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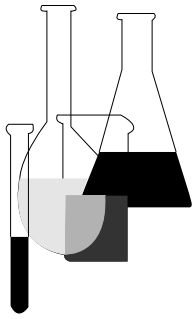
Microbial Pesticide Test Guidelines

OPPTS 885.4000 Background for Nontarget Organism Testing of Microbial Pest Control Agents



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

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OPPTS 885.4000 Background for nontarget organism testing of microbial pest control agents.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*).

(2) **Background**. The source material used in developing this harmonized guideline is OPP guideline 154A–1.

(b) **Overview.** The purpose of nontarget organism testing is to develop data necessary to assess potential hazard of microbial pest control agents (MPCAs) to terrestrial wildlife, aquatic animals, plants, and beneficial insects. The test standards and definitions in this guideline apply to OPPTS Series 885, Group D.

(1) **Approach.** The Agency has concluded that at least some test data on terrestrial and aquatic organisms should usually be evaluated, regardless of the pesticide's site of outdoor application and apparent potential for exposure. These data would be necessary for the following reasons:

(i) When a microorganism is applied as a pesticide, great numbers are placed in the environment apart from its host, at a discrete point in time (day of application), and spread over living and nonliving components of the target site. Often, there will be spread to adjacent areas, due to drift. Hence, in terms of numbers of nontarget organisms exposed, number of different species exposed, and the degree of exposure (number of microorganisms per nontarget organism), exposure may be greater than under natural conditions. In addition, data on toxic or pathogenic effects are essential for hazard assessment purposes when terrestrial or aquatic organisms are likely to be exposed to a MPCA, especially when no fate data will be required by the Agency in the first tier of testing.

(ii) Pathogenicity and toxicity appear to be the major effects of concern regarding exposure of terrestrial and aquatic organisms to microbial pesticides. Therefore, the Agency has developed guidelines that will allow hazard assessment of pathogenicity and toxicity problems to be made. The Agency desires a high level of confidence that no unreasonable adverse environmental effects will result from actual use of MPCAs. Toward this end, the guidelines in Tier I reflect a maximum hazard approach to testing. Negative results from tests using this approach would provide a high degree of confidence that no unreasonable adverse effects are likely to occur from the actual use of MPCAs.

(iii) If unacceptable adverse effects are identified in Tier I tests, Tier II tests are performed to attempt to quantify levels of the MPCA to which the susceptible nontarget species may be exposed. Prior to registration of MPCAs, applicants would submit Tier I data on nontarget organisms. Environmental expression data (Tier II) may also be required on a case-by-case basis for certain MPCAs which are determined to present unique con-

cerns. In addition, on a case-by-case basis, definitive Tier II data showing that the MPCA will not survive or persist in the environment to which it is applied, can be submitted as support for a request for waiver (40 CFR 158.45) of some or all of Tier I testing requirements. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(iv) If the results from Tier II tests show that the MPCA persists or survives in the environment at significant levels, Tier III studies are designed to show effects of chronic exposure to these levels on fish and wildlife. If it is indicated that there may still be a problem, Tier IV studies (simulated or actual field studies) may be able to determine if there is a problem under actual use conditions.

(2) **Major issues**—(i) **Maximum hazard dosage levels.** Unlike environmental levels of chemical pesticides, which generally decrease following application, the environmental levels of MPCAs and any associated toxins may, at least temporarily, increase when the product is effective. Therefore, the maximum hazard dose for Tier I testing will be based on some safety factor times the maximum amount of active ingredient (MPCA or its toxin) expected to be available to terrestrial and aquatic plants and animals in the environment. The target hosts (e.g. insects) are likely to contain the highest concentration of the MPCA that will be available to nontarget terrestrial wildlife and aquatic animals following a pesticide application.

(A) Avian wildlife will be exposed, most commonly, through the diet (via infected insects) or through the respiratory tract (via spray drift or aerosolization). The maximum amount of MPCA a bird in the wild may consume is difficult to determine, but as much a 1×10^9 units/mL is possible. Due to anatomical constraints, the Agency recognizes that dosing at this level cannot always be achieved. Thus, the recommended daily oral or injected dose should be calculated as follows:

MDD (units) = [MPCA] in TGAI $\times$ 5 mL/kg BW $\times$ weight of bird (kg)

where

MDD = maximum daily dose expressed as units per volume

[MPCA] = concentration of MPCA

TGAI = technical grade of the active ingredient

BW = body weight

Therefore, for a product whose TGAI contains 1 x 10^9 units/mL and using a 25 g bobwhite quail, the maximum daily dose would be:

$(1 \times 10^9$ units/mL) (5 mL/kg) (0.025 kg) = 1.25×10^8 units of MPCA

(B) This dose should be administered over a 5–day period so that the total dose the bird would receive orally, over a 5–day period, would be 6.25×10^8 units.

(C) Maximum doses for the respiratory administration should be calculated in a similar manner except that the dosing volume should be reduced from 5 mL/kg to 0.2 mL/kg.

(D) Maximum hazard aquatic exposures must, in some way, account for the fact that fish and aquatic invertebrates, are less mobile than terrestrial species and less able to avoid the pesticide. In addition, under conditions of nutrient influx or the presence of alternate hosts or target pests in aquatic ecosystems, aquatic organisms may be exposed to elevated numbers of microbial pesticides. It is recommended that the maximum hazard exposure be in the range of 1×10^6 units of MPCA per milliliter of water or in a concentration 1,000× the calculated concentration after direct application to a 6–in layer of water at label rates if the microorganism produces a toxin. Aquatic exposure will simultaneous expose fish by the dietary route.

