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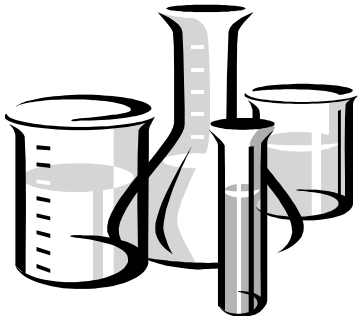
Ecological Effects Test Guidelines

OCSPP 850.2100: Avian Acute Oral Toxicity Test



NOTICE

This guideline is one of a series of test guidelines established by the United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a). Prior to April 22, 2010, OCSPP was known as the Office of Prevention, Pesticides and Toxic Substances (OPPTS). To distinguish these guidelines from guidelines issued by other organizations, the numbering convention adopted in 1994 specifically included OPPTS as part of the guideline's number. Any test guidelines developed after April 22, 2010 will use the new acronym (OCSPP) in their title.

The OCSPP harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA, and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. At places in this guidance, the Agency uses the word "should." In this guidance, the use of "should" with regard to an action means that the action is recommended rather than mandatory. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about these test guidelines and to access these guidelines electronically, please go to http://www.epa.gov/ocspp and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the guidelines in http://www.regulations.gov grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 850.2100: Avian acute oral toxicity test.

(a) **Scope-**

(1) **Applicability**. This guideline is intended to be used to help develop data to submit to EPA under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).

(2) **Background**. The source materials used in developing this harmonized OCSPP test guideline include the OPPT guideline under 40 CFR 797.2175 Avian Acute Oral Toxicity Test; the OPP 71-1 Avian Single-Dose Oral LD50 Test (Pesticide Assessment Guidelines Subdivision E); the Avian Single-Dose Oral LD50 Standard Evaluation Procedure; and OPP Pesticides Reregistration Rejection Rate Analysis: Ecological Effects.

(b) **Purpose**. This guideline is designed to develop data, specifically both a median lethal dose (LD_{50}) and slope of the dose-response relationship, for acute oral toxicity to upland game birds (*e.g.*, northern bobwhite (*Colinus virginianus*)), water fowl (*e.g.*, mallard duck (*Anas platyrhynchos*)), or passerine species (*e.g.*, house sparrow (*Passer domesticus*), zebra finch (*Taeniopygia guttata*), red-wing blackbird (*Agelaius phoeniceus*)) of chemical substances and mixtures ("test chemicals" or "test substances") subject to environmental effects test regulations. While the study is specifically designed to allow calculation of the LC_{50}, the study can be used to obtain information regarding sublethal effects which are used in Agency evaluations. This guideline prescribes methodologies to determine both the LD_{50} and slope of the dose-response in the same study. The Agency can consider data generated that provides a LD_{50} value but no slope information but such a study would be insufficient to meet the purpose of this guideline unless it is a limit test (see paragraph (d)(3) of this guideline). The use of a test based primarily on lethality is justified because it presents or insures a consistent, unbiased endpoint for assessment purposes and has unambiguous ecological relevance to adverse effects.

(c) **Definitions.** The definitions in the OCSPP 850.2000 guideline apply to this test guideline. In addition, the following more specific definition also applies to this guideline:

Observation period is the portion of the test that begins after the test birds have been dosed and extends at least 14 days, but to continue until overt evidence of toxicity has subsided.

Frank sublethal effects for the purpose of this study refers to overt or frank toxicological effects for birds and include, but are not limited to, decreased body weight, loss of coordination, or lethargy. Less significant sublethal effects such as ruffled appearance or muted color are not considered frank toxicological effects.

(d) **General considerations—**

1) **Summary of the test.** Birds are administered the test substance as a single oral dose, either by capsule or gavage. Birds are observed regularly for mortality or any signs of intoxication throughout the observation period. Birds are weighed and feed consumption is estimated at least weekly thereafter. The mortality response pattern is examined and subjected to the appropriate statistical analysis to derive the LD_{50}, confidence limits, and

slope of the dose-response line. Sublethal effects should also be monitored, for example, gross appearance and behavior of the birds, weight changes, and changes in food consumption. Histopathological and physiological changes should be monitored. Moreover, delayed mortality and differences in sensitivities of each sex should be assessed.

(2) **General test guidance.**

(i) The general guidance in OCSPP 850.2000 applies to this guideline except as specifically noted herein. Studies should not be conducted with endangered or threatened species.

(ii) Successful adherence to elements in paragraph (h) of this guideline when testing passerine species may depend upon special provisions regarding husbandry, diet, acclimation of wild-caught birds, handling and age at dosing. For tests conducted with passerine species it is recommended, prior to test initiation, to submit protocols to the regulatory Agency for approval that identify husbandry, dietary, holding and acclimation methods, age at dosing and any other special provisions planned (see in particular references in paragraphs (j)(2) and (j)(4) through (j)(9) of this guideline). To be complete, the protocol should also contain information on all of the items discussed in this guideline.

(3) **Range-finding test.** Unless the approximate toxicity of the test substance is known already, a range-finding test should be conducted to determine the dosage levels of the test substance to be used in the definitive test. Refer to paragraph (e)(4)(ii) of this guideline for details on dosage levels for definitive tests. Procedures for range-finding tests may vary, but generally, groups of a few birds are administered three to five widely-spaced doses. A series of 2, 20, 200, and 2,000 milligrams per kilogram of body weight (mg/kg-bw) is suggested.

