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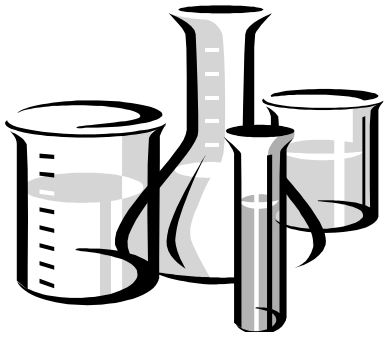
Ecological Effects Test Guidelines

OCSPP 850.2200: Avian Dietary 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 850.2200: Avian dietary 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 797.2050 Avian Dietary Toxicity Test; OPP 71-2 Avian Dietary LC50 Test (Pesticide Assessment Guidelines Subdivision E); Avian Dietary LC50 Test Standard Evaluation Procedure; OECD 205, Avian Dietary Toxicity Test; and ASTM E 857-05e1, Standard Practice for Conducting Subacute Dietary Toxicity Tests with Avian Species.

(b) **Purpose.** This guideline is intended for use in developing data, specifically both a median lethal concentration (LC_{50}) and slope of the concentration-response, on the dietary toxicity to young northern bobwhite (*Colinus virginianus*) and mallard (*Anas platyrhynchos*) 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 specific guidance for the testing of northern bobwhite and mallard, which are the Agency's preferred test species. The Agency will use these data to assess acute hazard to birds. 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 definitions also apply to this guideline:

Exposure period is the 5-day period during which test birds are offered a diet containing the test substance.

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.

Post-exposure period is the portion of the test that begins with the test birds being returned from a treated diet to the basal diet. This period is typically 3 days in duration, but may be extended if birds continue to die or demonstrate other toxic effects.

Test period is the combination of the exposure period and the post-exposure period, or the entire duration of the test.

(d) **General considerations**—

(1) **Summary of the test.** Birds are administered the test substance in their daily diet for five consecutive days. Birds are observed regularly for mortality and any signs of

intoxication (*e.g.*, abnormal behavior) during the exposure period and for a period of at least 3 additional days until the test is completed. Food consumption is estimated throughout the test. Body weights are determined prior to initiation of exposure and at the end of the test period (at test termination). The mortality response pattern is examined and subjected to the appropriate statistical analysis to derive the LC_{50}, confidence limits, and slope of the concentration-response line. Sublethal effects are also monitored, for example, gross appearance and behavior of the birds, body weights and feed consumption. Histopathological and physiological changes should be monitored.

(2) **General test guidance.** The general guidance in the OCSPP 850.2000 guideline applies to this guideline except as specifically noted herein.

(3) **Range-finding test.** Unless the approximate toxicity of the test substance is known already, a range-finding test should be conducted to help determine the test substance concentrations to be used in the definitive test. Refer to paragraph (e)(4)(ii) of this guideline for details on concentrations for definitive tests. Procedures for range-finding tests may vary, but generally, groups of a few birds are fed three to five widely-spaced concentrations for 5 days. A series of 5, 50, 500, and 5,000 parts per million (ppm) of diet (or milligrams per kilogram of diet mg/kg-diet) is suggested.

(i) If there is no mortality at the 5,000 ppm level, and the test procedures and numbers of birds per concentration level are the same as would be used in a definitive test, and also meet the elements of an acceptable limit test (see paragraph (d)(5) 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 concentrations.

(ii) If a test substance is expected to be of low toxicity, it may be useful to first conduct a limit test at 5,000 ppm as described under paragraph (d)(5) of this guideline. If mortality occurs at this concentration 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 concentration levels.

(4) **Definitive test.** The goal of the definitive test is to determine the concentration-response curve for avian mortality after dietary exposure, and to establish the subacute median lethal concentration (LC_{50}) (and its 95 percent (95%) confidence limits, as well as the slope of the concentration-response curve and its standard error). The definitive test consists of a minimum of five dietary levels of the test substance, plus appropriate controls. The dietary 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.

