

**TOXICITY TEST GUIDELINES FOR NON-TOXIC SHOT
FOR HUNTING MIGRATORY BIRDS**

**Canadian Wildlife Service
Conservation and Protection
Environment Canada
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TOXICITY TEST GUIDELINES FOR NON-TOXIC SHOT

Introduction

These guidelines are provided for the information of persons who may wish to develop or market non-toxic shot for migratory bird hunting in Canada.

It is important that contact be made early during consideration of a new product. These guidelines are subject to modification as scientific knowledge improves, and early contact will ensure that the current version is being used. Also, modifications to the procedures may be indicated, according to the toxicological properties of the substance under consideration.

Application to follow the Guidelines

Applications from parties wishing to follow these guidelines should be sent to:

Director General
Canadian Wildlife Service
Environment Canada
Ottawa, Ontario K1A 0H3.

The applicant should be prepared to provide to Environment Canada:

- i) A minimum of 25 pounds (11.6 Kg) of the candidate shot in size equivalent to #4.
- ii) A description of the chemical composition of the candidate shot and a statement of the range of composition variability during large-scale production and a description of the analytical methods used for determination of composition.
- iii) The applicant's assessment, based on a review of the scientific literature, of the potential toxicity of the candidate shot to migratory birds, other wildlife species, and humans which may be exposed to the shot directly or indirectly;
- iv) The applicant's assessment of the risks to overall health of migratory game bird environments into which the candidate shot is discharged based on a review of available scientific information concerning the environmental fate and toxicology of the spent material and its mostly likely decomposition products.

Objective of the Guidelines

- (1) Environment Canada shall determine that a specific shot material is acceptable as non-toxic for the purpose of hunting migratory game birds if, after a review of an application and supporting data submitted, together with other relevant evidence, including public comment, it is concluded that the spent shot material does not pose a significant toxic threat to migratory birds, or to their ecosystems.

The process for application of the Guidelines

- (2) The candidate shot will be chemically analyzed by Environment Canada or an independent laboratory whose analytical accuracy and precision are acceptable to Environment Canada, at the applicant's expense, and the results compared to the applicant's description of shot composition and composition variability. If Environment Canada concludes, on the basis of the technical and scientific information contained in the applicant's submission, that the candidate shot might qualify as a non-toxic shot, the applicant will be requested to submit a plan for conducting the acute toxicity test outlined below. Environment Canada shall specify what portion of the tests are required, and any modifications to the procedures that will be necessary. Depending on the variability in the composition of the shot and information on the stability and environmental fate of the shot, the applicant may be required to conduct independent toxicity tests on specific mixtures of the substances composing the shot. In addition, Environment Canada may require the applicant to submit plans for conducting other tests as prescribed by Environment Canada in order to fill essential data gaps required to complete the assessment of the risks to the health of the environments receiving the spent shot. The composition of the shot that will be produced commercially after the shot is granted a conditional or full approval for use in non-toxic shot zones shall not be statistically different from the composition of the shot used in the tests outlined below. Environment Canada will evaluate the applicant's testing plan with respect to timing, format, content, and the suitability of the facilities at which the proposed testing is to be done.
- (3) Upon approval of the plan by Environment Canada, the applicant will implement the acute toxicity and other tests and, upon completion, the applicant shall record, analyze and report the results in writing in accordance with Environment Canada requirements.
- (4) Environment Canada will review the results, analytical procedures, and conclusions from the acute test. Within 90 days of receipt of the report, on the basis of its review, Environment Canada will conditionally approve, unconditionally reject, or require additional information or testing of the candidate material, and will provide a written explanation for these decisions.
- (5) If the applicant is able to show:
 - (a) by way of the required comprehensive written assessment and literature review [1(iv)] that the spent shot product is stable and will not decompose and give rise to degradation products that are harmful to the long-term health of the receiving environment;
 - (b) that no significant negative effect has been detected in the acute test or its equivalent, and;
 - (c) that accumulation of the major constituents of the shot in blood and internal organs in ducks undergoing the acute test is negligible,

then the product may be given a conditional approval.

- (6) Coincident with the granting of conditional approval, the applicant will be advised to develop and submit a plan for conducting, within 24 months of receipt of conditional approval, a chronic-reproductive toxicity test and other tests as judged necessary by Environment Canada. Upon submission of the plan, Environment Canada or its authorized representatives may inspect the test facilities, and will review the proposed test procedures.
- (7) The applicant, upon receiving Environment Canada approval of the plan for conducting the chronic reproductive test, shall conduct the test and shall record, analyze, and report the test results to Environment Canada, as stipulated for the acute test.
- (8) Environment Canada shall review the results of the chronic reproductive test and within 90 days shall approve or unconditionally reject the candidate shot as a 'non-toxic shot' in Canada, or require further information or testing of the candidate material before granting final approval or rejection, and shall provide the applicant with a written explanation for these decisions.