(i) If there is no mortality at the 2,000 mg/kg-bw dose level, and the test procedures and numbers of birds per dosage are the same as would be used in a definitive test, and also meet the elements of an acceptable limit test (see paragraph (d)(4) of this guideline), then the range-finding test may provide sufficient information to negate the need for a definitive test. If mortality does occur, than the results of the range-finding tests may then be used to help establish the definitive test dose levels.

(ii) If a test substance is expected to be of low toxicity, it may be useful to first conduct a limit test at 2,000 mg/kg-bw as described under paragraph (d)(5) of this guideline. If mortality occurs at this dose level, then further range-finding at lower levels is suggested. The results of the range-finding test then may be used to establish the definitive test dosage levels.

(4) **Definitive test.** The goal of the definitive test is to determine a dose-response curve for avian mortality after oral dosing and an observation period of at least 14 days to establish the acute LD_{50} (standard error and 95 percent (95%) confidence limits), as well as the slope of the dose-response curve (and its standard error and 95% confidence

limits). The definitive test consists of a minimum of five dose levels of the test substance, plus appropriate controls. The dosage levels are confirmed by chemical analysis under test conditions. A summary of test conditions is provided in Table 2 and validity elements for an acceptable definitive test are listed in Table 3. The Agency should be contacted prior to testing with nanomaterials.

(5) **Limit test.** For test substances expected to have relatively low toxicity, a limit test may be conducted with a single dose level at 2,000 mg/kg-bw or the maximum expected environmental residue concentration, whichever is higher, plus a control group. For pesticides, if the expected environmental residue concentration exceeds 2,000 mg/kg-bw the test should be conducted at a higher level equivalent to the maximum expected environmental concentration (EEC) on food items (see paragraph (e)(4)(ii)(B) of this guideline). Based on the results of the limit test, the acute oral LD_{50} may be reported as greater than the limit dose provided that the following conditions are met: first the limit treatment group and control group each contain a minimum of 10 birds; second no mortality or frank sublethal effects occurs in the limit dose group; third except for the number of dosage levels the test procedures and duration are the same as in the definitive test; fourth the dosage level is confirmed by chemical analysis under test conditions; and fifth, for pesticides, the limit dose is 2,000 mg/kg-bw or the maximum expected environmental residue concentration, whichever is higher. Clinical signs of toxicity, if any, should be reported. Conduct the full definitive test when any mortality is observed at the limit dose. If sublethal effects are suspected in the study than the Agency should be consulted for discussion on the appropriate dose and conduct of the limit test.

(e) **Test standards—**

(1) **Test substance.** The substance to be tested should be technical grade unless the test is designed to test a specific formulation, mixture, or end-use product. For pesticides, if more than one active ingredient constitutes a technical product the technical grade of each active ingredient should be tested separately, in addition to the combination, if applicable. The OCSPP 850.2000 guideline lists the type of information that should be known about the test substance before testing and discusses methods for preparation of test substances.

(2) **Test duration.** The definitive and limit tests consist of the administration of the test substance followed by an observation period of at least 14 days. If mortality occurs during the last 3 days of the 14-day period, or if signs of intoxication are not clearly in remission, or if the test substance is expected to have delayed effects, then extend the observation period until mortality or signs of intoxication are not observed for 72 hours.

(3) **Test organism—**

(i) **Species.** Data on both a passerine species and either an upland game bird or a waterfowl are generally required for 40 CFR Part 158. These test protocols and standards describe tests specific to using the northern bobwhite *(Colinus virginianus* (L.)), for an upland bird, the mallard *(Anas platyrhynchos* L.) for a waterfowl. At this time, there is no identified preferred passerine species; examples of potential passerine species that have been used in acute oral testing

include the house sparrow (*Passer domesticus*), zebra finch (*Taeniopygia guttata*) and red-wing blackbird (*Agelaius phoeniceus*). Regardless of the passerine species chosen, a protocol should be submitted to the Agency prior to test initiation for review. In addition to these species, pigeon (*Columba livia*), Japanese quail (*Coturnix coturnix japonica*), ring-necked pheasant (*Phasianus colchicus*), and red-legged partridge (*Alectoris rufa*), have also be used as upland game birds. The Agency will use these and other data to assess acute hazards and risks to birds. Appropriate husbandry standards for species used in this test should be consulted, examples include references in paragraphs (j)(1), (j)(2) and (j)(4) through (j)(9) of this guideline.

(ii) **Source.** Birds may be reared in the laboratory or purchased from a breeder. For a satisfactory test, all control and treatment birds used in a test should be from the same source and breeding population and be in the same plumage. Birds should be obtained only from sources whose colonies have known breeding histories. Test birds should be phenotypically indistinguishable (except for size) from wild stock. It is recommended that birds be obtained from flocks that have been out bred periodically with genetically wild stock in order to maintain a genetic composition that approximates the natural heterogeneity of the species. Birds purchased from a breeder should be certified as disease-free or as bred from disease-free stocks. The Agency recommends against the use of wild-caught birds. However, where suitable cultured supplies of passerine species may not be available, protocols submitted in advance should detail why wild stock must be used and have the necessary elements identified in this guideline addressed (see paragraph (d)(2)(ii) of this guideline).