(5) **Limit test.** For test substances expected to have relatively low toxicity, in place of a definitive test a limit test may be conducted with a single dietary level of 5,000 ppm test substance or the maximum expected environmental residue concentration, whichever is higher, plus a control group. For pesticides, if the expected environmental residue concentration exceeds 5,000 ppm 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 subacute dietary LC_{50} may be reported as greater than the limit concentration (*i.e.*, 5,000 ppm) 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 treatment group; third except for the number of treatment levels the test procedures and duration are the same as in the definitive test; fourth the concentration level in the limit treatment diet is confirmed by chemical analysis under test conditions; and fifth for pesticides, the limit dose is 5,000 ppm or the maximum expected environmental residue concentration, whichever is higher. Signs of intoxication, if any, should be reported. Conduct the full definitive test when any mortality is observed at the limit concentration. If sublethal effects are suspected in the study then 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, then 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 the test substance in the diet for use in testing. The Agency should be contacted prior to testing with nanomaterials.

(2) **Test duration.** The definitive and limit tests consist of 5 days of exposure to the test substance in the diet (exposure period) followed by at least 3 days of additional observation (post-exposure period) while the test birds are receiving an untreated diet. If any test birds die during the second or third day of the post-exposure period, if toxic signs are evident on the third day of the post-exposure period, or if the test substance is expected to have delayed effects, the test period should be extended until there are two successive post-exposure days without mortality and one day without signs of intoxication.

(3) **Test organism—**

(i) **Species.** Data on both an upland game bird and 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 game bird, and the mallard (*Anas platyrhynchos* (L.)) for a waterfowl. In addition to these species, which are tested at a minimum, additional species, such as pigeon (*Columba livia*); Japanese quail (*Coturnix coturnix japonica*); ring-necked pheasant (*Phasianus colchicus*); and red-legged partridge (*Alectoris rufa*), may also be used as upland game birds. The Agency will use these and other data to assess acute hazards and risks to birds. Other sources should be consulted for appropriate husbandry standards for use of these other species in this test. For tests with species other than the northern bobwhite or mallard, it is important that they are responsive to the conditions of the test and cannot avoid exposure to the test substance through fasting. See paragraph (e)(3)(iii) of this guideline for additional guidance.

(ii) **Source.**

(A) 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 hatch. 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 also be certified as disease-free or as bred from disease-free stocks.

(B) If northern bobwhites are purchased, it is preferable that they be obtained as eggs which then are hatched and reared in the testing facility. During incubation, a temperature of 39 degrees Celsius (^{o}C) and relative humidity of 70% are recommended for northern bobwhite. It is feasible to purchase live young northern bobwhite chicks if they can be obtained locally, however, young northern bobwhite may suffer adverse effects if shipped by air or other commercial means. Young mallard ducklings normally can be shipped without undue adverse effects.

(iii) **Age.**

(A) Young birds that are not too old to be able to avoid eating during the exposure phase of the test (i.e. 5 days). This is typically achieved if at the beginning of the exposure period mallards are 5 days old and northern bobwhites are 10 to 14 days old. If additional test species are used, it is important that the test animals are not able to survive for five days without eating. This will insure that the animals cannot fast during the test, and that the test actually measures the toxicity of the test substance, rather than avoidance. Prior to conducting any testing on alternate species, confirm through studies or a literature search that the test animals are not old enough to survive without feeding for five days.

(B) For a satisfactory test all birds used in a test should be the same age, plus or minus (±1) a day. Due to the difficulty of sexing young birds, the sex of the test animals is not determined.

(iv) **Acclimation.** Test birds should be acclimated to test facilities and basal diet for a minimum of 3 days for mallard and 7 days for northern bobwhite. 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. During the 3-day period preceding testing, the health of the populations should be monitored and mortalities recorded. Birds should not be used for a test if more than 5% of the individuals in the total test population die during this time. Birds should not have

been selected in any way for genetic resistance to toxic substances or have been used in a previous test, either in a treatment or 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 as little as possible to conform to test procedures.

(vii) **Diet and feeding.**

(A) A standard commercial game bird (for northern bobwhite) or duck (for mallard) starter mash, or the nutritional equivalent, should be used for diet preparation. Guidance for recommended nutritional values is provided in Table 1.

