repository

Routine animal care facility procedures will also be followed. Data reporting will be in conformance with:

EDSP.D-003-01	Transmission of Information to the EDSP Data Coordination Center (DCC)
EDSP.D-013-01	Information to be Delivered for QA Documentation to the EDSP Data Coordination Center (DCC)

A Training Assignment matrix will be used to define the required training for each project participant based on the required work activities.

Deviations from SOPs will be documented using the Deviation Documentation Form from MSL-A-005, Deviations from Established Requirements. The form provides tracking and documentation of both miscellaneous issues and deviations from SOPs. All issues documented using this form must be assigned a deviation number by the Battelle-Sequim QA Unit Manager.

9.4 DATA REPORTING PACKAGE

Records for the data report package are specified in the Study Plan for WA 2-17, and are listed below. Except for the animal room environmental conditions and the statistical analysis records, the data are recorded manually on data forms. All data forms include a title identifying the type of data to be recorded, the unique study name, and the initials and date of the data recorder(s) to authenticate the records:

- Protocol and any Amendments
- List of any Protocol Deviations
- List of applicable Standard Operating Procedures
- Temperature and Humidity Records for the Animal Rooms
- Animal Research Facility Room Log(s)
- Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results
- Dosage Code Records (generation-treatment-concentration-animal-pen) number
- Dose Formulation Receipt, Transfer, and Use Records
- Sample Collection Records, including coding of samples
- Weight Sheets (Feed/Dosing, body weight, necropsy – organs collected)
- Specimen Sample Collection Records and Chain of Custody
- Statistical Analysis Records (see Section 19.3 for software information)

- Correspondence

9.4.1 Draft and Final Reports

Draft reports will be generated by the Principal Investigator within three months of the completion of the various phases of the studies. The draft report will be reviewed by project staff and the Battelle QA unit. Final reports will be reviewed by the Work Assignment Leader and EDSP Program Management before being forwarded to EPA.

General format requirements include:

1. ***"Draft or Final Report" and "Page 1 of _n", where n is the total number of report pages on the title page***

2. ***Contract number in the header, right justified***

3. ***Text, which includes the following sections:***

- Abstract
- Objectives
- Materials and Methods
- Results
- Discussion
- Conclusions
- References
- Protocol Deviations, if any
- Summary in-life and endpoint data with statistical analyses when applicable.

4. ***Appendices containing the individual animal data (in-life and necropsy), the protocol and any amendments, and historical control dataset. A histopathology report will also be provided.***

9.4.2 Data Package Report Contents

Data package report contents will include the following in addition to the data package contents specified in Section 19.1:

9.4.2.1 Test Substance. The report will include a detailed description of the test substance, including information on the CAS number, source, lot number, and purity. However, it needs to be stated that test substance will be provided by the EDSP Chemical Repository, which is responsible for purchasing, testing, and preparing the test substance at the request of the WA 2-17 PI.

9.4.2.2 Protocol and Any Amendments. The data package will document the methods (protocol) used, list of any protocol deviations, provide a data summary, and the results of any statistical analysis performed, The recommended test protocol is intended to be a product of

WA 2-17; this is the basis of this WA. However, there may be unforeseen circumstances that might lead to a protocol deviation.

9.4.3 QA Assessment Reports

QA assessment reports (see Section 20) are maintained as confidential files by the QA Unit Manager.

9.4.4 Status Reports

Monthly status reports will be provided to the EDSP Program Manager and WAL.

9.5 ENVIRONMENTAL CONDITIONS

There are specific requirements for environmental conditions for Japanese quail eggs, hatchlings, juvenile and breeding birds. Refer to Table 9-1 below.

9.5.1 Housing and Environmental Conditions

Adult birds will be housed indoors in stainless steel or galvanized cages designed to house one pair of quail. Cage size is 20.25" L x 10" W x 10" front height, 8" back height. The cages have sloping floors, individual cage feeders, and automatic water. Only birds associated with this study will be maintained in the study room. The requirements of MSL-T-048, Daily Care of Bioassay Animals and Cleaning of Exposure Rooms will be followed. The study room has controlled light, temperature, and humidity. The target conditions for light and temperature are the following:

Table 9-1. Summary of Environmental Conditions For Japanese Quail Eggs, Hatchlings, Juvenile And Breeding Birds

Test Organism	Japanese Quail **Eggs, Hatchlings to 21 –D**	Japanese Quail **Juveniles**	Japanese Quail **Breeding pairs**
Monitoring	Daily	Daily	Daily
Lighting	fluorescent	fluorescent	fluorescent
Photoperiod	16 hr light:8 hr dark with 2 hr gradual ramping between dawn and dusk	16 hr light:8 hr dark with 2 hr gradual ramping between dawn and dusk	16 hr light:8 hr dark with 2 hr gradual ramping between dawn and dusk
Temperature	35°C ± 2°C	22°C ± 1°C, but see below	22°C ± 3°C
Egg Rotation	6-10 rotations/24 hr period	NA	NA

The photoperiod for both adults and hatchlings will be 16 hours of light per day throughout the test. Birds will receive a minimum of 6 lux of illumination at the level of the bird. Light will be provided by fluorescent lights that emit a spectrum simulating that of daylight. The photoperiod shift is not abrupt, but rather will be attenuated to mimic as much as possible, natural sunrise and sunset. The photoperiod in the rooms housing both the adults and hatchlings will be maintained by time clocks. The temperature for adult birds ranges is 22°C ± 3°C. There is a relatively wide range of acceptable overall relative humidity; 40 to 95%.

Hatchlings will be housed in brooding pens by treatment group. Thermostats in the brooding compartment of each pen will be set to maintain a temperature of approximately 38°C for one week, and then temperature will be lowered 3 to 5°C per week over the following three weeks. Hatchlings will be housed in brooding pens until 14 days of age. Although, thermometers will be used to monitor temperatures, the spatial relationship of the hatchlings to the heat source is more important than the actual temperature reading. When hatchlings are clustered near the heat source, this indicates that the temperature is too low and needs to be adjusted higher. When the hatchlings cluster away from the heat source, it indicates that it is too high and needs to be adjusted downward. Temperatures will be assessed twice each day in over 50 brooding pens.

9.5.2 Egg Collection and Storage

Eggs will be collected daily and marked with a soft lead pencil or permanent ink according to the pen from which they were collected. Eggs then will be stored at an average temperature of 10 to 16 °C and an overall relative humidity of 40 to 95%. All eggs laid in weekly intervals will be considered as one lot. Lots will be identified by a lot code.

9.5.3 Incubation and Hatching

Eggs will be set weekly for incubation. The eggs will be incubated at 37.5°C, and an average relative humidity of approximately 60%. The incubator/brooder is equipped with a fan and blades that produce a mild breathing air movement that is designed to eliminate intra-cabinet temperature and humidity variation during incubation. To prevent adhesion of the embryo to the shell membrane, the incubator also is equipped with an automatic egg rotation device, designed to rotate the eggs from 50° off of vertical in one direction to 50° off of vertical in the opposite direction (a total arc of rotation of 100°) every two to four hours.