(iii) **Age, sex, and size.**

(A) Typically, test birds should be young adults of both sexes, not yet mated, and are at least 16 weeks old at the time of dosing. A less preferred alternative is for the use of first-year birds that may have been mated, as long as the birds are brought completely out of production through reduced light cycles. In addition, there may be situations where in order to address concerns associated with a chemical-specific pattern of use younger or older birds (*e.g.*, altricial nestlings), or birds of only one sex (*e.g.*, breeding females), are more appropriate for testing. The need for this testing should be determined on a case-by-case basis after the basic testing is completed.

(B) For a satisfactory test, all upland and waterfowl birds used in a test should be the same age, plus or minus (±) 1 week. Currently, due to culturing and/or husbandry practices of Passeriformes, passerine birds used in a test may not be ± 1 week; however this may change if husbandry practices evolve. More consistent responses may be attained if the range of body weights is no greater than ±10% of the mean body weight for the test population.

(iv) **Acclimation.** Test birds should be acclimated to test facilities and basal diet for a minimum of 14 days. Acclimation to test pens may be either in the actual pens used in the test or in identical pens.

(v) **Health status.** Birds used in the test should be in apparent good health. Deformed, abnormal, sick, or injured birds should not be used. Birds should not be used for a test if total mortality during the 14-day acclimation period is more than 5% for cage-reared birds or more than 10% for wild-caught birds. Birds should not have been selected in any way for genetic resistance to toxic substances. Birds should not have been used in a previous test, either in a treatment or a control group.

(vi) **Care and handling.** During holding, acclimation, and testing, birds should be shielded from excessive noise, activity, or other disturbance. Test birds should be handled only as much as is necessary to conform to test procedures.

(vii) **Diet and feeding.**

(A) A standard commercial game bird (for northern bobwhite) or duck (for mallard) feed, or the nutritional equivalent, should be used as the diet. Guidance for recommended nutritional values for these species is provided in Table 1. Passerine species may require more complex diets. Feed should be withheld from all test groups for a minimum of 15 hours prior to administration of the test substance but feed should be available *ad libitum* during the study. This fasting period should be decreased, as appropriate, for the selected passerine species.

Table 1.—Recommended nutritional values for feed

Nutritional Component	Recommended Range (%)
Crude protein	27 to 29
Crude fiber	3.5 to 5.0
Crude fat	2.5 to 7.0
Calcium	2.6 to 3.6
Phosphorus	0.9 to 1.1

(B) Feed should not be used past its normal shelf life. Antibiotics or other medication should not be used in the diet during the acclimation period or the test. It may not be possible to obtain feed that is completely free of pesticides, heavy metals, and other contaminants. Therefore, diets should be analyzed periodically, as described under paragraph (e)(9)(ii) of this guideline, and selected to be as free from contaminants as possible. Extra precautions should be taken when fish meal or oil is a major ingredient, since fish are often contaminated with high levels of chlorinated hydrocarbons.

(viii) **Water.** Clean water should be available *ad libitum*. Only clean, unmedicated water should be offered during the acclimation and testing periods. Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water should be changed at least once a day.

(4) **Administration of test substance.** After acclimation under paragraph (e)(3)(iv) of this guideline, feed should be withheld from all test groups for a minimum of 15 hours prior, except potentially for Passeriformes, which may require less time to clear the digestive system of confounding substances, to administration of the test substance. Dosing by gelatin capsule is preferred, but when dosing with capsules is not feasible, doses may be administered by gavage. Doses are based on the individual body weight (bw) of each bird. Body weights are typically determined at the time of dosing, but may be taken, especially when capsules are used, within 24 hours prior to dosing. Dosing should be done in the early morning hours. The Agency should be contacted prior to testing with nanomaterials.

(i) **Preparation of gavage mixtures.** If dosing is by gavage and a carrier is used to administer the test substance, the preferred carrier is distilled or deionized water unless the test substance is known to hydrolyze readily. Other acceptable carriers include corn oil, propylene glycol, 1% carboxymethyl-cellulose, and gum acacia. Materials with known toxic or emetic properties should not be used. The absence of the crop in many passerine species requires using smaller, softer tubing than with waterfowl (see paragraph (j)(12) of this guideline). The dosing volume of test substance plus vehicle in a test should be constant for all birds with respect to individual body weights and should not exceed 5 milliliters per kilogram of body weight (mL/kg-bw). For those unusual test substances that might require a larger dosing volume (*e.g.* liquids with low purity), a dosing volume up to 8 mL/kg-bw may be used; steps should be taken to ensure that birds do not regurgitate the dose. In choosing between the bobwhite quail and mallard, it is recommended to use the bobwhite when a dosing volume up to 8 mL/kg-bw is needed.

(ii) **Treatment concentrations.**

(A) At a minimum, five dosage levels of the test substance are tested for definitive testing, plus the appropriate control. These dosage levels should be spaced geometrically in such a manner so that the entire dose-response curve (LD_{10} to LD_{90}) is adequately characterized. Taking into account results of the range-finding test(s), dosage levels should be spaced so that at least three doses cause mortality between, but not including, 0% and 100%. For a scientifically sound estimate of a point on the curve (*e.g.*, LD_{50}), responses should immediately bracket the point estimate of concern. For some test substances, it may be necessary to use more than five dose levels to achieve these results.