(E) The Agency realizes that it would be very difficult to establish specific LC50, ED50, or LD50 values (e.g. LD50 = 1,000 mg/kg) and 95 percent confidence limits for most MPCAs whose mechanism of action is pathogenicity, because test data are not likely to exhibit a log-probit dose-response relationship that is typical of chemical pesticides. Therefore, data that establishes an LC50, ED50, or LD50 that is greater than the maximum hazard dosage level (e.g. LD50 >1,000 mg/kg) would often be adequate for the purposes of hazard assessment. In most cases, testing at one maximum hazard dosage level is expected to be sufficient to evaluate effects for these MPCAs. MPCAs that are toxin-producing are more likely to produce a log-probit response. In most cases multiple groups would be necessary in order to quantify the hazard of these organisms. If there are no effects at the maximum hazard dose, low doses will not be necessary.

(ii) **Maximum hazard routes of administration.** (A) Various routesof administration (dosing) are provided for in these guidelines and are chosen to reflect ‘‘natural’’ exposure routes. The Agency believes that these routes—oral and respiratory for birds, aquatic and food exposure for aquatic organisms, and the oral route for insects—can best define the hazard to nontarget organisms in the wild.

(B) Parenteral dosing, such as intravenous and intraperitoneal injection, would provide a high degree of confidence that a particular microbial

pesticide would not cause adverse effects, if negative. Positive results, on the other hand, given the complex and undefined components (exogenous protein, metabolic byproducts, etc.) of microbial pesticide preparations and the environmentally unrealistic nature of the route, would be difficult to translate to effects on species in the environment. Due to the high degree of confidence an injection test gives, it is being suggested as an alternate exposure route in OPPTS 885.3050 whenever the microbial dosing preparation is sufficiently free from exogenous protein and other contaminating substances so that the test will not be confounded.

(iii) **Age of the test animals.** The Agency considers that sufficient immunological and physiological differences exist between immature animals and mature animals to suggest that immature animals are potentially more susceptible to infection and possibly to the effects of any toxin produced by the MPCA. The Agency has developed age guidelines for the test animals in Tier I tests, and recommends the use of immature animals in keeping with the hazard approach to testing.

(iv) **Methods for detecting MPCAs.** Unlike toxicity tests where mortality can usually be determined by observation, infectivity tests often require sophisticated assessment methods for detecting sublethal pathogenic effects. These methods may include serological or nucleic acid technology.

(v) **Detailed test protocols.** No standard, widely accepted, laboratory validated, test protocols are available at this time to evaluate the safety of MPCAs to terrestrial and aquatic animals. In the meantime, the draft and final protocols, as they become available, may be obtained on request from the Biopesticides and Pollution Division of OPP.

(vi) **Length of tests.** (A) The guidelines provide that the duration of all Tier I tests be about 30 days long. This should permit time for incubation, infection, and manifestation of effects in the test organisms for most MPCAs. Some test species, notable nontarget insects, may be difficult to culture and the test duration has been adjusted accordingly. Recommended test durations are included for each testing guideline.

(B) Various authors have proposed test duration times for toxicity and pathogenicity tests ranging from 14 to 35 days (see paragraphs (g)(9), (g)(11), and (g)(24) of this guideline). The Agency realizes that the test duration period may be unnecessarily long, or may not be sufficiently long enough to detect effects such as viral diseases that recur after prolonged intervals of latency, e.g. *Herpes zoster* (under paragraph (g)(5) of this guideline). At the present time, however, the Agency is not aware of an accurate method to predict whether a virus detected in a test organism will manifest latent effects. The Agency invites comments on the proposed test duration period and the probability of encountering MPCAs with latent effects.

(vii) **Control groups.** Appropriate control groups are addressed in the recommended guidelines for each test.

(c) **Terrestrial wildlife**—(1) **Approach.** These guidelines call for two tests on birds for all MPCAs: an avian acute oral toxicity and pathogenicity test (OPPTS 885.4050) and an avian respiratory pathogenicity test (OPPTS 885.4100). The avian acute oral toxicity and pathogenicity test would provide data on any toxic effects to avian wildlife from exposure to the microorganism or any toxin it may produce. This test would also provide data on pathogenic effects following an acute exposure either by the oral (or injection) route. The duration of the study would be about 30 days to allow for an incubation period prior to onset of symptoms.

(i) The avian respiratory pathogenicity test would provide data on the pathogenic effects of the MPCA on birds following exposure due to drifts or aerosolation. The guidelines for the duration of the test and gross necropsies are similar to the avian acute oral toxicity and pathogenicity test.

(ii) In both the acute dose and inhalation tests, gross necropsy, histopathological examination and culture and isolation should be performed on exposure site tissues and other organs showing anatomical or physiological abnormalities. In some cases, such as viruses, there is a preference for certain cell or tissue types. In cases where tissue preferences are known or suspected, those tissues should be examined whether or not gross anatomical or physiological changes are seen.

(2) **Tier progression**—(i) **Tier I.** (A) If no toxic or pathogenic effects are observed after exposing birds to the MPCA via two different routes of administration (oral and respiratory) at the maximum hazard dosage levels, no further testing of birds would be indicated. If toxic or pathogenic effects are observed at the maximum hazard dosage levels, Tier II, environmental expression tests (OPPTS 885.5200, 885.5300, and 885.5400), would be indicated. In some cases, a subchronic test may serve to better understand the effects served at the Tier I level and might alleviate the need for Tier II testing.