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. For bobwhite only, an antibiotic demonstrated to fully depurate in 72 hours may be added to the drinking water up to 72 hours before test exposure, if necessary, for birds up through 10 days of age. It may not be possible to obtain food that is completely free of pesticides, heavy metals, and other contaminants; however, 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.** Only clean unmedicated water should be offered during the 96 hours preceding the exposure period and during the test period. Clean water should be available *ad libitum*. 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.** The test substance is administered in the diet with *ad libitum* feeding.

(i) **Preparation of diet treatments.**

(A) The test substance is mixed into the diet in a manner that will result in even distribution of the test substance throughout the diet. Diets can be mixed by commercial, mechanical food mixers. For many test substances, it is recommended that treated diets be mixed under a hood. Mashes and test substances should be mixed freshly just prior to the beginning of the test. Certain volatile or other test substances may require preparation of fresh diets at frequent intervals. The Agency should be contacted prior to testing with nanomaterials. Analysis of the diet for test substance concentrations is conducted as described under paragraph (e)(9)(i) of this guideline.

(B) If possible, test substance should be added to the diet without the use of a diluent. If a diluent is needed, the preferred diluent is distilled water, but water should not be used as a diluent for test substances known to hydrolyze readily. When a test substance is not water soluble, it may be dissolved in a reagent grade evaporative diluent (*e.g*. acetone or methylene chloride) and then mixed with the test diet. The diluent should be completely evaporated prior to feeding. Other acceptable diluents may be used; these include table grade corn oil, propylene glycol, and gum arabic (acacia). If a diluent is used, it should not comprise more than 2% by weight of the treated diet, and an equivalent amount of diluent should be added to control diets for untreated birds.

(ii) **Treatment concentrations.**

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

(B) For a limit test, there is single dietary level, plus the appropriate control (see paragraph (d)(5) of this guideline). A limit dose of 5,000 ppm is used unless environmental residues are expected to result in a higher dietary concentration. To calculate the dietary limit concentration (mg a.i./kg-diet) for spray applications of pesticides Equation 1 of this guideline can be used where a use is limited to a single application per year and Equation 2 of this guideline can be used where there are multiple applications per year. 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)(3) and (j)(4) of this guideline). If there are multiple uses this study is supporting for registration, the limit concentration for the study should be based on the one resulting in the highest dietary exposure.

where:

for a pesticide use with a single application per year:

$$Dietary\ \ Limit\ \ Concentration\,(mga.i./kg-diet) = (ApRate)(240) \quad \textbf{Equation 1}$$

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

$$Dietary\ \ Limit\ \ Concentration = \left(\sum_{i=1}^{n}\left((ApRate)(240)\left(e^{-\left(\frac{0.6931}{halflife}\right)(n-1)(\text{int}\,erval)}\right)\right)\right) \quad \textbf{Equation 2}$$

ApRate = maximum single application rate (in lb a.i./acre);

Halflife = the foliar halflife (default is 35 days);

Interval = the minimum application interval (in days);

i = application event from 1 to n;

n = total number of applications

(5) **Controls.**

(i) Every test includes a negative control group. 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 added to the diets of control birds. If a vehicle is used in preparation of the test diets, the same vehicle should be added to the diets of control birds in the highest concentration used for test diets.

(ii) Controls serve as a monitor of bird husbandry practices and 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 unacceptable if more than 10% of the negative control birds die during the test period.

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

(i) The minimum number of birds for each dietary test substance level and the control is 10 young birds. Birds may be divided into two replicates with a minimum of five birds per replicate. Equal numbers of birds should be used for

each treatment level. When a test substance is known or expected to result in high experimental variation, it may be appropriate or necessary to use additional birds.

(ii) For a satisfactory test, birds should be randomly assigned to treatment and control pens without respect to sex. Randomization may be done either at the initiation of the acclimation period or at the time when the birds are weighed at the beginning of the exposure period. 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 for housing young birds should have a floor area of at least 300 square centimeters per bird (cm^2/bird) (approximately 50 square inches per bird (in^2/bird)) for northern bobwhite quail and at least 600 cm^2/bird (approximately 100 in^2/bird) for mallards.