(B) For a limit test, there is single dose level, plus the appropriate control (see paragraph (d)(5) of this guideline). A limit dose of 2,000 mg/kg-bw is used unless environmental residues are expected to result in a higher

dosage. Equation 1 of this guideline can be used to calculate the acute avian oral limit dose (mg a.i./kg-bw) for spray applications of pesticides. The dietary residue estimates are based on a nomogram that relates food item residues to pesticide application rate; for an application rate of 1 lb a.i./A, the highest residue level expected is with short grass (nomogram value of 240). The nomogram is based on an EPA database called UTAB (Uptake, Translocation, Accumulation, and Biotransformation), a compilation of actual measured pesticide residue values on plants (see references in paragraphs (j)(5) and (j)(11) of this guideline). If there are multiple uses this study is supporting for registration, the limit dose for the study should be based on the one resulting in the highest dose. If the resulting limit dose exceeds the digestive capacity of the test organism, consult with the Agency prior to conducting the study to determine the appropriate dose to use.

$$Limit\ \ dose\,(mga.i./kg-bw)=\left(\frac{\left(C_{\max-diet}\right)}{\left(AW/TW\right)^{(SF-1)}}\right) \qquad \textbf{Equation 1}$$

where:
for a pesticide use with a single application per year:

$$C_{\max-diet}=\left(ApRate\right)(1.14)(240) \qquad \textbf{Equation 2}$$

for a pesticide use with more than one application per year:

$$C_{\max-diet}=\sum_{i=1}^{n}\left(\left(ApRate\right)(1.14)(240)\left(e^{-\left(\frac{0.6931}{halflife}\right)(n-1)(\text{int}\,erval)}\right)\right) \qquad \textbf{Equation 3}$$

ApRate = maximum single application rate (in lb a.i./acre);
Halflife = the foliar half-life (default is 35 days);
Interval = the minimum application interval (in days);
i = application event from 1 to n;
n = total number of applications;
AW = the body weight (in g) of the assessed bird – for pesticides, use 20 grams as this is most conservative value in screening level assessments;
TW = the body weight (in g) of the test bird;
SF = body weight allometric scaling factor (Mineau et al. scaling factor of 1.15 is the default (see paragraph (j)(10) of this guideline)); and
1.14 = a dose conversion factor assuming that a 20 gram bird consumes 114% of its body weight daily.

(5) **Controls.**

(i) Every test includes a negative control group where control birds receive a sham dose consisting of the same vehicle or capsule as received by the test

substance dosed birds. For a satisfactory test, negative control birds should be from the same hatch as the test substance dosed groups and be kept under the same experimental conditions. The test procedures should be the same for control and treated birds, except that no test substance should be administered to the control birds.

(ii) Controls serve as a monitor of bird husbandry practices, an indicator of possible problems due to handling, and test substance administration and aid in separating treatment related effects from non-treatment related effects. Controls are important in assessing background mortality and disease. Background mortality is never presumed to be negligible.

(iii) A test is not acceptable if more than 10% of the negative control birds die during the test period.

(iv) A concurrent positive control with a reference substance of known toxicity may be included in the test, as discussed in the OCSPP 850.2000 guideline.

(6) **Number of test organisms.**

(i) The minimum number of birds per dosage level of the test substance and the control is 10 birds. Typically, equal numbers of young adult birds of both sexes are used. However, when necessary to address chemical- and site-specific concerns, the sex and age of the birds used for testing may be modified if this is justified (*e.g.*, breeding females may be used when the expected exposure pattern indicates they may be at risk). Equal numbers of birds should be used for each dosage level.

(ii) Birds used in the test should be assigned randomly to treatment and control pens. Birds at a dosage level may be divided into two pens of five birds each. If this is done, dividing the groups by sex is recommended. Randomization should be done either at the initiation of the acclimation period or at the time of weighing just prior to dosing. The latter is recommended, because it avoids additional handling stress.

(7) **Facilities, apparatus and supplies.** Normal laboratory equipment and supplies, and items especially listed in (e)(7)(i) through (e)(7)(iv).

(i) **Facilities.** Pens should be kept indoors to control lighting, temperature, and other environmental variables.

(ii) **Pens—**

(A) **Size.** Pens should have a floor area of at least 500 square centimeters (cm^2) per bird (approximately 75 square inches (in^2) per bird) for northern bobwhite and at least 1,000 cm^2 per bird (approximately 150 in^2 per bird) for mallards and should be at least 24 centimeters (cm) (approximately 9.5 inches) high for northern bobwhite and 32 cm (approximately 12.5 inches)

high for mallard. Appropriate sized pens for passeriformes potentially may be different to accommodate bird size and social behaviors.

(B) **Construction materials.** Tests should be conducted with birds being maintained in commercial brooder or holding pens or pens of similar construction. Pens should be constructed of galvanized metal, stainless steel, or perfluorocarbon plastics. Materials that are toxic, may affect toxicity, or may sorb test substances should not be used. Wire mesh should be used for floors and external walls. Solid sheeting should be used for common walls and ceilings. Wire mesh for floors should be fine enough so as to not interfere with the normal movement of birds yet coarse enough to allow fecal material to fall through.

(C) **Cleaning.**

(1) Between tests, pens should be disassembled (if feasible) and thoroughly cleaned to prevent disease transmission and cross-contamination. Steam cleaning of cages is recommended. Cages may be hosed, brushed thoroughly, and hosed again, as an alternative method. The use of detergents or bleach is acceptable, but other chemical disinfectants such as quaternary ammonium compounds should not be used. When disease vectors have to be controlled, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended.