10.0 EXPERIMENTAL DESIGN

10.1 TEST SUBSTANCE

17β-estradiol (CAS 50-28-2), a compound with low hatching toxicity, but established *in ovo* transfer and endocrine-mediated effects during both maturation of juveniles and egg formation will be used to evaluate the appropriateness of pre-breeding vs. proven breeder exposure regimens. Range-finding tests will be conducted to define the appropriate test concentrations. Range-finding test concentrations will be determined from the literature in consultation with EPA. In the definitive testing stage, four treatment groups will be used for all P1 and F1 populations. A concurrent control group will be used for each of the two P1 test populations and each the two F1a populations. F2 chicks will not receive dietary treatment. The test concentrations will be geometrically spaced between the highest and lowest doses. The highest concentrations will be below levels shown to cause mortality or severe signs of parental toxicity as determined in the range finding test, but will be of a level that is expected to reveal significant effects on reproductive and endocrine endpoints. Control birds will be from the same hatch as the test groups and will be kept under the same experimental conditions as the test birds.

Control diets will consist of the same basal diets that the test birds receive with no test substance added. The carrier will be added to the control diet in the highest concentration used for the test diets.

The test substance will be analytically pure, and the purity will be reported, along with the percentage of each impurity.

10.2 TEST ANIMALS

The species to be tested will be the Japanese quail (*Coturnix japonica*). Birds used in this test will be obtained from a local commercial source as eggs and reared at Battelle Northwest Laboratory. All treatment and control birds used in a test will be from the same hatch for both P1 populations. F1 breeders will be obtained from the eggs produced during the eighth week of egg laying by the P1 birds. Control and test birds will be kept under the same experimental conditons. All birds will be in good health and free of abnormalities or injuries that may affect test results at test initiation. Daily observations and health records will be maintained from hatch until test termination.

The group of birds will not be used if more than 3-5% of either sex becomes debilitated in the 7-day period immediately prior to test initiation. If greater than 5% debilitation is observed, then the EPA Project Officer and Work Assignment Manager will be consulted.

The quail will be acclimated to the test facilities and an untreated diet until test initiation. Acclimation typically will occur in brooding pens. Birds will be weighed and randomly assigned to treatment and control pens. To avoid pairing siblings, within control and treatment group F1 birds will be randomly assigned to pens by pairs with males from odd-numbered pen parents being paired with females from even-numbered pen parents and males from even-numbered pen parents being paired with females from odd-numbered pen parents. The sex of the birds will be determined by a visual examination of the plumage. However, if birds in a pen are incompatible, they may be replaced or rearranged within a control or treatment group at any time prior to egg laying.

Identification: All birds will be identified by individual leg or wing bands. Each pen will be identified with a unique number. Groups of pens will be identified by exposure type (e.g., established breeder, P1B, or during maturation, P1A) and concentration. All eggs laid during the study will be marked with a soft lead pencil or permanent ink marker for identification.

Feed and Water: All birds and their offspring will be given feed and water *ad libitum* during acclimation and testing. Basal diet used to prepare the treated and control diets of both adults and offspring will be obtained from Purina Mills, in Spokane, WA. The basal ration will contain at least 27% protein and 2.5% fat and will contain no more than 6.5% fiber. During the test, adults will receive a basal diet supplemented with calcium (Layena) for proper eggshell formation. Offspring will receive diets prepared without the addition of limestone (Startena).

All birds will receive filtered tap water. Water is supplied by the City of Richland municipal water system. All offspring will receive a water-soluble vitamin and electrolyte mix

(Durvet, Incorporated, Blue Springs, MO 64015) in their water. Neither the adults nor offspring will receive any form of medication in their feed or water during the test. Birds will not be medicated beginning seven days prior to the start of the treatment until the test is terminated.

Diet Preparation and Chemical Handling: Test diets will be prepared by dissolving or suspending the test substance in a solvent or vehicle prior to mixing with the feed. If a diluent such as corn oil is used, it will not comprise more than 2% by weight of the treated diet.

A pre-mix of test chemical and feed may be used to facilitate diet preparation. All treatment and control premixes will be prepared under the auspices of the EDSP Chemical Repository at Battelle, Sequim by the Analytical Chemistry Group, National Security Division, Battelle, Richland and shipped locally to the testing room in Building 331. Diet pre-mixes will be prepared in a Hobart mixer or equivalent. Data will be generated in the purity analysis stability testing phase to indicate whether or not the test substance degrades or volatilizes. The assay used to determine test substance stability will be reported. Based on the results of stability testing, the pre-mix will be refrigerated until used. Pre-mixes will be prepared as frequently as necessary to assure stability of the test substance (less than 20% loss of test substance).

Once each week or more frequently, aliquots of the pre-mix will be blended into bulk quantities of the basal ration to achieve the desired dietary concentrations of the test substance. Bulk diet mixing will be done in a Hobart mixer or equivalent. Homogeneity of the test substance in the diet will be evaluated prior to the test. Samples of diets fed to the birds will be collected every time new diet is mixed during the treatment period to allow measurement of the actual concentration of the test substance.

Dr. Jocelyn Penner is a staff veterinarian supporting Battelle, Richland projects; she is a member of the IACUC. Teresa Luders, who is in charge of animal care for the project will have primary responsibility for day-to-day care. Crystal Driver, the Principal Investigator, is listed as an emergency contact. There are internal, department facility standard operating procedures for routine care that include cleaning, feeding, daily checking, health monitoring, and procedures for recognizing and dealing with sick animals.

10.3 TOTAL NUMBER AND AGE OF ANIMALS

The total number and age of animals are shown in Figure 6-2, Number of Adults, Eggs, and Hatchlings Used During Different Phases of the Dosing Study. As previously stated the study will begin with 300 eggs. It is estimated that there will be 558 adults, 6832 eggs and 2325 hatchlings during the duration of the study.

10.4 DURATION OF TEST

The duration of the test may be impacted by the strain of birds and their variability in maturation. The expected duration of the in-life portion of the test will be approximately 40 to 44 weeks. The primary phases of the study and their approximate durations are:

P1A
Incubation and hatch of eggs: about 18 days

Growth of P1A birds: from hatch until approximately 3 weeks of age.
Pre-laying exposure P1A: about 4 to 5 weeks
Egg-laying exposure of P1A: about 8 to 9 weeks (4 weeks of egg laying of paired birds)
Incubation of F1; set eggs weekly and incubate for 8 days and terminate: about 4 weeks
Incubation and hatching eggs for F1a and F1b population and mating behavior tests: set the fourth week of eggs and incubate 18 days.

F1a and F1b from P1A population (F1a is not treated, F1b receives treatment from hatch)
Selection and rearing, of F1a and F1b: about 3 weeks
Pre-laying period of F1a and F1b: about 4-5 weeks
Egg-laying period of F1a and F1b: about 5 weeks
Incubation and hatching of F2: eggs set weekly; about 18 days of incubation per hatch or about 13 weeks total.
Brooding of F2 to 14 days of age: about 2 weeks per hatch or about 8 weeks total.