(B) **Construction materials.** Tests should be conducted with birds being maintained in commercial brooder 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 young birds.

(C) **Pen placement to prevent cross-contamination of feed.** Where feasible, it is recommended that pens not be stacked upon each other. If pens are stacked, only one test substance is allowed in any single stack. Pens should be randomly arranged, whether or not in a stack, with respect to dose levels and controls. Pens, such as stacked, unmodified, commercial pens with external feeders, that allow food to be spilled from one pen to a lower pen, should be avoided. Any modifications that prevent cross contamination of concentration levels are acceptable. For example, commercially available, 30 cm (1 ft) long chick feeders may be placed inside the pens and be covered with 1.27 cm (0.5 in) mesh hardware cloth over the food, for northern bobwhite. The same feeders covered with approximately 2.5 cm (1 in) mesh wire are appropriate for mallards. For either species, external feeders can be covered with the appropriate size wire mesh and a solid piece of metal extended from the bottom of the cage to a point exterior to the feeder. Spillage may occur, but the added metal will prevent food from spilling into another feeder.

(D) **Pen placement to prevent cross-contamination of volatile substances.** In order to avoid cross-contamination for test substances that volatilize or otherwise forms aerosols or vapors in the air; no more than one test substance is tested in a room.

(E) **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, take care 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—**

(i) **Temperature.** Testing is done indoors to control temperature and other environmental variables. Pens should be heated, preferably by thermostatic control. A temperature gradient in the pen of approximately 38 degrees Celcius (^{o}C) to approximately 22 ^{o}C will allow young birds to seek a proper temperature. Temperature requirements for young birds typically decline over this range from birth through the first several weeks of life.

(ii) **Humidity.** Relative humidity is not as critical as some other variables, but ideal test conditions include a relative humidity between 45 and 70%.

(iii) **Lighting and photoperiod.** A photoperiod of 14 hours light and 10 hours dark provides sufficient light to promote the feeding of the young birds and is recommended. Continuous lighting is also acceptable. Lighting may be either incandescent or fluorescent. Pens and lights should be positioned so that all pens will receive similar 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 diet—**

(A) Sampling. Samples of treated diets are analyzed to confirm proper dietary concentration of the test substance under test conditions using analytical methods that are validated before beginning the test as described in the OCSPP 850.2000 guideline. Representative samples of test feed should be taken at a minimum from feeders of the high, middle, and low test concentrations, at the beginning and end of the test.

(B) **Stability.** If it is observed that the stability or homogeneity of the test substance in the diet cannot be maintained, care should be taken in the interpretation of the results of the study and note made that the results may not be reproducible. For a satisfactory test, test substance concentration in the diet should be maintained at least at 80% of the nominal concentration throughout the exposure period (*i.e.*, first 5 days of the test).

(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 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. Most commercial feed companies provide both the analysis and the list of supplements on the basal feed label. The analysis should include percent by weight of protein, fat, fiber, ash, calcium, and phosphorus.

(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 constant basis in at least one representative location.

(v) **Measures of effect—**

(A) **Monitoring of birds.** Observation of test birds should be made, at a minimum, 3 times on the first day of the exposure period. Observations also should be made at least daily throughout the remainder of the test period; twice daily observations are recommended, where feasible.

(B) **Mortality, intoxication, and other abnormal behavior.** Throughout the test period, all signs of intoxication, other abnormal behavior, and mortality are identified, enumerated and recorded by treatment level 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, *etc*. Record all signs of intoxication and any other abnormal behavior, such as excessive aggression, toe-picking, *etc*. that may or may not be attributed to the test substance. Among survivors, remission of signs of intoxication and cessation of abnormal behavior is identified and recorded by treatment level and by day. An estimate of the number of birds exhibiting such signs should be recorded for each treatment level.

(C) **Body weight.** Average body weight of birds is determined and recorded for each pen within each test substance treatment and control group at the beginning and end of the exposure period and at the end of the normal 3-day post-exposure period. If the study is continued beyond 8 days, body weight data should be recorded at least weekly and at the termination of the test. Body weight data at 72 hours before the exposure period are not essential, but would provide valuable base-line data.