Following completion of steps 1 to 8 or their equivalent Environment Canada will publish a summary of the results for public information and comment. If Environment Canada approves the candidate shot, it will announce this to the public.

There will be ongoing review of possible toxic side effects from any shot used for migratory bird hunting

Environment Canada will remain vigilant regarding the toxicity of any shot in use, and may revoke the approval of any given shot, or put forward regulations to control the use of any shot, if such a step becomes necessary.

These guidelines only deal with toxicity as regulated under the Migratory Birds Regulations. The applicant is responsible to ensure that other regulatory requirements are met

This assessment of the toxicity of bird hunting shot relates to the Migratory Birds Regulations. The applicant is responsible to ensure that any other requirements relating to federal or other regulations that apply to the candidate shot are met. For example, the Canadian Environmental Protection Act may apply in some cases.

Outline of the toxicity tests

For the purpose of these tests, 'significant', 'significantly greater' and 'significantly lower' refer to a significance probability threshold of 0.05. Statistical tests are one-tailed tests.

The two tests described in this section represent an evaluation of three major types of toxic effects: short-term acute exposure, chronic effects on health, and chronic exposure impact on reproduction. The details of the experimental procedure, including variables which must be measured, may be adjusted to suit the substances involved, particularly if the candidate shot is not a metal or a metalloid. Statistical tests will generally be performed on all data from each test, or on subsets of the data where appropriate.

Acute toxicity test

Materials

A minimum of forty male and forty female hand-reared Mallards approximately 6 to 8 months old.

These Mallards must have physical characteristics that resemble wild Mallards. At least eighty elevated, outdoor or indoor 1-meter square pens of vinyl-coated wire equipped with food containers and water dispensers.

Full spectrum lighting, if test is conducted in an indoor aviary.

Laboratory equipped to perform fluoroscopy, required blood and tissue analyses, and necropsies.

Commercial duck food.

Whole Kernel corn.

Steel shot #4 and candidate shot #4.

Procedures

Mallards will be housed individually in pens and given access to commercial duck food and water provided ad libitum. After three weeks, birds will be switched to a corn diet provided ad libitum. At the same time, males and then females will be randomly assigned to one of two groups (20 males and 20 females per group) and dosed with 6 steel shot or 6 candidate shot. Birds will be fluoroscoped 1 week after dosage to confirm retention of the six shot pellets/bird. Birds with less than six shot will be redosed with the required number of shot immediately after fluoroscopy. Birds will be observed daily for obvious behavioral signs of intoxication and mortality over a 30-day period. Body weight will be determined at the time of dosing, and at days 15 and 30 of the test. On days 0, 15 and 30, heparinized blood will be collected by venipuncture for determination of haematocrit. Remaining whole blood will be centrifuged to separate cells and plasma. Plasma Ca, P, Mg, Zn, and Cu will be determined. The concentrations of the major elements ($\geq 1\%$ by weight) comprising the candidate shot will also be measured in plasma and cells. All birds that die before the end of the experiment will be necropsied by a qualified veterinarian to attempt to determine the cause of death. Upon completion of the test, all remaining birds (up to 40 males and 40 females including birds that die during the experiment) will be sacrificed by decapitation using a small animal guillotine. Critical organs (gizzard, liver, kidney) from at least 10 males and 10 females per dosing group will be examined by a qualified veterinary pathologist for the presence of gross lesions and by standard light microscopy for histopathological changes. The concentrations of the major elements comprising the candidate shot, as well as essential metals Fe, Zn, Cu and Mn, and tissue water content, will be determined in liver and kidney tissue from all individuals.

Statistical Data Analysis and Information to be Submitted for the Acute Toxicity Test

General Notes:

1. For each variable on which the effect of the candidate shot is tested, a separate analysis should be carried out for the data from male and female birds. Environment Canada may

further analyze the data by comparing the effects for male and female birds, and if the difference is not significant Environment Canada may then test the effect of the candidate shot by a combined analysis using data from both male and female birds. If they wish, labs may run combined analyses themselves and submit the results.

