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Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms

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Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms

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Abstract

The intent of this document is to provide guidance on preparing for and conducting single-species tests to measure and evaluate the pathogenicity and/or toxicity of new microbial substances to aquatic and terrestrial organisms. It focuses on the information requirements with respect to laboratory tests for measuring the potential ecological effects of new microbial substances on the following six categories of test (host) organisms, that might be required by Environment Canada as part of the New Substances Notification (NSN) Regulations under the Canadian Environmental Protection Act*, 1999: (1) an aquatic plant; (2) an aquatic invertebrate; (3) an aquatic vertebrate; (4) a terrestrial plant; (5) a terrestrial invertebrate; and (6) a terrestrial vertebrate. The guidance herein is intended for notifiers, environmental consultants, study directors, and principal investigators. It will assist in the selection of an appropriate series of biological test methods for measuring the pathogenicity and/or toxicity of new microbial substances, as well as in the planning, execution, and reporting phases associated with each test.*

Background information is provided on the NSN requirements for testing new microbial substances for ecological effects, together with a description of the purpose and scope of the document. The overview (Section 2) addresses pertinent issues including the use of a series of appropriate test methods, considerations when measuring infectivity as well as pathogenic and/or toxic effects, the need for appropriate controls, and the worth of related findings that demonstrate the environmental expression of the new microbial substance under varying laboratory conditions. Other sections address the following topics: characterizing, preparing, and administering new microbial substances; control treatments in tests (including negative and positive controls); testing for infectivity; applicable (OECD) Principles of Good Laboratory Practice; laboratory biosafety; appropriate animal care and use; considerations when choosing the series of biological test methods to be applied to a particular new microbial substance; and guidance on reporting requirements.

Besides the foregoing, six sections of this guidance document provide guidance when performing a test for pathogenic and/or toxic effects of new microbial substances using each of the six categories of host (test) organisms. Each of these sections includes a description of previous tests performed with micro-organisms or microbial products using this category of test organisms, recommended biological test methods (including procedural specifics when testing a new microbial substance), and a consideration of alternate methods or procedures other than those recommended herein.

The Series 885 test guidelines for testing the pathogenicity and/or toxicity of microbial pest control agents published by the United States Environmental Protection Agency in 1996 influenced the selection of appropriate category-specific test methods. The availability (and adaptability) of certain biological test methods published by Environment Canada also influenced this selection process, as did the existence of specific test methods or standard guidelines published by international agencies (OECD, ASTM, ISO, USEPA). Recommended test methods are those which, with appropriate modifications as defined herein, are amenable to measuring the pathogenic and/or toxic effects of new microbial substances on selected species of organisms within each of these six categories of test (host) organisms.

Résumé

Le présent document renferme des conseils sur les étapes préparatoires et la conduite d'essais monospécifiques servant à mesurer et à évaluer la pathogénicité et/ou la toxicité de nouvelles substances microbiennes pour les organismes aquatiques et terrestres. Il est axé sur les exigences en matière d'information applicables aux essais de laboratoire visant à mesurer les effets écologiques possibles de nouvelles substances microbiennes sur chacune des six catégories suivantes d'organismes cobayes (organismes hôtes), information susceptible d'être demandée par Environnement Canada aux termes du Règlement sur les renseignements concernant les substances nouvelles *(RSN), pris en application de la* Loi canadienne sur la protection de l'environnement *de 1999 : 1) une plante aquatique; 2) un invertébré aquatique; 3) un vertébré aquatique; 4) une plante terrestre; 5) un invertébré terrestre; 6) un vertébré terrestre. Les conseils fournis ici s'adressent aux personnes qui effectuent les déclarations, aux consultants en environnement, aux directeurs d'études et aux experts principaux. Ils faciliteront la sélection d'une série adéquate de méthodes d'essais biologiques, de même que le déroulement des phases de planification, d'exécution et de rapport associées à chaque essai.*

Des renseignements de base sont fournis sur les dispositions du Règlement sur les RSN en matière d'essais visant à déterminer les effets écologiques de nouvelles substances microbiennes, de même qu'une description de l'objet et de la portée du présent document. La section 2 (Aperçu) traite de questions pertinentes, notamment l'utilisation d'une série de méthodes d'essai adéquates, les éléments à prendre en compte dans la mesure de l'infectivité et des effets pathogènes et/ou toxiques des substances en cause, la nécessité d'effectuer des contrôles adéquats, la valeur des conclusions connexes sur l'expression, dans le milieu naturel, de nouvelles substances microbiennes en fonction de diverses conditions de laboratoire. D'autres sections portent sur les sujets suivants : caractérisation, préparation et administration de nouvelles substances microbiennes; traitements de contrôle (dont des contrôles négatifs et positifs); essais d'infectivité; Principes de l'OCDE relatifs aux bonnes pratiques de laboratoire *applicables; biosécurité en laboratoire; soins des animaux et utilisation adéquate de ceux-ci; éléments à prendre en compte lors du choix d'une série de méthodes d'essais biologiques à appliquer à une nouvelle substance microbienne particulière; conseils sur la production de rapports.*

Outre les sections susmentionnées, six autres sections renferment des conseils relatifs à la conduite des essais portant sur les effets pathogènes et/ou toxiques de nouvelles substances microbiennes sur chacune des six catégories d'organismes cobayes. Chaque section renferme une description d'essais déjà effectués à l'aide de microorganismes ou de produits microbiens pour la catégorie en question; les méthodes d'essai biologique recommandées (y compris des spécifications procédurales lors d'essais portant sur une nouvelle substance microbienne); un examen des méthodes ou procédures de rechange à celles recommandées dans le présent document.

Les lignes directrices intitulées Series 885 *pour les essais de pathogénicité et/ou de toxicité d'agents pesticides microbiens, publiées par la United States Environmental Protection Agency (USEPA) en 1996, ont influé sur le choix des méthodes d'essai convenant à des catégories d'organismes données. La disponibilité (et l'adaptabilité) de certaines méthodes d'essais biologiques publiées par Environnement Canada a aussi été prise en compte dans le processus de sélection, tout comme l'existence de méthodes d'essai spécifiques ou de lignes directrices normalisées publiées par des organismes internationaux [Organisation de coopération et de développement économiques, American Society for Testing and Materials, Organisation internationale de normalisation (ISO), USEPA]. Les méthodes d'essai recommandées sont celles qui, avec les modifications pertinentes définies dans le présent document, conviennent aux mesures des effets pathogènes et/ou toxiques de nouvelles substances microbiennes sur des espèces choisies dans chacune des six catégories d'organismes cobayes.*

Foreword

*This is one of a series of supporting **guidance documents** published by Environment Canada (EC), that relate to recommended or standardized biological test methods for measuring and assessing the adverse toxic and/or pathogenic effects on single species of aquatic or terrestrial organisms, caused by their exposure to samples of test substances (in this instance, new microbial substances) under controlled and defined laboratory conditions. Recommended methods are those that have been evaluated by Environment Canada (EC), and are favoured:*

- *for use in EC environmental toxicity laboratories;*
- *for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *as a foundation for the provision of very explicit instructions as might be required in a regulatory protocol or standard reference method.*

The different types of tests included in this series were selected because of their acceptability for the needs of programs for environmental protection and management carried out by Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on the toxicity and/or pathogenicity to aquatic or terrestrial life of specific new microbial substances destined for release to the environment.

This guidance document includes 14 tables summarizing the procedures and conditions to be followed when undertaking biological test methods recommended herein for measuring the pathogenic and/or toxic effects of new microbial substances, along with related descriptions in the associated text. Certain of these biological test methods (i.e., EC, 2004a,b,c) have yet to be published at the time of this writing, and others might be modified and revised in future years. In instances where a specific test procedure or condition referred to in these summary tables and the associated text differs from that defined in one of these publications, the corresponding procedure or condition given in the most current biological test method published by the authoritative agency (i.e., EC, USEPA, or OECD) applies and should be substituted for that indicated herein.

Schedule XV of the New Substances Notification (NSN) Regulations under CEPA 1999 specifies the information that must be provided by a notifier wishing to import or manufacture within Canada a new microbial substance (micro-organism) for release to the environment. Included in that Schedule is a listing of data required from laboratory tests to determine the potential ecological effects of a new microbial substance on aquatic or terrestrial plant, invertebrate, and vertebrate species. In keeping with the NSN Regulations, EC and Health Canada (HC) jointly published a document entitled "Guidelines for the Notification and Testing of New Substances: Organisms*" (EC and HC, 2001). These Guidelines include (Section 4.2.7.1) a brief description of prospective biological test methods and study components when deriving data from tests conducted to determine the pathogenic and/or toxic effects of a new microbial substance on plant, invertebrate, or vertebrate species. The present guidance document expands on this, and is intended to supplement EC and HC (2001) in these respects. Proponents, including notifiers and their delegates (e.g., the Study Director and Principal Investigator(s); see Section 6.1) are advised to meet with representatives of EC and HC as part of a Pre-Notification Consultation (see Section 1.1.6), when designing and planning the series of biological test methods to be performed as part of the notification process in keeping with the NSN Regulations and the Notification Guidelines (EC and HC, 2001).*

*Appendix A lists the supporting **guidance documents**, the generic (universal) multi-purpose **biological test methods**, and the standardized **reference methods** published to date by Environment Canada's Method Development and Applications Section in Ottawa, ON. These documents are available from Environmental Protection Publications, Environmental Protection Service, Environment Canada, Ottawa, ON, K1A 0H3, Canada. Contact information for the Regional and Headquarters Offices of Environment Canada's Environmental Protection Service, which shares and applies the guidance herein, is provided in Appendix B.*

Words defined in the Terminology section of this document are italicized when first used in the body of the report according to the definition. Italics are also used as emphasis for these and other words, throughout the report.

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List of Abbreviations

AOACI	Association of Official Analytical Chemists International
APHA	American Public Health Association
ASTM	American Society for Testing and Materials
AWWA	American Water Works Association
CCAC	Canadian Council on Animal Care
CBER	Center for Biologics Evaluation and Research (USFDA)
CDER	Center for Drug Evaluation and Research (USFDA)
CEPA	Canadian Environmental Protection Act
CFIA	Canadian Food Inspection Agency
CFU	colony forming units
cm	centimetre(s)
DO	dissolved oxygen (concentration)
DSL	Domestic Substances List
E	embryo (test)
EA	embryo/alevin (test)
EAF	embryo/alevin/fry (test)
EC	Environment Canada
EC50	median effective concentration
ED50	median effective dose
EP	end-use (microbial) product
Fri	Friday
g	gram(s)
GLP	good laboratory practice
h	hour(s)
HC	Health Canada
HEPA	high efficiency particulate arrestance
ICp	inhibiting concentration for a (specified) percent effect
IC25	inhibiting concentration for a 25% effect
IDp	inhibiting dose for a (specified) percent effect
ID25	inhibiting dose for a 25% effect
ISO	International Organization for Standardization
kg	kilogram(s)
L	litre(s)
LC50	median lethal concentration
LD50	median lethal dose
LOEC	lowest-observed-effect concentration
LOED	lowest-observed-effect dose
m	metre
MDAS	Method Development and Applications Section (Environment Canada)
mg	milligram(s)
MHC	maximum hazard concentration
MHD	maximum hazard dose
mL	millilitre(s)
mm	millimetre(s)
Mon	Monday
MPCA	microbial pest control agent
NOEC	no-observed-effect concentration
NOED	no-observed-effect dose
NSB	New Substances Branch

NSN	New Substances Notification
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides, and Toxic Substances (USEPA)
pH	hydrogen ion concentration
PMRA	Pest Management Regulatory Agency (Health Canada)
PNC	Pre-Notification Consultation
QA	quality assurance
rpm	revolutions per minute
s	second
SD	standard deviation
SNAc	Significant New Activity
SOP	standard operating procedure
sp.	species (singular)
spp.	species (plural)
TM (™)	Trade Mark
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
UV	ultraviolet (light)
WBC	white blood cell
Wed	Wednesday
WEF	Water Environment Federation
wt	weight
YCT	yeast, Cerophyll™, and trout chow
µL	microlitre(s)
µmol	micromole(s)
°C	degree(s) Celsius
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
%	percentage or percent
‰	parts per thousand (salinity)
=	equals
+	plus
−	minus
±	plus or minus
×	times
÷	divided by
/	per; alternatively, “or” (e.g., holding/acclimation)
~	approximately

Terminology

Note: all definitions are given in the context of this report, and might not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

Might is used to express the possibility that something could exist or happen.

Technical Terms

Acute means occurring within a short period of exposure (e.g., seconds, minutes, hours, or a few days) in relation to the life span of the test organism.

Acute toxicity is a discernible adverse effect (lethal or sublethal) induced in the test organisms within a short period (minutes, hours, or a few days) of exposure to a *test substance.* [See also "*test substance*".]

Acclimation is the physiological adjustment to a particular level of one or more environmental factors such as temperature. The term usually refers to the adjustment to controlled laboratory conditions over a specified period of time.

Aerosolization refers to the mixing and dispersal of a test substance in the air as a fine spray of colloidal-sized particles.

Alevin is a recently hatched, non-feeding salmonid fish with an evident yolk sac for nutritive requirements. This life stage of trout or salmon is often referred to as a yolk-sac fry.

Anaesthetic is a chemical agent (i.e., a drug) that produces partial or complete insensitivity to touch or pain. A general anaesthetic affects the whole body, usually with loss of consciousness; whereas a local anaesthetic causes a regionalized insensitivity to pain.

Analgesic is a drug which, when taken orally, relieves systemic or localized pain.

Antimicrobial refers to any or all types of natural, semi-synthetic, or synthetic substances capable of killing or inhibiting the growth of micro-organisms. Antimicrobial agents include antibiotics, antivirals, antifungals, disinfectants, sanitizers, food or feed preservatives, antimicrobial pesticides or biocides, and wood preservatives, among others. [See also "*micro-organism*".]

Arthropods are aquatic or terrestrial animals within the phylum Arthropoda, which includes insects, spiders, crabs, centipedes, etc. These animals have a hard, jointed exoskeleton, a segmented body, and paired, jointed legs.

Batch means the total amount of a particular *test material* (or specific concentration thereof) or *test substrate* prepared for each treatment (concentration) in a test. A *batch* is any substance(s) or concentration ready for separation into replicates. [See also "*test material*".]

Benthic refers to those animals or plants living on the surface of ("i.e., *epibenthic*" organisms) or within (i.e., "*infaunal*" organisms) the sediment at the bottom of a waterbody. [See also "*epibenthic*" and "*infaunal*".]

Biological test method is a standardized practice or protocol for conducting a test under controlled laboratory conditions, to measure and evaluate the adverse effect(s) of a *test substance* (including a *new microbial substance*) on living plants or animals of a particular species and life stage. To be classified as a biological test method, the standardized practice or protocol must have been published by a regulatory agency (e.g., EC or USEPA) or other authority responsible for testing guidelines (e.g., ASTM). Depending on its design and intent, a biological test method may be a multi-purpose (generic, universally applied) test method intended for research or other purposes such as routine environmental monitoring, or it may be a standardized *reference method* (i.e., a rigid testing protocol) intended for regulatory applications. [See also "*new (microbial) substance*", "*reference method*", and "*test substance*".]

Biomarker means a specific biochemical, genetic, *immunologic*, or physiological measurement that is used to indicate the potential for an adverse effect of a *new microbial substance* on the health and condition (e.g., survival, growth, development, infection) of test (host) organisms. [See also "immunologic" and "*new (microbial) substance*".]

Bioremediation refers to the use of selected *remediation*-adapted micro-organisms to destroy, reduce, or remove *toxic substances* at a contaminated site. [See also "*microbial product*", "*remediation*", "*substances*", and "*toxic*".]

Blend (of micro-organisms) means a mixture containing two or more pure cultures of micro-organisms. As per the New Substances Notification Regulations of CEPA 1999, a mixture of pure cultures of micro-organisms (i.e., a blend) is not a single *substance*; rather, it is a mixture of two or more substances each of which is notified separately. For testing purposes, a *blend* may be used as the *test material*. However, *notifiers* should also consider the known characteristics of each pure culture in a blend, and in instances where there is reason to suspect masking of ecological effects, the *notifier* might be required to test some pure cultures separately (EC and HC, 2001). [See also*"consortium*", *"new (microbial) substance*", *"notifier*", *"substance*", and "*test material"*.]

Brood sac is an epithelial pouch connected to the oviducts of a female mysid in which embryos develop. [See also "*oviduct*".]

Carcinogenic means producing cancer. [See also "*carcinogenicity*".]

Carcinogenicity refers to the ability or tendency to produce cancer. [See also "*carcinogenic*".]

Chlorosis is the condition of the green parts of plants in which chlorophyll concentration is depressed and the leaves are pale green or yellow in colour. This may result from disease, toxic substances, nutrient deficiencies, genetic mutation, or senescence.

Chronic means occurring during a relatively long period of exposure, usually a significant portion of the life span of the organism such as 10% or more.

Chronic toxicity refers to discernible adverse long-term effects that are related to changes in biological endpoints such as reproduction, growth, metabolism, or ability to survive, which are measured for groups of test

organisms exposed to a *toxic substance*. The duration of the exposure can be *acute* or *chronic*; whereas the biological endpoints are measured during and/or at the end of a long-term (chronic) test. [See also "*acute*", "*chronic*", "*substance*", and "*toxic*".]

Cladoceran refers to a water flea (i.e., a species of daphnid)

Clean (water, sediment, or soil) means uncontaminated substrate to be used as *test substrate* which by itself does not contain any concentration of any substance(s) known to cause discernible adverse effects on test organisms during a controlled test for pathogenic and/or toxic effects. [See also "(*test) substrate*".]

Clearance of micro-organisms refers to the removal of microbes from an internal site in an animal's body, by means of cellular or extracellular mechanisms. For both primitive and advanced immune systems found in invertebrates and vertebrates, this is a term used to describe a complex (space and time) process beginning with macrophages or monocyte- (mammalian) like cellular interactions associated with adherence, engulfment, and killing or degradation of the invading microbes. This process leads to the inactivation and disposal (translocation) of "foreign" micro-organisms introduced by one or more exposure routes.

Commissioning refers to processes normally undertaken to verify that the design of a laboratory facility meets applicable codes and standards, and that it has been constructed in accordance with the design intent.

Concentration is the quantity of a *substance* contained in a unit quantity of a given medium. In the context of this guidance document, the substance is typically a *new microbial substance*, and the medium is a *test substrate* (e.g., *test water*, *test sediment*, *test soil*, or test food). [See also "*dose*", "*microbial*", "*new (microbial) substance*", "*substance*", "*test sediment*", "*test soil*", "*(test) substrate*", and "*test water*".]

Consortium (of micro-organisms) means a complex, unformulated mixture of micro-organisms isolated from the environment. A consortium of micro-organisms is considered to be a single *substance*, and is included in the definition of a micro-organism. [See also "*blend*", "*micro-organism*", "*new (microbial) substance*", "*substance*", "*test material*", and "*test substrate*".]

Contaminant is a substance that is present in a natural system, or present at increased concentration, because of some direct or indirect human activity. The term is frequently applied to substances that are present at concentrations having the potential to cause adverse biological effects.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect results, except the specific condition being studied. In tests for pathogenic and/or toxic effects, the control must duplicate all the conditions of the exposure treatment(s), but must contain no *test substance*. The *control* is used as a check for the absence of pathogenicity and/or toxicity due to basic test conditions such as temperature, health of test organisms, or effects due to their handling. *Control* is synonymous with *negative control*, unless indicated otherwise. [See also"*negative control*", "*non-infectious control*", "*positive chemical control*", "*positive control*", "*positive microbial control*", "*sterile filtrate control*", and "*test substance*".]

Control/dilution water is the *test water* used for preparing the *controls* as well as the concentration(s) of the *new microbial substance* to be included in a particular test. [See also "*control*", "*new (microbial) substance*", and "*test water*".]

Cotyledon is a primary leaf of the developing plant embryo; only one in monocotyledonous plants, and two in dicotyledonous plants. In many dicotyledonous species, such as the bean, they emerge above ground and appear as the first leaves. [See also "*monocotyledon*" and "*dicotyledon*".]

Cystocarp is a bulbous, structural growth on red macroalgae (e.g., *Champia parvula*) which occurs following sexual reproduction. This structural growth, which is readily identifiable, produces spores.

Dicotyledon in the classification of plants, refers to those species having two seed leaves. [See also "*cotyledon*" and "*monocotyledon*".]

Disease is a definitive morbid process having a characteristic train of symptoms. It might affect the whole body of the organism or any of its parts, and its etiology, pathology, and prognosis might be known or unknown. [See also "*pathology*".]

Domestic Substances List (DSL) is a compilation of substances that have been reported to the Government of Canada under subsections 66(1) or 105(1) of CEPA 1999, or have been added to this List by amendment under subsections 87(1), 112(1), or paragraph 87(5)(a) of CEPA 1999. This list indicates which *substances* are considered to exist in Canadian commerce. A substance on this list is not required to be notified prior to import or manufacture unless the *Significant New Activity* provisions are in effect for this substance as indicated on the DSL, and the notifier is proposing that the substance is now intended to be used for a new activity. [See also "*substance*".]

Dose is the total amount of a *substance* that is administered to, taken, or absorbed by an organism. In the context of this guidance document, the substance is typically a *new microbial substance*, and the term "dose" applies to the total amount of that substance administered to birds or small mammals by *gavage* or inhalation on one (if rodents) or more (if birds) occasions. [See also "*concentration*", "*gavage*", "*microbial*", "*new (microbial) substance*", and "*substance*".]

Ecozone means a particular geographic region demarked by specific and defined ecological characteristics. Appendix 2 in EC and HC (2001) provides a map of the fifteen regions of Canada defined as distinct ecozones.

EC50 is the *median effective concentration.* This is the concentration (i.e., in the present context, the number of microbial units per millilitre or gram dry weight of *test substrate*) of the *test substance* (in the present context, a *new microbial substance*) administered in a test substrate (water, sediment, or soil), that is estimated to cause some defined harmful effect on 50% of the test organisms. In most instances, the EC50 and its 95% confidence limits are statistically derived by analyzing the percentages of organisms affected (e.g., that percentage showing an adverse effect such as signs of tissue damage or gross anomalies, an avoidance response for motile organisms, or evidence of atypical behaviour) at various test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 72 h; or 30 d). The EC50 describes *quantal* effects, lethal or sublethal, and is not applicable to continuous (i.e., *quantitative*) effects. [See also "*concentration*", "*ICp*", "*new (microbial) substance*", "*quantal*", "*quantitative*", "*test substance*", and "*(test) substrate*".]

ED50 is the *median effective dose*. This is the dose of the *test substance* (in the present context, a *new microbial substance*) administered orally or by inhalation on one or more occasions, that is estimated to cause some defined harmful effect on 50% of the test organisms. This term applies to a test involving birds or small mammals exposed to multiple doses of an aqueous suspension of a new microbial substance, each of which is administered orally (by gavage) or by inhalation. In most instances, the ED50 and its 95% confidence limits are statistically derived by analyzing the percentages of organisms affected (e.g., that percentage showing an adverse effect such as signs of tissue damage or gross anomalies, or evidence of atypical behaviour) at various test doses, after a fixed period of exposure. The duration of exposure must be specified (e.g., 30 days). The ED50 describes *quantal* effects, lethal or sublethal, and is not applicable to *quantitative* effects. [See also "*dose*", "*IDp*", "*new (microbial) substance*", "*quantal*", "*quantitative*", and "*test substance*".]

Effect means a change in the state or dynamics of a system caused by the action of an agent. In the present context, "system" refers to the *test (host) organism*(s), and "agent" refers to a *new microbial substance* or, in certain instances, a *microbial product*. [See also "*host (organism)*", "*microbial product*", "*new (microbial) substance"*, and "*organism*".]

Emergence is a term and life process reserved herein for a terrestrial plant. It occurs following the germination of a plant, where the early growth of a seedling pushes the *epicotyl* through the soil surface. [See also "*epicotyl*".]