(D) **Food consumption.** Measure and record food consumption daily in control pens and pens with the low, middle, and high test substance concentration levels for both the exposure period and the normal 3-day post-exposure period. For other pens, estimate food consumption for the exposure period and for the post-exposure period. If the study is continued beyond 8 days measure and record food consumption weekly. Estimate and record any significant amount of food spilled onto litter pans.

(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 test substance treatment level and control group by observation day should be summarized in tabular form.

(iii) **Body weight.** For a given observation time, calculate the average body weight for a given pen using Equation 3. Calculate the change in average body weight for each pen between observation periods (see Equation 4) and calculate the total change in average body weight between test initiation and test

termination (Equation 4 where time *j* is test termination and time *i* is test initiation). Calculate and plot treatment mean body weight change and standard error by observation interval to assess effects on the pattern of weight change. Calculate and plot treatment mean total body weight change and standard error.

$$\overline{w}_t = w_t / n \qquad \textbf{Equation 3}$$

where:

$\overline{w}_t$ = average weight of a bird in a given pen at observation time *t*;

w_t = total weight of birds in the pen at time *t*;

n = total number of birds in the pen at time *t*.

$$\overline{d}_{i-j} = \overline{w}_j - \overline{w}_i \qquad \textbf{Equation 4}$$

where:

$\overline{d}_{i-j}$ = difference or change in average bird weight for a pen between observation time *i* and *j*;

$\overline{w}_i$ = average weight per bird in the pen at time *i*;

$\overline{w}_j$ = average weight per bird in the pen 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 pen. Tabulate among survivors, remission of signs of intoxication and cessation of abnormal behavior by test substance treatment level, pen, 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 test substance treatment level and pen.

(2) **Percent mortality.** Calculate the cumulative percent 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) **LC_{50} value.** At test termination, if no birds die at the limit concentration, the subacute dietary LC_{50} is considered to be greater than the limit concentration (*i.e.*,

LC_{50} > dietary limit concentration). This is because the Binomial theorem predicts that when 10 organisms are tested, the probability of seeing no mortality if the true LC_{50} is at or below the limit concentration is $\leq$0.001. Conversely the probability of seeing one or more dead birds if the true LC_{50} is at or below the limit concentration is $\geq$0.999.

(ii) **Proportion of mortality ($\hat{p}$).** The Binomial Theorem can also provide both 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)(2) of this guideline). For small sample sizes the interval may be large. For example, for no mortalities in 10 birds ($\hat{p} = 0$), the upper 99% confidence bound on the estimate of $\hat{p}$ is (0.00, 0.41) and 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 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 5,000 ppm or the maximum EEC, whichever is greater), a definitive LC_{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; 3) there are also no frank sublethal effect(s) at the limit dose; 4) the concentration level in the limit treatment diet is confirmed by chemical analysis under test conditions; 5) except for the number of treatment levels the test procedures and duration are the same as in the definitive test; 6) for pesticides, the limit dose was 5,000 ppm or equivalent to the maximum expected environmental concentration on food items, whichever is higher.

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

(i) **Concentration-response curve, slope and LC_{50}.** Statistical procedures are

employed to calculate the LC_{50} (standard error and 95% confidence limits). If a concentration-response curve model (*e.g.*, probit) was fit to the data to determine the LC_{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) **NOEC.** While calculation of a NOEC and LOEC is usually not part of this test design for acute tests, reporting these values is useful when testing pesticides 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 treatment levels, Table 2 also lists the important conditions that will 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 Subacute Dietary Toxicity Test