2. All tests of the effect of the candidate shot should be one-tailed tests at the 5% significance level. The critical values for these tests are the same as those for two-sided tests at the 10% level. It is assumed that the candidate shot has a negative effect on all variables. If the effect is positive for any variable, no test of significance is required for it.
3. All t-tests between treatment means can be standard t-tests, which assume that the variance is equal within both treatments. Environment Canada may further analyze the data for any variable by comparing the variances within treatments. If a significant difference is found, Environment Canada may run a modified t-test that allows for unequal variances. If they wish, labs may run modified t-tests themselves and submit the results.
4. Whenever a t-test is specified to compare treatment means, labs may choose instead to carry out an equivalent one-way ANOVA and perform an F-test.
5. The data set may be rejected if the variation between birds of the same sex within a treatment is considered to be excessive for one or more variables. This will be assessed by the size of the coefficients of variation (the standard error divided by the mean).
6. The raw data should be submitted for each test variable. The data should be submitted in ASCII code on a diskette, accompanied by a printout of the data, a list of the data files and a description of their format.
7. If a situation arises in which a treatment mean or variance can not be calculated for a variable or the required analysis can not be carried out (for example if some measurements are below the detection limit for the measurement procedure employed), Environment Canada should be consulted concerning how to proceed with the analysis of the data.

Mortality

Analysis: Mortality during the test period for male birds dosed with steel shot and candidate shot is to be compared by a chi-square test (without correction for continuity), and similarly for female mortality.

To be submitted for each sex: Submit the number of birds in each treatment at day 0, the number that died during the test, and the chi-square value.

Post-Mortem Variables (element concentrations and water content in liver and kidney tissues and histopathology data)

Analysis: For each variable a log transformation should be applied prior to the analysis if appropriate. The mean for male birds dosed with steel shot and the mean for those dosed with candidate shot are to be compared by a t-test. In addition if 5 or more birds die prior to the end of the test in either treatment, the mean of the birds that died in that treatment is to be compared with the mean of those that survived using a t-test. Histopathology data are to be analyzed using

a chi square analysis comparing steel-dosed and candidate-dosed birds and comparing steel-dosed birds and candidate-dosed birds that died during the experiment. The entire procedure is to be repeated using the data from female birds.

To be submitted for each sex: Submit raw data values and the transformation applied if any. For each treatment submit the mean, standard error, the coefficient of variation (coeff. of var. = [standard error/mean] x 100%), the mean for the birds that died prior to the end, the mean of those that survived, and the t-value to compare these latter two means (if any test was carried out). In addition submit the relative effect of the candidate shot (relative effect = [(difference in steel and candidate means) / (mean for steel shot)] x 100%), and the t-value to compare steel and candidate means.

Variables Measured Throughout the Test (weights, haematocrit, and elements in plasma)

Analysis: For each variable, apply a log transformation prior to the analysis if it is appropriate. For each male bird, calculate the difference between the value at day 0 and that at day 15. If a bird dies prior to day 15, use the difference between day 0 and death. Calculate the mean of this difference for birds dosed with steel shot and the mean for those dosed with candidate shot, and compare these means using a t-test. In addition if 5 or more birds die in either treatment prior to day 15, calculate the mean difference from day 0 to death for the birds that died in that treatment and also the mean difference from day 0 to day 15 for the birds that survived. Compare these latter two means using a t-test. Carry out the same analysis on the differences between values at day 0 and day 30. Repeat the entire procedure for the data from female birds.

To be submitted for each sex: Submit raw data values (including values at death for birds that die during the test), the transformation applied if any, and the means, standard errors and coefficients of variation at days 0, 15 and 30 for each treatment. For the day 0 - day 15 differences, submit for each treatment the mean, the standard error, the mean difference of the birds that died prior to day 15, the mean difference for those that survived, and the t-value for the test to compare these latter two means. Submit also the relative effect of the candidate shot (here relative effect = [(mean difference for steel shot minus mean difference for candidate shot) / (mean for steel shot at day 0)] x 100%) and the t-value to compare steel and candidate mean differences. Corresponding quantities should be submitted for the day 0 - day 30 differences.

Reproductive test including chronic health effects

Materials

Forty male and forty female hand-reared Mallards approximately 6 to 8 months old and weighing between 1135 and 1365 grams. These Mallards must have physical characteristics that resemble wild Mallards, and must have undergone 30 day dosing conditions similar to those described in the acute 30-day test (above). Eighty elevated, indoor 1-meter square pens of vinyl-coated wire equipped with food containers and water dispensers.

Full spectrum lights.

Laboratory equipped to perform fluoroscopy, required blood and tissue assays, blood metal, tissue metal and enzyme analysis, and necropsies and histopathology analyses.

Breeding duck food having not more than 2900 Kcal/kg of metabolizable energy, 16% protein, 0.32% methionine, 1.5% calcium, 0.4% phosphorous and 0.10% magnesium.

Commercial starter duck food

Steel shot #4 and candidate shot #4.