Endpoint means the measurement(s) or value(s) that characterize the results of a test. For a single-concentration test, this could be represented by calculated values such as percent survival, percent decline in dry weight at test end, or percent decrease in number of young produced. For a multi-concentration (or, in the case of birds or small mammals, a multi-dose) test, the endpoint could be an LC50, LD50, EC50, ED50, ICp (e.g., IC50 or IC25), or IDp (e.g., ID50 or ID25). The term *endpoint* also means the response of the test organisms that is measured (e.g., death, young produced, growth, histopathologies).

End-use Product: see "*EP*".

Environmental expression refers to the extent and manner in which the micro-organisms are influenced by environmental variables such as temperature, pH, and light intensity under controlled laboratory conditions. Testing for environmental expression typically determines, for each variable under investigation, the optimum and range of values that affect the survival, growth, and replication of the micro-organisms.

Environmental risk assessment is the process of identifying and quantifying *risks* to nonhuman organisms and determining the acceptability of those risks.

Environmental toxicology is a branch of *toxicology* with the same general definition; however, the focus is on ecosystems, natural communities, and wild living species, without excluding humans as part of the ecosystems. [See also "*toxicology*".]

EP (end-use product) is a commercially formulated *microbial product* containing a *micro-organism* together with other ingredients (e.g., UV stabilizers, suspending agents, carriers, encapsulating substance or substances, wetting agents). This term is synonymous with *microbial product*, and is typically used by USEPA (1996a-nn) and PMRA (2001) when referring to the final ("end-use") product containing a *MPCA*. The EP should have labelling that includes directions for use or application according to its intended purposes. [See also "*micro-organism*", "*microbial product*", and "*MPCA*".]

Ephippia are egg cases that develop under the postero-dorsal part of the carapace of a female adult daphnid in response to adverse culture or test conditions. The eggs within are normally fertilized (i.e., sexual reproduction has taken place).

Epibenthic means living upon or above the sediment.

Epicotyl is that portion of an embryo or seedling of a terrestrial plant that contains the shoot. It is delineated anatomically by the transition zone which separates the epicotyl from the *hypocotyl*. [See also "*emergence*" and *hypocotyl*.]

Estuarine is from a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

Etiology means the study or theory of the causation of any disease; or the sum of knowledge regarding causes. [See also "*disease*".]

Euthanasia means an easy or painless killing of an animal.

Euryhaline means able to adapt to a range of salinities.

Fecundity means the ability to produce offspring.

Fingerling is a young (underyearling) salmonid fish that is actively feeding and has progressed beyond the "swimup" stage of development.

Frond is the individual leaf-like structure of a duckweed plant. It is the smallest unit capable of reproducing.

Gavage means feeding through a tube into the stomach.

Germination means the physiological events associated with the mobilization of stored nutrients and initiation of growth from a dormant organism such as a spore (e.g., if a bacterium, fungus, or marine plant such as kelp) or seed (if a terrestrial plant) of an embryo. The emergence of the seedling radicle from the seed coat defines the end of germination and the beginning of early seedling growth. A similar but less complex event for bacteria, fungi, or yeasts is the emergence of vegetative cells upon spore germination. [See also "*seed*" and "*seedling*".]

Good Laboratory Practice (GLP) refers to a quality system concerned with the organizational process and the conditions under which environmental studies conducted in a laboratory are planned, performed, monitored, recorded, archived, and reported.

Growth is the increase in size or weight of an organism as the result of proliferation of cells which add to existing tissue or form new tissues. In these biological test methods, it refers to an increase in dry weight.

Hematopoeietic means pertaining to or affecting the formation of blood cells.

Host (organism) is a living plant or animal that harbours or nurtures a micro-organism. In the present context, a host is a test organism (i.e., a specific species of aquatic or terrestrial plant or animal) that is exposed to a *new microbial substance* to measure its ability and extent to cause pathogenic and/or toxic effects under controlled laboratory conditions. [See also "*new (microbial) substance*", "*organism*", and "*test organism*".]

Hypocotyl is that portion of an embryo or seedling containing the root or radicle. It is delineated anatomically by the transition zone which separates the *epicotyl* from the hypocotyl. [See also "*epicotyl*".]

ICp is the *inhibiting concentration for a (specified) percent effect*. It represents a point estimate of the concentration of the *test substance* (i.e., in the present context, the number of microbial units of the *new microbial substance* per millilitre or gram dry weight of *test substrate*) that causes a designated percent inhibition (*p*) compared to the control, for a *quantitative* biological measurement such as number of young produced or size attained by individuals at the end of the test. The ICp is frequently calculated and reported as an IC25 (i.e., the concentration of a test substance that causes a 25% inhibition in growth or other quantitative measurement compared to the control) or an IC20. [See also "*concentration*", "*new (microbial) substance*", "*test substance*", and "*(test) substrate*".]

IC25: see "*ICp*".

IDp is the *inhibiting dose for a (specified) percent effect*. This is the dose of the *test substance* (i.e., in the present context, a *new microbial substance*) administered orally (by gavage) or by inhalation on one or more

occasions, that causes a designated percent inhibition (*p*) compared to the control, for a *quantitative* biological measurement such as number of young produced or size attained by individuals at the end of the test. This term applies to a test with birds or small mammals exposed to multiple doses of an aqueous suspension of a new microbial substance, each of which is administered orally (by gavage) or by inhalation. The IDp is frequently calculated and reported as an ID25 (i.e., the dose of a test substance that causes a 25% inhibition in growth or other quantitative measurement compared to the control) or an ID20. [See also "*dose*", "*new (microbial) substance*", and "*test substance*".]

ID25: see "*IDp*".

Immunologic (processes) refers to the expression of acquired (i.e., adaptive) and/or innate (i.e., constitutive) mechanisms of the immune system resulting from exposure of higher vertebrates (mainly mammals) to foreign substance(s). Acquired (adaptive or clonal) immunity is a specific cell-mediated defence to eliminate antigenic substance(s) introduced by an exposure. Innate immunity includes non-specific components of *clearance*, including those associated with inflammation and mucous barriers. [See also "*clearance*".]

Inactive control: see "*non-infectious control"*.

Indigenous means, with respect to a micro-organism, occurring naturally in the ecozone into which that micro-organism is intended to be introduced.

Infaunal means frequenting or living within sediment.

Infectivity is the ability of a micro-organism to cross or evade natural host barriers to infection. This term describes the capability of the micro-organisms to escape *clearance* measures and to invade and persist in a viable state or multiply within or on an organism, with or without disease manifestation. [See also "*clearance*" and "*pathogenicity*".]

Insects are invertebrates within the phylum Arthropoda and class Insecta, which include bees, ants, beetles, butterflies, fleas, lice, flies, greenflies, termites, earwigs, cockroaches, silverfish, and springtails. Most insects are terrestrial and breathe air by means of tracheae.

Intranasal instillation refers to the injection of a mist or fine spray of colloidal-sized particles (e.g., a liquid suspension of a *new microbial substance*) directly into the nares (nostrils). [See also "*new (microbial) substance*".]

Intratracheal instillation refers to the injection of a mist or fine spray of colloidal-sized particles (e.g., a liquid suspension of a *new microbial substance*) directly into the trachea. [See also "*new (microbial) substance*".]

Isotonic saline is a salt solution prepared in the laboratory which has the same concentration of solutes as that in the blood of a particular species of test organism.

Killed control: see "*non-infectious control*".

LC50 is the *median lethal concentration.* This is the concentration (i.e., in the present context, the number of microbial units per millilitre or gram dry weight of *test substrate*) of the *test substance* (in the present context, a *new microbial substance*) in a test substrate (water, sediment, or soil), that is estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of percent mortalities for each of five or more test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 7-day LC50; or 30-day LC50). [See also "*concentration*", "*new (microbial) substance*", "*test substance*", and "*(test) substrate*".]

LD50 is the *median lethal dose*. This is the dose of the *test substance* (in the present context, a *new microbial substance*) administered orally (by gavage) or by inhalation on one or more occasions, that is estimated to be lethal to 50% of the test organisms. This term applies to a test involving birds or small mammals exposed to multiple doses of an aqueous suspension of a new microbial substance, each of which is administered orally (by gavage) or by inhalation. The LD50 and its 95% confidence limits are usually derived by statistical analysis of percent mortalities for each of five or more test doses, after a fixed period of exposure. The duration of exposure must be specified (e.g., 30-day LD50; or 90-day LD50). [See also "*dose*", "*new (microbial) substance*", and "*test substance*".]

Lethal means causing death by direct action. Death of test organisms is defined as the cessation of all visible signs of movement or other activity indicating life.

Living organism, when applied according to the NSN Regulations and CEPA 1999, means a *substance* that is an animate product of biotechnology. [See also "*substance*".]

LOEC is the *lowest-observed-effect concentration*. This is the lowest concentration of the *test substance* (in the present context, a *new microbial substance*) for which a statistically significant effect on the test organisms was observed, relative to the control. [See also "*concentration*", "*new (microbial) substance*" and "*test substance*".]

LOED is the *lowest-observed-effect dose.* This is the lowest dose of the *test substance* (in the present context, a *new microbial substance*) for which a statistically significant effect on the test organisms was observed, relative to the control. This term is synonymous with the term *LOEL* (i.e., the *lowest-observed-effect level*). [See also "*dose*", "*new (microbial) substance*" and "*test substance*".]

LOEL: see "*LOED*".

Lot means a particular *batch* of a *microbial product* or an unformulated *new microbial substance*, which is identified by a specific code or number. [See also "*batch*", "*microbial*", "*microbial product*", "*new (microbial) substance*", and "*substance*".]

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux. Also, one lux $\cong$ 0.015 µmol/($m^2 \cdot s$) or one klux $\cong$ 15 µmol/($m^2 \cdot s$). Light conditions or irradiance are properly described in terms of quantal flux (photon fluence rate) in the photosynthetically effective wavelength range of approximately 400 to 700 nm. The relationship between quantal flux and lux or foot-candle is highly variable and depends on the light source, the light meter used, the geometrical arrangement, and possible reflections.

Marine is from or within the ocean, sea, or inshore location where there is no appreciable dilution of the seawater by natural fresh water derived from land drainage.

Maximum Hazard Concentration (MHC) is based on some safety factor times the highest *concentration* of the *test substance* (expressed as microbial units per millilitre or kilogram dry weight of *test substrate*) to which groups of aquatic or terrestrial organisms could be exposed in the environment following product application at the maximum recommended rate. See Section 3.3 for further information including test-specific definitions of the MHC. [See also "*concentration*", "*test substance*", and "*(test) substrate*".]

Maximum Hazard Dose (MHD) is based on some safety factor times the highest *dose* of the *test substance* to which birds or small mammals are exposed on one (if rodents) or more (if birds) occasions. See Section 3.3 for further information including test-specific definitions of the MHD. [See also "*dose*" and "*test substance*".]

Mesocosm: see "*microcosm*".

Mesophilic refers to a micro-organism whose optimum temperature for growth is ~20 to 45 °C and which tolerates a minimum temperature no lower than ~15 to 20 °C. Most micro-organisms fall within this category. [See also "*psychrophilic*".]

Microbe means micro-organism. [See also "*microbial*" and "*micro-organism*".]

Microbial means comprised of micro-organisms. [See also "*micro-organism*".]

(*Microbial*) *unit* is a discrete living entity of a *new microbial substance*. This unit is used when calculating and measuring the MHC (or, in the case of tests with birds or small mammals, MHD) and other concentrations (doses) of micro-organisms to which host organisms are exposed in a laboratory test for pathogenic and/or toxic effects. See Section 3.3.1 for a more detailed and explicit definition. [See also "*MHC*", "*microbial*" and "*new (microbial) substance*".]

Microbial product is a commercial formulation that contains one or more *micro-organisms*. It might also contain one or more inanimate inorganic and/or organic *substance*s (e.g., UV stabilizers, suspending agents, carriers, encapsulating substances, wetting agents). [See also "*end-use product*", "*microbial*", "*micro-organism*", and "*substance*".]

Microcosm (testing) involves an experimental setup in the laboratory that includes one or more environmental media such as water, sediment, soil, vegetation, and leaf litter, together with various groups of sensitive organisms found in differing environmental media (e.g., selected species of aquatic plants and invertebrates, fish, and terrestrial plants and invertebrates). The experimental setup is designed in an attempt to physically simulate some portion of the ambient environment. A similar setup, when established in the field, is termed a *mesocosm*.

Micro-organism means a microscopic *living organism* that is: (a) classified in the Bacteria, Archaea, or Protista (which includes protozoa and algae) or the Fungi (which includes yeasts), (b) a virus, virus-like particle, or sub-viral particle[1]; (c) a cultured cell of an organism not referred to herein other than as a cell used to propagate such an organism; or (d) any culture other than a pure culture (NSN Regulations of CEPA 1999; Government of Canada, 1997). The preceding item (d) refers to a *consortium*. [See also "*consortium*" and "*living organism*".]

Morbidity means the condition of being diseased. [See also "*disease*".]

Monocotyledon, in the classification of plants, refers to those species having a single seed leaf. [See also "*cotyledon*" and "*dicotyledon*".]

MPCA (microbial pest control agent) is a pure culture or consortium (i.e., a complex, unformulated natural combination) of micro-organisms that, when mixed with other ingredients (e.g., UV stabilizers, suspending agents, carriers, encapsulating substances, wetting agents), results in an *end-use product (EP)* suitable for application in pest control. [See also "*consortium*", "*EP*", and "*substance*".]

Necropsy means a post-mortem examination (i.e., an autopsy), involving a careful inspection of the external and internal surfaces and parts of the dead organism for any signs of an atypical appearance. Histological examination of certain organs or tissues might be included as part of the examination.

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[1] Although classified as a micro-organism for the purpose of these Regulations, it is recognized that a virus is in reality a sub-cellular, infectious genetic entity totally dependent on a living host cell or cells for its propagation.

Necrosis refers to the sum of the morphological changes indicative of cell death (excluding programmed cell death; i.e., apoptosis), that is caused by the progressive degradative action of enzymes. These changes might affect groups of cells or a part of a tissue or organ.

Negative control is a *treatment* in a test that contains no *test substance* that could adversely affect the survival, behaviour, reproduction, growth, or other biological *endpoint(s)* of a specific *biological test method*. Each test must include this treatment, which serves as a check for the absence of pathogenicity and/or toxicity due to basic test conditions such as temperature, health of test organisms, or effects due to their handling. For the results of a test to be considered meaningful and acceptable, the negative control must meet the test-specific criterion or criteria for validity. [See also "*biological test method*", "*control*", "*endpoint*", "*substance*", and "*treatment*".]

Neonate is a newly born or newly hatched individual (e.g., a first-instar daphnid, $\leq$24-h old).

New (microbial) substance is a (microbial) *substance* that is not included on the Domestic Substances List (DSL). [See also "*microbial*" and "*substance*".]

NOEC is the *no-observed-effect concentration*. This is the highest concentration of the *test substance* (in the present context, a *new microbial substance*) in the *test substrate*, at which no statistically significant effect on the test organisms was observed, relative to the control. [See also "*concentration*", "*new (microbial) substance*", "*test substance*", and "*(test) substrate*".]

NOED is the *no-observed-effect dose*. This is the highest dose of the *test substance* (in the present context, a *new microbial substance*) in the *test substrate*, at which no statistically significant effect on the test organisms was observed, relative to the control. This term is synonymous with the term *NOEL* (i.e., the *no-observed-effect level*). [See also "*dose*", "*new (microbial) substance*", "*test substance*", and "*(test) substrate*".]

NOEL: see "*NOED*".

Non-infectious control is a *control treatment* included in a test, that consists of the *test material* at its *MHC*, after its treatment to inactivate (e.g., by heating) viable micro-organisms therein while preserving their structural integrity. This control is included in a test to determine if the attenuated (non-infectious) MHC (or, in the case of tests with birds or small mammals, MHD) is responsible for any adverse effect(s) on the test organisms once its ability to cause an infection and subsequent pathogenic effects has been removed. Synonymous terms are "*inactivated control*", "*killed control*", and "*non-viable control*". [See also "*maximum hazard concentration (MHC)*", "*test material*", and "*treatment*".]

Non-viable control: see "*non-infectious control"*.

Notifier is a person residing in Canada that proposes to import or manufacture a *new substance* within Canada, and provides to Environment Canada's New Substances Branch the information specified in a schedule to the New Substances Notification Regulations. Where the person is not resident in Canada, the person shall provide the name and address of an agent resident in Canada. [See also "*new (microbial) substance*".]

Nulliparous means having never given birth to a viable offspring.

Organism is an individual living animal or plant.

Overlying water is *test water* placed over *test sediment* in a test chamber. [See also "*test water*" and "*test sediment*".]

Oviduct is a tube within a female mysid in which unfertilized eggs (ova) develop.

Pathogen means any disease-producing micro-organism.

Pathogenic means giving origin to disease or morbid symptoms caused by micro-organisms. [See also "*morbid*" and "*pathogenicity*".]

Pathogenicity refers to the ability of a micro-organism to infect a *host* (e.g., a test organism), establish itself and multiply there, and subsequently inflict injury or damage that might or might not lead to death. The effect on the host might be sublethal or lethal, and depends on the *virulence* of the pathogen (i.e., the micro-organism) as well as on host resistance or susceptibility. [See also "*host*", "*pathogenic*", and "*virulence*".]

Pathology means either the branch of science that deals with bodily diseases, or the symptoms of a disease. It represents the structural and functional changes in tissues and organs of the body which cause or are caused by disease.

Pelagic describes an aquatic organism which remains free-swimming or free-floating.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 indicating increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24-h period.

Pore water is the water occupying space between particles of sediment. The term is synonymous with *interstitial water*.

Positive chemical control (test) is a multi-concentration test with a toxic chemical, performed on or about the time of a definitive test for pathogenic and/or toxic effects of a *test substance*. This test uses a *reference toxicant* known to adversely affect the survival, behaviour, reproduction, growth, or other biological *endpoint(s)* measured for the *test* (*host*) *organism* in a specific *biological test method*, according to a predictable and demonstrable manner. [See also "*biological test method*", "*endpoint*", "*host (organism)*", "*positive control*","*reference toxicant*", "*test organism*", and "*test substance*".]

Positive control is a *treatment* (or, in the case of a *positive chemical control*, a series of treatments) in a test that contains a *test substance* known to adversely affect the survival, behaviour, reproduction, growth, or other biological *endpoint(s)* measured for the *test* (*host*) *organism* in a specific *biological test method*, according to a predictable and reliable manner. [See also "*biological test method*", "*endpoint*", "*host (organism)*", "*new (microbial) substance*", "*positive chemical control*", "*positive microbial control*", "*reference toxicant*", "*test organism*", "*test substance*", and "*treatment*".]

Positive microbial control is a *treatment* in a test that contains a *pathogen* known to adversely affect the survival, behaviour, reproduction, growth, or other biological *endpoint(s)* measured for the *test* (*host*) *organism* in a specific *biological test method*, according to a predictable and demonstrable manner. A positive microbial control used in a test with a *new microbial substance* consists of a single concentration of a *pathogen* other than the *micro-organism* (i.e., the *new microbial substance*) under investigation, that is known to affect the test organisms adversely and predictably during the test method to be employed. [See also "*biological test method*", "*endpoint*", "*host (organisms)*", "*micro-organism*", "*new (microbial) substance*", "*pathogen*", "*positive control*", "*test organism*", and "*treatment*".]

Precision refers to the variation associated with repeated measurements of the same metric variable, i.e., the degree to which data generated from repeated measurements are similar. It describes the degree of certainty around a result, or the tightness of a statistically derived endpoint such as an ICp or IDp.

Principal Investigator means an individual who acts on behalf of the Study Director and has defined responsibility for delegated phases of the study. The Study Director's responsibility for the overall conduct of the study cannot be delegated to the Principal Investigator(s). [See also "*Study Director*".]

Protocol is an explicit set of procedures for a test or an experiment, formally agreed upon by the parties involved, and described precisely in a written document.

Psychrophilic refers to a micro-organism whose optimum temperature for growth is ~15 °C or lower. The term means "cold loving". [See also "*mesophilic*".]

Quality assurance (program) means a defined system including personnel, operating within a test laboratory, which is independent of the conduct of the study and is designed to assure management and others compliance with the Principles of Good Laboratory Practice.

Quality control consists of specific actions within the program of *quality assurance*. It includes standardization, calibration, replication, control samples, and statistical estimates of limits for the data. [See also "*quality assurance*".]

Quantal effects in a test for pathogenic and/or toxic effects are those in which each *test organism* responds or does not respond. For example, an animal might respond by dying in or avoiding a contaminated test water, sediment, or soil. Generally, quantal effects are counts. [See also "*quantitative*" and "*test organism*".]

Quantitative effects in a test for pathogenic and/or toxic effects are those in which the measured effect is continuously variable on a numerical scale. Examples would be number of young produced, or dry weight of young at test end. Generally, quantitative effects are measurements. [See also "*quantal*".]

Raw data means all original records and documentation obtained and entered by the testing laboratory, or verified copies thereof, which are the result of the original observations and activities in a study. Raw data might include photographs, computer readable media, dictated observations, recorded data from automated instruments, or any other data storage medium that has been recognized as capable of providing secure storage of information.

Reference method refers to a specific protocol for performing a biological test method in a regulatory context, i.e., a biological test method with an explicit set of test procedures and conditions, formally agreed upon by the parties involved and described precisely in a written document.

Reference pathogen is a standard infectious *micro-organism* used as a *positive microbial control* in a test with a *new microbial substance*. It is included in the test to provide assurance that the *test organisms* and the associated test method are responsive to a known *pathogenic* micro-organism. [See also "*micro-organism*", "*new (microbial) substance*", "*positive microbial control*", "*pathogenic*", and "*test organism*".]

Reference toxicant is a standard chemical used to measure the sensitivity of the *test organism*s to establish confidence in the toxicity data obtained for a *test substance*. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test substance is evaluated, and the precision and reliability of results obtained by the laboratory for that chemical. [See also "*positive chemical control*", "*test organism*", and "*test substance*".]

Reference toxicity test is a test conducted using a *reference toxicant* in conjunction with a definitive test intended to measure the pathogenic and/or toxic effects of a *new microbial substance*. The purpose of a reference toxicity test is to appraise the sensitivity of the organisms and the precision and reliability of results obtained by the laboratory for that reference chemical at the time the new microbial substance is evaluated. Deviations outside an established normal range indicate that the sensitivity of the *test organisms*, and the performance and precision of the test, are suspect. [See also "*new (microbial) substance*", "*reference toxicant*", and "*test organism*".]

Remediation is the management of a contaminated site to prevent, minimize, or mitigate damage to human health or the environment. *Remediation* can include both direct physical actions (e.g., removal, destruction, and containment of toxic substances) and institutional controls (e.g., zoning designations or orders). It can also include the use of a *new microbial substance* or *microbial product* to destroy, reduce, or remove toxic substances at a contaminated site (i.e., *bioremediation*). [See also "*bioremediation*", "*microbial product*", and "*new (microbial) substance*".]

Replicate means an identical entity (e.g., a replicate group, a replicate test chamber, or a replicate treatment). [See also "*replicate treatment*" and "*(test) treatment*".]

Replicate treatment refers to a single test chamber containing an identical number of test (host) organisms from the same group (population) within a culture or holding chamber(s) , together with a concentration of the same *test substance* and a measured amount of test substrate(s) which are identical to other *replicate* chambers representing this same treatment. A *replicate* of a treatment must be an independent test unit; therefore, any transfer of test organisms, test substance, or test substrate from one test chamber to another would invalidate a statistical analysis based on the replication. [See also "*replicate*", "*test substance*", and "*(test) treatment*".]

Risk is the probability or likelihood that an adverse effect will occur.

Risk assessment: see "*environmental risk assessment*".

Root refers to that part of a vascular plant that usually grows downwards into the soil or other substrate, anchoring the plant and absorbing water and nutrients.

Salinity is the total amount of sea salts, in grams, dissolved in 1 litre of (sea)water; and is traditionally expressed as parts per thousand (‰). It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means.