P1B (concurrent with P1A)
Incubation and hatch of eggs: about 18 days
Growth of P1B birds: from hatch to pairing: about 12 weeks of age
Pre-laying acclimation of P1B birds: 4 to 5 weeks
Egg-laying of P1B: about 4 weeks to establish baseline production (no treatment)
Exposure during peak egg laying of P1B: 4 weeks
Incubation of F1; set eggs weekly and incubate for 8 days and terminate: about 4 weeks
Incubation and hatching eggs for F1a and F1b population and mating behavior tests: set fourth week of eggs and incubate18 days.

F1a and F1b from P1B population (concurrent with F1a and F1b from P1A population: F1a is not treated, F1b receives treatment from hatch)
Selection, rearing, and pairing of F1a and F1b: about 3 weeks
Pre-laying period of F1a and F1b: about 4-5 weeks
Egg-laying period of F1a and F1b: about 5 weeks
Incubation and hatching of F2: eggs set weekly; about 18 days of incubation per hatch or about 13 weeks total.
Brooding of F2 to 14 days of age: about 2 weeks per hatch or about 8 weeks total.

10.5 OBSERVATIONS OF RECORD

Eggs will be candled on Day 8 and again on Day 15 of incubation (for those that will be incubated to hatch) to determine early and late embryonation (embryo viability), respectively. On Day 15, the eggs will be will be transferred to another incubator and placed in pedigree baskets where they will be allowed to hatch. Eggs will not be rotated during hatching, and the pedigree baskets will be designed to keep hatchlings separated by their parental pen or origin. The incubator will be set to maintain a temperature of 37.5°C (and an average relative humidity of approximately 60%). Wet and dry bulb temperatures in the incubator will be recorded twice daily during incubation and hatching.

Hatchlings will be removed from the incubator over an approximately 24-hour period beginning on approximately Day 18. All unhatched eggs and eggshells will be removed from the hatcher by the end of Day 20.

Eggs: Eggs will be collected daily and marked with a soft lead pencil or permanent ink according to the pen from which they were collected. At the end of the weekly interval, all eggs will be removed from the cold room, counted, and selected eggs taken for eggshell quality measurements (1 egg per pen per week). The remaining eggs will be candled with an egg-candling lamp to detect eggshell cracks or abnormal eggs. Cracked or abnormal eggs will be recorded and discarded.

F2 eggs will be set weekly for incubation. F1 eggs collected from the beginning of the 5th week after the onset of laying will be set weekly for incubation.

Early embryo viability: Eggs will be candled on Day 8 of incubation to determine early embryonation (embryo viability). Eggs set for F1a and F1b breeding populations and male mating behavior trials will also be candled on Day 15.

Hatching success: On Day 15, viable eggs will be placed in pedigree baskets and transferred to another incubator where they will be allowed to hatch. Those chicks that have not hatched within about 24 hours of the majority of chicks, will be considered unhatched. The number hatched and unhatched will be recorded.

Survivability of hatchlings: F1 offspring (F2) will be observed over a 14-day period beginning when birds are first removed from the incubator. The number surviving to 14 days will be recorded. The observation period will be extended if late mortality occurs that appears to be treatment related. Survivability of chicks hatched for F1a and F1b populations and male mating behavior trials will be also recorded.

Clinical Observations: All adults and offspring will be observed daily throughout the test for overt signs of toxicity or abnormal clinical observations. A record will be maintained of all mortalities and observations.

Body Weight: Individual body weights of the adults will be measured at start and end of treatment. Body weights will not be measured during egg laying because of the possible adverse effects that handling may have on egg production. Individual body weights of offspring by parental pen will be recorded at hatching and on Day 14 post hatch.

Feed consumption: Feed consumption for each pen will be measured at least weekly and at test termination. More frequent measurements will be made if feed must be changed more often than weekly to maintain 80% of the original concentration under the test conditions. Feed consumption is determined by weighing the freshly filled feeder on Day 0, recording the amount of any additional diet added during the week and weighing the feeder and remaining feed at the end of the seven-day feeding period. The accuracy of feed consumption values may be affected by unavoidable wastage of feed by birds. Since wasted feed normally is scattered and mixed

with water and excreta, no attempt will be made to quantify the amount of feed wasted by the birds. Therefore, feed consumption will be presented as an estimate.

Reproductive Parameters: The following reproductive parameters will be measured and recorded by pen:

- Body weight of F2 14-Day-old Survivors and chicks raised for F1a and F1b populations and male mating behavior trials
- Dead embryos (Day 8 for F1; Day 8 and Day 15 for F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- Viable embryos (Day 8 for F1; Day 8 and Day 15 for F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- Unhatched eggs (F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- Hatchlings (F2 and for eggs set for F1a and F1b populations and mating behavior trials)
- 14-day-old survivors (F2 and 14-day-old chicks raised for F1a and F1b mating behavior trials).

Eggshell Strength Measurements: Each week's eggs will be selected from those eggs laid during that week for eggshell thickness measurement. One egg will be collected from each of the odd-numbered pens during odd-numbered weeks (1, 3, 5, etc.), and one egg will be collected from each of the even-numbered pens during even-numbered weeks (2, 4, 6, etc.). Shell strength will be measured with a universal testing instrument. The egg will be placed on its side on the test stand so that the compression head will contact the egg at the equator between two parallel stainless steel surfaces advancing at a constant rate of 4 mm/min with a 50 Newton maximum load range. The load (± 1%) will be recorded in Newtons.

Eggshell Thickness Measurements: Following the shell strength test, the same eggs will be prepared for shell thickness measurements. Each egg will be cut open using a scalpel or scissors at the waist, the contents removed, and the empty shell rinsed with tap water. The shells then will be allowed to air dry with the membrane intact for at least 48 hours at room temperature. The mean thickness of the dried shell, including membranes, will be determined by measuring five points around the waist of the egg with a micrometer. Measurements will be made to the nearest 0.002 mm. The average of the five measures will be reported.

Offspring Body Weights: The mean weight of all surviving offspring will be determined both at hatch and at 14 days of age. Mean weights may be determined from either individual or group body weight measurements and will be determined from all offspring originating from a given parental pen during a specific week of egg laying.

Necropsy, Organ Weights, Histology: All adult test birds that die during the course of the test and all adults remaining at the termination of the adult portion of the test will be subjected to a gross necropsy. The necropsy will include an examination of the overall condition of the birds, as well as any external or internal observations. The examination will include, but not be limited to, gross observations of the liver, gonads, and general condition of the organs. Gonads, oviduct, thyroid, adrenal glands, liver, brain and cloacal gland will be excised and their weight recorded. Thyroid and adrenal glands will be excised with adjoining tissue and injected with fixative prior to trimming and organ weight determination to diminish handling damage to the tissues and decrease time required for excisement. All lesions will be recorded.