Procedures

After undergoing the acute exposure test conditions, ducks will be brought into breeding condition by gradually (over 2 weeks) adjusting the photoperiod under which they are maintained. Birds within each dosing group will then be randomly paired, redosed with 6 shot pellets of the appropriate type, and fed for three weeks on breeding duck food. Ducks will be fluoroscoped after 1 week to confirm retention of the six shot, and redosed if required. Males will be returned to their pens after initiation of laying. Nests will be checked daily. For each nest, data on clutch initiation time, clutch size, egg fertility, egg hatchability, and overall reproductive success (number of chicks produced per dosing group) will be recorded. After initiation of egg laying, a clutch will be deemed to be complete when 2 consecutive days have passed during which no egg is laid. At that time, all eggs from that pen will be counted and weighed. The size (weight) of all eggs will also be measured. All eggs will be candled to determine fertility. Fertile eggs will then receive an individual mark and be placed in an incubator. After chicks have hatched, shell thickness will be measured on each properly marked egg. Ducklings will be provided with starter mash after hatching. Body weight of adult females will be determined at days 0 and then every two weeks until sacrifice. All hatchlings will be weighed and sacrificed at seven days of age. Blood will be collected at sacrifice from adults and chicks for determination of haematocrit, and concentrations of major elements constituting the shot, and major nutritionally essential elements. All birds that die before the end of the experiment will be necropsied to determine the cause of death. Internal organs (liver, kidney, ovaries) from at least 10 females from each dosing group will be examined for both gross and microscopic lesions. Internal organs of ducks dosed with the candidate shot will be analyzed for elements contained in the shot, as well as major essential trace elements.

Statistical Data Analysis and Information to be Submitted for the Reproduction Test

Variables Measured on Adult Birds

General Notes

See general notes for the analysis of data for the acute toxicity test.

Mortality

See the section on mortality data in the acute toxicity test.

Post-Mortem Variables (haematocrit, element concentrations in the blood)

See the section on post-mortem variables in the acute toxicity test.

Variables Measured Throughout the Test (weight of female birds)

For each bird calculate the weight difference from day 0 to day 14, the difference from day 0 to day 28, the difference from day 0 to day 42, etc. For the analysis and material to be submitted, see the section on variables measured throughout the test in the acute toxicity test.

Variables Measured on Eggs or Chicks

General Notes - See general notes 2 to 7 for the analysis of data for the acute toxicity test.

Count Variables (number of eggs laid, number of eggs hatched, number of live chicks after 7 days)

Analysis: Calculate the count for each pair of birds in each treatment. Apply a square root transformation to the counts prior to the analysis if appropriate. Calculate the mean for each treatment, and compare treatment means by a t-test.

To be submitted: Submit the value of the count for each pair of birds, and the transformation applied if any. For each treatment submit the mean, standard error, and the coefficient of variation. In addition submit the relative effect of the candidate shot and the t-value to compare the treatment means.

Proportional Variables (proportion of eggs laid that are fertile, of fertile eggs that hatch, and of hatchlings that live 7 days)

Analysis: Calculate the proportion for each pair of birds in each treatment, leaving out proportions for which the denominator is less than 3. Apply a transformation (angular or logit) to the proportions prior to the analysis if appropriate. Calculate the mean for each treatment, and compare treatment means by a t-test. Environment Canada may reanalyse the data to take into account differences in denominator from one data value to another. This would be achieved by employing weighted treatment means in place of the regular means, with the weights being derived by the method described in Cochran (1943) or by an equivalent method. If they wish, labs may run this or an equivalent analysis themselves and submit the results.

To be submitted: Submit the value of the proportion for each pair of birds, including both numerator and denominator, and the transformation applied if any. For each treatment submit the mean, standard error, and the coefficient of variation. In addition submit the relative effect of the candidate shot and the t-value to compare the treatment means.

Measurement Variables (shell thickness, egg size, egg weight, chick weight, chick haematocrit and element concentrations in chicks)

Analysis: Apply a transformation prior to the analysis if appropriate (such as a log transformation for the element concentration values). Calculate the mean value for the eggs or chicks from each pair of birds. Then treating these pair means as individual data values, calculate

a mean for each treatment and compare these means by a t-test. Environment Canada may reanalyse the data to compare the treatment means by running a nested one-way ANOVA. The sources of variation would be the variation between treatments, between pairs within a treatment, and between individual eggs or chicks within a pair. If they wish, labs may run this nested analysis themselves and submit the results.

To be submitted: Submit the raw data values for each egg or chick and the transformation applied if any. For each treatment submit the mean for each pair of birds, and the mean, standard error and coefficient of variation of these pair means. In addition submit the relative effect of the candidate shot and the t-value to compare the treatment means.

References

Cochran, W.G. (1943). Analysis of variance for proportions based on unequal numbers. *JASA* 38, 287-301.