Sediment is natural particulate matter, which has been transported and deposited in water and then deposited on the floor below the water. The term can also describe matter that has been experimentally prepared (formulated) using selected particulate matter (e.g., sand of particular grain size, bentonite clay, etc.) and within which benthic test organisms can burrow.

Seed is the propagule of a plant derived from an ovule. It consists of an embryo and a protective covering (i.e., the seed coat), and might also contain storage tissue (i.e., endosperm).

Seedling is a young (sexually immature) plant that originates from a seed rather than a cutting. [See also "*seed*".]

Series (of tests) refers to a combination of several tests for measuring pathogenic and/or toxic effects of a *new microbial substance*, normally using different species of host (test) organisms representing differing categories of organisms found in the aquatic or terrestrial environment (e.g., one or more tests involving

aquatic plants, aquatic invertebrates, aquatic vertebrates, terrestrial plants, terrestrial invertebrates, and terrestrial vertebrates). [See also "*new (microbial) substance*".]

Shoot is the above-ground portion of a plant, consisting of stems, leaves, as well as any reproductive parts that might be attached.

Soil is whole, intact matter representative of the terrestrial environment, that has had minimal manipulation following collection or formulation. In the natural environment, it is formed by the physical, chemical, and biological weathering and disintegration of rocks and the decomposition and recycling of nutrients from organic matter originating from plant and animal life. Its physicochemical characteristics are influenced by microbial, invertebrate, and plant activities therein, and by anthropogenic activities.

Sponsor means an entity which commissions, supports, and/or submits an environmental safety study.

Standard Operating Procedure (SOP) refers to documented "in-house" laboratory procedures and conditions which describe how to perform a particular test or activity within that facility. A SOP describes procedures and conditions related to the performance of a particular test or activity within a laboratory that are normally not specified in detail in study plans or test guidelines.

Static describes a test using aquatic plants or animals in which the test concentrations are not renewed during the test.

Static renewal describes a test using aquatic plants or animals in which the test concentrations are renewed (replaced) at designated intervals during the test. Synonymous terms are "renewed static", "renewal", "static replacement", and "semi-static".

Stenohaline means unable to tolerate a wide variation in salinity.

Sterile filtrate control is a *control treatment* included in a test, that consists of a sterile filtrate prepared from the *test material* (e.g., a *new microbial substance*) suspended at its *maximum hazard concentration*. This control is included in a test to determine if the sterilized filtrate of the MHC (or, in the case of tests with birds or small mammals, MHD) is responsible for any adverse effect(s) on the test organisms. [See also "*control*", "*maximum hazard concentration*", "*new (microbial) substance*", "*test material* ", and "*treatment*".]

Study means an experiment or set of experiments in which a *test substance* is examined under laboratory conditions to obtain data on its properties, and for which the results are intended for submission to the appropriate regulatory authority or authorities. This term is synonymous with "*test*" in instances where a single experiment is involved. [See also "*test*" and "*test substance*".]

Study Director means the individual responsible for the overall conduct of the study. [See also "*study*".]

Swimup fry refers to a young, post-alevin salmonid fish which has commenced active feeding.

Sublethal means detrimental to the organism, but below the level that directly causes death within the test period.

Sublethal effect is an adverse effect on an organism due to *pathogenicity* and/or *toxicity*, below the level which directly causes death within the test period. [See also "*pathogenicity*" and "*toxicity*".]

Substance is any distinguishable kind of organic or inorganic matter, whether animate or inanimate. Included in this definition is (a) a pure culture of a *micro-organism* and (b) a *consortium*. A *blend* of micro-organisms, or a *microbial product*, is a mixture of substances rather than an individual substance. [See also "*blend*", "*consortium*", "*microbial product*", "*micro-organism*", and "*new (microbial) substance*".]

Substrate: see "*(test) substrate*".

Test means an experiment performed in the laboratory under defined and controlled procedures and conditions. This term is synonymous with *study* in instances where a single experiment is involved. [See also "*study*".]

Test material is, in the context of this guidance document, either a pure culture of a *micro-organism*, a *consortium*, a *blend* of micro-organisms, or a *microbial product*, to which *host* (*test*) organisms are exposed in a controlled study. [See also "*blend*", "*consortium*", "*host*", "*micro-organism*", "*new (microbial) substance*", "*microbial product*", and "*test*".]

Test organism means, in the present context, a *host organism* in which an effect is measured under controlled laboratory conditions, during or following its exposure to a *test material* (i.e., a *new microbial substance* or, in certain instances, a *blend* of *micro-organisms* or a *microbial product*). [See also "*blend*", "*host (organism)*", "*microbial product*", "*micro-organisms*", "*new (microbial) substance*", "*organism*", "*substance*", "*test*", and "*test material*".]

Test sediment is a sample or subsample of *clean* whole sediment which is used as *test substrate* in a laboratory test designed and intended to measure the pathogenic and/or toxic effects of a *new microbial substance*. It is either collected from an uncontaminated site within the natural environment, or formulated in the laboratory using a specific ratio of natural constituents of uncontaminated sand, silt, and/or clay-sized particles. In certain instances, the term "test sediment" also applies when referring to any sediment being prepared for or within test chambers, including a sample or subsample of negative control sediment, reference sediment, or any treatment containing a particular concentration of a *test material*. [See also "*clean*", "*new (microbial) substance*", "*test material*", and "(*test) substrate*".]

Test soil is a sample of *clean* whole soil which is used as *test substrate* in a laboratory test designed and intended to measure the pathogenic and/or toxic effects of a *new microbial substance*. It is either collected from an uncontaminated site within the natural environment, or formulated in the laboratory to simulate a natural soil using a specific ratio of natural constituents of sand, silt, clay, and peat. In certain instances, the term "test soil" also applies when referring to any soil being prepared for or within test chambers, including a sample or subsample of negative control soil, reference soil, or any treatment containing a particular concentration of a *test material*.[See also"*clean*", "*new (microbial) substance*", "*test material*", and "(*test) substrate*".]

Test substance is an animate or inanimate substance that is studied under controlled laboratory conditions, to measure its pathogenic and/or toxic effects. [See also "*new (microbial) substance*", "*pathogenic*", "*substance*", "*test*", "*test material*", and "*toxic*".]

(Test) substrate is defined as any environmental substrate (e.g., water, sediment, or soil) used in a test for pathogenic and/or toxic effects of a *new microbial substance*, once that matter is homogenized as a *batch* and divided into *replicates*. A *test treatment* is prepared by mixing a particular concentration of the test material with a *test substrate*. In a laboratory test whereby a test material is mixed in food offered to the test organisms, that food is also considered to be a *test substrate*. [See also "*batch*", "*new (microbial) substance*", "*replicate*", "*test material*", and "(*test) treatment*".]

(Test) treatment refers to a specific concentration of a *test substance* in a *test substrate*, which is prepared in the laboratory and to which *replicate* groups of *test organisms* are exposed. Each set of specific *control* groups (e.g., *negative control*, positive *non-infectious control; sterile filtrate control,*) included in a test for pathogenic and/or toxic effects also represents a discrete *treatment*. [See also "*non-infectious control*", "*replicate*", "*replicate treatment*", "*sterile filtrate control*", "*test organisms*", "*test substance*", and "(*test) substrate*".]

Test water is the estuarine, marine, or fresh water used in a test for pathogenic and/or toxic effects of a *new microbial substance* on aquatic plants, invertebrates, or vertebrates. It represents a sample or subsample of *clean* water which is used either as *test substrate* or, for certain tests involving both test water and test sediment, as *overlying water* in the test. The *test water* is either drawn or collected from an uncontaminated source of natural water, or formulated in the laboratory using a specific mixture of uncontaminated salts mixed in deionized or distilled water in a ratio which achieves the desired characteristics (e.g., hardness or salinity) suitable for a specific test organism and biological test method. In certain instances, the term "test water" also applies when referring to any water being prepared for or within test chambers, including a sample or subsample of negative control water, reference water, or any treatment containing a particular concentration of a *test material*. [See also "*clean*", "*new (microbial) substance*", "*test material*", "*test substrate*", and "*overlying water*".]

Toxic (substance) means a substance that enters or might enter the environment in a quantity or concentration or under conditions that (a) have or might have an immediate or long-term harmful effect on the environment or its biological diversity; (b) constitute or might constitute a danger to the environment on which life depends; or (c) constitute or might constitute a danger in Canada to human life or health. [As defined in Article 64 of CEPA 1999.]

Toxicant is a toxic substance.

Toxicity refers to the ability of a *test substance* to cause adverse effect(s) on living plants or animals due to its poisonous (toxic) nature. These effect(s) could be lethal or sublethal. For a test with a *new microbial substance*, toxicity could be associated with *toxin* production by the micro-organism and/or with its metabolic products or metabolites or its structural components (e.g., cell walls, if bacteria). For a test with a *microbial product*, toxicity could also be associated with dissolved or particulate substance(s) (e.g., a carrier culturing medium and/or "inert" filler) found in that product together with the micro-organisms therein. [See also "*lethal*", "*microbial product*", "*new (microbial) substance*", "*sublethal*", "test *substance*", and "*toxin*".]

Toxicity test is a determination of the poisonous effect of a *substance* on a group of selected organisms (e.g., a particular species of aquatic or terrestrial plant or animal), under defined conditions. An environmental toxicity test usually measures: (a) the proportions of *test organism*s affected (*quantal*); and/or (b) the degree of effect shown (*quantitative* or *graded*), after exposure to one or more specific *test substance*s (e.g., a *new microbial substance*), or to one or more concentrations thereof. [See also "*lethal*", "*new (microbial) substance*", "*quantal*", "*quantitative*", "*sublethal*", "*substance*","*test organism*", "*test substance*", and "*toxicity*".]

Toxicology is a branch of science that studies the *toxicity* of *substances* or conditions. There is no limitation on the use of various scientific disciplines, field or laboratory tools, or studies at various levels, whether molecular or encompassing an ecosystem. Applied toxicology would normally have a goal of defining the limits of safety of chemical or other substances including *new microbial substances*, based on *toxicants* therein. [See also "*environmental toxicology*" "*new (microbial) substance*", "*substance*", "*toxicant*", and "*toxicity*".]

Toxigenicity refers to the ability of a micro-organism to produce a *toxin*. [See also "*toxin*".]

Toxin is a substance produced by a micro-organism that might have a harmful effect on a host (test) organism, irrespective of the presence of the living micro-organism.

Treatment: see "*(test) treatment*".

Virion is a complete viral particle, consisting of RNA or DNA surrounded by a protein shell and constituting the infective form of a virus.

Virulence refers to the degree of *pathogenicity* of a *micro-organism*, as indicated by demonstrable *sublethal* and/or *lethal* effect(s) on a host organism and/or its ability to invade the tissues of the host. Within the context of this guidance document, the virulence of a micro-organism is measured under controlled laboratory conditions in a series of multi-concentration tests involving differing host organisms. The degree of pathogenicity caused by the micro-organism (i.e., its virulence) is determined and expressed using statistical endpoints such as the LC50, ICp, and/or LOEC/NOEC (or, for controlled tests involving birds or small mammals, the LD50, IDp, and/or LOED/NOED). [See also "*host*", "*ICp*", "*IDp*", "*LC50*", "*LD50", "lethal", "LOEC*", "*LOED*", "*micro-organism*", "*NOEC*", "*NOED*", "*pathogenicity*", and "*sublethal*".]

Warning chart is a graph used to follow changes in the endpoints for a *reference toxicant* over time. Date of the test is on the horizontal axis and the effect-concentration is plotted on the vertical logarithmic scale. [See also "*positive chemical control*" and "*reference toxicant*".]

Warning limit is plus or minus two standard deviations, calculated logarithmically, from a historic geometric mean of the endpoints from tests with a *reference toxicant*. [See also "*positive chemical control*" and "*reference toxicant*".]

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Introduction

1.1 Background

1.1.1 Canadian Environmental Protection Act, 1999

The *Canadian Environmental Protection Act*, 1999 (CEPA 1999) was promulgated in 1988 and revised in 1999.[2] Since the inception of the New Substances Notification (NSN) Regulations (Government of Canada, 1997)[3] under CEPA 1999, the Ministers of Environment and Health have been required to assess the notification information provided by the company or individual that proposes to import or manufacture a *new substance* in Canada. As part of this process, notification information is assessed by the New Substances Programs within Environment Canada and Health Canada, to determine whether there is potential for adverse *effect*s of the new substance on the aquatic and/or terrestrial environment. Under the CEPA 1999 legislation, such assessments can lead to (i) no restrictions on import and/or manufacturer of a new substance; or (ii) control measures on or prohibition of manufacture and/or import of a new substance, which could include a) a requirement to submit additional information determined to be required by the departments, or b) restrictions on limiting the purpose for which the new substance may be used.

1.1.2 New Substances Notification Regulations

Consistent with the CEPA 1999 legislation, data on adverse environmental effects are systematically considered as part of the *environmental risk assessment* of *new substances* that are notified for environmental release (as per Schedule XV of the NSN Regulations). The NSN Regulations for chemicals and polymers came into force within Canada on July 1, 1994. On September 1, 1997, Part II.1 of the NSN Regulations prescribed the process for notification of new substances that are *living organisms* including *micro-organisms* and organisms other than micro-organisms (Government of Canada, 1997).[4] Health Canada conducts an assessment of the new living organism for potential human health effects, while Environment Canada conducts an assessment to determine potential environmental effects. The Ministers of Environment and Health assess the prescribed information provided by the *notifier* along with other information available to them in respect of the living organism, to determine whether it is *toxic vis-à-vis* CEPA 1999, or capable of becoming toxic.

1.1.3 Guidelines for the Notification and Testing of New Substances: Organisms

In 2001, Environment Canada and Health Canada revised the document entitled "*Guidelines for the Notification and Testing of New Substances: Organisms*" (EC and HC, 2001)[5], to reflect the legislative changes in CEPA 1999. These Guidelines help notifiers understand and meet their responsibilities under the NSN Regulations before importing or manufacturing a new living organism. Micro-organisms, whether naturally occurring or genetically modified, require notification of the prescribed information prior to import or manufacture when the micro-organism[6] does not

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[2] This *Act* may be accessed through the Internet at http://laws.justice.gc.ca/en/C-15.31/, and then selecting "Canadian Environmental Protection Act, 1999". The *Canadian Environmental Protection Act*, 1999 is also available at www.ec.gc.ca/substances/.

[3] These Regulations are available through the Internet at http://laws.justice.gc.ca/en/C-15.31, and then choosing "New Substances Notification Regulations" under "Related Regulations". The Regulations are also available at www.ec.gc.ca/substances/.

[4] Part II.1 of the NSN Regulations (Government of Canada, 1997) was amended on March 31, 2000 to reflect the legislative changes in CEPA 1999.

[5] This document is available through the Internet at www.ec.gc.ca, by selecting "CEPA Registry" and then "Guidelines/Codes of Practice", and scrolling to and selecting the title. Additional guidelines and information related to *new substances* are available at www.ec.gc.ca/substances/.

[6] Note that the definition of *micro-organism* herein and in the NSN Regulations includes a *consortium*.

appear on the *Domestic Substances List (DSL)*. Such micro-organisms are considered to be *new substances*, and these Guidelines (EC and HC, 2001) provide general descriptions of the information requirements which are to be met by the notifier.

1.1.4 Microbial Substances Subject to the New Substances Notification Regulations

Microbial substances that are not listed on the DSL are subject to the NSN Regulations before they are imported or manufactured in Canada unless they meet the research and development exemption criteria, or are manufactured or imported for a use that is regulated under Acts and Regulations listed in Schedule 4 of CEPA 1999. To avoid duplication of regulation, Schedule 4 of CEPA 1999 lists other Federal Acts and Regulations that provide for notice and assessment of *toxicity* and/or *pathogenicity* prior to import, manufacture, or sale. Currently, the following Acts and Regulations are listed in Schedule 4 of CEPA 1999 for which micro-organisms used in the following products may be regulated:

- pesticides regulated under the *Pest Control Products Act* and *Pest Control Products Regulations*;
- livestock feeds regulated under the *Feeds Act* and *Feeds Regulations*;
- supplements such as inoculants regulated under the *Fertilizers Act* and *Fertilizers Regulations*; and
- veterinary biologics regulated under the *Health of Animals Act* and *Health of Animal Regulations*.

All other uses of micro-organisms are regulated under CEPA 1999 and the NSN Regulations. Examples of applications and products involving micro-organisms, which might be subject to the NSN Regulations, include but are not limited to the following:

a. bioremediation/bioaugmenation of contaminated soil, water, or other matter;

b. desulphurization processes associated with mining and metal extraction;

c. effluent treatment systems including bio-filters;

d. enhanced oil recovery operations;

e. degradation of proteins or fats such as those used in restaurant grease traps;

f. septic tank activators;

g. manure processing products

h. compost enhancers;

i. cleaning products;

j. production of bio-products such as enzymes, proteins, amino acids, vitamins, or biofuel/bioenergy; and

j. water/pond/dugout clarification products.

1.1.5 Current Guidance Document

As part of the assessment process under the NSN Regulations, information is required to determine whether there is a potential for *new microbial substances* to cause adverse effects on the aquatic and/or terrestrial environment. These environmental assessments must necessarily be based on state-of-the-art procedures and methodologies for characterizing adverse effects. To the extent possible, the approach adopted therein must also be harmonized with that of other Federal departments and international agencies. The current guidance document is intended to meet these needs, while at the same time increasing public transparency of the scientific data that underpins decisions related to the NSN Regulations.[7]

A number of diversified procedures for detecting potential *pathogenic* and/or toxic effects on aquatic or terrestrial organisms (plants, invertebrates, and vertebrates), caused by a micro-organism or a *microbial product*, are now being developed by the

[7] This is consistent with recommendation 9.2 made in 2001 by the Royal Society of Canada's Expert Panel on the future of biotechnology within Canada, in their report entitled *"Elements of Precaution: Recommendations for the Regulation of Food Biotechnology in Canada"*. This report is available at: http://www.rsc.ca/foodbiotechnology/indexEN.html.

international scientific community. As research in these and related areas increases, there is an increasing need for systematic application of current knowledge to *risk assessment* including (where appropriate and applicable) the use of standardized *biological test methods* for such an assessment. In keeping with this need, the New Substances Branch of Environment Canada approached the Method Development and Applications Section (MDAS) of the Environmental Technology Centre (Environment Canada, Ottawa, ON) to further develop detailed guidance on how to measure the pathogenicity and/or toxicity to aquatic and terrestrial organisms of new microbial substances that are subject to the NSN Regulations. This document represents the results of that undertaking.

Under the NSN Regulations, the notification process is specific to (new) microbial substances rather than (new) microbial products. However, for the purpose of testing a new microbial substance (i.e., a specific micro-organism, or a consortium of micro-organisms) in the laboratory for its potential ecological effects, the *test material* might, in some instances and preferably with prior agreement with the New Substances Program, be a microbial product containing that substance. Where applicable, the test material might also be a *blend* of two or more micro-organisms. Section 3.2 provides additional information and guidance on the test material.

1.1.6 Pre-Notification Consultation

A Pre-Notification Consultation (PNC) with Environment Canada and/or Health Canada is strongly recommended and advised before commencing *test*s to satisfy the information requirements in respect of the ecological effects of a new microbial substance. Such discussion will assist the notifier in determining the acceptability of proposed biological test methods, proposed test (*host*) organisms, and the proposed test material. In the event that the notifier wishes to use surrogate data, data from a blend of micro-organisms, or data on the microbial product, the acceptability of the rationale for such a choice would also be discussed. Request for a PNC (i.e., consultation prior to a formal regulatory submission) should be made through the New Substances Information Line at 1-800-567-1999 (in Canada), 1-819-953-7156 (outside Canada), by fax at 1-819-953-7155, or by e-mail at nsn-infoline@ec.gc.ca.

1.2 Purpose and Scope of this Guidance Document

This guidance document builds on the general guidance on new substance notification with respect to the ecological effects of new microbial substances, that is included in the "*Guidelines for the Notification and Testing of New Substances*" (EC and HC, 2001) pursuant to the NSN Regulations. It is intended to assist notifiers in choosing appropriate test (host) organisms and associated biological test methods (see Sections 8 to 14 inclusive) when testing for these effects. Additionally, this document will serve as a working tool for environmental consultants designing an appropriate testing regime as well as laboratory personnel undertaking the testing program. As such, this guidance document clarifies the appropriate pre-test and *test* procedures and conditions to be applied when testing new microbial substances under consideration for pathogenic and/or toxic effects to each of the following six categories of organisms:

- aquatic plants,
- aquatic invertebrates,
- aquatic vertebrates,
- terrestrial plants,
- terrestrial invertebrates, and
- terrestrial vertebrates.

Emphasis is on the adaptation and subsequent use of existing biological test methods which have been standardized by environmental regulatory agencies (including Environment Canada) for these and other purposes (e.g., EC, 1990a–c; EC, 1992a–g; EC, 1997a,b; EC, 1998a,b; EC, 1999a; EC, 2000a,b; EC, 2001a; EC, 2002; EC, 2004a–c). Environment Canada has reviewed the scientific literature on this subject (Douville, 2001) and that review has provided background information for the development of this guidance document.

The scope of this guidance document is restricted to single-species tests[8] for adverse effects of new

[8] Support for single-species tests and their validity in terms of studies of *environmental toxicology* and associated *environmental risk assessment*s is found in *"Guidance Document on Application and Interpretation of Single-Species Tests in Environmental Toxicology"* (EC, 1999b). This (i.e., EC, 1999b) guidance document is highly recommended as a knowledgeable and useful

microbial substances on aquatic or terrestrial *organism*s, which are performed in the laboratory. Guidance is therefore on the use of a *series* of biological test methods intended to measure the toxic and/or pathogenic effects of new microbial substances on sensitive test species and life stages of organisms representing aquatic and terrestrial plants, invertebrates, and vertebrates common to the Canadian environment.

Environmental effects on *microcosms* are not addressed herein, nor is guidance provided on field study design to directly evaluate the effects of new microbial substances applied to designated experimental sites.[9] Additionally, the document does not provide guidance on tests for the fate (e.g., dispersal and persistence) of a new microbial substance.[10] A further limitation in scope is that this document does not provide guidance on how to measure and define the influence of environmental variables (such as temperature and *pH*) on the survival, *growth*, and replication of the micro-organisms within a new microbial substance (i.e., *environmental expression*); although this issue is addressed in a general sense in Section 2.

1.3 Topics Addressed

Section 2 is an overview of various issues pertaining to the testing of new microbial substances for pathogenic and/or toxic effects on aquatic and terrestrial organisms. This section briefly introduces and addresses:

(i) the use of an approach involving a series of tests with selected species and groups of aquatic and terrestrial plants, invertebrates, and vertebrates;

(ii) the inability of many or most standardized biological test methods to distinguish pathogenic effects from toxic ones;

(iii) the focus herein on pathogenic and/or toxic effects rather than tests for *infectivity*;

(iv) the need for proper *controls* in each test applied; and

(v) the associated need for laboratory tests which define the *environmental expression* of the new microbial substance under controlled laboratory conditions.

Section 3 addresses the need for information on the physical, chemical, and biological characteristics of the test material and provides guidance on preparing test *concentration*s and administering them to the test (host) organisms. Section 4 gives guidance on the various types of controls that should be included in each definitive test for pathogenic and/or toxic effects. Information on various approaches and procedures for testing for infectivity, coincident with tests for pathogenic and/or toxic effects, is addressed in Section 5. Section 6 considers the (OECD-derived) Principles of *Good Laboratory Practice* associated with *quality assurance* and *quality control*, that should be implemented during all definitive tests for pathogenic and/or toxic effects to ensure high quality, valid test results. The issues of safety to laboratory workers, and humane practices for tests with animals, are considered in Section 7. Guidance on choosing an appropriate series of biological test methods is provided in Section 8. Sections 9 to 14 are dedicated to guidance on recommended biological test methods for each category of test organisms (i.e., aquatic or terrestrial plants, invertebrates, and vertebrates) on a section-by-section basis. Guidance on reporting the results of each test for pathogenic and/or toxic effects is dealt with in Section 15.