Necropsies will be conducted on a subset of the F2 chicks. One-third of the chicks from each pen will be necropsied. In one-third of the chicks from the Week 5 eggs, the gonads, oviduct, and thyroid will be examined histologically. The wing or leg length will be measured in F2 chicks and the bone removed and measured.

Organ weights will be normalized by body weight (100 X organ weight/body weight) and the testis weight asymmetry (left testis weight/right testis weight) calculated.

Sexual Maturation: Sexual maturation of males will be determined by the protrusion and secretion of foam from the cloacal gland. Cloacal gland measurements (size, foam weight) will be taken. Female maturation will be recorded as the day the first egg is laid. The number of follicles in rapid development (>4mm in diameter and yellow in color) will be determined at termination of the study.

Feather Color and Pattern: Appearance of feather dimorphism will be recorded. Apparent gender will be confirmed at necropsy and recorded.

Steroid Content of Fecal-Urate Samples: Fecal-urate matter (0.2-2 g) will be collected from the drop pans under each of the breeder cages at termination of the P1 birds. Samples will be collected to avoid contamination by feed and adjoining cage occupants as described by Brewer et al. (in prep). The samples will be prepared for analysis as described by Tell and Lasley (1991) and as modified by Brewer et al. (in prep) and Wasser et al. (2000). The hormone content of the fecal-urate sample preparations will be determined using commercially available Enzyme Immunological Assay kits for testosterone and estradiol.

Steroid Content of Eggs: The steroid content of a subset (1 egg per pen per week) of eggs collected during the 5^{th} and 8^{th} weeks of egg laying of the P1 birds and one egg per pen during week 5 of the F1 parents will be determined by Enzyme Immunoloical Assay. Four eggs from each group will be composited for the analyses (2 composites of 4 eggs each per group). Yolks will be separated from albumin by differential thawing and the yolks homogenized and mixed. Free steroids will be extracted with petroleum and diethyl ethers, the proteins and excess lipids precipitated, the steroids extracted in ethanol and the extracts cleaned using chromatography columns as described by Schwabl (1993) and modified by Lipar et al. (1999). The eluents will be analyzed for steroid content using Enzyme Immunological Assay kits for testosterone and estradiol.

Male Sexual Behavior: A receptive egg-laying female will be placed in a 50 x 40 x 30 cm-high test arena. The male will be introduced and sexual interaction observed for 2 minutes. Mount attempts will be recorded when a male, while grabbing neck of the female, places one leg over the female's back. One test will be performed each day for 4 consecutive days. Mounts and cloacal contact movements will also be noted. However, because they are more dependent on the receptivity of the female than the mount attempt (Halldin et al. 1999), only the mount attempt will used in statistical analysis of the behavioral data.

A sample of blood or feather pulp will be obtained from the hatchlings of about one half of the Week 5 F2 chicks for genetic sex determination by PCR.

Disposition of Test Birds: At test termination, all surviving adults will be euthanized using carbon dioxide gas, cervical dislocation, or any other appropriate methods. Following measurement of body weight at 14 days of age, all chicks also will be euthanized. All euthanasia methods will be documented in the raw data. All birds will be disposed of by incineration or other appropriate methods.

10.6 QUARANTINE

Quarantine of all incoming animals upon arrival until released by the veterinarian will follow requirements specific in MSL-T-049, Receiving Eggs and Birds, and "Quarantine" signs on the door or doors to and from the room. Any animals found sick or dead during the quarantine period will be reported to the Principal Investigator who will report them to the attending veterinarian. Dead animals will be bagged and refrigerated for possible necropsy

10.7 ANIMAL IDENTIFICATION

Animal identification will follow the requirements specified in MSL-T-044, Avian Identification with Wing Clips and Leg Bands. Animals will be uniquely identified. The method of identification and the animal number assignments will be either wing clips or leg bands Groups of pens will be identified by exposure type (e.g., established breeder, P1B, or during maturation, P1A) and concentration. All eggs laid during the study will be marked with a soft lead pencil or permanent ink marker for identification.

Animals with lost tags are to be re-tagged with the same number or assigned a new number. The wing clip is routinely placed in the right wing. However, if the right wing is torn or damaged in any way, place the tag in the left wing. Leg bands are attached to right shank, above the toes of the bird.

10.8 LIMITATION OF DISCOMFORT

Although not anticipated, some toxicity may occur with exposure to the test chemicals. Discomfort or injury to quail animals will be limited, in that if any animal becomes severely debilitated or moribund at any time during the study, it will be humanely terminated by use of cervical dislocation or CO_2 asphyxiation.

10.9 BREEDING

The Japanese quail used in this study will be as randomly bred as possible within the confines of the bird population available. The breeding birds will be maintained in an environmentally-controlled facility under the conditions stated in Table 9-1. All birds are monitored daily for health. All chicks are monitored for health and any possible abnormalities.

11.0 SAMPLING METHODS

Sampling methods have been discussed in detail in Sections 6.1.2, 10.2, and 10.5

12.0 SAMPLE HANDLING AND CUSTODY

Sample handling is specified in the study protocol. Custody of samples is addressed in MSL-A-002, Sample Chain of Custody.

12.1 DIET SAMPLING AND ANALYSIS

Samples of the treated and control diets will be collected and analyzed to evaluate the homogeneity of the test substance in avian diet and to confirm test concentrations in the prepared diets. Samples from all test substance concentrations will be collected from food troughs within each treatment concentration at the end of the first feeding period (before the diet in the food trough is renewed) and again at the end of the last feeding period of the study. The Chemical Repository at MSL will analyze samples. The nutrient analysis supplied by the manufacturer will be reported. Phytoestrogen and mycotoxin content of the diet will be determined if a laboratory can be found to perform these analyses. The composition of the vitamin supplement added to the water provided to the chicks will also be recorded.

12.2 HISTOLOGY SAMPLES

Organ samples will be collected and weighed at necropsy, with the exception of thyroid and adrenal glands. Thyroid and adrenal glands will be removed and fixed prior to weighing to minimize tissue damage. Histology of adult thyroid, adrenal glands, reproductive tissue (oviducts, ovaries, and testes), liver, and brain tissue is needed to meet project objectives. In addition, thyroid, oviducts, ovaries, and testes will be collected from F2 chicks. Samples will be preserved in the appropriate fixative, the fixative drained off and the samples will be placed in a Ziploc™ or Whirl Pac™ bag with an absorbent moistened with the appropriate fixative. The self-locking bags can be vacuum packed for shipping. Samples will be embedded and analyzed by AquaTechnics, Sequim, WA.

12.3 EGG YOLK AND FECAL URATE SAMPLES

Egg yolk and fecal urate samples will be collected as described in Section 10.5. Samples will be frozen, then air- or freeze-dried, and steroids extracted for analysis by the EIA methods specified in MSL-T-055, Steroid Content of Eggs and Fecal/Urate Samples.

12.4 EGGSHELL STRENGTH AND THICKNESS

Eggshells will be collected, processed and analyzed for strength and thickness as described in Section 10.4. Eggshell thickness is a simple measure made by using a micrometer. Eggshell strength is measured using a Universal material testing instrument.