(B) Data on wild mammal toxicity and pathogenicity (OPPTS 885.4150) are required on a case-by-case basis when data indicate that there is considerable variation in the sensitivity of different mammalian species to the effects of a MPCA or where wild mammals would be heavily exposed to the MPCA under normal use. The toxicity and pathogenicity data in OPPTS 885.3050 through 885.3650 for evaluating hazard to humans and domestic animals are normally adequate to indicate hazard to wild mammals. If no toxic or pathogenic effects are observed in these tests, no further testing of wild mammals would follow. If any effects are observed in tests on wild mammals, Tier II, environmental expression testing (OPPTS 885.5200, 885.5300, and 885.5400) would be indicated.

In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(ii) **Tier II.** The data outlined in Tier II are described in the environmental expression testing guidelines (OPPTS 885.5200, 885.5300, and 885.5400). If the expression characteristics preclude exposure of the MPCA to nontarget birds and mammals, no further testing of these animals would be indicated. If Tier II tests indicate that birds and mammals will be exposed to the MPCA, testing at Tier III would follow.

(iii) **Tier III.** (A) The types of effects reported in the Tier I tests would determine which Tier III tests would apply. If adverse effects are reported in Tier I tests, and Tier II tests indicate exposure, Tier III testing would be required. If reproductive or fertility effects, or oncogenicity are reported in tests in OPPTS 885.3650 for evaluating hazards to humans and domestic animals, a long-term avian pathogenicity and reproduction test would apply. This test would provide data on pathogenic effects of the MPCA on birds during a critical period in their life—breeding and reproduction. It would also provide data on the effects of the MPCA on avian reproduction. If no pathogenic or reproductive effects are observed, the Agency would, at this time, review all the data and determine if decisions regarding registration can be made.

(B) Pathogenic effects occurring at Tier III and beyond raise serious questions concerning the registration of any MPCA. Also, testing at Tier IV, simulated and actual field testing for mammals and birds (OPPTS 885.4900) may not be feasible, since it may not be possible to confine the MPCA to a test area and prevent it from contaminating adjacent areas. In such a case, simulated field testing may be possible but would necessitate a very complex design.

(iv) **Tier IV.** Simulated and actual field testing (OPPTS 885.4900) would provide data on the pathogenic effects of the MPCA on birds and mammals following field applications at actual label use rates. This test would be indicated when pathogenic effects are reported in Tier III testing (OPPTS 885.4600) at levels equal to actual or expected field residue exposure levels, and when the Agency is reasonably confident that quarantine methods will prevent MPCA dispersal to pen, large-pen, or full-scale field tests) should be discussed with the Agency before beginning the study. Protocols for such studies must be submitted to the Agency and must receive Agency approval prior to test initiation.

(3) **Major issues.** In the process of developing the guidelines for terrestrial animals, the Agency recognized many important areas that require outside input and comment. The Agency invites scientific input and comments on the following issues of concern:

(i) **In vivo testing.** (A) The guidelines outline in vivo testing of birds and mammals. in vitro testing may be considered in the future. Wolf, under

paragraphs (g)(30) and (g)(31) of this guideline a two-pronged testing approach for safety testing of baculoviruses has been suggested, using both in vivo and tissue culture testing. There are established or permanent cell lines for duck embryo fibroblasts, chicken embryo fibroblasts, and representative mammalian cell lines from a bat, rabbit, mouse, and deer. Ignoffo (under paragraph (g)(10) of this guideline) reported that at least 12 viruses—including all major viral types—have been tested in vitro in either avian egg embryo fibroblasts (chicken or turkey), fish, or mammalian cell lines. Virus multiplication or cytopathic effects were reported for one nuclear polyhedrosis virus in chicken embryo cells and human amnion tissue, and for one noninclusion virus in chicken embryo cells and mouse sarcoma tissue. In contrast, no effects were observed in vivo when rabbits and mice were injected or fed the latter virus. More recently it has been shown (under paragraph (c)(4)(i) of this guideline) that *Autographica californica* NPV can penetrate the nucleus of three poikilothermic vertebrate cell lines, although no productive infection was demonstrated.

(B) The Agency is not convinced at this time that the results of in vitro tests can be used exclusively to determine potential adverse effects to individual terrestrial animals (e.g. endangered species) or populations of terrestrial animals in the environment.

(ii) **Test substance.** (A) Microorganisms used as pesticides could be applied in any one of a combination of naturally existing forms. It is preferable that the test organism be exposed to the most infectious form whenever infectivity is the primary hazard of concern. Similarly, when toxicity (e.g. a microbial toxin) is the hazard of concern, the test organism should be exposed to a form of the MPCA in which the toxin would be produced in the greatest amount and most readily available. Unfortunately, there is no easy way to determine which is the most infectious or toxic form of the microorganism to the test organisms. The route of administration may also play an important role in determining which form should be tested. For example, if the route of administration is intravenous, the active vegetative cells of a bacterium, or the infectious hemolymph may be more appropriate than vegetative cells or polyhedryda, respectively.

(B) For these guidelines, testing the technical grade of the active ingredient applies in all tests except the simulated and actual field testing (OPPTS 885.4900), where the use of the formulated product applies in order to simulate or reproduce actual field use. The Agency realizes that in some cases the technical grade of the active ingredient and the formulated product may be identical.

(iii) **Route of administration.** (A) These guidelines outline testing by oral gavage or by injection and via the respiratory tract. It is important to note that the administration of test material to 14– to 28–day old birds by oral gavage will likely require the use of small needles or cannulae with ball-tipped ends in order to prevent injury to the birds. It has been

reported under paragraph (g)(9) of this guideline that the following groups of terrestrial animals have been tested in vivo for effects caused by entomopathogens:

Routes of Administration in Terrestrial Animals

Group	Route
Mammals—primarily laboratory populations	Diet, oral, inhalation, subcutaneous, dermal application, intradermal, intraperitoneal, intravenous, intracerebral, intranasal, intramuscular, eye application.
Birds—chickens and laboratory populations of quail and ducks that are phenotypically similar to wild species	Oral, diet, intraperitoneal (chickens)

Since the gut normally provides such a radically different environment from that in the rest of the bird or mammal body, and since insectivorous birds and mammals can be expected to ingest large quantities of actively growing microorganisms when they feed on diseased insects, the Agency believes that the oral route would be appropriate.