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source of information when designing and applying laboratory tests for adverse effects of new microbial substances, and when interpreting their results.

[9] The reader is directed to Section 4.3.5.1 of EC and HC (2001) as well as USEPA (1996w) when considering microcosm or mesocosm tests, and to Sections 4.2.4 and 4.2.5 of EC and HC (2001) when considering experimental field studies. Parts 8.2.2 and 10.2.2 in PMRA (2001) provide further guidance when performing microcosm tests or when field-testing microbial pest control agents and products, respectively.

[10] The reader should consult Section 4.2.6 of EC and HC (2001) when considering the environmental fate of a new microbial substance. Part 8 *"Environmental Fate"* in PMRA's (2001) guidelines for the registration of microbial pest control agents and products also provides guidance in this respect.

Sections 9 to 14 provide guidance on recommended biological test methods for measuring the pathogenic and/or toxic effects of new microbial substances on aquatic and terrestrial plants and animals. Each of these six sections is specific to a particular category of test organisms (i.e., aquatic plants, aquatic invertebrates, aquatic vertebrates, terrestrial plants, terrestrial invertebrates, and terrestrial vertebrates). In each, a brief summary is included on historic procedures and methods used by various investigators to determine the effects of micro-organisms or microbial products on that category of test organisms. Thereafter, the standardized biological test method(s) for measuring toxic and/or pathogenic effects of various new microbial substances on that category of test organisms recommended herein for testing their environmental effects are described. Details on appropriate modifications and adaptations for each recommended biological test, when testing for pathogenic and/or toxic effects of new microbial substances, are provided. Mention is also made of other methods or specialized test procedures that could be performed using these or other *host organism*s within each general category, when testing for the adverse effect(s) of a particular new microbial substance.

Overview

The guidance herein addresses the following wording in Section 4.2.7 of the guidelines for the notification and testing of new substances including micro-organisms, published jointly by Environment Canada and Health Canada (EC and HC, 2001):

> "*Data on the effects of the notified micro-organism on appropriate plant, invertebrate, and vertebrate species from the aquatic and terrestrial environments should be provided. Data from six tests (aquatic plant, vertebrate, and invertebrate species as well as terrestrial plant, vertebrate, and invertebrate species) should be provided for notifications under subsection 29.11 (1)* [of the NSN Regulations under CEPA 1999], *whereas three tests (aquatic plant, vertebrate, and invertebrate species or terrestrial plant, vertebrate, and invertebrate species) should be provided for notifications under paragraph 29.11(2)(a) of the Regulations. These data should be provided from* in vivo *animal or plant tests*."

Accordingly, six tests involving both aquatic and terrestrial plants, invertebrates, and vertebrates are required in most instances when a new microbial substance is proposed by a notifier for import or manufacture in Canada and the potential receiving environment is not limited to any one particular *ecozone* (Government of Canada, 1997).[11] Only three tests are necessary when the new microbial substance will only ever be introduced into one ecozone wherein the micro-organism is not *indigenous* to that ecozone (EC and HC, 2001).[12] No tests for pathogenicity and/or toxicity are required when the new microbial substance is intended to be introduced to one or more specified ecozones wherein the micro-organism is indigenous (Government of Canada, 1997). Other circumstances might also apply whereby it is not necessary for the notifier to conduct any such tests (see Section 4.2.7.1 in EC and HC, 2001).

This guidance document is intended to complement and expand on the guidance found in "*Guidelines for the Notification and Testing of New Substances: Organisms*" (EC and HC, 2001) for testing the pathogenicity and/or toxicity of new substances that are micro-organisms. In particular, it expands on Section 4.2.7 "*Information in respect of the ecological effects of the micro-organism*" in that document, while relying on the guidelines in EC and HC (2001) regarding related information requirements. For instance, the following technical information requirements in Section 4 (EC and HC, 2001) are pertinent when testing new microbial substances for pathogenic and/or toxic effects to aquatic or terrestrial plants or animals:

(i) information pertaining to the biological, physical, and chemical characteristics of the new microbial substance;

(ii) known information on the biological and ecological responses and adaptability of the new microbial substance (including its *environmental expression*);

(iii) information on any field studies for environmental effects;

(iv) information on the environmental fate of the new microbial substance; and

(v) information on the human health effects of the new microbial substance.

The guidelines for notification and testing described in EC and HC (2001) should be consulted and followed when addressing these information requirements.

While complementing and expanding on the guidance in EC and HC (2001), the present report

[11] A map of the 15 ecozones of Canada is provided in Appendix 2 of EC and HC (2001).

[12] The rationale for requiring a lesser number of tests in this instance is that the receiving environment is restricted and so is the number of potentially exposed plants, invertebrates, and vertebrates.

has benefited from a number of key guidance documents related to measuring pathogenic and/or toxic effects of new microbial substances on aquatic or terrestrial organisms. In 1995, Environment Canada held a workshop entitled "*Workshop on Guidance for Notification of Hazard Information on Microbial Products of Biotechnology Under the Canadian Environmental Protection Act*" (EC, 1996). Much of the guidance agreed to at that workshop is found in Section 4.2.7.1 of EC and HC (2001). The *"Series 885 Microbial Pesticide Test Guidelines"*[13] published by the USEPA's Office of Prevention and Toxic Substances in 1996 provides a wealth of information specific to testing microbial pesticides for pathogenic and/or toxic effects on selected species of aquatic or terrestrial plants and animals (USEPA, 1996a-v). Health Canada's Pest Management Regulatory Agency (PMRA, 2001) relied on this series of test guidelines when outlining requirements for the registration of microbial pest control agents (MPCAs) or their *end-use products* (*EPs*). A 1991 European Directive for registering pesticides including microbial products also relied on unspecified test guidelines (*"e.g., the USEPA testing guidelines"*) accepted by a competent authority using a series of tests involving aquatic and terrestrial organisms, without providing any specific guidance for individual biological test methods (Douville, 2001). Using an approach harmonized with USEPA (1996a,b), Japan has adopted testing guidelines for MPCAs or EPs which are based on a series of single-species tests with aquatic and terrestrial plants and animals (Katoh, 2001). The guidance herein is largely consistent with these international approaches.

The focus of this guidance document is on recommended biological test methods and associated procedures for measuring the pathogenicity and toxicity of new microbial substances to potentially sensitive species and life stages of aquatic and terrestrial plants and animals. *Pathogenicity* refers to the ability of a micro-organism to infect a host (in the current context, a test organism), establish itself and multiply there, and subsequently inflict injury or damage that might or might not lead to death. *Toxicity* refers to the ability of a test substance to cause adverse effects (*sublethal* or *lethal*) on living plants or animals due to its poisonous (toxic) nature. These two terms are inextricably linked, as is the measurement of pathogenic or toxic effects in host (test) organisms due to a new microbial substance.

Pathogenic and toxic substances are both capable of causing gross and microscopic anomalies (i.e., damage to tissues or organs, as observed by *necropsy* or histological examination). These substances can also cause *sublethal effects* such as growth retardation or impaired reproductive success, which are common biological *endpoints* in sublethal *toxicity tests*. Both pathogenic and toxic substances can cause the death of host organisms. Additionally, certain micro-organisms produce *toxin*s that can affect the host (test) organisms by way of a toxic response (i.e., *toxigenicity*). Certain microbial products can also contain organic or inorganic substances within their formulation that could cause a toxic response by themselves or in combination with the micro-organisms therein. The USEPA (1996b,n) recognized this, in stating that recommended biological test methods for measuring the adverse effects of microbial pest control agents (or their end-use products) are designed to simultaneously measure both toxicity and pathogenicity. Inasmuch as the biological endpoints measured in standardized laboratory tests for adverse effects (e.g., reduced growth, impaired reproductive success, behavioural alterations, gross or microscopic pathologies, or death) of micro-organisms are incapable of distinguishing if these effects are due to pathogenicity or toxicity, such test methods are referred to herein as *"tests for pathogenic and/or toxic effects"*.

An important distinction between effects due to toxicity or pathogenicity is that host organisms respond differently to substances whose primary adverse effects are either due to their toxic (i.e., poisonous) or pathogenic (i.e., *disease* causing) properties. Test (host) organisms respond to a range of concentrations of a toxic chemical substance in a generally predictable manner (i.e., according to a log-concentration response curve), whereas this is not normally the case for microbial substances. The concentration-dependent (and time-related) responses of test organisms to pathogenic

[13] Each of the Series 885 test guidelines can be accessed through the Internet at www.epa.gov/docs/OPPTS_Harmonized/, by selecting "885 - Microbial Pesticide Test Guidelines".

substances can differ markedly from those caused by toxic chemical substances. Toxic chemicals can be diluted to harmless concentrations, while micro-organisms that have successfully infected the host (test) organism can multiply and cause sublethal or lethal effects beginning with a few invading microbial units, given adequate time, appropriate conditions, and susceptibility of the host. Accordingly, the occurrence of pathogenic effects in host organisms exposed to infectious new microbial substances is frequently unrelated to the initial exposure concentrations in a predictable (log concentration) manner typical of effects caused by toxic chemical substances. Suter (1985) succinctly and correctly stated "*It is generally not possible to identify a concentration of an introduced organism with a level of effect....*". Exceptions occur when pathogenic micro-organisms exert their adverse effect(s) by producing endotoxins or toxic metabolites, or when other (non-biological) substances associated with a new microbial substance (e.g., those formulated within a microbial product) prove harmful depending on concentration.

Toxic sublethal or lethal effects can occur rapidly, depending on the exposure concentration, nature of the toxicant, susceptibility of the test organism, etc. Infectious micro-organisms invading a host organism in small numbers, however, might multiply (replicate) in tissues and organs over an extended period of time before their pathogenic effects are realized. Other differences in modes of effect on test (host) organisms caused by pathogenic micro-organisms versus toxic chemicals have been addressed in overview articles (Suter, 1985; Dean-Ross, 1986; Briggs and Sands, 1992; Spacie, 1992). Such differences require modification of certain procedures, when applying standardized biological test methods designed for measuring toxic effects, to new microbial substances that can exert both pathogenic and toxic effects. Recommendations regarding appropriate changes in procedures and conditions for existing biological test methods designed initially for measuring toxic effects of chemical substances, are included in this guidance document on using single-species tests for measuring adverse pathogenic and/or toxic effects of new microbial substances on aquatic or terrestrial organisms.

As a further corollary on the inextricable link between pathogenic and toxic effects and the inability to clearly distinguish such from each other, Article 64 of the *Canadian Environmental Protection Act*, 1999 defines "*toxic*" as:

> *.....a substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions that:*
> *(a) have or may have an immediate or long-term harmful effect on the environment or its biological diversity;*
> *(b) constitute or may constitute a danger to the environment on which life depends; or*
> *(c) constitute or may constitute a danger in Canada to human life or health.*

According to this definition, a new microbial substance that exerts or is capable of exerting a harmful effect on (or danger to) the environment is classified as "toxic *vis-à-vis* CEPA 1999" , whether it causes an adverse effect due to its pathogenicity or toxicity (or both).

The biological and physicochemical characterization of a new microbial substance to be tested in the laboratory for pathogenic and/or toxic effects on host organisms is highly recommended as an essential prerequisite to such a testing program. Section 3 refers to key guidance found in EC and HC (2001), that applies in this respect. Included here is guidance on preparing certain new microbial substances to be administered in water, *sediment*, soil, and food at the start of (and, in some instances, during) tests for their pathogenic and/or toxic effects on certain categories of test (host) organisms. Recommended concentrations to be applied when performing either a single-concentration test or a multi-concentration test are also considered in Section 3. Additionally, guidance on quantifying the concentration of viable micro-organisms in individual tests at their initiation and, depending on the test method, as the test proceeds and/or at its end, is provided (Section 3).

For the results of any laboratory test for pathogenic and/or toxic effects to be valid and meaningful, a *treatment* involving a *negative control* must be included as an integral component of the test. *Positive control* treatments (i.e., use of a *positive chemical control* and/or a *positive microbial control*) are also warranted for inclusion in certain tests, to assist in providing *quality assurance* and *quality control*. A *sterile filtrate control* and/or a

non-infectious control is also useful and recommended for inclusion in most tests to assist in interpreting the findings and especially in providing insight into the causative nature (i.e., pathogenicity and/or toxicity) of adverse effects observed during a particular laboratory test with a new microbial substance. Section 4 addresses the various types of control treatments that are recommended or required when testing for such effects.

Infectivity means the ability of a micro-organism to cross or evade natural host barriers to infection. The measurement of infectivity is not a prerequisite to any controlled test intended to measure the pathogenic and/or toxic effects of a new microbial substance, although it provides useful information that aids in distinguishing if adverse effects observed are likely attributable to an infection and ensuing symptoms of *disease* (i.e., pathogenicity). As such, concurrent measurements for infectivity should be considered when planning the biological test methods (and associated test procedures) to be applied for measuring the pathogenicity and/or toxicity of a new microbial substance. Section 5 provides guidance on measuring infectivity during certain tests; further guidance in this respect is found in the sections dedicated to biological test methods recommended for specific categories of test (host) organisms (i.e., Sections 9 to 14 inclusive).

An understanding of the behaviour of a new microbial substance, when it is applied to or comes in contact with various environmental compartments (i.e., air, water, sediment, or soil), is very useful if not essential when choosing the appropriate series of biological test methods to apply for measuring and evaluating the adverse environmental effects of this substance. Laboratory tests that investigate the *environmental expression* of micro-organisms have been described in USEPA (1996x,y,z,aa). These "expression" tests determine, under controlled conditions, the effect of varying certain environmental variables (e.g., for the aquatic environment — temperature, pH, conductivity or *salinity*, light intensity, dissolved oxygen, turbulence; for the terrestrial environment — temperature, pH, light intensity, humidity, precipitation, nutrients) on the survival and replication of the micro-organisms. Item (v) in Section 4.2.1.6 of EC and HC (2001) advises the notifier that information should be provided to describe ranges and optima for significant environmental variables, pertaining to the survival and replication of micro-organisms. Parts 2.7.2 and 8.2 of Health Canada's guidelines for the registration of MPCAs and EPs (PMRA, 2001) also list information requirements on the biological responses of microbial pesticides to certain environmental variables, that should be provided as part of the registration process. Persons choosing the series of tests for pathogenic and/or toxic effects to be applied to a particular new microbial substance should have on hand basic information on the *environmental expression* of that substance before making decisions on the test methods to be used. Such information includes an understanding of the influence of variations in key testing conditions (e.g., temperature, pH, lighting conditions, conductivity or salinity if testing aquatic organisms; or humidity and/or moisture content if the substance is administered directly or indirectly to terrestrial organisms) including those representative of such conditions that could or would be applied when performing each biological test method under consideration, on the survival, growth, and replication of the new microbial substance under consideration.

Boxes with bullets identifying the salient points preface the wording for certain sections and subsections of this document to assist in summarizing and highlighting key guidance. These "key guidance" boxes and bullets also serve to introduce the more important elements of the guidance provided under the headings for those sections. Since such summary points cannot explain in full the various recommendations and their implication, the reader pursuing guidance on various subjects is urged to read the subsequent text in full.

Characterizing, Preparing, and Administering New Microbial Substances

3.1 Known Characteristics of the Test Material

Key Guidance

- *The biological and physicochemical characteristics of the test material should be known before selecting and undertaking an appropriate series of biological test methods to measure pathogenic and/or toxic effects of a new microbial substance.*
- *The technical information requirements for characterizing a test material detailed in Section 4.2 of EC and HC (2001) should be consulted, and such information obtained, reviewed, and considered.*
- *Information on the environmental expression of the new microbial substance is particularly relevant and important.*

The biological and physicochemical characteristics of each new microbial substance to be evaluated for pathogenic and/or toxic effects should be known before the laboratory tests for ecological effects are undertaken. Persons responsible for the selection and performance of the appropriate series of biological test methods to be applied to the test material (i.e., a new microbial substance or, in some instances, a blend of micro-organisms or a microbial product containing that substance) should carefully review and consider this information before the testing program is initiated.

Part II.1 and its associated Schedule XV of the *New Substances Notification Regulations* in effect under CEPA 1999 specify the information requirements with respect to micro-organisms.[14] Section 3.2 in EC and HC (2001) describes how to identify the information necessary to comply with Part II.1 of the Regulations. Section 4.2 in EC and HC (2001) expands on this by detailing technical information requirements specific to the characteristics of a new microbial substance:

- taxonomic identification of the micro-organism(s) (Section 4.2.1.1).
- history of micro-organism(s) (including culture and storage conditions; name of strain, if applicable; method of isolation and identification; and any genetic modifications) (Section 4.2.1.3).
- description of any modifications to the micro-organism (Section 4.2.1.4).
- description of methods that can be used to distinguish and detect the micro-organism (Section 4.2.1.5).
- description of the known biological and ecological characteristics of the micro-organism, including: life cycle; infectivity, pathogenicity, toxicity, and toxigenicity to non-human species; tolerance to heavy metals, pesticides, and *antimicrobials*; involvement in biogeochemical cycling; conditions required for and conditions that limit survival, growth, and replication[15]; and

[14] These Regulations are available through the Internet at http://laws.justice.gc.ca/en/C-15.31, by choosing "New Substances Notification Regulations" under "Related Regulations". Part II.1 of the Regulations, as well as Schedule XV, are each identified separately in the associated listing, for easy access.

[15] As stated in this section of EC and HC (2001), information should be provided describing ranges and optima for significant environmental parameters such as pH, temperature, salinity (if *estuarine* or *marine* water), oxygen, and particle size (if sediment or soil), that influence the survival, growth, and/or replication of the micro-organism. If survival, growth, or replication is known to be limited by specific parameters, this information should also be provided. Section 2 herein refers further to such measurements of environmental expression and their importance when designing an appropriate set of biological tests for measuring the pathogenic and/or toxic effects of new microbial substances and when interpreting the findings of these laboratory tests relative to potential ecological effects.

mechanisms of micro-organism dispersal (Section 4.2.1.6).

- description of the known mode of action in relation to the intended use (e.g., how the micro-organism functions by altering the physical, chemical, or biological environment) (Section 4.2.1.7).

- description of the reasonably expected by-products following introduction to the environment (Section 4.2.1.8).

- description of the potential for, and mechanisms by which, genetic transfer to other organisms can occur for traits such as pathogenicity, toxigenicity, and antimicrobial resistance (Section 4.2.1.10).

- description of the geographic distribution of the micro-organism (Section 4.2.1.11).

- physical state of the microbial product (e.g., powder, suspension, mist; particle size; nature of any carrier medium) (Section 4.2.2.4).

- concentration of the micro-organism in the microbial product (Section 4.2.2.5).

- identification and concentration of other ingredients and of any *contaminants* in the microbial product (Section 4.2.2.6).

- viability of the micro-organism in the microbial product (Section 4.2.2.7).

- description of any recommended storage and disposal procedures (Section 4.2.2.8).

- information pertaining to the environmental fate of the micro-organism (e.g., potential plants and animals that could be exposed to the micro-organism based on its intended introduction to the environment; description of habitats where the micro-organism might persist or proliferate; estimated quantities of the micro-organism in the air, water, and soil at points of introduction) (Section 4.2.6).

- existing information regarding the ecological effects of the micro-organism (Section 4.2.7).

- information concerning the human health effects of the micro-organism (e.g., any documented involvement of the micro-organism in adverse human health effects; a description of the characteristics of the micro-organism that distinguish it from known human *pathogens*; data from tests of antimicrobial susceptibility; data from tests of pathogenicity for related micro-organisms; potential for adverse *immunologic* reactions in persons exposed to the micro-organism; estimated number of persons that might become exposed and the degree of exposure to the micro-organism) (Section 4.2.8).

Persons responsible for the selection, design, and conduct of laboratory tests for pathogenic and/or toxic effects of a new microbial substance on aquatic or terrestrial organisms should consult the pertinent sections of EC and HC (2001) for a further description of these information requirements. All relevant information on the biological and physicochemical characteristics of a new microbial substance to be evaluated should be reviewed before proceeding with these biological tests for ecological effects. This background information should also be considered when interpreting the results of the laboratory tests for pathogenic and/or toxic effects.

3.2 Test Material and Route(s) of Exposure

Key Guidance

- *When testing for pathogenic and/or toxic effects, the test material used in each biological test method may be one of the following: a pure culture of a particular micro-organism; a consortium of micro-organisms; a blend of two or more pure cultures of particular micro-organisms; or a representative sample of the commercially formulated microbial product.*

- *Before finalizing or commencing the testing program, a pre-notification consultation with the NSB is strongly recommended to discuss and decide on the appropriate test material to be used with each intended test for ecological effects.*

- *Test (host) organisms should be exposed to a new microbial substance using the route(s) of exposure anticipated to be the most significant means of their exposure in the natural environment.*

- *If a single-concentration test using two exposure routes at their respective MHCs proves harmful to the test (host) organisms, and no adverse effects are found in a multi-concentration test using one of these exposure routes at the MHC and lower concentrations, a second multi-concentration test must be performed using the other exposure route(s) included in the single-concentration test.*

- *For a given test, the route(s) of exposure can influence as well as depend on the type of test organism and the associated biological test method to be applied. Appropriate (test dependent) exposure routes include suspension in water, mixing in sediment or soil, and mixing in food to be offered to the test organisms. For terrestrial vertebrates (i.e., birds or small mammals), the suitable route of exposure might, depending on the nature of the test material and its intended manner of entry to the environment, be orally (by gavage) or by inhalation.*

- *Multiple routes of exposure should be restricted to single-concentration tests with plants, invertebrates, or fish, and are only recommended in instances where more that one type of exposure of the test organism (e.g., in water and food; in water and sediment; in water and soil) would be possible or anticipated in the natural environment following the intended use of the new microbial substance.*

Schedule XV within the NSN Regulations addresses the requirement for data from laboratory tests conducted to determine the ecological effects of a micro-organism designated as a *new substance*. In keeping with these Regulations and their definition of a new substance (EC and HC, 2001), the notification process (including registry of micro-organisms in Canadian commerce on the Domestic Substances List) is specific to new micro-organisms rather than new microbial products. However, for the purpose of testing a new microbial substance in the laboratory for its potential ecological effects, the test material might, in some instances, be a microbial product.[16] Where applicable, the test material might also be a blend of two or more micro-organisms.[17] Accordingly, the test material might be one of any of the following:

- a pure culture of a micro-organism,
- a *consortium*,
- a *blend* of two or more pure cultures, or
- a microbial product.

Before proceeding with any definitive laboratory tests intended to measure the pathogenic and/or toxic effects of a new microbial substance, the notifier (and, as appropriate, involved consultants and the Study Director responsible for each test; see Section 6.1) should participate in a Pre-Notification Consultation with representatives of Environment Canada's New Substances Branch (see Section 1.1). One of the objectives of this consultation would be to discuss the proposed testing program and, for each test method to be applied, the proposed test material and test (host) organism. The choice of test material depends on a number of considerations, including the known physicochemical characteristics of the new microbial substance, the ingredients used to formulate the microbial product, and the biological test method under consideration.

For tests with aquatic or terrestrial host organisms (see Sections 9 to 14 inclusive), the new microbial substance is the test material for which notification is required under the NSN Regulations. Notifiers, however, must be aware that there are certain instances whereby the formulated microbial product (rather than the new microbial substance alone)

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notification of the new additive or carrier is also required. In this instance, see "*Guidelines for the Notification and Testing of New Substances: Chemicals and Polymers*" (Government of Canada, 2001) for additional information.

[17] Each micro-organism within a blend that does not appear on the DSL must be notified separately. However, it might be possible to use data generated on the blend as surrogate data for each micro-organism notified. Before proceeding with the tests, the notifier is advised to seek guidance from Environment Canada (see *"Pre-Notification Consultation"* in Section 1.1), and consider the known characteristics of each pure culture in the blend. In instances where there is reason to suspect a masking (reduction) of possible ecological effects on host (test) organisms due to their interaction, the notifier should consider testing some if not all of the pure cultures separately, for pathogenic and/or toxic effects.