12.5 GENETIC SEX

Genetic sex samples will be collected as described in Section 6.1.2. Genetic sex can be determined from either blood or feather pulp using a gel electrophoreses method. Sample preparation and analysis is described in MSL-T-058, Determining Genetic Sex.

13.0 ANALYTICAL METHODS

The assays will be performed as specified in the study protocol presented in the approved study plan. No deviations from the protocols are expected, but if deviations occur, they will be documented and reported to the EDSP and EPA managers.

The analytical method to test for 17β-estradiol and phytoestrogens in the feed prior to beginning the study and verification of spiking levels of 17β-estradiol in the feed during the study has yet to be determined. The method will be documented when developed and become part of the data documentation. It is likely that the test substance, (e.g., 17ß-estradiol) will be analyzed by gas chromatography/mass spectroscopy (GC-MS). The food will be solvent extracted; the extract will be derivitized to form compounds of estradiol and degradation products of estradiol that can be quantified by GC-MS. The caloric content of the feed and the amount of natural endocrine-active compounds in untreated diet will also be determined.

All test substance calculations will be based on the purity of the test substance as received or will be corrected for purity of the active ingredient in the test substance. Dietary concentrations will be adjusted for purity of the test substance expressed as ppm (active ingredient, a.i.). Although the estradiol is minimally volatile (1.26 E-008 mm Hg), there may be potential for dermal and respiratory exposure of staff through handling the diet and/or from airborne fines from the feed. Therefore staff handling the preparation and distribution of the treated diets and accessing the animal rooms will be trained relative to the hazards and safe handling of this potent estrogen and will wear appropriate personal protective equipment as determined by an Environmental Safety and Health subject area expert. Area access is restricted by a proximity access card system. Access criteria require completion of all required training and approval of the Cognizant Space Manager of the space within the Integrated Operations System. To minimize cross-contamination of the test diets, the dietary treatments will be mixed in increasing concentration from control to high dose. All mixing equipment will be cleaned with

ethanol prior to the control feed and between each dose. An initial "control" diet will mixed and discarded, and a second batch made following cleaning of the equipment to assure no transfer of estrogen to the control diet. Where possible dedicated mixing vessels will be used per dose. Cages in the animal rooms will be positioned to minimize contamination of feed.

14.0 QUALITY CONTROL MEASURES

The experimental design (Figure 6-2) was determined to provide a statistically-valid sample size for all phases of the study. Refer to Section 19.2, Statistical Analyses.

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

The following equipment is needed to perform the work at Battelle, Richland:

Brooders/Incubators
Animal Cages
Test Rooms
Eggshell Cracks – Egg Candling System
Eggshell Strength – Universal Material Tester
Eggshell Thickness – micrometers
Calipers
Pipettes
Balances – body, tissue weights, feed
pH meter/electrode
Microcentrifuge
Thermocyclers
Gel Electrophoresis System for genetic sex determination
Refrigerators for storing feed
Refrigerators/freezers for storing samples (histological samples, egg content, fecal material).

All equipment will be evaluated to ensure that it is functional and operable before use. Equipment requiring calibration will be calibrated as specified below.

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

The necessary project equipment is summarized in Table 16-1. Each time a calibration or calibration verification is made it needs to be recorded – either in the instrument calibration book, on an appropriate data sheet. The record will note the date, initials of who made the calibration, the result, the expected result, and whether or not the calibration was within the acceptable limit. If the result is not within the acceptable limit, the corrective action must be documented.

Table 16-1. Project Equipment Calibration and Frequency Requirements

Measure/ Use	Measuring or Test Equipment	Manufacturer/ Model	Serial Number or Unique ID	Location	Primary Calibration	Secondary Calibration
Weight (mg/g)	Balance	Mettler AE240	Standard Lab Code # 505-06-022		Semi-Annual QCS*	With use, Std. Weight Set
Weight (mg/g)	Balance	Sartorius QS400	Standard Lab Code # 505-01-032		Semi-Annual, QCS*	With use, Std. Weight Set
Weight (mg/g)	Balance	Ainsworth scale			Semi-Annual QCS*	With use, Std. Weight Set
Weight (mg/g)	Calibration Verification Standard Weight Set (2 mg-100 g)	Troemner 1139	Standard Lab Code # 505-86-02-003		Annual QCS*	NA
pH (pH units)	pH meter/electrode	Fischer Accumet Basic			With use; pH 4, 7 and 10 buffers, chose one closest to expected measure	NA
Sample/ Reagent Preparation	Microcentrifuge (2)	Eppendorf 5415C			NA	NA
Sample/ Reagent Preparation	Thermocyclers (3)	Tecne Genemate Genius			NA	NA
Volume transfer	Pipetman (set range, 2 µl - 1000 µl)	Gilson			Gravimetric checks with use	Return to Manufacturer when needed
Cloacal gland size	Digital Slide Calipers	L.S. Starrett Co., Athol, MA/ Model 721 (digital)	Serial No. 02205209		Calibrated by Manufacturer; letter of certification	Checked using thickness gauge sets
Eggshell thickness	Outside diameter Micrometer	L.S. Starrett Co., Athol, MA/ Model 733M (digital)	Serial No. 01524128		NIST –traceable Certificate of Calibration, annual recalibration	Thickness gage set, check with use
	Thickness gauge set	L.S. Starrett Co., Athol, MA/ Model 66MA	Serial No. 03021222		Calibrated by Manufacturer	
	Thickness gauge set	L.S. Starrett Co., Athol, MA/ Model 173MA	Serial No. 02511242		Calibrated by Manufacturer	
Eggshell Cracks	Egg Candler	Lyon Electric Company, Inc.200	NA			NA