(B) Inhalation, or rather intranasal or intratracheal instillation has been chosen as the second exposure route because birds may be exposed by this route during spraying operations or by the MPCA made airborne through the effects of wind or animal movement during feeding or other activities. In addition, the respiratory tract is a major portal of disease acquisition in avian species.

(C) The Agency recognizes that a combination of administrations in one test (e.g. oral and intravenous or intraperitoneal injection) may be possible. It would certainly be in keeping with the maximum hazard testing philosophy and would reduce testing time and expense. However, combined exposures could unduly traumatize the test animals so as to cause mortality, or in some other way cause spurious results.

(iv) **Avian test species.** (A) These guidelines provide that young bobwhite quail or mallard ducks be tested in Tier I tests. Birds between 14 and 28 days of age at the beginning of the test period should be used in the avian oral toxicity and pathogenicity test and in the avian inhalation pathogenicity test. Within a given test, all birds should be the same age.

(B) The Agency prefers bobwhite quail, but will accept ringneck pheasants, and mallard ducks as acceptable test species for avian acute toxicity tests of chemical pesticides.

(C) In support of testing immature birds in Tier I, the Agency notes that insects are vital to immature birds during the first 2 or 3 weeks of life and make up a much larger proportion of their diet during this time than at other times in their life. Thus, they are functionally insectivorous birds at this age. Also, for the purposes of pathogenicity testing, the Agency feels that sufficient immunological and physiological differences exist between immature birds and adult birds to warrant considering the immature bird as potentially more susceptible to infective challenge and so proposes their use in the maximum hazard testing approach.

(v) **Selection of dose levels.** For Tier I tests, the Agency suggests that a maximum hazard dosage be administered. For all testing, the maximum dose should be no less than the maximum hazard dose as defined in the testing guidelines (OPPTS 885.4000(h) and OPPTS 885.4050, 885.4100, 885.4150, 885.4200, 885.4240, 885.4280, 885.4300, 885.4340, and 885.4380). If the MPCA produces significant toxic or pathogenic effects at the maximum hazard dose level, testing at lower doses would be indicated. Sufficient doses and test organisms would be required to determine an LD50 value, if possible.

(vi) **Protocols.** Interim protocols for some ecological effects testing have been developed by EPA's Office of Research and Development. Although these protocols have not been validated, they are available on request from EPA in order to provide guidance for applicants and testing laboratories in developing protocols for testing microbial pesticides on nontarget organisms.

(d) **Aquatic animals** (1) **Approach.** (A) The Agency has considered several criteria that could be used to determine the extent of testing for effects an aquatic animals in Tier I—the site of application and resulting potential for aquatic exposure, the natural geographic distribution of the microorganism, the natural population level of the microorganism compared with population levels likely after application, and the ability of the MPCA to survive and replicate after application.

(B) While all of these criteria are important, the Agency has chosen site of application and its resulting potential for aquatic exposure as the key criteria for establishing the extent of initial effects testing far MPCAs. The rationale for selecting these criteria is that they directly address the most critical issue regarding potential hazard—likelihood of exposure. Furthermore, other criteria would be implicitly considered in connection with the criterion for site of application.

(C) The Agency recognizes that considerable judgment will be required to properly employ site of application as a criterion. While many uses obviously entail direct application to water (e.g. mosquito control and aquatic weed control), the Agency also intends that less obvious or borderline uses will be considered aquatic uses. Some examples that fall into

the latter category are applications to forests, drainage ditches, riverbanks, and partially aquatic crops such as rice. Widespread applications to major crops such as cotton, soybeans, and corn could also warrant expanded testing if these crops are grown near bodies of water. To the extent possible, the Agency will rely on its experience with the classical chemical pesticides in distinguishing between terrestrial and aquatic use patterns in borderline situations.

(2) **Tier progression** (i) **Tier I.** (A) For MPCAs applied in terrestrial use patterns (where direct aquatic exposure is not anticipated), one freshwater fish (OPPTS 885.4200) and one freshwater aquatic invertebrate (OPPTS 885.4240) should be tested to assess toxicity and pathogenicity. For MPCAs applied directly to fresh, estuarine, or marine waters, one additional fish species and one additional invertebrate species should be tested in Tier I. These tests should be conducted as 30–day static renewal bioassays using one or a combination of methods to administer the pesticide (e.g. aqueous or dietary) These tests should be designed to simultaneously assess both toxicity and pathogenicity as to detect and quantify the microbial agent in the test animal. The concentration of MPCA in the water or food must be monitored to ensure that the test organisms are exposed to a sufficient MPCA level throughout the test period.

(B) No further testing would be indicated if:

(*1*) Results of the Tier I tests indicate no toxic or pathogenic effects.

(*2*) Host range testing indicates that the MPCA has a narrow host range such that crossover into nontarget aquatic invertebrates is unlikely.

(*3*) If toxic or pathogenic effects are observed, environmental expression testing (Tier II) would generally be required. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(C) If host range testing implies crossover into nontarget aquatic invertebrates, additional aquatic invertebrate species (those expected to be susceptible or likely to be exposed) would have to be tested in Tier I, or as an alternative, Tier II testing would have to be conducted. If tests on these additional species indicate toxic or pathogenic effects, testing at Tier II would be indicated; if otherwise, no further testing would be necessary.