[16] In the event that an additive or carrier in a microbial product acts to enhance the toxicity and/or pathogenicity of a new microbial substance, the microbial product might be considered to be the preferred test material. Additives and carriers used in a microbial product must also appear on the DSL. If they are *new substances* themselves,

should be tested. For instance, if an additive or carrier is known or thought to enhance the toxicity and/or pathogenicity of a new microbial substance, the formulated microbial product might be considered as the preferred test material.[18] Conversely, if the inanimate carrier used in the microbial product has a high oxygen demand when mixed in water, which (based on preliminary studies) contributes to a depressed dissolved oxygen in test chambers containing invertebrates (e.g., *Daphnia magna*; see Section 10.1.2) or fish (see Section 11) and results in adverse effects unrelated to the product's pathogenicity and/or toxicity, the use of the new microbial substance(s) alone in such aquatic tests would be a preferred choice. The influence of inert additives used to formulate a product on its solubility in water should also be considered, when choosing the test material. If these carriers and the resulting microbial product are not very water soluble, consideration should be given to performing the laboratory tests for ecological effects using the new microbial substance alone. For tests with birds or small mammals (see Sections 14.1 and 14.2), the use of the new microbial substance(s) alone (rather than the microbial product) is also preferred in most instances, since the volume of inanimate inorganic and/or organic matter used in formulating certain microbial products can contribute to undue stress (and adverse effects unrelated to the product's pathogenicity and/or toxicity) when administered orally or by inhalation.

For a given test, any test material applied on more than one occasion must be from the same *lot* unless the new microbial substance or its formulated product is known or likely to be unstable during storage. If this is the case, fresh preparations of the new microbial substance, each prepared using an identical procedure, should be used during each administration of the test material. The test material used in each of a series of biological test methods to measure and evaluate the pathogenic and/or toxic effects of a new microbial substance should ideally be from the same lot. If this proves unfeasible, all lots of the test material should be prepared using an identical procedure (and formulation, if a product), and must be as similar in physicochemical and biological characteristics (see Section 3.1) as possible.

When choosing the test material to be used in a particular biological test method (see Sections 9 to 14 inclusive), an important consideration is the state of the new microbial substance. If, for example, the test material contains bacterial or fungal spores, consideration should be given to whether the spore state or its vegetative state is more likely to cause pathogenic and/or toxic effects on the test (host) organisms. If existing information for this new microbial substance or similar substances indicates that the vegetative state is more likely to cause adverse effects on a particular host organism than the spore state, consideration should be given to performing a test with that host using a culture of the vegetative state of this substance. If, however, existing evidence suggests that the spore state is more likely to cause pathogenic and/or toxic effects (e.g., for a test involving a particular fungal spore and terrestrial plants as hosts), a particular test might best be performed using the spore state of the new microbial substance. A tentative decision to use a particular state (i.e., vegetative, spore, or cyst) of a new microbial substance in one or more planned biological test methods for pathogenic and/or toxic effects should be discussed with Environment Canada's New Substance Branch as part of the Pre-Consultation Notification process (see Section 1.1).

The manner in which test (host) organisms are exposed to a new microbial substance depends on a number of considerations, such as:

(1) the type of host organism;

(2) the biological test method to be conducted;

(3) the nature of the test material (e.g., a liquid suspension or a pelletized solid); and

(4) the intended mode of application of the new microbial substance (e.g., as a spray or by distribution in a powdered or pelletized form).

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[18] A notifier might not have an end-use microbial product available when developing the notification submission, in which instance the notifier must demonstrate that the intended additives will not enhance any potential pathogenic and/or toxic effects of the notified substance.

As a general rule, host organisms should be exposed to a new microbial substance (alone or within the microbial product) using the route(s) of exposure anticipated to be the most significant means of exposure to this substance in the natural environment. It is essential to test the most challenging form of the new microbial substance (or, if appropriate, the microbial product containing this substance), in terms of its potential pathogenic and/or toxic effects on aquatic and terrestrial plants and animals.

A single-concentration test (see Section 3.3.1) is performed at one test concentration only, for each of the one or, sometimes, two exposure routes included in the test. For certain test methods, such as a test using aquatic plants (see Sections 9.1.2 and 9.2.2, certain aquatic invertebrates (see Section 10.1.2), or terrestrial vertebrates (see Sections 14.1.2 and 14.2.2), a test-specific single-concentration test will include only one exposure route (e.g., in the water, or, for a test with birds or small mammals, by gavage or inhalation). In other instances, a single-concentration test might include two exposure routes applied simultaneously to the test (host) organisms (e.g., in the water and sediment if a certain type of aquatic invertebrate; in the food and water if shrimp or fish; in the soil and water if a terrestrial plant; in the soil and the food for certain soil-dwelling invertebrates). This "combined exposure" approach is cost effective, and is recommended for certain single-concentration tests when more than one exposure route (e.g., in the food and in the water, if shrimp or fish; in the water and in the sediment, if an *infaunal* aquatic invertebrate such as a species of amphipod or polychaete worm) is possible or likely following the intended use of a particular new microbial substance. For a multi-concentration test (see Section 3.3.2), the use of more than one route of exposure during an individual test is impractical[19]; therefore, only one route of exposure should be applied. In instances where more than one route of exposure to a particular new microbial substance is possible or of concern in the environment, a separate multi-concentration test should be performed using each of these routes of exposure alone. For example, for multi-concentration tests involving a species of fish, one test should be performed exposing groups of test (host) organisms to a range of concentrations of the new microbial substance in water, and a second test performed exposing groups of test organisms to a range of concentrations of the new microbial substance mixed in their food. A test result that indicates the absence of an adverse effect at the MHC (and lower concentrations) for a given multi-concentration test suggests that the exposure route used was probably not the route responsible for the adverse effect(s) observed in the previous single-concentration test involving two concurrent exposure routes. In such an instance, a second multi-concentration test using the other exposure route used concurrently in the preceding single-concentration test (for which an adverse was found) should be performed. The findings for each multi-concentration test performed using a differing exposure route will provide useful information regarding the harmful route(s) of exposure as well as the concentration-dependent effect(s).

Sections 9 to 14 inclusive provide a description of the route(s) of exposure that are to be used when performing a specific biological test method recommended therein. For tests involving aquatic plants, invertebrates, or vertebrates (i.e., fish), suspension of the new microbial substance in the *test water* is the primary route of exposure. Single-concentration and multi-concentration tests involving aquatic plants or *pelagic* or certain *epibenthic* invertebrates (e.g., daphnids or mysids) should only use this route of exposure (see Sections 9 and 10). In instances where single-concentration tests involve shrimp or fish that are offered food during the test, the new microbial substance should be administered in the food as well as in the water (see Sections 10 and 11). Exposure by mixing the test material in the sediment as well as suspending it in the overlying water is recommended (see Section 10) for single-concentration tests with *infaunal benthic* invertebrates (e.g., *Hyalella azteca* or midge larvae if freshwater tests; marine or estuarine polychaete worms if seawater tests) that incorporate a sediment *substrate* together with *overlying water*.

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[19] A multi-concentration test is intended to define the effect of a range of concentrations of the new microbial substance administered to the test organisms in a discrete and consistent manner. For a given test, determining the effect of the new microbial substance on a concentration-dependent basis (and, where calculations permit, determining an endpoint such as an IC25 or *EC50*) can only be achieved using one route of exposure.

Exposure routes for single-concentration tests involving terrestrial plants might include both the mixing of the new microbial substance in the *test soil* to be used, as well as mixing the new microbial substance in the test water to be used throughout the test (see Section 12). The route of exposure for single-concentration tests with terrestrial invertebrates might be by mixing the new microbial substance in food (if honey bees), or by mixing it in food and soil (if earthworms) (see Section 13). For tests involving birds or small mammals, the exposure route should be that most likely to cause pathogenic and/or toxic effects during or subsequent to the application of the new microbial substance within the terrestrial environment. Accordingly, the recommended exposure route for tests with terrestrial vertebrates is orally by *gavage* or by inhalation (see Section 14). Only one exposure route should be used in any test with a terrestrial vertebrate, to minimize undue stress associated with the administration of the test material.

Researchers have used various routes for administering micro-organisms to host (test) organisms, other than those recommended herein. These include the following exposure routes (Douville, 2001):

- for aquatic plants—by wounding *shoots* of Eurasian water milfoil, followed by immersion of plants in a suspension of micro-organisms in water.

- for aquatic macro-invertebrates—by direct injection (intrahemocoelic); or by feeding diseased prey.

- for aquatic vertebrates (fish)—by direct injection (intramuscular or intraperitoneal) of the micro-organism; by feeding live prey previously exposed to the micro-organism under investigation; by dermal abrasion associated with aqueous exposure; or by gavage feeding via a tube inserted into the stomach.

- for terrestrial plants—by wounding leaves, stems, or *roots*, followed by plant exposure to the micro-organism under investigation by spraying or dipping; by hypodermic or pin-prick injections of leaves or stems; by treatment of *seeds*; or by injection of micro-organisms into tubers or bulbs.

- for terrestrial invertebrates—by direct injection; by feeding infected leaves or infected pollen; by feeding infected prey; or by topical application.

- for terrestrial vertebrates (birds or small mammals)—by intraperitoneal injection; by intravenous injection; by subcutaneous injection; by intramuscular injection; by intranasal injection; by intracerebral injection; by dermal application; or by infected food.

Such exposure routes are, in most instances, not recommended when testing new microbial substances for potential adverse environmental effects in keeping with the NSN Regulations in effect under CEPA 1999. These exposure routes have not been chosen due to a number of considerations, including:

(i) their lack of "environmental realism";

(ii) the absence of standardized biological test methods for measuring the environmental effects of new microbial substances using such exposure routes;

(iii) cost-benefit considerations; and/or

(iv) the availability of appropriate standardized test methods using (other) environmentally realistic routes of exposure to the new microbial substance.

Exceptions do exist, however, such as in the case where a specific biological test method recommended in Sections 9 to 14 is not appropriate, in which instance a modified approach such as described in separate sections herein might be preferable and appropriate for use. Such a preference could, for example, be justified by the characteristics of the new microbial substance and/or host (test) organism known to be necessary to cause a pathogenic effect. For example, a modified approach might include wounding of a particular species of terrestrial plant at a specified period of time (and a particular developmental stage) prior to infection, or else a requirement for the exposure of a

particular plant part or life stage that differs from that in the recommended test method described in Section 12.2.

3.3 Determining and Expressing Test Concentrations or Doses

Key Guidance

- *As a cost-savings measure, it is recommended that definitive tests for pathogenic and/or toxic effects initially be performed using a single* "maximum hazard concentration (MHC)". *Additional testing at lower concentrations is not necessary if no adverse effects are evident at this concentration.*
- *A multi-concentration test, which includes the MHC as its highest concentration, should be performed if the MHC proves harmful in a single-concentration test (or if the investigator chooses to proceed directly to a multi-concentration test). Choice of test concentrations should attempt to include lower ones that cause no harmful effects, and be selected to enable the appropriate statistical endpoint(s) for a multi-concentration test to be calculated, data permitting. A preliminary range-finding test is recommended to establish an appropriate range of concentrations.*
- *With the exception of tests involving terrestrial vertebrates, each test concentration used in a single-concentration test or a multi-concentration test should be expressed and reported as the nominal number of microbial units per millilitre or gram (dry weight) of test substrate in which the test material is mixed.*
- *For tests with birds, each test dose including the MHD should be expressed and reported as the nominal number of microbial units per millilitre in the aqueous suspension of the test material administered × quantity of test material consumed or inhaled/body wt × wt of test organism. For these tests, the dose(s) is/are administered daily for five consecutive days only, at the start of the test.*
- *For tests with rodents, each test dose including the MHD should be expressed and reported as the nominal number of microbial units per millilitre in the aqueous suspension of the test material administered. For these tests, the dose(s) is/are administered once only, at the start of the test.*

A biological test method may be performed as a single-concentration test or as one involving multiple concentrations. Initially, a single-concentration test is frequently performed using a single "*maximum hazard concentration (MHC)*" of the test material (see Section 3.3.1). Depending on the test method, this single-concentration test may involve a single exposure route or, in some instances, two exposure routes. If two exposure routes are used in a single-concentration test, the test-specific MHC (see Section 3.3.1) is applied concurrently during the test via each of these routes, as a cost-effective approach intended to maximize the exposure. If, for the exposure route(s) used, no adverse effects are observed at this concentration, additional testing of lower concentrations will not be necessary (EC and HC, 2001). However, if adverse effects are observed in a single-concentration test at the maximum hazard concentration(s) employed, a multi-concentration test which includes the MHC and lower concentrations (applied using a single exposure route) is recommended to determine the threshold-effect concentration that affects the biological endpoint(s) being measured (e.g., survival, growth, reproductive success, incidence of gross or histological pathologies). Paragraph 8 in Section 3.2, as well as Section 3.3.2, provide guidance when undertaking a multi-concentration test.

3.3.1 Single-Concentration Test and MHC (or MHD)

Single-concentration tests should be performed using the *maximum hazard concentration (MHC)* of the test material. The MHC is based on a safety factor × the highest concentration of viable micro-organisms to which groups of aquatic or terrestrial organisms could be exposed in the environment following the application of a particular microbial product at its maximum recommended rate.

When determining the MHC, the concentration of a new microbial substance in a particular test material or substrate is derived using calculations and measurements of the number of *microbial units* in a given quantity of that material or substrate. The definition of a microbial unit depends on the nature of the micro-organism, as follows (from USEPA, 1996o, with modifications):

(i) *Bacterial or fungal spore, bacterial or protozoan cyst.* A microbial unit is an intact, viable spore or cyst, as determined microscopically or by other means. It is also usually the viable entity that produces a single colony forming unit (CFU) on an appropriate germination medium. Viability can be determined using a viability stain, or a germination test on appropriate medium.

(ii) *Fungal mycelium.* A microbial unit is equivalent to 10^{-9} g dry weight, after using standardized preparatory procedures involving a viable mycelium-producing entity produced on a semi-solid growth medium.

(iii) *Protozoan.* A microbial unit is an intact, viable vegetative organism, spore, or cyst of the members in the various classes of this phylum.

(iv) *Vegetative bacterium*. A microbial unit is a single, viable organism, usually the entity that produces a single CFU on an appropriate semi-solid growth medium.

(v) *Virus.* A microbial unit is an intact, complete *virion* or a polyhedral body as determined by electron microscopy. It is also usually the entity that produces an infective unit on appropriate host cells or tissues.

3.3.1.1 Administering MHC in test water to aquatic organisms. The USEPA (1996d,e,g) provides a worthwhile standard approach for calculating and administering the MHC in water, that is applicable to tests involving aquatic plants, invertebrates, or vertebrates. Using this approach, which was also recommended by EC and HC (2001), the MHC is defined as:

> *The maximum hazard concentration is 10^6 units/mL, or 1000 times the expected micro-organism concentration in the aqueous environment, whichever is greater and readily attainable.*

The number of microbial units applied per measured volume of medium (i.e., 10^6 microbial units/mL) refers to the concentration of viable micro-organisms within the *test water* to which a measured quantity of the *test material* is applied, following mixing (see Section 3.4.1). When adding a test material as a suspension to the test water (see Section 3.4.1), the target concentration of viable micro-organisms in the test water following mixing would be 10^6 microbial units/mL.

In instances where 1000 times the expected micro-organism concentration in the aquatic environment is greater than 10^6 units/mL (see preceding paragraph), this higher concentration should be applied in the test as the MHC, provided that such a concentration is readily attainable under laboratory conditions[20] (USEPA, 1996d,e,g). This concentration is determined by multiplying, by 1000, the maximum calculated concentration of viable micro-organisms that would be anticipated to occur in water immediately following the direct application of a microbial product to the aquatic environment at the maximum application rate identified by the notifier for the product. If the test material is to be mixed in a given quantity of *test water* before starting the test for pathogenic and/or toxic effects, the calculated concentration of viable micro-organisms representing 1000 times the expected micro-organism concentration in the aquatic environment is determined. This calculation is based on 1000 times the maximum calculated

[20] The term "readily attainable under laboratory conditions" refers to the maximum concentration of the micro-organism that can be maintained in a test system without lowering its quality to an unacceptable level relative to that needed to support the test organisms. Particularly in aquatic test systems, the use of such a high concentration as represented by the MHC might be impractical in certain instances due to its adverse effect on water quality such as oxygen depletion and the production of metabolic by-products by the micro-organisms. The use of excessively high concentrations could result in non-specific adverse effects on the host (test) organisms unrelated to the test material's pathogenicity and/or toxicity, thus confounding the objectives and results of the study. However, in instances where such non-specific adverse effects might "constitute a danger to the environment on which life depends" (wording within the definition of "toxic" in CEPA 1999), the use of such a high concentration should be considered as practical and suitable for inclusion in the test. This is particularly true in instances where the species of test micro-organism might find itself under optimal conditions for multiplication and growth within the environment.

density of microbial units in a 15-cm layer of water immediately following the direct application of the end-use microbial product at its maximum application rate (USEPA, 1996d,e,g) and complete mixing immediately thereafter.

If added to test water, the maximum hazard concentration of micro-organisms to which test organisms are exposed in a single-concentration test should be expressed and reported as the nominal number of microbial units per millilitre of water in which the test material is mixed.

There are numerous cases where the MHC in test water, calculated as indicated here, causes problems in a particular test due to resulting adverse effects on the host (test) organisms that are unrelated to the test material's pathogenic and/or toxic effects. For instance, in certain tests with aquatic algae, the MHC might result in a competition for nutrients between the relatively high concentration of viable micro-organisms within the test material and the algae, with a resulting algal growth inhibition that is unrelated to the pathogenicity and/or toxicity of the test material. If results from preliminary testing indicate that this is the case, the concentration used in a definitive single-concentration test might be reduced to that representing the maximum application rate as opposed to 1000 times this rate (USEPA, 1996c). Similarly, in certain tests with daphnids (i.e., *Daphnia magna*), the MHC in the test water might adversely affect daphnid survival and/or reproductive success due to high turbidity caused by the large quantity of micro-organisms at this concentration. Another confounding factor might be the excessive oxygen depletion in a test with fish performed at the MHC, which is unlikely to occur within a well-oxygenated receiving environment. If preliminary tests at the MHC identify such problems that are both unrelated to the inherent pathogenicity and/or toxicity of the test material and unlikely to *"constitute a danger to the environment on which life depends"* (CEPA 1999), the concentration used in a definitive single-concentration test might once again be reduced from 1000 times the maximum application rate to 10 or 100 times this rate. A decision concerning this should be reached during or following a Pre-Notification Consultation with Environment Canada's New Substances Branch (see Section 1.1) which considers and discusses such test-specific confounding factors and their potential environmental implications.

3.3.1.2 Administering MHC in test sediment to aquatic invertebrates. When administering the MHC in *test sediment*, the approach used is much the same as that described previously for *test water*. For this application (see biological test methods recommended in Sections 10.1.3 and 10.2.3) the MHC is defined as:

> *The maximum hazard concentration is 10^6 units/g sediment, or 1000 times the expected micro-organism concentration in sediment within the aqueous environment, whichever is greater and readily attainable.*

The number of microbial units applied per measured weight of medium (i.e., 10^6 microbial units/g) refers to the concentration of viable micro-organisms within the *test sediment* to which a measured quantity of the *test material* is applied, following mixing (see Section 3.4.2). When adding a test material to the test sediment (see Section 3.4.2), the target concentration of viable micro-organisms in the test sediment following mixing would be 10^6 microbial units/g dry wt.

In instances where 1000 times the expected micro-organism concentration in the aquatic environment is greater than 10^6 microbial units/g sediment (dry wt), this higher concentration should be applied in the test as the MHC, provided such a concentration is readily attainable under laboratory conditions. This concentration is determined by multiplying, by 1000, the maximum calculated concentration of viable micro-organisms that would be anticipated to occur in sediment immediately following the direct application of a microbial product to the aquatic environment at the maximum application rate identified by the notifier for the product. If the test material is to be mixed in a given quantity of test sediment before starting the test for pathogenic and/or toxic effects, the calculated concentration of micro-organisms representing 1000 times the expected micro-organism concentration in the aquatic environment is determined. This calculation is based on 1000 times the maximum calculated density of microbial units in a 15-cm layer of sediment immediately following the direct application of the end-use microbial product at its

maximum application rate and complete mixing immediately thereafter.

If added to test sediment, the MHC of micro-organisms to which test organisms are exposed in a single-concentration test should be expressed and reported as the nominal number of microbial units per gram (expressed as dry weight) of test sediment in which the test material is mixed.

3.3.1.3 Administering MHC in test food to aquatic animals. Certain single-concentration tests with aquatic invertebrates or vertebrates recommended herein include the administration of the test material in the diet as well as the water (see Section 10.2.2 for tests with shrimp, and Sections 11.1.2 and 11.2.2 for tests with fish). The following guidance pertains for a test whereby a test material is applied to the test food offered to the test (host) organisms (see Section 3.2):

> *The maximum hazard concentration is 100 times the expected micro-organism concentration in the aquatic environment.*

This approach, including the definition of the MHC for test food offered to shrimp or fish during the test, is consistent with that prescribed by USEPA (1996e,g) when administering a microbial test material to shrimp or fish in the diet.

If the test material is to be mixed in a given quantity of food offered to shrimp or fish, the concentration of micro-organisms representing 100 times the expected micro-organism concentration in the aquatic environment should be calculated according to USEPA (1996e,g). This calculation is based on 100 times the maximum density of microbial units estimated for a 15-cm layer of water, immediately following the direct application of the microbial product to the water at its maximum application rate and complete mixing immediately thereafter. Section 3.4.4 provides guidance when mixing and administering a test material in food.

3.3.1.4 Administering MHC in test water to terrestrial plants. The recommended biological test method for single-concentration tests with terrestrial plants involves the administration of test material in both the *test water* sprayed on the plants and soil within each test chamber at regular intervals throughout the test, as well as dosing (mixing) of test material in the soil (once only) when setting up the test (see Section 12.2). The MHC specified by both USEPA (1996c) and PMRA (2001) for exposing terrestrial plants is to be followed when administering the MHC in the test water. This exposure concentration is defined as:

> *The maximum hazard concentration is to be equivalent to (or no less than) the maximum concentration of micro-organisms specified by the notifier for the final tank mix of a microbial product, when it is applied at the "maximum label rate".*

The term "*maximum label rate*", refers to the maximum concentration of end-use microbial product (and its corresponding microbial units/mL) recommended by the notifier for dispensing in a carrier such as water to a specified area of land (or, in some instances, water) (USEPA, 1996c). In practice, the MHC in test water to be applied to terrestrial plants represents the microbial units/mL (or, for granular non-aqueous mixes, units/g) in the final tank mix of the microbial product to be dispensed to the environment according to the maximum rate of application.

The MHC should be prepared by mixing a measured quantity of the *test material* in deionized water, using a quantity equivalent to that specified by the notifier for use when administering the maximum label rate (see Section 3.4.1). The MHC of viable micro-organisms to which terrestrial plants are exposed in a single-concentration test should be calculated, expressed, and reported as the nominal number of microbial units per millilitre of water in which the test material is mixed.

3.3.1.5 Administering MHC in test soil to terrestrial plants or soil-dwelling invertebrates. The MHC to be applied to terrestrial plants or soil-dwelling invertebrates (e.g., earthworms or springtails) is consistent with that prescribed by USEPA (1996c) and PMRA (2001) when administering a microbial substance in soil. It is also consistent with the approach defined herein when calculating and preparing the MHC for test materials administered to aquatic organisms via test

water or test sediment. Recommended biological test methods using this approach are described in Sections 12.2 (for terrestrial plants) and 13.2.2 (for earthworms).