(2) Depending upon the type of pens used, pens may be cleaned during a test as needed to maintain good animal husbandry; however, care should be taken to minimize disturbance of the birds.

(iii) **Disposal.** After the test is terminated, treated and positive control birds should be sacrificed and disposed of properly. Negative control birds may be kept as breeding stock, but they should not be used in any other tests.

(iv) **Cleaning.** All materials that will come in contact with the test organisms and test substance should be cleaned before use. Cleaning procedures should be appropriate to remove known or suspected contaminants.

(8) **Environmental conditions.** Environmental conditions should be appropriate to the study species. For mallards and northern bobwhite quail environmental conditions during the test should be maintained as follows (conditions should be modified as appropriate for passerine species):

(i) **Temperature.** Testing is done indoors to control lighting and other environmental variables. Temperatures for adult birds should be maintained at normal indoor temperatures, preferably between 15 degrees Celsius (^{o}C) and 27 ^{o}C (70 to 80 degrees Fahrenheit (^{o}F)).

(ii) **Humidity.** Relative humidity is not as critical as some other variables, but the test room should be maintained at a relative humidity between 45 and 70%.

(iii) **Lighting and photoperiod.** A photoperiod of 10 hours light and 14 hours dark is recommended in order to prevent birds from coming into reproductive condition for upland and waterfowl species. However, this may potentially be different for passerine species and should be adjusted as appropriate to ensure birds are not entering reproductive condition. Lighting may be either incandescent or fluorescent. Pens and lights should be positioned so that all pens will receive approximately equal illumination.

(iv) **Ventilation.** It is recommended that ventilation be sufficient to supply 10 to 15 air exchanges per hour.

(9) **Observations—**

(i) **Measurement of test substance in dosing media.** Analytical confirmation of the dosing media concentration at test initiation is performed as described in the OCSPP 850.2000 guideline using analytical methods that are verified before beginning the test, to measure the amount of test substance in a sample.

(ii) **Contaminants in feed.** Contaminated feed may compromise study results, therefore, feed should be analyzed periodically to identify background contaminants such as heavy metals (*e.g.*, arsenic, cadmium, lead, mercury, and selenium) and persistent pesticides, especially chlorinated insecticides. A broader pesticide screen to include other chemicals that are known to be acutely toxic to birds may be useful.

(iii) **Basal diet composition.** A nutrient analysis of the basal diet should be included in the test report. The analysis should include percentages by weight of protein, fat, fiber, ash, calcium, and phosphorus. In addition to these analyzed components, a list of expected amounts of vitamins, minerals or other supplements should also be recorded. Most commercial feed companies provide both the analysis and the list of supplements on the label.

(iv) **Environmental conditions—**

(A) **Temperature.** Temperature should be monitored on a constant basis in at least one representative location.

(B) **Humidity.** Humidity should be monitored on a continual basis in at least one representative location.

(v) **Measures of effect—**

(A) **Monitoring of birds.** Birds are monitored closely for the first 60 to 120 minutes after dosing. Any regurgitation should be noted. Additional

observations of test birds are made a minimum of three times on the day of dosing and at least daily (where feasible, twice daily observations are recommended) throughout the remainder of the test period.

(B) **Mortality, intoxication, and other abnormal behavior.** Throughout the test period, all signs of intoxication, other abnormal behavior, and mortality are identified, counted, and recorded by dosage level, by sex, and by day. Signs of intoxication are those behaviors apparently due to the test substance and may include a wide array of behaviors, such as labored respiration, leg weakness, hemorrhage, convulsions, and ruffled feathers. Record all signs of intoxication and any other abnormal behavior, such as excessive aggression, toe-picking, *etc*. Among survivors, remission of signs of intoxication and cessation of abnormal behavior is identified and recorded by dosage level and by day. An estimate of the number of birds exhibiting such signs should be recorded for each dosage level.

(C) **Body weight.** Individual body weights of birds are recorded for control and treated birds at the time of calculating the dosage to be administered and weekly thereafter until the test is concluded. An extra weighing on the third day after dosing may provide useful information, especially on anorexia. Body weights of birds a week prior to dosing are recommended to provide valuable base-line data.

(D) **Food consumption.** Measure and record food consumption at least weekly in each pen throughout the test. Valuable additional information can be obtained by monitoring food consumption daily, especially for the first few days following dosing.

(E) **Gross pathology.** Gross pathology examinations are conducted on all birds that die, as well as a sufficient number of survivors selected randomly in all test substance treatment groups as well as at least three control survivors in order to provide characterization of lesions.

(f) **Treatment of results—**

(1) **Descriptive summary statistics—**

(i) **Environmental conditions.** Calculate descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) temperature and humidity.

(ii) **Mortality.** Cumulative number of dead for each dosage level and control group by observation day should be summarized in tabular form. If birds are separated by sex, provide cumulative number of dead birds by sex for each dosage level and control group by observation day.