Test duration	Minimum of 8 days
Exposure period	Five days
Temperature	Gradient in a pen of 22 ^{o}C to 38 ^{o}C
Light quality	Ambient: incandescent or fluorescent
Photoperiod	14 hours light: 10 hours dark
Pen size	>300 cm^2 (approximately 50 in^2) per bird for northern bobwhite and >600 cm^2 (approximately 100 in^2) per bird for mallards
Number of pens	One or two per dose level
Test species	Northern bobwhite and mallard are preferred
Age of test organisms at test initiation	Northern bobwhite: 10 - 14 days Mallard: 5 days
Sex of test organisms	Typically both sexes should be tested; however it is not practical to determine the sex of young birds
Number of birds per concentration level	Minimum of 10, randomly assigned
Concentration levels	Minimum of five, plus control for definitive test (for limit test, the limit concentration (5,000 ppm or higher) plus a control)
Administration of test substance	Through diet
Measures of Effect (Measurement Endpoint)	Death (LC_{50}), body weight, food consumption and other signs of clinical toxicity

(h) **Test validity elements.** This test would be considered to be 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, communication between the investigator and the Agency is important in order 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 are identified, reasons for these changes given, and any resulting effects on test endpoints noted and discussed.

Table 3.—Test Validity Elements for Avian Subacute Dietary Toxicity Test

1. Birds were not randomly assigned to treatment and control pens.
2. More than 10% of the control birds died or became moribund during the test.
3. Concentrations of the test substance were not satisfactorily maintained in the diet (levels should be at least 80% of the nominal concentration) throughout the exposure period (*i.e.*, the first 5 days).
4. Birds were not administered the test substance in their daily diet for 5 consecutive days.
5. A minimum of 10 young birds were not used for each dietary concentration of the test substance.
6. The test substance was not administered in the diet.
7. In the definitive test a minimum of five dietary levels of the test substance, plus appropriate controls, 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, stock solutions, and the treatment concentrations 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.

(v) Name of toxicant used for positive control (if applicable), dietary treatment 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) Age of birds (in days) at the beginning of the test.

(iv) Average body weight for each pen at the beginning of the test, at the end of the exposure period, and at the end of the test.

(v) Acclimation procedures and conditions, including housing and environmental conditions (if different from test conditions), feeding procedures, and health observations (including mortality).

(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 (see 850.2000 (h) Reference toxicants) tests.

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

(ii) Description of feed and water dispensing apparatus.

(iii) Description of placement of pens to eliminate cross-contamination, due either to food cross-contamination or volatility of test substance. Include a description of any modifications of food dispensers that prevent cross-contamination.

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

(v) Detailed description of basal diet, including source/type, percent 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.

(vi) 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.

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

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

(ix) Methods of assigning birds to test pens and test pens to treatment levels.

(x) Date of initial exposure of birds to the test substance, dates of exposure, duration of test, including length of exposure period (in days) and length of the post-exposure period on "clean" feed (in days).

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

(xii) Methods and frequency of measuring test substance to confirm dietary exposure levels of the test substance and in control feed.

(xiii) 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).

(xiv) 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.

(xv) Estimated food consumption per pen daily in control pens and pens with the low, middle, and high test concentration levels. For other pens, food consumption should be estimated for the exposure period and for the post-exposure period. Indicate whether food was regurgitated or spilled and in which treatments.

(6) **Results.**

(i) Tabulation of dietary test substance analytical results by pen and treatment (provide raw data). Include a statement about the stability and homogeneity of the test substance in the diet throughout the exposure period.

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

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

(v) For the definitive test, tabulation by test pen and treatment of the number of dead birds at each observation time during the test (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 test 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 by test pen, treatment gross morphology of dead birds and samples of surviving birds at test termination (provide raw data).

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

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

(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 method(s) used for point estimates, including software package, for determining the LC_{50} value, fitting the dose-response model, and the basis for the choice of method. 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) Conover, W. 1980. Practical Nonparametric Statistics, 2nd Edition. John Wiley & Sons, Inc., New York, NY. 493 pp.

(3) 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.

(4) 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.

(5) Organization for Economic Cooperation and Development (OECD), 1984. Guidelines for testing of chemicals, Guideline 205, Avian Dietary Toxicity Test, Adopted April 1984.

(6) 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.

(7) U.S. Environmental Protection Agency, 1985. Hazard Evaluation Division Standard Evaluation Procedure: Avian Dietary LC50 Test. Office of Pesticides Programs, U.S. Environmental Protection Agency, Washington, D.C. EPA-540/9-85-008, June 1985.