For this application, the MHC is defined as:

> *The maximum hazard concentration is 10^6 units/g soil, or 1000 times the expected micro-organism concentration in soil within the terrestrial environment, whichever is greater and readily attainable.*

The number of microbial units applied per measured weight of medium (i.e., 10^6 microbial units/g) refers to the concentration of micro-organisms within the *test soil* to which a measured quantity of the test material is applied, following mixing (see Section 3.4.3). The target concentration of micro-organisms in the test soil following mixing would be 10^6 microbial units/g dry wt when adding a test material to the test soil (see Section 3.4.3).

In instances where 1000 times the expected micro-organism concentration in the terrestrial environment is greater than 10^6 microbial units/g soil (dry wt), this higher concentration should be applied in the test as the MHC, provided that such a concentration is readily attainable under laboratory conditions. This concentration is determined by multiplying, by 1000, the maximum calculated concentration of micro-organisms that would be anticipated to occur in soil immediately following the direct application of a microbial product to the terrestrial environment at the maximum application rate identified by the notifier for the product. If the test material is to be mixed in a given quantity of test soil before starting the test for pathogenic and/or toxic effects, the calculated concentration of micro-organisms representing 1000 times the expected micro-organism concentration in the terrestrial environment is determined. This calculation is based on 1000 times the maximum calculated density of microbial units in a 15-cm layer of soil immediately following the direct application of the end-use microbial product at its maximum application rate and complete mixing immediately thereafter (PMRA, 2001).

If added to test soil, the maximum hazard concentration of viable micro-organisms to which test organisms are exposed in a single-concentration test should be expressed and reported as the nominal number of microbial units per gram (expressed as dry weight) of test soil in which the test material is mixed.

3.3.1.6 Administering MHC to plant-dwelling invertebrates. The MHC to be administered to plant-dwelling invertebrates (i.e., foliar or pollinating insects such as honey bees, ladybird beetles, or green lacewings; see Sections 13.2 and 13.3) is defined as:

> *The maximum hazard concentration is to be equivalent to 100 times the maximum concentration of micro-organisms specified by the notifier for the final tank mix of a microbial product, when it is applied at the "maximum label rate".*

Health Canada (PMRA, 2001) prescribes this approach when administering the MHC of a microbial test material topically or in the diet to insects that frequent the foliage or petals of terrestrial plants, and USEPA also endorses this (Belliveau and Vaituzis, 2001).

This MHC applies when administering the test material to plant-dwelling terrestrial invertebrates in the diet, topically, by full immersion, or by spray application (see Section 13.3). The term "*maximum label rate*", refers to the maximum concentration of end-use microbial product (and its corresponding microbial units/mL) recommended by the notifier for dispensing in a carrier such as water to a specified area of land (or, in some instances, water). In practice, the MHC to be applied to plant-dwelling terrestrial invertebrates represents the microbial units/mL (or, for granular non-aqueous mixes, units/g) in the final tank mix of the microbial product to be dispensed to the environment according to the maximum recommended rate of application, times a safety factor that is 100× this field concentration (PMRA, 2001; Belliveau and Vaituzis, 2001).

The MHC of micro-organisms to which plant-dwelling terrestrial invertebrates are exposed in a single-concentration test should be calculated,

expressed, and reported as the nominal number of viable microbial units per millilitre (if an aqueous mix) or gram (if a granular non-aqueous formulation) of substrate in which the test material is mixed.

3.3.1.7 Administering MHC in test food to soil-dwelling invertebrates. The recommended biological test method for earthworms described herein (see Section 13.2) involves, for a single-concentration test, the administration of the test material in supplemental food offered during the test. The MHC for this application is the same as that described in the preceding subsection "*Administering MHC to plant-dwelling invertebrates*". Guidance provided in Section 3.4.4 applies here as well.

3.3.1.8 Administering MHD to birds by gavage. If an aqueous suspension of a test material is to be administered orally by gavage to birds, the maximum hazard *dose* (MHD) for that substance and suspension thereof is determined as:

> *The MHD is the expected micro-organism concentration (microbial units/mL) in the test material or aqueous suspension thereof, × 5 mL/kg body weight × weight of bird (kg). This dose is administered once per day during the first five days of the test.*

This approach is consistent with that used by USEPA (1996k) and PMRA (2001) when administering the MHD for a microbial pest control agent (MPCA) orally by gavage to birds during a 30-day test (see Section 14.1.2), and takes into account the maximum volumes of an aqueous suspension per body weight that should be provided to birds at any one time using this exposure route (i.e., 5 mL/kg body weight or, if necessary, up to 10 mL/kg body weight; see Section 3.4.5). If the test material is a solid substance (e.g., a powder), preliminary analyses of various quantities of this test material mixed in water will be required to determine the appropriate quantity to be mixed in deionized or distilled water to achieve the MHD. Guidance provided in Section 3.4.5 should be followed when administering a test material to birds orally by gavage.

3.3.1.9 Administering MHD to birds by inhalation. If an aqueous suspension of a test material is to be administered to birds by inhalation (i.e., through the respiratory tract via intranasal or intratracheal instillation), the maximum hazard dose (MHD) for that substance and suspension thereof is determined as:

> *The MHD is the expected micro-organism concentration (microbial units/mL) in the test material or aqueous suspension thereof, × 0.2 mL/kg body weight × weight of bird (kg). This dose is administered once per day during the first five days of the test.*

This approach is consistent with that used by USEPA (1996l) and PMRA (2001) when administering the MHD for an MPCA to birds by inhalation during a 30-day test (see Section 14.1.2), and takes into account the maximum volumes of an aqueous suspension per body weight that should be provided to birds at any one time using this exposure route (i.e., no more than 0.2 mL/kg body weight; USEPA, 1996l). If the test material is a solid substance (e.g., a powder), preliminary analyses of various quantities of this substance mixed in water will be required to determine the appropriate quantity to be mixed in *isotonic saline* (see Section 3.4.6) to achieve the MHD. Guidance provided in Section 3.4.6 should be followed when administering a test material to birds by inhalation.

3.3.1.10 Administering MHD to rodents by gavage. If an aqueous suspension of a test material is to be administered orally by gavage to rodents, the maximum hazard dose (MHD) for that test material and suspension thereof is determined as:

> *The MHD is 10^8 units of the new microbial substance, administered as a single dose at the start of the test.*

This approach is consistent with that used by USEPA (1996o) when administering the MHD of an MPCA orally by gavage to rodents during a 30-day test for pathogenic and/or toxic effects. Section 14.2.2, herein, recommends the use of this test method, including this approach for determining and administering the MHD. When administering this dose, the volume of the aqueous suspension should

not exceed 20 mL/kg body weight (USEPA, 1996o). If the test material is a solid substance (e.g., a powder), preliminary analyses of various quantities of this test material mixed in water will be required to determine the appropriate quantity to be mixed in deionized or distilled water to achieve the MHD. Section 3.4.5 provides this and additional guidance for administering a test material to birds orally by gavage.

3.3.1.11 Administering MHD to rodents by inhalation. If an aqueous suspension of a test material is to be administered by inhalation to rodents, the maximum hazard dose (MHD) for that substance and suspension thereof is determined as:

> *The MHD is 10^8 units of the new microbial substance, administered as a single dose at the start of the test.*

This approach is consistent with that used by USEPA (1996p) when administering the MHD of an MPCA to rodents by inhalation during a 30-day test for pathogenic and/or toxic effects. Section 14.2.2, herein, recommends the use of this test method, including this approach for determining and administering the MHD. When administering this dose, the volume of the aqueous suspension (i.e., the test material suspended in isotonic saline; see Section 3.4.6) should not exceed 3.0 mL/kg body weight (USEPA, 1996p). If the test material is a solid substance (e.g., a powder), preliminary analyses of various quantities of this substance in saline suspensions will be required to determine the appropriate quantity to be mixed in isotonic saline to achieve the MHD. Section 3.4.6 provides additional guidance for administering a test material to rodents by inhalation.

3.3.2 Multi-Concentration Test

A multi-concentration test can be used to measure the *virulence* (i.e., degree of pathogenicity) of a new microbial substance and/or its toxicity. This test should be performed using a series of concentrations of the new microbial substance up to and including that representing the MHC or MHD. For tests with aquatic or terrestrial plants or invertebrates, or aquatic vertebrates, each test concentration (including the MHC) should be expressed and reported as the nominal number of microbial units per millilitre or gram dry weight of test substrate in which the test material is mixed (see Section 3.3.1). The MHD and lower doses of a test material administered orally or by inhalation during tests with birds, should be expressed and reported as the nominal number of microbial units consumed or inhaled based on body weight, on each day that the test material is administered (see Section 3.3.1). The MHD and lower doses of a test material administered orally or by inhalation during tests with rodents, should be expressed and reported as the nominal number of microbial units administered as a single dose at the start of the test (see Section 3.3.1).

A logarithmic series of concentrations is recommended to achieve a broad range of test concentrations and encompass adverse effects that might be attributable to toxicants (e.g., endotoxins, toxic metabolites, or other non-biological substances) associated with the new microbial substance (see Appendix D). The range of concentrations (or doses) included in the test should be selected to enable the calculation of the appropriate statistical endpoints for multi-concentration tests (e.g., LC50 or LD50, EC50 or ED50, IC25 or ID25, NOEC/LOEC or NOED/LOED). Environment Canada's guidance document on statistical methods applicable to single-species tests for adverse effects of environmental contaminants (EC, 2004d) should be consulted when choosing test concentrations for a multi-concentration test and when calculating the appropriate statistical endpoints.

If the adverse effect(s) on test organisms noted in a single-concentration test performed using the MHC or MHD are caused by the pathogenicity of the new microbial substance, there is a good likelihood that lower concentrations (doses) will not show a log-concentration (or, in the instance of a test with birds or small mammals, log-dose) response typical of that caused by toxic chemical substances (see Section 2). This being the case, there is also a good likelihood that statistical endpoints such as the *LC50, LD50, EC50, ED50, ICp, IDp, NOEC/LOEC*, or *NOED/LOED*, which are frequently calculated for multi-concentration tests with toxic substances, cannot be calculated. Nonetheless, the performance of a multi-concentration test is useful in all instances where adverse effects are evident at the MHC or MHD, to determine the response of the test

organisms to a graded (e.g., logarithmic) series of lower test concentrations (doses) and the extent to which low concentrations of the new microbial substance, which might be found in the environment, exert a harmful effect.

When preparing for a definitive multi-concentration test, a preliminary range-finding test is recommended to establish an appropriate range of concentrations to be used in the definitive test. Typically, the range-finding test would cover a broad range of test concentrations (e.g., each concentration in a series differing by an order of magnitude from the next one). The findings of the range-finding test would indicate the types of adverse effects caused by the new microbial substance, and whether or not they respond in a log-concentration manner. Final test concentrations for the definitive test would be selected accordingly.

3.4 Preparing and Administering Test Concentrations

Procedures used to prepare concentrations of a particular test material to be administered to aquatic or terrestrial plants, invertebrates, or vertebrates using a particular biological test method, should be as standardized and similar as possible, to enable the meaningful comparison of test results. However, since the route(s) of exposure (i.e., via water, sediment, soil, food, gavage, or inhalation) varies depending on the type of test organism and the biological test method to be applied (Section 3.2), the procedure for preparing and administering test concentrations or doses varies when mixing a test material in each of these differing *test substrates*. The nature of the test material (e.g., liquid or powder) and its behaviour when mixed in a test substrate (water, sediment, soil, food) or by gavage or inhalation also influence the procedures to be used when preparing test concentrations.

Guidance when preparing and administering various types of test materials in a specific test substrate (i.e., water, sediment, soil, or food) or by gavage or inhalation (if birds or small mammals) is provided here in separate subsections. Section 4.13.1 "*Testing Substances with Troublesome Properties*" in EC (1999b) provides additional guidance on mixing and administering substances with difficult properties (e.g., low water solubility, tendency for sorption, volatility, instability), which might apply to certain test materials.

3.4.1 Mixing and Administering in Water

Key Guidance

- *The test water in which a test material is mixed must be* clean, *and have physicochemical characteristics suitable for use as control/dilution water in the intended biological test method.*
- *Each test concentration should be prepared by mixing a measured quantity of the test material in test water, using a volume of test water sufficient for all replicates of that concentration to be included in the test.*
- *No solvent other than test water is to be used when preparing test concentrations. The use of ultrasonic dispersion or other procedures potentially harmful to the new microbial substance must also be avoided when preparing and mixing the test material in test water.*
- *Time for mixing should be adequate to ensure the homogeneous distribution of the test material, and may be from minutes up to 24 h. Once mixed, each test concentration and the control treatment(s) must be transferred to the test chambers for the start of the test or renewal of suspensions/solutions.*
- *Tests involving the static renewal of each test concentration (including the controls) must prepare a fresh set of test concentrations at the time of each renewal.*

Any fresh, estuarine, or marine water used as *test water*[21] must be uncontaminated. A history of its basic physicochemical characteristics (e.g., pH, hardness or salinity, dissolved oxygen, suspended solids, ammonia, nitrite, dissolved metals, pesticides) should be known by the testing laboratory before choosing it as the test water in a particular biological method with a new microbial substance. Additionally, it must be *clean* according to the test method for which it is used; that is, it

[21] Depending on the biological test method, the test water is *clean* marine, estuarine, or fresh water from an uncontaminated source or formulated in the laboratory. In certain instances (i.e., tests using terrestrial organisms), it is deionized water.

must enable the *negative control* groups included in the test to meet the test-specific criterion or criteria for validity, and must not cause discernible adverse effects on test organisms using the intended test method. The test water may be from a suitable natural source, or formulated in the laboratory (i.e., artificial water) to meet test-specific requirements for characteristics such as hardness (if fresh water), salinity (if estuarine or marine water), and pH.

For a single-concentration test involving the addition of a new microbial substance to test water (see Sections 3.2 and Sections 9 to 14 inclusive), a measured quantity (by volume or weight) of the test material representing the MHC (Section 3.3.1) should be mixed thoroughly in a suitable quantity of *test water*. Mixing may be by hand (e.g., using a clean spatula or glass rod) or by using a mechanical stirring device (e.g., a teflon-coated bar in a container of test water on a magnetic stirrer; or, for larger quantities, a stainless steel vortex mixer in a plastic pail or other nontoxic mixing chamber containing test water). The MHC and each lower test concentration used in a multi-concentration test, should be prepared in the same manner, on a concentration-by-concentration basis.[22]

The quantity of each test concentration prepared for use in tests with aquatic organisms should be sufficient to set up the appropriate number of *replicate* test chambers required for each treatment (concentration) by the biological test method to be applied (see Sections 9 to 11), with a surplus of 10% or more. Once mixing is completed, a measured aliquot of each prepared test concentration must be transferred to each replicate test chamber. Exposure of test organisms to these freshly prepared suspensions must begin then (e.g., if starting a test with aquatic plants or fish, or if renewing test concentrations) or the next day (e.g., if starting a test using amphipods or midge larvae), according to procedures defined for the biological test method (see Sections 9 to 11). As necessary, the suspension within the mixing chamber should be restirred just before taking each aliquot.

Each test concentration should be mixed thoroughly with sufficient turbulence and duration to attain a homogeneous suspension of the test material in the test water. A solvent (other than the test water) must not be used to prepare any test concentration, since it could prove toxic to the the new microbial substance, or might cause or contribute to toxic effects on the test organisms. Additionally, any procedure such as ultrasonic dispersion, which might prove harmful to the new microbial substance, must not be used in an attempt to achieve a homogeneous suspension when preparing the test concentration(s). Temperature during mixing should be standardized for each treatment, and should be kept low to minimize effects on the new microbial substance. Time for mixing should be adequate to ensure the homogeneous distribution of the test material, and may be from minutes up to 24 h. Each control treatment included in a test (see Section 4) must be mixed using the identical procedure used to prepare the *test treatment*(s).

The following procedure is recommended when preparing aqueous suspensions of test material that are somewhat hydrophobic or require vigorous stirring to achieve a suspension.[23] Weigh 50 g (or proportionately more or less) of the test material in a sterile blender jar. Add 450 mL (or proportionately more or less) of diluent (i.e., fresh water, seawater, or isotonic saline). Blend for two minutes in a high-speed blender at 10 000 to 12 000 rpm. Alternatively, a Stomacher™ can be used to blend the suspension. No more than 15 minutes should lapse from the time that each test suspension is blended until each is added to the test chambers.

In tests with aquatic plants, invertebrates, or fish, where renewal of the test water during the test is feasible, each concentration (including each control treatment) must be renewed during the test at a specified (test-specific) interval (see Sections 9, 10, and 11). This is achieved by the *static renewal* of each test concentration including the controls. At

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[22] The preparation of a stock suspension, followed by the addition of measured aliquots to test water, is not recommended when preparing a range of test concentrations for a multi-concentration test. Depending on the nature of the test material including particle size(s), appreciable errors in preparing test concentrations using a stock suspension might occur.

[23] This procedure is provided as Method 966.23B in AOACI (2000). Although prescribed for preparing suspensions of food in water, it is also suitable for preparing microbial suspensions in water.

each renewal period, the test concentration(s) must be freshly prepared when they are required. The procedure used to prepare each fresh test concentration (including the controls) required for the static renewals must be identical to that used when setting up the test (i.e., for the initial exposure).

3.4.2 Mixing and Administering in Sediment

Key Guidance

- *The test sediment in which the test material is mixed must be* clean, *and have physicochemical characteristics that are suitable for use as negative control sediment in the intended biological test method.*
- *Each test concentration should be prepared by mixing a measured quantity of the test material in test sediment, using a volume of sediment sufficient for all replicates of that concentration to be included in the test.*
- *No solvent (other than test water, if used to dilute the test material before mixing in a measured amount) is to be used when preparing the test concentration(s) of a new microbial substance mixed in test sediment.*
- *Identical procedures for mixing the test material in test sediment must be used when preparing each test concentration and the control(s).*
- *Time for mixing should be adequate to ensure the homogeneous distribution of the test material, and may be from minutes up to 24 h. Once mixed, each test concentration and the control treatment(s) must be transferred to the test chambers, and the test started within 24 h thereafter.*

Any fresh, estuarine, or marine sediment used as *test sediment* must be uncontaminated. The testing laboratory should know the basic physicochemical characteristics of the sediment (e.g., for whole sediment — particle size distribution, percent water content, total organic carbon content; for *pore water*—pH, ammonia, salinity/hardness, dissolved metals, pesticides) before choosing a particular source of sediment as the test sediment in a particular biological test method with a new microbial substance. Additionally, the sediment must be *clean* according to the test method for which it is used; that is, it must enable the *negative control* groups included in the test to meet the test-specific criterion or criteria for validity, and must not cause discernible adverse effects on test organisms using the intended test method. The test sediment may be from a suitable natural source, or formulated in the laboratory (i.e., artificial) to meet test-specific requirements for characteristics such as particle size distribution, porewater salinity (if estuarine or marine sediment), and porewater pH. Provided that a *clean* natural sediment with suitable properties (including particle size and organic carbon content) can be obtained, the use of natural sediment is preferred over artificial sediment when testing a new microbial substance for its pathogenic and/or toxic effects. The use of sterile sediment (natural or artificial) is not recommended, since most sterilization processes alter the physicochemical characteristics of the sediment.

For a single-concentration test involving the addition of a new microbial substance to test sediment (see Sections 3.2 and 10), a measured quantity (by volume or weight) of the test material representing the MHC (Section 3.3.1) should be mixed thoroughly in a suitable quantity of *test sediment*.[24] The MHC and each lower test concentration used in a multi-concentration test should be prepared in the same manner, on a concentration-by-concentration basis. Mixing may be by hand (e.g., a plastic or stainless steel spoon) or by using a mechanical mixing device (e.g., a drum roller). Pertinent guidance on preparing mixtures of test materials in sediment is found in each of the recommended biological test methods using sediment (see Section 10) which apply here. Environment Canada's guidance document on collecting and preparing sediments for physicochemical characterization and biological testing (EC, 1994) should also be consulted for practical advice.

[24] If and as necessary to achieve one or more test concentrations, a measured quantity of the test material may be mixed in a suitable volume of *test water* to dilute it, before mixing it in a measured weight of the *test sediment*. If this is done, the final calculation of each nominal concentration of test material mixed in test sediment must take this dilution into account.

The quantity of each test concentration prepared should be sufficient to set up the appropriate number of replicate test chambers required for each treatment (concentration) by the biological test method to be applied (see Section 10), with a surplus of 10% or more. Once mixing is completed, a measured aliquot of each prepared test concentration must be transferred to each replicate test chamber. Exposure of test organisms must commence the following day, unless specified otherwise in the biological test method to be applied (see Section 10).

Each test concentration should be mixed thoroughly, with sufficient duration to attain a homogeneous dispersal of the test material throughout the test sediment. The procedure and duration of mixing should be the same for each test concentration and the controls. A solvent (other than the test water) must not be used to prepare any test concentration, since it could prove toxic to the new microbial substance, or might cause or contribute to toxic effects on the test organisms. Additionally, any procedure which might prove harmful to the new microbial substance, must not be used when preparing the test concentration(s) in an attempt to achieve a homogeneous suspension. For instance, temperature during mixing should be kept low to minimize thermal effects on the new microbial substance and changes in the physicochemical characteristics of the mixture. Time for mixing should be adequate to ensure homogeneous distribution of the test material, and may be from minutes up to 24 h. Analyses of subsamples of the mixture, for concentrations of the new microbial substance, are advisable to determine the degree of mixing and homogeneity achieved. Each control treatment included in a test (see Section 4) must be mixed using the identical procedure used to prepare the test treatment(s).

3.4.3 Mixing and Administering in Soil

Key Guidance

- *The test soil in which a test material is mixed must be* clean, *and have physicochemical characteristics that are suitable for use as negative control soil in the intended biological test method.*

- *Each test concentration should be prepared by mixing a measured quantity of the test material in test soil, using a volume of soil sufficient for all replicates of that concentration to be included in the test.*

- *No solvent (other than test water, if used to dilute the test material before mixing in a measured amount) is to be used when preparing the test concentration(s) of a new microbial substance mixed in test soil.*

- *Identical procedures for mixing the test material in test soil must be used when preparing each test concentration and the control(s).*

- *Time for mixing should be adequate to ensure the homogeneous distribution of the test material, and may be from minutes up to 24 h. Once mixed, each test concentration and the control treatment(s) must be transferred to the test chambers, and the test started within 24 h thereafter.*

Any soil used as *test soil* must be uncontaminated. The testing laboratory should know the basic physicochemical characteristics of the soil (e.g., particle size distribution, percent water content, percent organic carbon, pH, metals, pesticides, petroleum hydrocarbons) before choosing a particular source of soil as the test soil in a particular biological test method with a new microbial substance. Additionally, the test soil must be *clean* according to the test method for which it is used; that is, it must enable the *negative control* groups included in the test to meet the test-specific criterion or criteria for validity, and must not cause discernible adverse effects on test organisms using the intended test method. The test soil may be from a suitable natural source, or formulated in the laboratory (i.e., artificial) to meet test-specific requirements for characteristics such as particle size distribution, percent water content, percent organic carbon, and pH. Choice of test soil depends on the

required or recommended physicochemical characteristics for soil that are specified in the biological test method to be followed using a mixture of the test material in soil as a test substrate (see Sections 12 and 13). Provided that a *clean* natural soil with suitable properties (including particle size and organic carbon content) can be obtained, the use of natural soil is preferred over artificial soil when testing a new microbial substance. If artificial soil is used, the formulation provided in EC (2004a,b,c) as well as in ISO (1998, 1999b) and OECD (2000c) is recommended. The use of sterile soil (natural or artificial) is not recommended.[25]

For a single-concentration test involving the addition of a new microbial substance to test soil (see Sections 3.2, 12, and 13), a measured quantity (by volume or weight) of the test material representing the MHC (Section 3.3.1) should be mixed thoroughly in a suitable quantity of *test soil*.[26] The MHC and each lower test concentration used in a multi-concentration test, should be prepared in the same manner, on a concentration-by-concentration basis. Mixing may be by hand (e.g., a plastic or stainless steel spoon) or by using a mechanical mixing device (e.g., a drum roller). Pertinent guidance on preparing mixtures of test materials in soil is found in each of the recommended biological test methods using soil as a test substrate (see Sections 12 and 13) that applies here.