(iii) **Body weight.** Calculate the change in body weight for an individual bird between observation periods (see Equation 4) and calculate the total change in body weight between test initiation and test termination (Equation 4 where time *j* is test termination and time *i* is test initiation). For the control and each test substance dosage level, calculate and plot the mean body weight change and standard error by observation interval to assess effects on the pattern of weight change. Calculate and plot for the control and each test substance dosage level, the mean total body weight change and standard error. Determine the mean body weight change for males and females separately.

$$d_{i-j} = w_j - w_i \qquad \textbf{Equation 4}$$

where:
d_{i-j} = difference or change in weight for an individual between observation time *i* and *j*;
w_i = weight of an individual at time *i*;
w_j = weight of an individual at time *j*.

(iv) **Food consumption.** Calculate and plot the mean food consumption by treatment level and observation period.

(v) **Appearance and behavior.** Number of birds with appearance and behavioral symptoms should be summarized in tabular form by time of observation, treatment, and sex (if applicable). Tabulate among survivors, remission of signs of intoxication and cessation of abnormal behavior by dosage level and by observation day.

(vi) **Gross pathology.** Types of observed pathologies, and the number of dead or examined surviving birds with these lesions should be summarized in tabular form by treatment and sex.

(2) **Percent mortality.** Calculate the cumulative percentage of dead birds at each test substance treatment level and in the controls at test termination. Test substance treatment data should be adjusted to account for any control mortality.

(3) **Limit test—**

(i) **LD_{50} value.** At test termination, if no birds die at the limit dose, the acute oral LD_{50} is considered to be greater than the limit dose (*i.e.*, LD_{50} > limit dose). This is because the Binomial Theorem predicts that when 10 organisms are tested, the probability of seeing no mortality if the true LD_{50} is at or below the limit dose is $\leq$0.001. Conversely the probability of seeing one or more dead birds if the true LD_{50} is at or below the limit dose is $\geq$0.999.

(ii) **Proportion of mortality ($\hat{p}$).** Assuming mortality follows the binomial distribution, an estimate of the true proportion of mortality ($\hat{p}$) in the laboratory test population as well as confidence bounds on that estimate (see Table A4 of the reference in paragraph (j)(3) of this guideline) can be obtained. For small sample sizes, the interval may be large. For example, for a limit test resulting in no

mortality in 10 birds ($\hat{p}$ = 0), the 99% confidence interval on the estimate of $\hat{p}$ is (0.00, 0.41) and the 95% confidence interval is (0.00, 0.31). Using the 95% confidence interval as an example, the true (unknown) proportion of mortality will be covered by the calculated confidence interval in 95% of repeated trials. For assessing risks, the confidence in the estimated proportion impacted is considered in determining acute effects at environmental exposure doses. If the uncertainty in $\hat{p}$ is high at the limit concentration, and the expected environmental exposure concentration is close to the limit concentration, risks to threatened and endangered species may not be able to be discounted.

(iii) **Multiple-dose definitive testing.**

(A) At test termination, if one or more mortalities occur among the 10 birds at the limit concentration (which was conducted at 2,000 mg/kg-bw or the maximum limit dose, whichever is greater), a definitive LD_{50} test should be conducted. If frank sublethal effect(s) are observed in one or more birds at the limit dose, despite an absence of mortality, then a full definitive test may be necessary. For pesticides, if frank sublethal effect(s) are observed in one or more birds and the limit dose tested was: 1) less than ten times the maximum expected EEC, then a full definitive study is necessary; or 2) was at least ten times the maximum EEC, but there is other evidence or data that indicate a risk to avian species, *e.g.*, pesticide use incident data, then a full definitive test is necessary.

(B) A multiple-dose definitive LD_{50} test may be waived if, at test termination: 1) the limit treatment group and control group each contain a minimum of 10 birds 2) no birds died at the limit dose; 2) and there are also no frank sublethal effects observed at the limit dose; 3) except for the number of dosage levels the test procedures and duration are the same as in the definitive test; 4) the dosage level is confirmed by chemical analysis under test conditions and 5) for pesticides, the limit dose was 2,000 mg/kg-bw or equivalent to the maximum expected environmental concentration on food items, whichever is higher.

(4) **Multiple-dose definitive test—**

(i) **Dose-response curve, slope, and LD_{50}.** Statistical procedures are employed to calculate the LD_{50} (standard error and 95% confidence limits). If a dose-response curve model (*e.g.*, probit) was fit to the data to determine the LD_{50}, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded. A statistical test for goodness-of-fit (*e.g.*, chi-square test) should also be performed to determine how well the data fit the computational model used.

(ii) **NOEL.** While calculation of a NOEL and LOEL is usually not part of this test design, reporting these values is useful when testing both pesticide and industrial chemicals.

(iii) **Statistical methods.** Statistical procedures for modeling quantal data are available and should be used. Additional discussion about endpoints and statistical procedures is found in the OCSPP 850.2000 guideline.

(g) **Tabular summary of test conditions.** Table 2 lists the important conditions that should prevail during the definitive test. Except for the number of dose levels, Table 2 also lists the important conditions that should prevail during a limit test. Meeting these test conditions will greatly increase the likelihood that the completed test will be acceptable or valid for the purposes of this test.