(ii) **Tier II.** The data for Tier II are described in environmental expression testing (OPPTS 885.5200, 885.5300, and 885.5400). If the environmental expression characteristics do not indicate exposure of the MPCA to nontarget fish or aquatic invertebrates, no further testing of these animals would be indicated. If Tier II tests indicate that fish and aquatic invertebrates will be exposed to the MPCA, testing at Tier Ill is indicated.

(iii) **Tier III.** (A) Whereas Tier I tests are designed to screen MPCAs using a maximum hazard testing scheme, Tier III tests are intended to evaluate and quantify the actual hazard associated with the MPCA more precisely. The types of effects reported in Tier I tests would help determine which Tier III tests would be required. If only toxic effects are observed in Tier I tests, OPPTS Series 850 (Ecological Effects Test Guidelines) would apply, and further testing would proceed. If pathogenic effects or both pathogenic and toxic effects are observed in Tier I, tests that could be indicated in Tier III are the following:

(*1*) Additional acute or subacute tests of fish or aquatic invertebrates to evaluate the spectrum of susceptible nontarget species, or determine the susceptible routes of exposure, or determine the dose-response relationship between the pesticidal agent and susceptible nontarget organism.

(*2*) Aquatic invertebrate range testing (OPPTS 885.4650) and fish life cycle testing (OPPTS 885.4700).

(*3*) Aquatic ecosystem disruption studies (OPPTS 885.4750).

(B) If results of Tier III tests indicate no pathogenic effects, no further testing would be indicated. Conversely, if results of Tier III tests, along with environmental fate data, indicate toxic or pathogenic effects, simulated or actual field testing may be warranted.

(3) **Major issues.** This section identifies and discusses issues regarding aquatic testing of MPCAs that may require further research and development. Most of the issues stem from there being no standard widely accepted test protocols available to evaluate the effects of MPCAs on nontarget aquatic animals. There are some potential hazards associated with the use of MPCAs that the Agency recognizes and for which practical methods of evaluation are not available. The role of in vitro testing and Tier IV testing is also discussed.

(i) **Issues associated with Tier I protocol.** Useful Tier I test protocols would simultaneously assess toxicity and pathogenicity in aquatic animals. The maximum hazard test philosophy would be exerted in terms of treatment level, method of pesticide administration, and age of the test animal.

(A) **Conduct of Tier I tests.** (*1*) A Tier I test should be conducted as a static renewal bioassay. The microorganisms should be administered as a suspension in the water (aqueous exposure), in the diet in the form of diseased host insects or treated feed, or as a combination of both routes of exposure.

(*2*) If any test animals die during the test, the cause of death (e.g. toxicity, pathogenicity) should be determined, if possible, and reisolation of the microorganism from test organism tissues should be attempted. This

information would be used to determine what further tests, if any, are warranted. Exposure and observation should extend for at least 30 days for fish and 21 days for aquatic invertebrates. Individual test animals should be removed periodically, if necessary, throughout the test period and at test termination for examination to assess pathogenicity.

(*3*) If a sublethal infection is observed in test animals prior to test termination, it may be necessary to continue the observation period in order to more adequately assess the significance of the infection (e.g. will it be lethal?). Several published studies address certain aspects of the protocol, under paragraph (g) of this guideline.

(ii) **Discussion of Tier I aquatic organism tests—(A) Test organisms.** (*1*) The guidelines provide that the species tested be selected from the list of species recommended with the exception of goldfish (warmwater species—bluegill sunfish, channel catfish, and fathead minnow; coldwater species—rainbow trout, brook trout, coho salmon). These species are desirable test organisms for several important reasons: They are used to evaluate chemical pesticides, and EPA has considerable background data on these species; standard methods for the care and handling of these species are available; and the species are widely distributed, are generally available, and have a variety of food habits and habitat requirements.

(*2*) Consideration should be given to testing species representative of the geographic region or ecosystem where the MPCA is to be applied. Species likely to prey upon or scavenge the diseased target host animals should be tested when appropriate.

(*3*) Unless there are other overriding considerations, the rainbow trout should be used as the freshwater fish test species. It is a desirable test animal because: It is partially insectivorous; no one species has been shown to be preferable in terms of sensitivity to MPCAs; there is considerable background data on this species pertaining to its microbial diseases; and standard tissue culture procedures are available for this species (under paragraph (g)(xix) and (g)(xx) of this guideline).

(*4*) Use of young fish (3 to 6 mon old) is preferable since they would be more likely to display a lethal pathogenic effect, whereas older fish may become carriers.

(*5*) Due to the broad phylogenetic spectrum from which the investigator may choose, it is difficult to select the most appropriate aquatic invertebrate. Generally, a test organism that is phylogenetically closest to the target host should be chosen. Such a test organism would be the most likely to be susceptible to infection by the MPCA. It would be appropriate to choose an aquatic insect (e.g. caddisfly) as the nontarget aquatic invertebrate test species when evaluating a MPCA whose target host is an insect.

(*6*) *Daphnia*, a *Cladoceran*, has the advantage of having considerable background data for comparative purposes. Pound (under paragraph (g)(18) of this guideline) exposed the entomopathogen *Mattesia* to *Daphnia* and observed a bioconcentration effect. This resulted from the filter feeding habits of *Daphnia* and is a desirable feature in terms of assuring that the test animal ingests the microorganism. Both *Daphnia* and certain other aquatic insects have the advantage of a short like cycle or aquatic phase, and both undergo periods of natural stress and potential susceptibility to the microorganism as a consequence of molting.

(B) **Method of MPCA administration.** (*1*) Two methods of pesticide administration should be considered:

(*i*) Suspension in the test water (aqeous exposure).