Measure/ Use	Measuring or Test Equipment	Manufacturer/ Model	Serial Number or Unique ID	Location	Primary Calibration	Secondary Calibration
Eggshell Strength	Universal Material Tester Nexygen applications software	Chatillon Inc LF Plus Series Lloyd Instruments, SW version 4.5 TickIT registration Certificate No. Q11777, Independently tested by applications engineers to ensure meets acceptance criteria, performs accurate calculations, is not affected by combinations of unusual events, and will fail-safe. External auditing of software by manufacturer	LF1179/ WD33560 40/0658	PSL 601	Certificate of Calibration, Weston Inc. Annual recal. specified ISO/IEC 17025 and ANSI/NCSL Z-540 compliant. All standards used will be traceable to NIST or other international standards.	Test system: calibrated on site. Standards used: All have NIST traceable numbers for: Ameteck WG-Q (weight) Innovative Time L333B (stop watch) Mitutoyo 182-163 (ruler) 50N Load cell : Ametek Wg-Q
PCR/ Genetic Sex	Gel box, electrophoresis	MPH	9529		NA	NA
PCR/ Genetic Sex	Gel visualization system with UV transilluminator and Sony digital graphic printer				NA	NA
PCR/ Genetic Sex	Balance	Mettler AE260 Delta Range	Standard Lab Code # 5I5-06-01-067		Semi-Annual QCS*	With use, Std. Weight Set
Animal care	Brooder cages 50+	GA Quail Farm Manufacturing Co.; Gamebird	Will assign cage numbers	331, Room 192	Thermometers are calibrated against NIST traceable standard, but only used for general monitoring	Initial heat settings are adjusted with age of the chicks, and according to thermal comfort behavior.
Animal care	Adult breeding cages (6/row, 5 rows/rack; 10 racks = 300)	GA Quail Farm Manufacturing Co	Will assign cage numbers	331, Room 194	Thermometers are calibrated against NIST traceable standard	Automated daily ambient temperature /humidity monitoring
Animal care	Incubator (4)	Natureform Hatchery Systems NMC-620	0802-IJIA-3126/WD3357 4	331, Room 192	Thermometer Dry Humidistat Wet bulb/dry bulb method (i.e., 2 thermometers)	Humidistat Calibrated by Manufacturer when new, but must be recalibrated on-site.
Animal care	Incubator (2)	Natureform Hatchery Systems NMC-620	WD33575	331, Room 192	Thermometer Dry Humidistat Wet bulb/dry bulb method (i.e., 2 thermometers)	Humidistat calibrated by Manufacturer when new, but must be recalibrated on-site.

Measure/ Use	Measuring or Test Equipment	Manufacturer/ Model	Serial Number or Unique ID	Location	Primary Calibration	Secondary Calibration
Animal care	Cold Room		NA	331, across from Rm 131	Annual, by PNNL Calibrations; NIST traceable thermometer	Daily by use of strip chart recorder
Animal care	Animal Rooms and Food Storage Room		NA	331, off of Rm 124	Annual, by PNNL Calibrations NIST traceable thermometer	Daily monitoring of humidity, temperature, (light cycle in animal rooms)
Sample/ Reagent Storage	Laboratory Walk-In Refrigerator	Baily	NA	331, cold room #1	External alarm External chart recorder External monitoring dials; F&O responsibility	NA
Sample Storage	Bio-Freezer -70 °C	Forma Scientific 8525	89933435		Semi-annual NIST-traceable digital thermometer	Daily monitoring
Behavioral Testing Times	Stopwatch	To be purchased	To be purchased			

*QCS Quality Control Services, Inc. Kelso, WA.

16.1 BALANCES

Battelle balances are calibrated by an external contractor (QC Services, Portland, OR) semiannually, and verified daily with each use as per requirements in the procedure, MSL-C-009, Use and Performance Checks of Balances. An external contractor verifies standard weight set calibration annually under the calibration program. Balance calibration is documented by placing a sticker directly on the balance or weight set to document the permissible calibration interval and written documentation is also provided for each item calibrated. The data sheets used for this purpose contain a field to record the calibration interval on the calibration sticker, thereby requiring staff to assure before use that the balance is within the stated semiannual calibration interval. If the calibration verification weight is not within the specified tolerance, two more measures are made, and an average weight computed. If this average is not within the specified tolerance, then corrective actions will be implemented. The first action is to ensure that the balance is level and that the certified weight is clean and free from degradation. Another weight from a different weight set might also be measured. If the calibration cannot be verified, then the balance is placed on a service call list. Battelle has back up balances that can be used if one balance fails.

Records from daily balance verification will be maintained in a logbook for that room. The person responsible for balances in each room will be listed on the front of the logbook.

16.2 REFRIGERATORS AND FREEZERS

Refrigerator and freezers are subjected to daily temperature monitoring (i.e., each working day) and documented as per the requirements specified in MSL-I-026, Use of Laboratory Refrigerators and Freezers. Readings are taken every day and reported on the Refrigerator/Freezer Temperature Log. Acceptable ranges have been established. Completed log sheets will be filed in a refrigerator/freezer notebook for that room. The person responsible for refrigerator and freezers in each room will be listed on the front of the logbook.

16.3 PIPETTES

A limited number of project pipettes are needed. Pipetman (1 set) calibration is verified gravimetrically with use, or at least quarterly as specified in MSL-C-010, Calibration and Use of Pipettes.

16.4 pH METER

The pH meter will be calibrated with use as per the requirements specified in MSL-C-004, pH in Water. National Institute of Standards and Technology (NIST) –traceable-buffer solutions for pH 4.0, 7.0, and 10.0 pH unit measures are available and the buffer used depends on the pH of the solution being measured. Traceability is documented either 1) directly on the buffer solution container, or 2) by documentation accompanying the solution when received.

16.5 CALIPER/MICROMETER

Calipers and micrometers used for measuring are calibrated by the manufacturer and will be newly purchased for this work. Verification of calibration is made using a thickness gage set as a standard to verify accurate measures. Accuracy will be verified with each use using a thickness gage set.

16.6 UNIVERSAL MATERIAL TESTER

The Universal Material Tester is used to assess eggshell strength. Primary calibration involves the use of a standard weight, a ruler, and a time recorder. A 50 N load cell is used in the test. The system will be calibrated prior to each use according to manufacturer's specifications.

17.0 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Upon receipt, purchased items are inspected for conformance to quality requirements prior to use. All expiration dates are expected to be within the expected use of the product, when applicable.

18.0 NONDIRECT MEASUREMENTS

No collection of any samples or sample data is obtained from nondirect measures such as computer databases or programs.

19.0 DATA MANAGEMENT

19.1 DATA MANAGEMENT OVERVIEW

Data will be collected to data sheets according to the end points listed above and according to the methods previously below. In general, data are entered into Excel spreadsheets and analyzed using SAS, Statview, or Minitab using the statistical and data presentation abilities of the applications.

19.2 STATISTICAL ANALYSES

The overall objective of the statistical analysis is to determine which dosing scenario for the P1 generation birds (during maturation or after proven breeding ability is established) and F1 birds (exposure from hatch or no additional exposure above *in ovo* exposure) are more biologically sensitive to chemically induced reproductive/endocrine disrupting impacts to species fitness. The study design will produce a time series of reproductive parameters for P1 adults under both dosing scenarios for each concentration, a dose-response curve for each generation, plus the pen mean responses for each concentration, dosing scenario, and generation. Thus, three statistical approaches will be used: a regression against time for a given concentration, dosing scenario, and generation; a regression against chemical concentration for a given dosing scenario and generation; and an ANOVA approach based on the mean pen responses for a given concentration, dosing scenario, and generation.

The time series produced by the P1 and F1 generation birds for a given concentration and dosing scenario will allow the evaluation of a possible delay in response time, the form of the time series response (i.e., linear, curvilinear, spline), and the potential carry-over effect of the reproductive response to the F1 generation.

The dose-response for each generation and dosing scenario will allow the estimation of the EC_{50} and slope of the response. The difference in the slope and EC_{50}s between generations will also allow the evaluation of a potential carry-over effect between generations. Curve fitting procedures will also be used as an alternative to the traditional NOAEL approach to better assess the appropriateness of the dosing regimens. Specific effect levels for quantal and continuous endpoints will be identified in the range of biologically observable data and confidence limits generated around this concentration. The Benchmark Dose (the lower confidence limit producing a specified percentage of change in a response) determined under each exposure regimen will be compared.