Table 2.—Summary of Test Conditions for Avian Acute Oral Toxicity Test

Test duration (observation period)	Minimum of 14 days
Temperature	15 ^{o}C to 27 ^{o}C (59 to 80 ^{o}F)
Light quality	Ambient: incandescent or fluorescent
Photoperiod	10 hours light: 14 hours dark
Pen size	≥500 cm^2 (approximately 75 in^2) per bird for northern bobwhite and ≥1,000 cm^2 (approximately 150 in^2) per bird for mallards. Pens for passerines should be appropriate for the study species.
Number of pens	One or two per dose level. Dividing by sex is recommended if using two pens
Test species	Northern bobwhite quail and mallard ducks are the preferred upland game bird and waterfowl species, respectively. Currently, no preferred passerine species has been identified. For passerines, protocols, which include the selected test species, should be submitted prior to test initiation.
Age of test organisms	Young adults, not yet mated. Other age groups may be tested to address a specific concern associated with a chemical-specific pattern of use on a case-by-case basis
Sex of test organisms	Typically, both sexes should be tested. Just one sex may be tested to address a specific concerns associated with a chemical-specific pattern of use on a case-by-case basis.
Number of birds per dose level	Minimum of 10 birds per dose, split evenly by sex if both sexes are tested
Dose levels	Minimum of five for definitive LD_{50} test, plus a control (for the limit test—the limit dose (2,000 mg/kg-bw or higher) plus a control)
Administration of dose	By gavage or capsule
Measures of Effect (Measurement Endpoint)	Death (LD_{50}), body weight, food consumption, and other signs of clinical toxicity

(h) **Test validity.** This test would be considered unacceptable or invalid if one or more of the conditions in Table 3 occurred. This list should not be misconstrued as limiting the reason(s) that a test could be found unacceptable or invalid. However, except for the conditions listed in Table 3 and in the OCSPP 850.2000 guideline, it is unlikely that a study will be rejected when there are slight variations from guideline environmental conditions and study design unless the

control organisms are significantly affected, the precision of the test is reduced, the power of a test to detect differences is reduced, and/or significant biases are introduced in defining the magnitude of effect on measurement endpoints as compared to guideline conditions. Before departing significantly from this guideline, the investigator should contact the Agency to discuss the reason for the departure and the effect the change(s) will have on test acceptability. In the test report, all departures from the guideline should be identified, the reasons for the changes given, and any resulting effects on test endpoints noted and discussed.

Table 3.—Test Validity Elements for the Avian Acute Oral Toxicity Test

1. Birds were not randomly assigned to treatment and control pens.
2. More than 10% of the control birds died during the test.
3. A minimum of ten birds were not used for each dose level of the test substance and control.
4. The test substance was not orally administered, via either capsule or gavage.
5. In the definitive test a minimum of five dose levels of the test substance, plus an appropriate control, were not tested.

(i) **Reporting—**

(1) **Background information.** Background information to be supplied in the report consists at a minimum of those background information items listed in paragraph (j)(1) of the OCSPP 850.2000 guideline.

(2) **Guideline deviations.** Provide a statement of the guideline or protocol followed. Include a description of any deviations from the test guideline or any occurrences which may have influenced the results of the test, the reason for these changes, and any resulting effects on test endpoints noted and discussed.

(3) **Test substance.**

(i) Identification of the test substance: common name, IUPAC and CAS names, CAS number, structural formula, source, lot or batch number, chemical state or form of the test substance, and its purity (*i.e.* for pesticides, the identity and concentration of active ingredient(s)), radiolabeling if any, location of label(s), and radiopurity.

(ii) Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.

(iii) Methods of preparation of the test substance and the treatment doses used in the range-finding and definitive test, or limit test.

(iv) If a diluent is used to prepare stock or test substance provide: the name and source of the diluent, the nominal concentration(s) of the test substance in the stock solution, and the diluent concentration(s) used in the treatments and diluent control. Provide a description of the dosing volume of test substance plus diluent for all birds with respect to individual body weights.

(v) Name of toxicant used for positive control (if applicable) and dosage levels.

(4) **Test organisms.**

(i) Name of species tested (including scientific name).

(ii) Information about the source: type, name, breeding history, certification of disease status.

(iii) Sex and reproductive history and condition at test initiation. If sexually mature birds that have been mated are used, describe the process used to bring the birds completely out of production.

(iv) Age (in weeks) of all birds at test initiation.

(v) Individual body weights at the beginning of the test (typically determined at time of dosing, but may be taken, especially for capsules, within 24 hours prior to dosing) and weekly thereafter.

(vi) Acclimation procedures and duration.

(5) **Test methods and conditions.** Provide a description of the test system and conditions used in the definitive or limit test, any preliminary range-finding tests, and any positive control tests.

(i) Description of housing containers: including type, size, and material of pens.

(ii) Description of housing environmental conditions: temperature, humidity, ventilation rate, photoperiod, and lighting source and intensity.

(iii) Detailed description of basal diet, including source/type, percentages by weight of protein, fat, fiber, ash, calcium, and phosphorus, a list of expected amounts of vitamins, minerals or other supplements. Most commercial feed companies provide both the analysis and the list of supplements on the label.

(iv) Describe the frequency and sample date(s) for documenting the contaminant status (heavy metals, persistent or chlorinated pesticides) of the feed and tabulation of the results of the analysis.

(v) Number of birds added to each pen at test initiation.

(vi) The number of pens per test substance dose level and control.

(vii) Methods of assigning birds to pens, and pens to dose levels and the control.

(viii) Date of dosing of test animals and test observation duration.

(ix) Methods and frequency of environmental monitoring performed during the definitive or limit study for temperature and humidity.

(x) Methods and frequency of measuring test substance to confirm exposure doses.