(*ii*) Dietary, in the form of diseased target host animals or incorporation of the MPCA into a standard feed.

(*2*) When possible, both routes should be used simultaneously in a single test to ensure that the most appropriate route of exposure has been tested and to ensure a maximum challenge. Different pathogens may be capable of infection by different routes of exposure so that no single route may adequately screen all microorganisms. Because each of the proposed routes has certain advantages and disadvantages, multiple routes of exposure would be extremely beneficial and cost effective in screening MPCAs.

(*3*) Addition of the microorganism directly to the test water is a routine procedure. It simulates the type of natural exposure that could occur immediately after application of a MPCA. It also simulates the routes of exposure by which many known pathogenic agents infect fish and aquatic invertebrates. However, care must be taken to assure that a high concentration of microorganisms be maintained in the test system and that this high concentration does not lower water quality to an unacceptable level. Therefore, the static revewal method is recommended. Use of this method will ensure that high MPCA concentrations and acceptable water quality can be maintained.

(*4*) Dietary exposure also simulates certain natural conditions. It is perhaps the most important means of infection for the normal hosts of entomopathogenic agents (under paragraph (d)(4)(xv) of this guideline), and its use in evaluating effects an nontarget fish and aquatic invertebrates is logical. This route offers a further advantage: It increases the possibility of exposing the test animals to a different life stage of the microorganism than may be present in the formulated product if diseased target hosts (e.g. insects) are used as the feed.

(*5*) Finally, oral intubation of fish is another possible route of exposure, and is one that has been used to evaluate microorganism effects in fish. This route has the advantage of assuring that a known amount of

test material is ingested. This advantage does not outweigh the risk of injury or undue stress that could result from using this method, so that the oral intubation method, though acceptable, is not recommended.

(C) **Test substance.** The substance to be tested will depend in part on the method of pesticide administration used in the study. It is essential to test the most challenging form of the microorganism (in terms of pathogenicity or toxicity). It is equally important to test the form of the microorganism to which nontarget aquatic animals are most likely to be exposed. These objectives should be achievable through the use of multipleof administration, provided it is known which form is most challenging and which form is most likely to be encountered by the nontarget animal. The technical grade of the active ingredient should be used for all exposures. The formulated product should be tested if it is to be applied directly to water.

(D) **Selection of treatment concentrations.** (*1*) Treatment concentrations must be related to the number of microorganisms to which aquatic animals nay be exposed under actual use conditions. In keeping with the maximum hazard philosophy, treatment concentrations must be relatively high. Consideration must be given to the level of exposure resulting from direct application as well as exposure resulting from consumption of diseased target host organisms (usually insects). Exposure in terms of frequency and number of microorganisms could be extremely high in the latter case.

(*2*) The highest feasible concentrations should be used in all exposures. At a minimum, the concentration for aqueous exposure $1x10^6$ units/mL H_2O or 100 $\times$ the theoretical concentration present in 6 in of water immediately after a direct application of the MPCA, at label rates, to 6 in of water, whichever is greater and attainable. Because the use of such a high concentration may be limited by its adverse effect on water quality such as oxygen depletion and production of metabolic wastes by the microorganisms, treated water in the test vessels should be renewed frequently enough to maintain water quality and microorganism concentration.

(E) **Test duration.** (*1*) Exposure and observation must be extended to at least 30 days (unless test animals die) to allow time for any potential infection, microorganism replication, or pathogenic or toxic effects to manifest themselves. If a sublethal infection is observed, the test should be extended to evaluate the significance of the infection. Similarly, if test animals begin to die near the end of the 30–day period, the test should be continued to determine the fate of the remaining test population.

(*2*) The 30–day test duration was selected on the basis of past research under paragraph (g)(xvii) of this guideline and the recommendation of Summers et al. (under paragraph (g)(23) of this guideline). Certain factors may dictate that this period be modified. For example, if infection and

death of target hosts is normally not evident for many days (i.e. 20 to 30), it would be logical to lengthen the period of exposure for the test animals. Conversely, a shorter period of exposure may be warranted in tests using animals with short life cycles (i.e. *Daphnia* or mysid shrimp).

(F) **Observation and examination of test animals.** (*1*) Daily observations are required to record mortalities and note any behavioral, pathogenic, or toxic effects. Test organisms must be examined for infection or any microorganism-related effects periodically throughout the study and at test termination. The most difficult aspect of this requirement is the verification of the presence or absence of an infection. The general methods of assessment that may be required to make this determination include histopathology, serology, and nucleic acid hybridization and reisolation and identification of the microorganism from organ tissue. These methods, and the situations in which their use may be appropriate, were presented in paragraph (c)(4) of this guideline under the general discussion of nontarget organism hazard testing.

(*2*) Undeen and Maddox (under paragraph (g)(26) of this guideline) used the following criteria in their work with *Nosema algerae* to distinguish between a true infection and microorganisms observed in the test animal. In a true infection:

(*i*) Both vegetative forms and spores had to be present in the test animal.

(*ii*) The number of spores recovered had to exceed the number injected by 100×. This type of approach may be useful for other microorganisms.

(iii) **Issues associated with Tier III test protocols.** (A) The aquatic invertebrate host embryo larvae, fish life cycle, and aquatic ecosystem tests in Tier III (OPPTS 885.4650, 885.4700, and 885.4750) are similar to the protocols that are referenced for these types of tests in OPPTS Series 850 (Ecological Effects Test Guidelines). Generally accepted standard protocols for conducting these studies with MPCAs have not been developed. In fact, few, if any, such tests have ever been conducted with MPCAs, and the Agency recognizes at the outset that new and different test designs and test parameters may be more appropriate than modified OPPTS Series 850 tests. Research and methods development are in progress and need to be completed in this area before the Agency can publish specific recommendations concerning protocols and Tier progression.