ANOVA analysis of the pen mean responses will be conducted to compare specific contrast between treatment groups. Specific parameters will be tested only against the control using Dunnett's Analysis. Appropriate data transformations will be applied to maintain homogeneity of the within class variances (i.e., data expressed as a percentage will be arcsine-square root transformed, counts will be square root transformed, and continuous data will be transformed to the natural logarithm). Nonparametric statistic will be used when the data transformation is not successful in controlling heterogeneity. Any pen in which an adult mortality occurs will not be used in statistical comparisons of the reproductive data.

Analyses will be performed on each of the following parameters:

1. Adult Body Weight- Individual body weight will be measured at the test initiation and at adult termination. Statistical comparisons will be made by sex between the control group and each treatment group at each weighing interval using dose-response and Dunnett's Analysis.

2. Adult Feed Consumption- Feed consumption expressed as grams of feed per bird per day will be examined by pen at weekly intervals during the test. Statistical comparisons will be made between the control group and each treatment group using time series analysis, dose- response, and Dunnett's Analysis.

3. Eggs Laid of Maximum Laid (%) - The number of eggs laid per hen divided by the largest number of eggs laid by any one hen. This transformation is used to convert the number of eggs laid to a percentile value less than or equal to 100. The value is correlated with eggs laid per pen per day. Statistical analysis of egg production will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

4. Eggs Cracked of Eggs Laid (%) - The number of cracked eggs (determining by candling) divided by the number of eggs laid per pen. Statistical analysis of the percentage of eggs cracked will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

5. Viable Embryos of Eggs Incubated (%) - The number of viable embryos as determined by candling on ~Day 10 divided by the number of eggs set per pen. Statistical analysis of the percentage of viable embryos will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

6. Hatchlings of Viable Embryos (%) - The number of hatchlings removed from the hatcher divided by the number of viable embryos per pen. Statistical analysis of the percentage hatching will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

7. Hatchlings of Fertile Eggs (%) - The number of live hatchlings divided by the number of fertile eggs per pen. Statistical analysis of the percentage hatching will

include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

8. 14-Day Old Survivors of Normal Hatchlings (%) - The number of hatchlings divided by the number of eggs set per week by pen. Statistical analysis of the percentage normal will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

9. Normal Hatchlings as a Percentage of the Maximum Number of Eggs Incubated- The number of hatchlings per hen divided by the largest number of eggs set from any one hen. This transformation is used to convert the number of hatchlings to a percentile value equal to or less than 100. Statistical analysis of the percentage normal will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

10. 14- Day Old Survivors of Eggs Set (%)- The number of 14-day old survivors divided by the number of eggs set per week by pen. Statistical analysis of the percentage surviving will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses

11. 14- Day Old Survivors of Maximum Set (%) - The number of 14-day old survivors per pen divided by the largest number of eggs set. Statistical analysis of the percentage surviving will include the time series analysis, dose-response, and ANOVA comparing the overall mean pen responses.

12. Hatchling Body Weight- The group body weights of surviving hatchlings and 14-day old survivors will be measured by parental pen group will be analyzed by dose-response and ANOVA.

13. 14- Day Survivor Body Weight- The group body weights of surviving hatchlings and 14-day old survivors measured by parental pen group will be analyzed by dose-response and ANOVA.

14. Eggshell Thickness and Eggshell Strength- The average eggshell thickness of indiscriminately selected eggs per pen will be measured and analyzed by dose-response and ANOVA.

15. Male Sexual Behavior- The average number of mountings per pen will be analyses by dose-response and ANOVA.

16. Hormone level in egg contents and fecal/urate matter- Concentrations of hormones averaged per group and per pen respectively will be analyzed by dose-response and ANOVA.

17. Sexual Maturation- The time to sexual maturation averaged per pen will be analyzed by dose-response and ANOVA. If significant photoperiod drift occurs in

the strain of Japanese quail used, onset of egg laying may be analyzed by comparing the percentage of the group laying eggs over time. Onset of lay will be recorded as the number of days to first egg laid in the group and days until 33% hen-day egg production is reached in each group (Lien et al., 1987).

18. Genetic Sex Ratio- The ratio of the number of males to females by blood analysis will be analyzed by dose-response, and ANOVA.

19. Incidence of Abnormal Reproductive Structures – The number of abnormal reproductive structures found in the adults and 14-day old chicks will be analyzed by regression analysis based on treatment.

20. Organ Weights – The absolute value, the somatic index of organ weight to body weight, and the organ weight to brain weight of 14-day old chicks and adults will be analyzed by dose-response, and ANOVA.

21. Oocyte Development – The number of oocytes in rapid development per adult female at termination will be analyzed by dose response and ANOVA.

22. Cloacal gland size – Coacal gland size will be calculated as the volume of the cloacal gland using the following formula:

$$4/3\pi ab^2$$

where a is half of the length of the long axis, and b is half of the length of the short axis. The cloacal volume will be analyzed by dose-response, and ANOVA

23. Gonad Lesions- Histological scores of testicular and ovarian abnormalities will be will be analyzed by dose-response, and ANOVA.

24. Spermatid Counts- Histological counts of spermatids will be analyzed by dose-response, and ANOVA.

19.3 DATA TRANSFER

Data will primarily be collected by hand-entering data on data sheets. Data will be transferred to Excel files and verified. 100% verification is required when transferring hand entered data to an electronic database. Excel data can then be analyzed using the applications available in SAS, Statview and Minitab.

20.0 ASSESSMENTS AND RESPONSE ACTIONS

The EDSP QA Unit may perform assessments on WA 2-17 activities and operations affecting data quality, but will at a minimum review the raw data and final report. Assessments of important phase activities and operations will be identified and scheduled by the PI and the QA Unit. Any findings will be reported to the PI and the Work Assignment Leader and other line management as necessary to ensure that the requirements in relevant SOPs, WA protocols

and QAPP, and the QMP are met. The assessments for this WA include Technical Systems Audits (TSA) and Audits of Data Quality (ADQ). Performance Evaluations do not apply to this QAPP.

20.1 TECHNICAL SYSTEMS AUDITS

A TSA is a process by which the quality of a WA is assessed through evaluating a WA activity's conformance with the protocols, applicable facility or program SOPs, the WA QAPP, and the EDSP QMP. The acceptance criteria are that WA activities and operations must meet the requirements of these planning documents or be explained and evaluated in a deviation report by the Principal Investigator.

20.2 TYPE, SCHEDULING, AND PERFORMANCE OF TECHNICAL SYSTEMS AUDITS

The QA Manager, with the concurrence of the Principal Investigator, will determine the critical phases of WA 2-17 if any, based on the activities specified in the protocols. Whenever possible, TSAs should be done at the commencement of the WA critical phase to ensure WA integrity based on compliance with the protocols and QAPP. Critical phases targeted for TSAs may include:

- QAPP and/or procedure review,
- Personnel training files for documentation that EDSP SOPs and WA QAPP have been read and understood by WA personnel before any activities begin, and
- Calibration and data records.