[25] Autoclaving can release toxic components that might adversely affect host (test) organisms or confound results. In addition, certain test micro-organisms introduced to sterilized soil could achieve unrealistically high populations that would never occur in the presence of natural populations of micro-organisms in unsterilized soil. Sterilization of test soil is also not recommended since this process would remove endemic micro-organisms in the soil which might otherwise affect the test micro-organisms within a new microbial substance by inhibiting their multiplication and growth.

[26] If and as necessary to achieve one or more test concentrations, a measured quantity of the new microbial substance may be mixed in a suitable volume of *test water* to dilute it, before mixing it in a measured weight of the *test soil*. If this is done, the final calculation of each nominal concentration of test material mixed in test soil must take this dilution into account.

The quantity of each test concentration prepared should be sufficient to set up the appropriate number of replicate test chambers required for each treatment (concentration) by the biological test method to be applied (see Sections 12 and 13), with a surplus of 10% or more. Once mixing is completed, a measured aliquot of each prepared test concentration must be transferred to each replicate test chamber. Exposure of test organisms must commence the following day, unless specified otherwise in the biological test method to be applied (see Sections 12 and 13).

Each test concentration should be mixed thoroughly, with sufficient duration to attain a homogeneous dispersal of the test material throughout the test soil. The procedure and duration of mixing should be the same for each test concentration and the controls. A solvent (other than the test water) must not be used to prepare any test concentration, since it could prove toxic to the new microbial substance, or might cause or contribute to toxic effects on the test organisms. Additionally, any procedure which might prove harmful to the new microbial substance, must not be used when preparing the test concentration(s) in an attempt to achieve a homogeneous suspension. For instance, temperature during mixing should be kept low to minimize thermal effects on the new microbial substance and changes in the physicochemical characteristics of the mixture. Time for mixing should be adequate to ensure homogeneous distribution of the test material, and may be from minutes up to 24 h. Analyses of subsamples of the mixture, for concentrations of the new microbial substance, are advisable to determine the degree of mixing and homogeneity achieved. Each control treatment included in a test (see Section 4) must be mixed using the identical procedure used to prepare the test treatment(s).

3.4.4 Mixing and Administering in Food

Key Guidance

- *Each test concentration of a new microbial substance in food should be prepared by mixing a measured quantity of the test material in the food on each of the days of the test during which food is offered.*
- *The food required for each treatment (concentration), including the controls, must be mixed under identical conditions (including time and temperature).*

- *No solvent (other than test water, if used to dilute the test material before mixing in a measured amount) is to be used when preparing the test concentration(s) of a new microbial substance mixed in food.*
- *When finalizing the mixing procedure to be used, preliminary analyses of subsamples of a mixture are recommended to ascertain the homogeneity of the mixture.*

For certain tests involving shrimp (Section 10.2.2), fish (Sections 11.1.2 and 11.2.2), or terrestrial invertebrates (Section 13), test organisms are exposed to the test material by mixing it in the food offered during the test. The type of food in which the test material is mixed is test dependent. In a test using shrimp or fish, the test organisms are fed a diet of appropriately sized commercial fish food (flakes or pellets)[27] at the beginning of and during the test (USEPA, 1996e,g). A test for effects of a new microbial substance on the prolonged survival and growth of earthworms should incorporate a measured quantity of the test material in the bolus of cooked oatmeal added to replicate test chambers at the start of and during the test (EC, 2004b). For a test involving honey bees, the test organisms should be exposed orally to the test material (USEPA, 1996j); e.g., by mixing it in a sucrose solution (OECD, 1998d). Guidance regarding the frequency of feeding during each of these tests is provided in Sections 10.2.2, 11.1.2, 11.2.2, and 13.2, along with other test-specific information.

In selected cases, the test material administered to birds or rodents may be incorporated in the food, rather than delivering it orally by gavage (see Section 3.4.5) or by inhalation (Section 3.4.6). If this procedure for delivery is feasible and appropriate (particularly for studies of chronic effects on birds or small mammals), the test material must meet the following criteria: (i) it must be palatable without disruption of the normal daily food intake; and (ii) it must be incorporated into the normal ration without adversely affecting the quality of the food or the stability of the test material.

When preparing the test concentration(s), a measured quantity of the test material should be mixed with the food according to guidance provided in Section 3.3. The procedure for mixing the test material with the food depends on the nature of both the test material (e.g., aqueous suspension, powder, or granular solid) and the food. When standardizing the mixing procedure to be used in a test, preliminary analyses of subsamples of a mixture for concentrations of the new microbial substance in the food (see Section 3.5) are recommended to confirm the suitability of the mixing procedure and to ascertain the homogeneity of the mixture.

The quantity of each test concentration prepared by mixing a test material in food should be sufficient to enable all replicates of that treatment (concentration) to be fed at the rate specified for the biological test method (see Sections 11 and 13), with a surplus of 10% or more. Each mixture of test material in food should be prepared on each feeding day.

Each test concentration should be mixed thoroughly, with sufficient duration to attain a homogeneous concentration of the new microbial substance throughout the *batch* of food. The procedure and duration of mixing should be the same for each test concentration and the controls. A solvent (other than the test water) must not be used to prepare any test concentration, since it could prove toxic to the

[27] A preferred approach, to minimize the loss of the test material from the food when it is added to water, is to mix the test material with the flake food and then pelletize it.

new microbial substance, or might cause or contribute to toxic effects on the test organisms. Additionally, any procedure which might prove harmful to the new microbial substance, must not be used when preparing the test concentration(s) in an attempt to achieve a homogeneous mixture. For instance, temperature during mixing should be kept low to minimize thermal effects on the new microbial substance and changes in the physicochemical characteristics of the mixture. Time for mixing should be adequate to ensure homogeneous distribution of the test material, and may be from minutes to a few hours. Each control treatment included in a test (see Section 4) must be mixed using the identical procedure used to prepare the test treatment(s).

3.4.5 Administering Orally by Gavage

Key Guidance

- *Care must be taken when administering a test material to birds or small mammals orally, by gavage.*
- *The use of cannulae with ball-tipped ends is recommended.*
- *If the test material is a solid, it should be administered by gavage as an aqueous suspension. Alternatively, it may be administered in a gelatin capsule.*
- *Preferably, no solvent (other than test water, if used to dilute the test material before mixing in a measured amount) should be used when preparing the test concentration(s) of a new microbial substance to be provided by gavage. If the test material is hydrophobic, corn oil or carboxymethylcellulose may be used.*
- *The control group(s) must be given the same quantity of liquid or aqueous suspension by gavage, as that volume of suspension given to test animals receiving the MHD.*

One of the recommended routes of exposure to a new microbial substance for tests with birds or small mammals, is orally by gavage (USEPA, 1996b,k,m,o,t,u) (see Sections 3.2 and 14). The use of cannulae with ball-tipped ends (USEPA, 1996b) is recommended to prevent injury to the test organisms. Guidance on calculating the quantity of the test material to be administered by gavage to each test animal in a *replicate treatment*, to achieve the MHD (and, if a multi-concentration test, lower doses), is given in Sections 3.3.1. For birds, the volume generally should not exceed 5.0 mL/kg body weight (USEPA, 1996b,k), although up to 10.0 mL/kg body weight may be used if necessary to achieve the MHD. For rodents, the volume should not exceed 20.0 mL/kg body weight (USEPA, 1996o).

The recommended test methods for administering a test material to birds or rodents by gavage specify five daily doses at the start of tests with birds (Section 14.1.2), and one dose only at the start of tests with rodents (Section 14.2.2). During each exposure, the test animals receiving each control treatment included in a test (see Section 4) must be subjected to the same gavage procedure as those administered the test dose(s) by gavage. For each treatment, care must be taken to not cause undue stress to the test organisms or injure them (Section 7.2). Handling techniques and procedures for cannulation should be identical for each animal included in a test.

If the test material is a liquid suspension, the quantity of test material necessary to achieve the test dose(s) may be either administered directly by gavage to each test organism or suspended in the appropriate volume of test water (see Section 3.4.1). If the test material is a solid, the quantity of test material to be administered by gavage to achieve the test dose(s) should be suspended in test water (i.e., deionized water) to facilitate its delivery. Alternatively, a solid test material may be administered using gelatin capsules. The control group(s) must be given the same quantity of liquid or aqueous suspension[28] by gavage as any aqueous suspension of the test material given to test animals receiving the MHD.

Unless the test material is hydrophobic, a solvent (other than the test water) should not be used to prepare any test concentration, since it could prove

[28] For a negative control or a positive chemical control, the liquid is test water or a concentration of a *reference toxicant* dissolved in test water, respectively. For a non-infectious control or a sterile filtrate control, the modified test material administered by gavage is an aqueous suspension or a liquid, respectively (see Section 4).

toxic to the new microbial substance or might cause or contribute to toxic effects on the test organisms. If a solvent other than test water is required (e.g., based on hydrophobic or other properties of the test material), corn oil or carboxymethylcellulose may be used to create a suspension for dosing. The control group(s) must be given the same quantity of any solvent used to administer the MHD.

Certain microbial products are viscous or contain proportionately large quantities of inorganic or organic carrier substances. For such products, the investigator is advised to administer the new microbial substance alone to birds or rodents by gavage, rather than using the end-use microbial product (see Section 3.2).

3.4.6 Administering by Inhalation

Key Guidance

- *Administration of a test material to birds or small mammals by inhalation should be a chosen exposure route, provided that the new microbial substance could contact terrestrial vertebrates in the natural environment as a fine mist or spray.*
- *The possible routes of exposure by inhalation are by intranasal or intratracheal instillation, or via aerosols. The preferred route of exposure is via intranasal instillation; dosing by aerosolization is typically the least acceptable route because of the difficulty in achieving an appropriate dose. Test animals receiving each control treatment included in a test must be subjected to the same procedure as that used to provide the MHD of the test material.*
- *Care must be taken when administering a test material to birds or small mammals by intranasal or intratracheal instillation.*
- *No solvent other than isotonic saline should be used to dilute the test material when preparing the test concentration(s) of a new microbial substance to be delivered by inhalation.*

In instances where a new microbial substance might enter the natural environment as a fine mist or spray of colloidal-sized particles, laboratory tests with birds or small mammals should involve the administration of this substance to test organisms by inhalation through their respiratory tract (see Sections 14.1.2 and 14.2.2). To achieve this, the possible routes of exposure are by *intranasal* or *intratracheal instillation*, or via aerosols. The intranasal route is the preferred route of exposure. The intratracheal route ensures that the test material will be delivered directly into the lower respiratory tract. This procedure for delivery is relatively invasive, however, and might require the use of analgesics to deliver the test material, particularly for larger test animals. The intranasal and/or aerosol routes most closely duplicate the normal routes of exposure in the environment.

Administering a test material to birds or small mammals by aerosolization is typically the least preferred route of exposure, for several reasons.[29] Depending on the physical properties of the test material and its manner of dispersal in the environment, it may in some instances be administered by *aerosolization* within a closed chamber (USEPA, 1996l; PMRA, 2001). If aerosolization is chosen by the applicant as a route of exposure, a written justification for choosing this procedure (rather than using intranasal or intratracheal instillation) should be submitted to Environment Canada's New Substances Branch in advance of the test (i.e., as part of the Pre-Notification Consultation; see Section 1.1).

The use of *isotonic saline* to dilute the test material, rather than deionized water, is recommended for the following reasons: (i) if infectious agents are under test, isotonic saline promotes the viability of the micro-organism; and (ii) it is much less irritating to the mucous membranes of the test (host) animal.

Guidance on calculating the quantity of any test material to be administered by inhalation to each test animal in a replicate treatment, to achieve the MHD (and, if a multi-concentration test, lower doses), is given in Sections 3.3 and 14. For birds, the volume generally should not exceed 0.2 mL/kg body weight (USEPA, 1996l). For rodents, the

[29] It is very difficult to deliver an equivalent dose via aerosol, compared to delivery by intratracheal or intranasal instillation. Also, rodents are not mouth breathers and have a nasal-pharyngeal geometry that is unsuitable for use with most aerosol delivery methods. Exposure by aerosolization can also result in preening or licking of fur or feathers contaminated with test material applied in this manner, possibly causing some transfer of the test material to the oral cavity.

volume should not exceed 3.0 mL/kg body weight (USEPA, 1996p).

Certain microbial products are viscous or contain proportionately large quantities of inorganic or organic carrier substances. For such products, it is advisable to administer the new microbial substance alone to birds or rodents by gavage, rather than using the end-use microbial product (see Section 3.2).

A solvent (other than isotonic saline) should not be used to prepare any test concentration, since it could prove toxic to the new microbial substance, and cause unnecessary irritation to the mucous membranes of the test animal. Guidance provided in Section 3.4.1 for preparing "difficult" suspensions of a new microbial substance should be consulted and applied as appropriate, when preparing aqueous suspensions of test material to be administered to birds or rodents by inhalation.

The recommended test methods for administering a test material to birds or rodents by inhalation specify five daily doses at the start of tests with birds (Section 14.1.2), and one dose only at the start of tests with rodents (Section 14.2.2). During each exposure to a test material by inhalation, the test animals receiving each control treatment included in a test (see Section 4) must be subjected to the same procedure as those administered the test dose(s) by inhalation. That is, the negative control groups must be given the same quantity of a fine spray of test saline as those receiving the MHD of the test material, using the same dosing procedure. Similarly, any replicate *non-infectious controls* or *sterile filtrate controls* included in a test (Section 4) must be administered a fine spray of the (modified) test material using the same dosing system and quantity of inhaled substance as that representing the MHD. Any *positive chemical controls* or *positive microbial controls* included in a test should be treated in the same manner.

For each treatment, care must be taken to not cause undue stress to the test organisms or injure them (Section 7.2). Handling techniques and procedures for intranasal or intratracheal instillation should be identical for each animal included in a test.

3.5 Quantifying the Concentration of Micro-organisms

Key Guidance

- *The concentration of viable micro-organisms in the* lot *of the test material must be known. The notifier must provide this information to the testing laboratory along with the analytical technique for quantifying the concentration of viable micro-organisms. A minimum of three aliquots of the test material should be re-analyzed for microbial concentration when laboratory testing begins.*

- *Analytical techniques permitting, the concentration of new microbial substance (i.e., viable micro-organisms) in the test concentration(s) administered to test organisms should be monitored during the test. Sampling and analytical procedures for quantifying the concentration or dose to which test organisms are exposed vary depending on the means of administration (i.e., in water, sediment, soil, food, orally by gavage, or by inhalation) and the frequency of administering (e.g., static renewal, if mixed in test water; at start of test only, if mixed in sediment or soil).*

- *Aliquots of the negative control substrate(s) should be taken and analyzed in an identical manner when analyzing the test substrate(s) (or, in the case of terrestrial vertebrates, aqueous suspensions of the test material administered by gavage or by inhalation) for concentration of new microbial substance. Similarly, for any test that includes a non-infectious control and/or a sterile filtrate control, the investigator(s) should sample and analyze aliquots of these treatments for concentration, using the same procedure as that applied to the test concentration(s).*

The concentration of viable micro-organisms in the *lot* of the test material investigated in the laboratory for pathogenic and/or toxic effects must be known and reported. The *notifier* or other responsible party should provide this information along with the lot submitted to the testing laboratory. Instructions or guidance concerning the appropriate analytical procedure to be followed when determining concentrations of the new microbial substance in the

test substrate(s) to which aquatic or terrestrial plants or animals are exposed in the laboratory should also be provided. Ideally, three or more aliquots of the lot received by a testing facility will be re-analyzed at the time that a definitive test for pathogenic and/or toxic effects is started, to determine its mean (± SD) concentration of viable micro-organisms in the test material at that time.

In its background document for testing the pathogenic and/or toxic effects of microbial pest control agents (MPCAs) on nontarget organisms, the USEPA (1996b) states:

> "*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*".

No further guidance or instructions in this respect are provided in USEPA (1996b) or in most other reports in the "OPPTS 885" series (USEPA, 1996c–n,p, r–u).[30] *Standard Operating Procedures* (*SOPs*) used by private laboratories undertaking laboratory tests for environmental effects of MPCAs in keeping with the OPPTS 885 series of test guidelines (e.g., USEPA, 1996a–u) frequently describe the collection of aliquots of *test substrate* at the beginning and during such tests, and their analysis for concentrations of the new microbial substance therein. Both EC and HC (2001) and PMRA (2001) do not discuss requirements or recommendations for quantifying the concentration of a new microbial substance to which aquatic or terrestrial plants or animals are exposed, during laboratory tests for its pathogenic and/or toxic effects.

Analytical techniques permitting, the concentration of new microbial substance in the test substrate(s) within which a test material is mixed (see Sections 3.4.1 to 3.4.4 incl.) should be measured at the time that these mixtures are prepared for use, as well as during and/or at the end of the test. Analytical procedures for determining the concentration of a particular new microbial substance within the *test substrate* (i.e., test water, test sediment, test soil, or test food) might, depending on the micro-organism (e.g., a bacterium, fungus, or protozoan that is readily quantifiable by a microbial plate count) be straightforward using a standard methodology such as that in Standard Methods (APHA *et al.*, 1998 or later version). Quantification of the concentration of certain other micro-organisms (e.g., a virus) in a test substrate (e.g., test sediment, test soil, or test food) might, however, prove difficult or impossible using available analytical techniques.

Each control treatment must be treated in the same manner, when analyzing the test substrate(s) for concentration of new microbial substance therein. Accordingly, for the *negative control* included in the test (Section 4.1), untreated aliquots of any test substrate(s) (i.e., water, sediment, soil, and/or food) within which the test material is mixed must be taken and analyzed for concentrations of the new microbial substance in a manner identical to that applied to the test treatment(s). Similarly, any test including a *non-infectious control* (Section 4.4) or a *sterile filtrate control* (Section 4.5) must sample and analyze this treatment for concentration of new microbial substance, in a manner identical to that applied to the test treatment(s). The number of replicate aliquots of any control treatment taken to quantify their concentration of new microbial substance at the start of and during the test must be identical to that for the test concentration(s). Measurements of concentration of the new microbial substance in any *positive chemical control* (Section 4.2) included in a test are not necessary, although an investigator might choose to do so to ensure that the findings for either of these treatments are not influenced by their unintended contamination with the new microbial substance. Analyses permitting, measurements of concentration of a particular micro-organism in any *positive microbial control* (Section 4.3) included in a test are recommended.

For certain biological test methods with aquatic organisms, the *test water* into which a new microbial substance is mixed (Section 3.4.1) is renewed at frequent and defined intervals throughout the test (see Sections 9, 10, and 11). Analytical techniques permitting, the concentration

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[30] In its test guidelines for determining the toxicity/pathogenicity of MPCAs to rodents exposed orally (by gavage) or by inhalation, USEPA (1996o,q) indicated that determinations of viable or potentially viable infective units in each dose should be made where possible; and that dose quantification should be done concurrently with testing.

of the new microbial substance in this test substrate should be measured at least at 0 h (i.e., start of test), at test termination, and at least once per week at the beginning and end of an intervening cycle (USEPA, 1996bb).[31] Alternatively, the concentration in both the fresh and aged test suspension(s) may be measured at the beginning and end of each static-renewal period. The mean (± SD) measured concentration for all analyses representing each treatment should be calculated and compared with the nominal concentration. Calculating and comparing the average concentration of new microbial substance in fresh versus aged suspensions of test water to which test organisms are exposed is also recommended to determine the variation in concentration during the static-renewal test. Additionally, a plotting and examination of the measured values for each fresh test concentration throughout the duration of the exposure is useful to ascertain the stability of the *lot* of test material used for preparing each test concentration.

For tests in which the test material is mixed in sediment or soil (see Sections 3.4.2 and 3.4.3), a minimum of three aliquots of each fresh mixture (including that for each control treatment) should be taken from the freshly prepared *batch* for analysis of the concentration of new microbial substance therein. Three or more aliquots of sediment or soil within the test chambers should also be taken from the replicates of each treatment upon completion of the test.[32] The mean (± SD) of the six analyses representing each treatment should be determined as a measure of the average concentration within the sediment or soil during the test. A comparison of the average concentration in the fresh test substrate versus that at the end of the test is also recommended, to gain an understanding of the extent to which the concentration in this substrate changes during the test.

For tests in which the test material is administered to test organisms by mixing it in their food (see Section 3.4.4), the concentration of new microbial substance in the food at each offering should be quantified, analytical techniques permitting. For this purpose, one or more aliquots of each batch of freshly prepared food administered to the replicate test concentration(s) should be taken for analysis of concentration on each occasion that the "substance-in-food" mixture is provided. The mean (± SD) of all analyses representing each treatment should be determined as a measure of the average concentration in the food during the test exposure.

The concentration of new microbial substance in each suspension of a test material administered orally by gavage (Section 3.4.5) or by inhalation (Section 3.4.6) to birds or small mammals should also be measured, if possible, concurrently with each treatment administered during the test. Accordingly, one or more aliquots of each aqueous suspension of the test material prepared for this purpose should be taken for analysis of microbial concentration on each occasion that the test dose(s) is (are) administered. For a test with birds, the mean (± SD) of all analyses representing each treatment should be determined as a measure of the average microbial concentration in each dose administered to the test organisms during the test.[33]

If the test material to which birds or rodents are

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[31] Aliquots of fresh suspensions may be taken from the mixture prepared for distribution to each test chamber (see Section 3.4.1). Aliquots of aged suspensions should be removed from the centre of the test water within the test chamber. Pooling of aliquots from replicate test chambers might be necessary or advisable, on each sampling occasion for aged suspensions, to attain a sufficient volume for analysis or as a cost-effective means of determining the average concentration for the replicates. If sampling only once per week at the beginning and end of a renewal cycle, and if variable renewal cycles (e.g., renew every two or three days) are employed, the longest cycle in the weekly sequence should be used when collecting fresh and aged aliquots (USEPA, 1996bb).

[32] If more information on the change in concentration of new microbial substance in the sediment or soil is desired, additional sets of test chambers representing each treatment should be set up at the start of the test for this purpose, and destructively sampled at the desired monitoring frequency.

[33] This applies only to the recommended test method for birds, which involves five daily doses of the test material at the start of the test (see Section 14.1.2). These measurements also provide an indication of the *precision* of the dose administered over time, as well as the stability of the test material during the exposure period. For the recommended test method using rodents (see Section 14.2.2), the animals are subjected to only one dose of the test material at the start of the test.

exposed is a liquid (e.g., an aqueous suspension), the liquid to be analyzed and administered would be an undiluted or diluted aliquot of the test material (see Sections 3.4.5 and 3.4.6). If the test material to which these host organisms are exposed is a solid (e.g., a powder), preliminary analyses of the concentrations of the new microbial substance in aqueous suspensions of various quantities of the test material in water will be necessary, following thorough mixing of each suspension. These preliminary analyses are required to determine the appropriate quantity of the (solid) test material to be mixed in test water to achieve the concentration (i.e., microbial units/mL) used to provide the MHD (see Section 3.3.1) and, if a multi-concentration test (see Section 3.3.2), lower concentrations. The MHD must be the highest concentration of microbial units in water that can be prepared as an aqueous suspension of the (solid) test material, that is suitable for dispensing to the test (host) organisms by inhalation, or orally by gavage.

Control Treatments in Tests

4.1 Negative Control

Key Guidance

- *To be meaningful and judged as valid, each test must include a negative control treatment.*
- *Conditions and procedures for the negative control must be identical to that for the test concentration(s), except that the negative control must not contain any test material or modified test material.*

A *negative control* is a treatment in a test that contains no substance that could adversely affect the survival, behaviour, reproduction, growth, or other biological endpoint(s) of a specific biological test method. Each test must include this treatment, which serves as a check for the absence of pathogenicity and/or toxicity due to basic test conditions such as temperature, health of test organisms, or effects from their handling. The negative control must meet the test-specific criterion or criteria for validity, for the results of a test to be considered meaningful and valid.