(iv) **Issues associated with in vitro testing.** (A) The Agency recognizes that there are in vitro tests available to assess the infectivity of certain microorganisms, one of which is tissue culture for viruses. Cell lines are established for several species of fish. and such a test might be a useful means of assessing infectivity in certain situations. However, the relationship between effects demonstrated by in vitro tests and effects likely to

occur under in vivo situations is uncertain. For example, Ignoffo, under paragraph (g)(9) of this guideline, states that ‘‘Tissue, completely non-susceptible in the intact organism, may support viral multiplication when explanted into a culture media.’’ Therefore, the results obtained from tissue culture tests could be useless in accurately predicting environmental hazard. Another potential drawback of tissue culture studies is that, often, no host cell culture (e.g. insect cell culture) has been developed. Such a study would have no positive control group and the validity of a negative result would always be subject to some doubt.

(B) The Agency has concluded that, at the present time, in vitro studies such as tissue culture cannot be substituted for the in vivo studies provided in Tier I. At the same time, the Agency recognizes the potential value of these studies for the following purposes:

(*1*) As a relatively inexpensive and rapid means to screen for potential infectivity in a broad spectrum of species.

(*2*) As a test to support or check the results of in vivo tests. A provision for cell culture studies is included in Tier III of the testing scheme.

(v) **Issues associated with Tier IV testing.** (A) The Agency recognizes the possible shortcomings in using simulated or actual field tests (Tier IV) as the final test of the safety of an MPCA. If an agent has progressed through the Tier system and requires a field test, it must have displayed significant adverse effects in some or all of the previously conducted laboratory tests. This fact might argue against the use of a field test, since such a test could release potentially hazardous microorganisms, with the potential to proliferate in the environment and pose widespread environmental risk, unless adequate quarantine measures could be taken. Before any Tier IV field test is to be undertaken, the applicant should discuss its plans with the Agency concerning potential hazards. If the Agency determines that a Tier IV field test would pose an unacceptable risk, the MPCA would not likely be acceptable for registration.

(B) The Agency recognizes the potential value of Tier IV simulated or actual field tests as a further check on the safety of MPCAs that demonstrate a hazard in Tier I tests, or that demonstrate a hazard that could be adequately controlled by quarantine methods in the field. These tests could be conducted concurrently with full-scale efficacy testing, and the Agency would strongly encourage such tests. This would provide the opportunity to evaluate pesticidal effects (both direct and indirect) on a much broader spectrum of nontarget species, under more natural exposure conditions than is possible in Tier I testing.

(vi) **Assessment of other potential hazards: opportunistic infections and latent viruses.** (A) Opportunistic infections in nontarget aquatic animals are recognized by the Agency to be a potential hazard. A similar concern is noted for latent viruses. Research indicates that aquatic animals

may be rendered significantly more susceptible to microbial infection, (e.g. by viruses and bacteria) when stressed by such factors as Aroclor 1254, copper, temperature, salinity, pesticides, and other pollutants. This increased susceptibility raises several important questions:

(*1*) What is the likelihood of an opportunistic infection (from an MPCA) occurring in a nontarget aquatic animal?

(*2*) What is the significance of the effect of opportunistic infections on individuals and populations?

(*3*) Will the proposed Tier I test adequately screen MPCAs for potential opportunistic effects? Or could a MPCA be noninfective in a Tier I test, but infect stressed nontarget animals?

(*4*) Will a latent virus be detected by a Tier I test and, if so, how can its significance be assessed?

(B) There is far too little background information and research on MPCAs to suggest an answer to the first question. However, the Agency believes that the potential for this type of problem should not be ignored. The Agency is confident that sublethal infections produced in Tier I tests can be detected if the proper methods of detection are employed. However, the potential for an apparently noninfective agent (in Tier I testing) to infect stressed animals is unknown. At present, the Agency is not aware of any practical, generally accepted, routine screening test that could be used in Tier I to determine the potential for such an occurrence. If a sublethal infection is observed in Tier I, further testing may be warranted. A microorganism/stress interaction test is proposed in Tier III as a means of assessing sublethal infections, but further research is needed to develop the protocol for such a test. With regard to latent viral infections, the Agency is not aware of a standard method to evaluate the potential for a latent virus to reactivate and cause adverse effects in aquatic animals. Further research is required.

(vii) **Oncogenic effects.** The Agency recognizes the potential for oncogenic effects that are associated with viruses and mycotoxins. The probability of oncogenicity in nontarget aquatic animals, as a result of exposure to a viral pesticide, is unknown. At this time, the Agency is unaware of any standard method that could be used to screen for such an effect. Further research is required to develop an appropriate test and determine when its use is justified.

(e) **Nontarget plant testing**—(1) **Approach.** The plant testing scheme proposed herein is based on the Tier testing scheme for testing other nontarget organisms. Tier I screening tests incorporate maximum hazard single dosing using a route of exposure most likely to show any potential plant toxicity or pathogenicity. The duration of the test should be sufficient to allow for manifestation of a delayed pathogenic response.

Tier II testing examines population dynamics to quantity persistence and survival of the MPCA in the environment. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing. Tier III testing is designed to record a dose response and determine if there is a minimum infective dose for any adverse effects identified in Tier I tests. Tier IV testing, if still needed for risk assessment, will include both exposure and hazard testing under simulated or actual field conditions.