During the TSA, EDSP QA team members record observations to be used later in preparing the audit report. EDSP QA team members observe the procedure, data recording, and any equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the WA protocols and QAPP, applicable SOPs, and the EDSP QMP. Any findings will be communicated to the technical personnel at the completion of the WA activity unless an error could compromise the WA (e.g., lost or broken samples). EDSP QA team members immediately notify the Principal Investigator in person or by telephone and/or e-mail of any adverse findings that could impact the conduct of the WA. This direct communication is also documented in the audit report.

20.3 AUDITS OF DATA QUALITY

An ADQ is a process by which the accuracy of data calculations and reporting is assessed to ensure that the reported results are of high quality and accurately reflect the raw data and that the executive summary accurately describes the materials used in the WA. The acceptance criteria for the ADQ are that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA protocols and QAPP, and the EDSP QMP or be explained and evaluated in a deviation report.

20.4 SCHEDULING AND PERFORMANCE OF AUDITS OF DATA QUALITY

Direct and frequent communication between the Principal Investigator and the QA Unit Manager will provide for sufficient time to perform an ADQ, so that the submission date of the audited executive summary meets that specified in the WA 2-17 protocols. The scheduling process should also allow for a reasonable amount of time for corrections and subsequent verification of the corrections by QA.

WA personnel submit all data and records for review at the time of the data and executive summary audit. An EDSP QA team member reviews the data package for completeness and, if incomplete, requests that the additional records needed for review be submitted. EDSP QA team members review a minimum of 10% of the raw data, the tabulated data, and WA records of performance and methods to ensure compliance with the planning documents mentioned previously. At least 10% of the raw data are compared to the tabulated individual data, and a minimum 10% of the summary data tables are checked. EDSP QA team members will also check all tabulated data designated as statistically significant. Findings are reported, and corrective actions undertaken as described earlier. EDSP QA team members review the executive summary using the audited data and corrected tables to ensure that the reported results are of high quality and accurately reflect the raw data, and that the summary accurately describes the materials used in the WA. Findings are then reported and corrective actions undertaken, as described earlier.

20.5 AUDIT REPORT FORMAT

The audit report consists of a cover page for WA information and additional page(s) with the audit findings. All pages have header information containing the WA protocol number, audit report date, and audit code. The audit report date is the date on which the EDSP QA Unit Manager signs the audit report and sends it to the Principal Investigator and EDSP management. The audit code is an abbreviation of the audit type (e.g., D = dosing, DA/RA = data audit/report audit).

The cover page contains the WA protocol title, number, and code; contract number; EPA Sponsor; PI; audit type; audit date(s); EDSP QA Unit; distribution list; the dated signature of the auditor; the date that the Work Assignment Leader received the audit report; and the dated signatures of the PI and management. The distribution list may include additional names for individuals who have findings pertaining to their area of responsibility and is used to ensure that the report is sent by e-mail attachment to all who will receive an electronic copy of the report. The official audit report is the hard copy containing corrective actions and dated signatures of the EDSP QA Unit, PI, and management. Subsequent page(s) contain the audit finding(s), any recommended remedial actions, and space for the PI to respond to the findings and document remedial actions taken or to be taken.

20.6 RESPONSE ACTIONS AND RESOLUTION OF ISSUES

The PI shall respond to a TSA report within five working days of receipt of the report. There is no deadline for the PI's response to an ADQ report except for the time constraint deriving from the submission date of the final WA 2-17 report. The PI forwards the audit report

to management for review. Management adds comments as necessary, signs and dates the report, and returns it to the EDSP QA Team Member. The EDSP QA Team Member assesses the responses and verifies the corrective actions. If a disagreement between the PI and EDSP QA team member arises over a finding, it will be discussed among the other EDSP QA team members. The EDSP QA team member will then present the majority opinion to the PI for further consideration. If the disagreement remains, the issue is reported to the PI's management and, if necessary, the Battelle, Sequim Laboratory Director. The action decided on by management will be documented in the QA files.

During an assessment, if the auditor determines that adverse health effects could result or WA 2-17 objectives of acceptable quality cannot be achieved, the auditor follows the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.7 INDEPENDENT ASSESSMENTS

The EDSP Battelle QAM or designee may conduct an independent TSA and ADQ during the conduct of this work assignment. Typically, one independent audit will be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits may be scheduled. The conduct and reporting of the audits will be consistent with the procedures described in the EDSP QMP (Section 3.3). In addition, the EDSP EPA QAM or designee has the option of conducting external TSAs/ADQs.

21.0 REPORTS TO MANAGEMENT

The Battelle QA Unit Manager routinely prepares and distributes quarterly reports. If this frequency is not considered adequate to convey EDSP concerns, the EDSP Ecotoxicology Project Leader will receive project-specific briefings so that project-specific reports can be made on a monthly basis. The QA Unit Manager will send copies of resolved assessment reports containing findings, responses, and corrective actions taken to the EDSP Battelle QA Manager as soon as they are completed.

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The data produced under this work assignment will be reviewed by the technical personnel for the validation process and by EDSP QA team members for the verification process (see Section 23.3). The criteria used for validation depend on the type of data. Compromised samples are not analyzed and any deviations resulting from such circumstances will be addressed.

23.0 VERIFICATION AND VALIDATION METHODS

23.1 CHAIN OF CUSTODY FOR TEST SOLUTIONS

Documentation for the preparation, purity and stability of test solutions is evaluated for accuracy and completeness as part of the EDSP Chemical Repository operations. Test solution/treated feed data maintained to support WA 2-17 must be traceable to the Chain of Custody documentation to provide the link to Chemical Repository data.

23.2 DATA VALIDATION

Data validation is a process by which the Principal Investigator and/or other technical personnel ensure that the processes for data entry, reduction, and reporting provide the desired product and meet project objectives. WA 2-17 personnel are responsible for reviewing the data, evaluating any technical deviations or non-conformances, and then determining the degree to which the data meet the quality criteria stated Section 7.0.

23.3 DATA VERIFICATION

Data verification constitutes part of the ADQ process performed by EDSP QA team members and described earlier. Verification ensures that 1) the data are of high quality and were collected according to the planning documents' requirements, and 2) the reported results accurately reflect the raw data. Each data type is evaluated against its collection and reduction requirements, using the data calculations required by the protocol as specified in the planning documents. Errors discovered during the data evaluation are corrected. The reported conclusions drawn from the data are verified by EDSP QA team members during the report audit to confirm that they are true and accurate. The procedure for resolving issues of data verification has been detailed in prior sections of this document.

24.0 RECONCILIATION AND USER REQUIREMENTS

Proposed methods for data analysis, which will include a test for statistical outliers, are specified above, in Section 19.2.

25.0 REFERENCES

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