(xi) For the definitive and limit test, all analytical procedures should be described. The accuracy of the method, method detection limit, and limit of quantification should be given (described in 850.2000 (b) Definitions).

(xii) Methods and frequency of measuring number of dead birds, observing signs of intoxication, including regurgitation, and other abnormal behavior, including time of onset, duration, severity, and number affected at each dose level and control.

(xiii) Feed consumption per pen at least weekly or as often as measured, if more frequently than weekly, along with an estimate of wastage.

(6) **Results.**

(i) Tabulation of test substance analytical results by treatment (provide raw data).

(ii) Environmental monitoring data results (temperature and humidity) in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, and maximum).

(iii) For preliminary range-finding tests, if conducted, tabulate the number and percentage of birds that died in each test pen, treatment level and in the control at each observation period. Provide a description and count of any other appearance or behavioral effects at each treatment level and in the control. Tabulate the results of gross pathological examinations in dead birds and samples of surviving birds.

(iv) For a limit test, tabulation of the number of dead birds in each pen at each observation time during the test for the limit dose and the control (provide the raw data).

(v) For the definitive test, the number of dead birds at each observation time during the test tabulated by pen and treatment (provide the raw data).

(vi) For the definitive test, tabulation of the treatment percent dead, adjusted for control mortality.

(vii) For the limit and definitive test, tabulation by pen, treatment, and observation time of abnormal appearance and behavioral signs of toxicity and recovery, if any (provide raw data).

(viii) For the limit and definitive test, tabulation of gross morphology of dead birds and samples of surviving birds at test termination by pen and treatment (provide raw data).

(ix) Graphs of the dose-response data for percent mortality.

(x) For a limit test, provide conclusion about the LD_{50} being above the limit concentration and the lack or presence of other signs of toxic effects at the limit dose.

(xi) For the definitive test, where sufficient data exist to fit a model (*e.g.* probit) the slope of the dose-response curve and its standard error and 95% confidence limits and any goodness of fit results

(xii) If determined for the definitive test, the NOEL for mortality.

(xiii) Description of statistical methods used to fit the dose-response model or determine point estimates and the LD_{50} (including software package), and the basis for the choice of methods. Provide results of any goodness-of-fit tests.

(xiv) Description of statistical method(s) used for NOEL and LOEL determination, including software package, and the basis for the choice of method.

(j) **References.** The following references should be consulted for additional background material on this test guideline.

(1) American Society for Testing and Materials. ASTM E 857-05e1, Standard Practice for Conducting Subacute Dietary Toxicity Tests with Avian Species, In Annual Book of ASTM Standards, Vol. 11.06, ASTM, West Conshohocken, PA. Reapproved 2005.

(2) Committee on Birds of the Institute of Laboratory Animal Resources, National Research Council, 1977. Laboratory Animal Management: Wild Birds. National Academy of Sciences, Washington, DC.

(3) Conover, W. 1980. Practical Nonparametric Statistics, 2nd Edition. John Wiley & Sons, Inc., New York, NY. 493 pp.

(4) Dorrestein, G, 2003. Diagnostic approaches and management of diseases in captive passerines. Seminars in Avian and Exotic Pet Medicine 12(1): 11-20.

(5) Fletcher, J., J. Nellessen, and T. Pfleeger. 1994. Literature review and evaluation of the EPA food-chain (Kenaga) Nomogram, an instrument for estimating pesticide residues on plants. Environmental Toxicology and Chemistry 13(9):1383-1391.

(6) Grue, C. and L. Franson, 1986. Use of captive starlings to determine effects of environmental contaminants on passerine reproduction: pen characteristics and nestling food requirements. Bulletin of environmental contamination and toxicology 37(5):655-663.

(7) Harding, C., 1999. Husbandry and care of passerine birds. Poultry and avian biology reviews 10(2): 79-83.

(8) Hayre, M., 1995. Guidelines for housing excruciatingly happy zebra finches. Lab Animal 24(6):43-44.

(9) Matheson, S., L. Asher, and M. Bateson, 2008. Larger, enriched cages are associated with 'optimistic' response biases in captive European starlings (*Sturnus vulgaris*). Applied Animal Behaviour Science 109(2-4):374-383.

(10) Mineau, P., B. Collins, and A. Baril. 1996. On the use of scaling factors to improve interspecies extrapolation to acute toxicity in birds. Regulatory Toxicology and Pharmacology 24:24-29.

(11) Nellessen, J. and J. Fletcher. 1992. UTAB: A computer database on residues of xenobiotic organic chemicals and heavy metals in plants. Journal of Chemical Information and Computer Sciences 32:144-148.

(12) Tully, T., M. Lawton, G Dorrestein, 2000. Avian Medicine. Elsevier Health Science.

(13) U.S. Environmental Protection Agency, 1982. Pesticide Assessment Guidelines Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms. Office of Pesticides and Toxic Substances, Washington, D.C. EPA-540/9-82-024, October 1982.

(14) U.S. Environmental Protection Agency, 1985. Hazard Evaluation Division Standard Evaluation Procedure, Avian Single-Dose Oral LD50. Office of Pesticides Programs, Washington, DC, EPA-540/9-85-001, June 1985.

(15) U.S. Environmental Protection Agency, 1994. Pesticides Reregistration Rejection Rate Analysis: Ecological Effects, Office of Prevention, Pesticides and Toxic Substances, Washington, D.C. EPA 738-R-94-035.