(2) **Major issues.** (i) Diseases of commercially important plants have been intensively studied for decades and many plant pathogens have been identified and subsequently well characterized. Some plant pathogens have a very narrow host range and may attack only one species of plant, other plant pathogens may attack a wide range of plant species, and still other microorganisms have never been identified in association with disease in plants.

(ii) A thorough taxonomic description of the MPCA should allow determination of its similarity to known plant pathogens. MPCAs that are similar to plant pathogens with very narrow host ranges may only need to be tested for adverse effects against plants similar to known hosts. MPCAs that are similar to wide range plant pathogens may need additional testing to identity the complete host range. A knowledge of the mode of action may assist in determining the extent of testing needed for potential plant pathogens. Finally, MPCAs that do not resemble any known plant pathogen may require little, if any, plant testing. Microbial herbicides are designed to be toxic or pathogenic to their target plants. This class of MPCAs will require close scrutiny to ensure that nontarget plants are not unreasonably affected, whereas microbial insecticides generally would not be expected to have phytopathogenic properties, and would not require as much testing,

(iii) A second factor in determining the extent of plant testing is the anticipated exposure of plants to the MPCA as determined by the use pattern, dissemination, and the persistence/survival in the environment. For example, MPCAs that will not be disseminated to, or do not survive in, aquatic environments will not need testing in aquatic plants. A related factor is whether the MPCA is to be used within its area of natural occurrence. Where an MPCA is proposed for use in an area where it does not naturally occur, additional plant testing may be warranted.

(iv) Another factor in selecting species of plants to be tested is that of susceptibility to plant diseases. Genetically diverse groups of plants are generally less susceptible as a species to any given plant pathogen since there is a greater chance that a variety of the species will be resistant to the disease. The most important group of genetically identical (monoculture) plants are the commercial agricultural crops. These plants

should be given priority in testing for plant pathogenicity because of both their potential susceptibility and their commercial importance.

(f) **Nontarget insects**—(1) **Terrestrial insects**—(i) **Approach.** Assessment of potential nontarget insect hazard from uses of MPCAS is made difficult by a number of factors:

(A) Most MPCAs will be specifically selected and/or designed for their ability to control pest insects. Nontarget insects are the organism group most at risk, being relatively closely related to the target organism in most cases.

(B) While there are few nontarget insects that have been shown to be economically important to humans, there are many nontarget insects which have an important rule in ecological processes and may benefit humans indirectly.

(C) Unlike chemical pesticides, many microbials will exert their effect through pathogenicity as well as toxicity. The acute, short duration, Tier I tests, which should suffice for hazard evaluation for some chemical pesticides, will not be appropriate for microbial agents. Adequate assessment of pathogenicity will demand time to evaluate the MPCA for infectivity and for its ability to reproduce or develop in the test insect.

(D) The host range is an important factor in hazard evaluation for a MPCA. A problem here is that extrapolation, even across species lines, is often not dependable. For this reason, the Agency will provide for testing with representatives from a number of ‘‘beneficial insect’’ taxa. Information from these tests will be used in conjunction with host range data (developed during efficacy testing) to develop a clearer idea of the overall insect host range.

(E) The Agency is aware that Tier I testing may be more extensive in some cases than the baseline data requirements in OPPTS Series 850. However, there should be very few microbials which require effects testing beyond the Tier I level.

(F) The tier-testing scheme for MPCAs is based on a fairly extensive first tier. The purpose of the Tier I testing is to assess toxicity and pathogenicity of the MPCA to the honey bee and to three species of predaceous and parasitic insects. Selection of the predator/parasite species to be tested should take into account such factors as the likelihood of exposure to the MPCA, phylogenetic proximity of the test species to target pest species, and similar relationships. A rationale for selection should be developed by the registrant.

(ii) **Tier progression**—(A) **Tier I.** Under these guidelines, toxicity/pathogenicity tests on the honey bee and insect predators and/or parasites are indicated for all MPCAs except Bts. Selection of predator and parasite

species for testing is made by the registration applicant. Rationale for selection is to be provided by the registrant. The main purpose of the Tier I testing is to determine presence of toxic or pathogenic effects an representatives of a few major orders of beneficial insects. As noted above, the representative test species selected, in addition to the honey bee, should be of some importance in the ecosystem to be exposed to the microbial control agent. Data derived from Tier I testing will be used in conjunction with available information on use pattern, host range (specificity), fate, and other similar factors, to assess potential for adverse effects. If data indicate no potential for affects, no further testing would be indicated. If the results of Tier I testing indicate toxic and/or pathogenic effects, Tier II testing (environmental expression) would follow. In some cases, a subchronic test may lead to a better understanding of the effects observed at the Tier I level and might alleviate the need for Tier II testing. The Agency should be consulted before making these decisions.

(B) **Tier** II. The data for Tier II are described in environmental expression testing (OPPTS 885, Group E) of these guidelines. If expression characteristics preclude exposure, no further testing would be indicated. If data indicate that nontarget insects will be exposed to the MPCA, the registration applicant should consult with the Agency regarding possible Tier III testing.

(C) **Tier III.** For all MPCAs, Tier III consists of advanced tests specifically responding to adverse effects identified in earlier Tier testing. Such tests may be simulated or actual field tests, but further research is needed to develop the protocols for such testing. In any case, Tier III testing would be preceded by consultation with the Agency.

(2) **Aquatic insects.** Tier I testing, as outlined in the ''Aquatic Animal Tier Testing Scheme for Microbial Pest Control Agents'' (under paragraph (d) of this guideline) will include toxicity/pathogenicity testing with Daphnia, or a species of aquatic insect, or both, depending on use pattern. Detection of pathogenicity/toxicity in Tier I testing will automatically lead to expanded testing which, if the impacted site is fresh water, will most likely involve testing with aquatic insects.

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