Treatment of replicate groups representing the negative control is identical to treatment of those groups exposed to the test concentration(s), except that they are not exposed to the test material or any modification thereof. For a test in which the new microbial substance is mixed in test water (see Sections 3.2 and 3.4.1), the negative control groups are exposed to test water alone. This test water is mixed using the same procedure as that applied to the test concentration(s). Similarly, for a test in which the new microbial substance is mixed in *clean* test sediment or test soil, the negative control groups are exposed to the same test substrate alone, after it has been mixed using the same procedure as that for the test concentration(s) (see Sections 3.2, 3.4.2, and 3.4.3). For any test in which the new microbial substance is mixed in test food, the negative control groups are fed the same ration of food that is mixed using a procedure identical to that for the test material, but with no addition of the test material or any modification thereof (see Sections 3.2 and 3.4.4). Negative control groups to be included in a test involving birds or small mammals administered the test material orally (by gavage) or by inhalation are provided the same quantity of test water alone, as those treated with an aqueous suspension of the new microbial substance in the test water (see Sections 3.2, 3.4.5, and 3.4.6).

The number of replicates and the number of test organisms per replicate for both the negative control treatment and the test treatment(s), must be identical. All other conditions and procedures applied to the negative control groups and those exposed to the test concentration(s) of the new microbial substance must also be identical. Test organisms placed in each test chamber, including those representing the negative control (and any other control groups; see Sections 4.2 to 4.5), must be from the same population and source, and be randomly assigned to each chamber.

4.2 Positive Chemical Control

Key Guidance

- *Some of the recommended biological test methods require the use of a positive chemical control in conjunction with a definitive test; others do not.*
- *A positive chemical control provides an internal check on quality control at the testing laboratory, in terms of the condition of the test organisms and the precision and reliability of test results using a reference toxicant.*

A *positive chemical control* is a multi-concentration test with a toxic chemical, that is performed on or about the time of a definitive test for pathogenic and/or toxic effects of a new microbial substance. This test uses a *reference toxicant* that is known to adversely affect the survival, behaviour, reproduction, growth, or other biological endpoint(s) of a specific biological test method, in a predictable and demonstrable manner. The reference toxicant is a standard chemical used repeatedly at a testing laboratory to measure the sensitivity of the test organisms and to establish confidence in toxicity data obtained for a test substance. In most instances, a toxicity test with a reference toxicant is

performed to assess the sensitivity of the organisms at the time the test substance is evaluated, and the precision and reliability of results obtained by the laboratory for that chemical.

Many of the biological test methods recommended in Sections 9 to 13 require or recommend the routine use of a positive chemical control, as an internal quality control. Depending on the test method, a test using a positive chemical may either be performed at the time of the definitive test, or at another time (e.g., within one month of the definitive test), provided that test organisms are taken from the same group (e.g., a culture container or one or more holding containers with organisms from the same population) as those used in the definitive test. Depending on the specifications in the biological test method, the procedures and conditions used to perform a positive chemical control test involving a reference toxicant might be identical to that defined for the definitive test, or might be simplified (e.g., of a shorter duration, as in EC 2004b; or using a simpler means of exposure to the test substance, as in EC 1992e; 1997a,b; 1998a; 2001a).

The results of a particular test using a reference toxicant are plotted on a *warning chart*, and compared against historic data obtained by the testing facility using the same chemical and the identical testing procedure. Any value for current data with this chemical which falls beyond the *warning limits* (± 2 SD of values obtained in previous tests) indicates to that laboratory that the sensitivity of the test organisms and/or the performance and precision of the test are suspect. Such findings trigger a check of all culturing or holding conditions to which the test organisms are subjected, as well as a check of the test conditions and procedures applied during the *reference toxicity test* for which results are in question. Depending on the findings, it might be necessary to repeat the reference toxicity test, and/or to obtain a new supply (or, depending on the test, a new culture) of test organisms, before undertaking further definitive tests using this method. Findings for a positive chemical control that fall beyond established warning limits for that chemical do not necessarily negate the results of a definitive test with the new microbial substance, although they might raise some questions or concerns in this regard.

Certain biological test methods recommended herein (i.e., tests using terrestrial vertebrates; Section 14) do not require the performance of a reference toxicity test in conjunction with the definitive test using a new microbial substance or other test material. This being the case, the inclusion of a positive chemical control as part of (or in conjunction with) such test methods is unnecessary and might serve no useful purpose unless historic performance data for that chemical and test method have been compiled and plotted (as a warning chart) by the testing laboratory. If, however, the testing laboratory has compiled historic performance data for a reference toxicant using any of the biological test methods conducted with a new microbial substance, the inclusion and reporting of the findings for a positive chemical control performed as part of (or in conjunction with) the test would be worthwhile.

4.3 Positive Microbial Control

Key Guidance

- *The inclusion of a positive microbial control in a test with a new microbial substance is not required and, for most applications, is presently not recommended due to cost considerations and the absence of a suitable pathogen with genetic relatedness similar to that micro-organism and known effects on the host (test) organism.*

- *A positive microbial control might prove useful in certain instances, in that it could provide assurance that the test organisms and the biological test method are responsive to a standard reference microbial pathogen. Given this consideration, the future identification and use of suitable pathogens as positive microbial controls in specific biological test methods might prove warranted.*

A *positive microbial control* is a treatment in a test that contains an infectious *pathogen* known to adversely affect the survival, behaviour, reproduction, growth, or other biological endpoint(s) of a specific biological test method, in a predictable and demonstrable manner. A positive microbial control used in a test with a new microbial substance consists of a single concentration of a micro-organism other than that in the test material under investigation, that is known to affect the test organisms adversely and predictably during the test method to be employed. Typically, any micro-

organism serving as a positive microbial control, and its concentration, would have been used repeatedly by the testing laboratory in previous tests involving the same biological test method as that to be applied to the new microbial substance under investigation.

The purpose of including a *positive microbial control* in a definitive test with a new microbial substance would be to ascertain that testing conditions and procedures used in the test are such that penetration, infection, and disease development are likely to occur in a susceptible host (i.e., the test organisms), in a predictable and reliable manner. When considering the possible use of a positive microbial control, attention should be given to the nature of the new microbial substance under investigation, and an attempt made to use a known pathogen that is similar to this substance.

The inclusion of a positive microbial control in a definitive test is seldom required or recommended in the USEPA Series 885 (USEPA, 1996a–u) biological test methods for measuring the pathogenicity and/or toxicity of microbial pest control agents (MPCAs) or related end-use products (EPs).[34] Consistent with this approach, private laboratories performing tests according to these (Series 885) test method guidelines typically do not mention or apply a positive microbial control as part of their SOPs.

The inclusion of a *positive microbial control* in a test performed to measure the *pathogenic* and/or toxic effect(s) of a new microbial substance, according to any of the biological test methods recommended herein (see Sections 9 to 14 incl.), is not required and, for most applications, is not recommended due to cost considerations and the absence of a suitable pathogen with genetic relatedness similar to the new microbial substance and known adverse effects on the host (test) organism. Suitable *positive microbial controls* have yet to be identified for most of the biological test methods recommended herein. However, in instances where a suitable pathogen is available (i.e., one with genetic relatedness and for which pathogenic and/or toxic effects are known to occur using a particular test method), incorporation of a positive microbial control as part of a test with a new microbial substance might be warranted.

4.4 Non-Infectious Control

Key Guidance

- *The inclusion of a non-infectious control in a test with a new microbial substance is strongly recommended for all tests intended to measure the infectivity of a new microbial substance.*
- *A non-infectious control is very useful as part of the test design, in that it indicates if any adverse effects on test organisms, caused by their exposure to a new microbial substance, are due to the pathogenicity of the substance rather than its toxicity.*
- *A decision as to whether or not to include a non-infectious control in any (or all) of the biological test methods used to evaluate a new microbial substance depends on the objective(s) of the testing program.*

A *non-infectious control* is a control treatment included in a test, that consists of the *maximum hazard concentration*[35] of the test material after its treatment (e.g., by heating) to inactivate viable micro-organisms therein while preserving their structural integrity (e.g., cell walls, if bacteria). This control is included in a test to determine if the attenuated (non-infectious) MHC is responsible for any adverse effect(s) on the test organisms once its ability to cause an infection (and subsequent pathogenic effects) has been removed. The non-

[34] An exception is the USEPA test guidelines for non-target plants (USEPA, 1996c), in which instance the use of a positive microbial control is required when testing a microbial herbicide or a MPCA similar to a known plant pathogen. When testing a microbial herbicide, USEPA (1996c) states that "*the positive control should consist of the target pest weed and the microbial herbicide*"; such an application (i.e., using the target test weed as the test organism subjected to the positive microbial control) might be counter to the possible application of a positive microbial control described herein, in that the test organism subjected to the positive microbial control could differ from that exposed to the new microbial substance under investigation.

[35] The MHC is used when preparing this treatment, since it represents the concentration of the test material to which test organisms are exposed in a single-concentration test (see Section 3.3.1) as well as the highest concentration of the test material included in a multi-concentration test (see Section 3.3.2).

infectious control measures the potential contribution to adverse effects on the test organisms caused by intact but non-viable micro-organisms within the test material, along with any metabolites and/or soluble or particulate carrier substances therein. By eliminating the potential for infectivity while retaining other characteristics of the test material, the non-infectious control assists in determining if any adverse effects on test organisms caused by the new microbial substance are attributable to its pathogenicity (rather than its toxicity).

The USEPA's Series 885 (USEPA, 1996a–u) biological test methods for measuring the pathogenicity and/or toxicity of microbial pest control agents (MPCAs) or related end-use products (EPs) recommend the inclusion of a non-infectious control in some, but not all, of its pesticide test guidelines. The "overview" guidance for this series of test methods (USEPA, 1996a) states that the inclusion of a non-infectious control in a test provides information useful in determining the mechanism of pathogenesis. For tests with nontarget *insects*, USEPA (1996i) recommends that either a non-infectious control or a "*microbe*-free control" (presumably meaning a sterile filtrate control; see Section 4.5) be included in the test. The same recommendation is given for tests with honey bees (USEPA, 1996j). For tests with birds, the USEPA either recommends (USEPA, 1996k) or requires (USEPA, 1996l,m) the inclusion of a control consisting of inactivated test material in which the structural (e.g., cell walls, if bacteria) integrity of the micro-organism is preserved (i.e., a non-infectious control). Similarly, for tests with rodents, USEPA states that the use of a non-infectious control "*may prove useful to evaluate toxic properties of the MPCA*" (USEPA, 1996o,q,t), or "*is recommended*" (USEPA, 1996u). Standard operating procedures used by private laboratories performing tests according to these (Series 885) test method guidelines frequently require or recommend the inclusion of a non-infectious control (and/or a sterile filtrate control; see Section 4.5) in tests involving aquatic invertebrates, fish, or terrestrial invertebrates. The PMRA (2001) states that a non-infectious control must be included in tests with MPCAs involving birds or mammals.

For any of the recommended biological test methods associated with this guidance document, the inclusion of a non-infectious control is not a test requirement. Nonetheless, its inclusion as part of each test design is strongly recommended for all tests intended to measure (and distinguish) the infectivity and resulting pathogenicity of a new microbial substance. If the objective of the test is restricted to meeting the information requirements with respect to potential ecological effects of a new microbial substance (Government of Canada, 1997; EC and HC, 2001), it is unnecessary to distinguish if any adverse effects observed during the test are due to the pathogenicity of the new microbial substance versus its toxicity. Notifiers wishing to know if any demonstrable adverse effects are caused by a substance's toxicity rather than (or in addition to) its pathogenicity should, however, consider the incorporation of a non-infectious control in the test design.[36]

A non-infectious control can be included as part of any of the recommended biological test methods identified in Sections 9 to 14 inclusive. If this control treatment is to be included in a test, the notifier should provide the *Study Director* or *Principal Investigator* (see Section 6.1) with either the quantity of attenuated (non-infectious) test material required for the test(s), or detailed written guidance on how this control material should be prepared (e.g., conditions such as temperature, time, and equipment for sterilization, if appropriate).

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[36] If the results for both the test material and the non-infectious control show a positive response, it can be concluded that the test material is toxic and might also be pathogenic. On the other hand, if the results for the test material show a positive response while those for the non-infectious control do not, it can be concluded that the test material is pathogenic but not toxic. If the findings for a test involving both a microbial product and a non-infectious control indicate that the attenuated (non-infectious) MHC is toxic to the test organisms, consideration could be given to reformulating the microbial product in an attempt to eliminate or reduce the cause(s) of this toxicity.

4.5 Sterile Filtrate Control

Key Guidance

- *The inclusion of a sterile filtrate control in a test with a new microbial substance is optional.*
- *A sterile filtrate control is useful in that it indicates if any adverse effects on test organisms, caused by their exposure to a new microbial substance, are due to toxicity associated with a sterile filtrate of an aqueous suspension of the substance rather than the substance's pathogenicity.*
- *A decision as to whether or not to include a sterile filtrate control in any (or all) of the biological test methods used to evaluate a new microbial substance depends on the objective(s) of the testing program.*

A *sterile filtrate control* is a control treatment included in a test, that consists of the maximum hazard concentration of the test material after its treatment by sterilization (to kill viable micro-organisms) and filtration (to remove suspended particulate matter including that associated with the killed micro-organisms as well as any suspended solids associated with one or more carrier substances within the microbial product). This control is included in a test to determine if the sterilized filtrate of the MHC causes any adverse effect(s) on the test organisms.

The sterile filtrate control determines if soluble metabolites or chemicals dissolved in the filtrate are toxic to the test organisms. A test that includes both a sterile filtrate control and a non-infectious control can, depending on the findings, distinguish any toxic effects that are attributable to suspended particulate matter (i.e., attenuated micro-organisms and carrier substance or substances) and/or soluble constituents associated with a microbial product.[37] If a test includes a sterile filtrate control but not a non-infectious control, it will not be possible to distinguish toxic effects on the test organisms caused by suspended solids within the test concentration(s). If a test includes a non-infectious control but no sterile filtrate control, it will not be possible to determine if any toxic effects identified for the non-infectious control are due to soluble (dissolved) constituents.

A recommendation for the use of a sterile filtrate control in tests for the pathogenic and/or toxic effects of pesticides on host organisms is included in some but not all of the USEPA Series 885 test guidelines (USEPA, 1996a-u). The USEPA guidelines for tests using freshwater, estuarine, or marine invertebrates, or fish state that a sterile filtrate control should be included in the test (USEPA, 1996d,e,i). When testing nontarget insects including honey bees, the USEPA (1996i,j) reports state that controls in the test should either be derived from "microbe-free material" (presumably meaning a sterile filtrate control) or contain nonviable micro-organisms (i.e., a non-infectious control). For Tier-I tests with birds treated orally with pesticides, both a sterile filtrate control and a non-infectious control should be included in the test (USEPA, 1996k). For other tests with birds (USEPA, 1996l,m) or rodents (USEPA, 1996o,q,t,u), no mention is made of a sterile filtrate control (although the inclusion of a non-infectious control in the test is recommended; see Section 4.4). Standard operating procedures for private testing laboratories performing tests with pesticides according to the Series 885 guidelines frequently specify that either a sterile filtrate control or a non-infectious control (or both) is (are) to be included in a particular test.

As is the case for non-infectious controls, the inclusion of a *sterile filtrate control* in any of the recommended biological test methods associated with this guidance document is not a test requirement. The information requirements regarding the potential ecological effects of a new microbial substance (EC and HC, 2001) can be fully met without distinguishing if any adverse effects observed during the tests with host organisms (i.e., aquatic or terrestrial plants or animals) are due to the pathogenicity of the new microbial substance versus its toxicity. If, however, a notifier wishes to determine if any adverse effects attributable to a particular new microbial substance are caused by

[37] If the results for the non-infectious control indicate toxicity but those for the sterile filtrate control do not, it can be concluded that the adverse effects noted for the non-infectious control are associated with suspended solids. If both of these controls cause adverse effects due to toxicity, it can only be concluded that soluble constituents of the test material are toxic to the host organisms and that the suspended solids therein might or might not be as well.

their infectivity (and resulting pathogenicity) or toxicity, or both, the incorporation of a sterile filtrate control along with an infectivity control in the *study* design should be considered.[38]

A sterile filtrate control can be included as part of any of the recommended biological test methods identified in Sections 9 to 14, inclusive. If this control treatment is to be included in a test, the notifier should provide the *Study Director* or *Principal Investigator* (see Section 6.1) with either the quantity of a sterile filtrate of the test material that is required for the test(s), or detailed written guidance on how this control material should be prepared (e.g., conditions such as temperature, time, and equipment for sterilization, along with those for filtration to remove suspended particulate matter).

[38] In a test, the inclusion of a non-infectious control without a sterile filtrate control can discern if a new microbial substance is toxic rather than pathogenic, although it cannot distinguish if any demonstrable toxicity is caused by suspended solids and/or dissolved solids in the test concentration(s). Depending on the type of micro-organism in a new microbial substance (e.g., a virus), it might be difficult or cost prohibitive to prepare a non-infectious control; whereas a sterile filtrate control could be more easily prepared. The use of both a sterile filtrate control and a non-infectious control in a test offers additional value in terms of providing an understanding of the cause(s) of adverse effects that might be found when testing a new microbial substance.

Testing for Infectivity

Key Guidance

- *Measurements for infectivity reflect the ability of a micro-organism to cross or evade natural barriers to infection in the host (test) organism.*
- *With the exception of tests using terrestrial vertebrates, the inclusion of measurements for infectivity during or at the end of a biological test method is optional, and subject to numerous considerations including the existence of a suitable analytical method, the amount of tissue, organ, or whole-body homogenate available for analysis, and cost-benefit considerations. For tests involving birds or rodents, measurements for infectivity are required at the end of the test, methodology permitting.*
- *Measurements for infectivity conducted as part of a biological test method are useful in distinguishing whether any adverse effect(s) discerned during the test are due to pathogenicity and/or toxicity, and in satisfying information requirements on the infectivity of the test micro-organism itself, which are implicit within the NSN Regulations.*

Infectivity has been variously defined as *"the ability of a micro-organism to cross or evade natural host barriers to infection"* (USEPA, 1996a), *"the capability of a micro-organism to become established within the host species"* (EC and HC, 2001), or *"the capability of an MPCA* [(a micro-organism)] *to invade and persist in a viable state or multiply within or on an organism, with or without disease manifestation"* (PMRA, 2001). Each of these definitions aids in defining this term herein (see Terminology section, starting p. xviii). Measurements for infectivity determine the abundance of the invasive micro-organism in tissues, organs, or whole-body homogenates of the host (test) organisms. Infection of tissue(s) or organ(s) can result in overt disease, in latent disease that might become evident at a later time, or in a carrier state in which viable micro-organisms are shed from healthy individuals to others.

If introduced into a host (test) organism by one or more exposure routes (see Section 3.2), a micro-organism might infect the host and affect the induction and regulation of its primary and secondary immune responses as well as its short or long-term immunity (i.e., various defense mechanisms). The immune system of animals is typically pervasive and complex; virtually all tissues and organs are interconnected and can be affected adversely in some way by a microbial exposure. The extent of adverse effects from an infection is inversely linked to the speed and effectiveness of various *clearance* processes (both immunologic and biomechanical). A number of attempts are now being made by researchers to model the dynamic aspects such as immune complex formation and clearance, which involve at least 19 known immunologic processes related to a micro-organism's pathologic processes and immune complex-mediated antigen production. The mechanisms that living micro-organisms use to avoid immune clearance are intricate and overlapped.

Any analytical values for host animals determined during or at the end of tests for pathogenic and/or toxic effects depend on the ongoing clearance processes and rates. During clearance, a complex (space and time) process beginning with macrophages (and, in vertebrates, other cells including monocytes, neutrophils, B-cells, and T-cells) results in their adherence to infective micro-organisms followed by engulfment, killing/degradation of the micro-organisms, antigen selection, and the inactivation and disposal (translocation) of the "foreign" substances together with parts of necrotic host cells. This mechanism is very primitive, and is shared with invertebrates and vertebrates. When "foreign" microbial substances are recognized within the host organism, and depending on pre-exposure history, they are frequently coated with antibodies and/or complement components that facilitate their adherence and uptake by macrophages (and also their deactivation by neutrophils). Depending on the scale of infection, the clearance of invasive micro-organisms usually involves some damage of host tissue(s) which leads to necrosis, inflammation, fibrosis, etc. Clearance is affected adversely by certain chemicals (e.g., environmental

contaminants) to which a host organism might be previously or concurrently exposed.

Symptoms of pathogenicity (e.g., specific histopathologies or tumour formation) do not provide reliable and acceptable ways of distinguishing an infection. Infectivity can occur without signs of pathogenicity, and toxic microbial substances or associated inanimate organic or inorganic test substances included in the formulation of a microbial product can also cause certain changes evident during autopsies and associated histological examinations that are identical to those due to infections. In some instances, viable infectious micro-organisms *per se* are not responsible for signs of pathogenicity, since these symptoms are caused by toxins (i.e., *toxigenicity*). Also, with some invasive micro-organisms, notably fungi, it might take weeks or months to establish a detectable infection (USEPA, 1996cc). Observations of infectivity without signs of pathogenicity might simply mean that more time is required before signs of disease are manifested.

Douville's (2001) review of test procedures for assessing the pathogenicity and toxicity of micro-organisms to aquatic and terrestrial wildlife, in laboratory exposures, does not address testing for infectivity. The report by EC and HC (2001) briefly addresses infectivity by stating that the specific biological test methods used should be capable of detecting both infectivity and disease symptoms, and by indicating that it might be necessary to extend the observation period to assess the significance of an infection observed during a test. The PMRA (2001) provides similar statements. In each of these guidance documents, no description of procedures for measuring and monitoring infectivity during a test are provided.

Separate guidance documents for determining the nature of residues of MPCAs in animals or plants are presented (USEPA, 1996cc,dd) together with guidelines on analytical methods (USEPA, 1996ee,ff) as part of the USEPA Series 885 test guidelines for microbial pesticides. Based on a review of these guidance documents, it is evident that analytical procedures for quantifying infectivity are varied and depend on the nature of the infectious micro-organism. The onus is on the notifier to provide appropriate analytical methods for quantifying infectivity, that are relatively simple, rapid, specific, and sensitive (USEPA, 1996ee,ff).

A number of the biological test methods for microbial pesticides published by the USEPA are designed to evaluate infectivity as well as pathogenicity and/or toxicity (USEPA, 1996a). As part of their background for tests with nontarget organisms, USEPA (1996b) states:

> "*Test organisms must be examined for infection or any micro-organism-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*."

According to USEPA (1996b), the general methods of assessment for infectivity that might be required to make this determination include histopathology, serology, or nucleic acid hybridization together with re-isolation and identification of the micro-organisms from tissue. Confirmation of viral infections might require a variety of techniques including histopathology, immunohistochemistry, and serology. Depending on the infectious agent under investigation, available serological techniques vary from the traditional agglutination tests for bacteria, to more sophisticated methods such as the polymerase chain reaction (PCR-based) tests. In Canada, specialized serology and other diagnostic services are available through both commercial and government-supported regional diagnostic laboratories. The American Association for Veterinary Laboratory Diagnosticians (AAVLD) provides useful information on current diagnostic techniques and services available in North Amercia at www.aavld.org/.

For Tier-I tests with aquatic invertebrates or vertebrates, USEPA (1996d,e,g) state that a report of the results of the test must include "*a detailed description of the steps taken to determine micro-organism dissemination, replication, or survival in the test animal tissues, organs, or fluids*." Similarly, for Tier-III life cycle studies with fish, USEPA (1996h) indicates that the test report shall contain information on the isolation, identification, and enumeration of micro-organisms responsible for any observed pathogenic effects. For Tier-I tests with nontarget terrestrial plants, USEPA (1996c) states

that observations should be continued for at least two years, and that roots, foliage, fruit, vascular tissues, etc. should be analyzed for the presence of the micro-organism under investigation at the end of the observation periods using sensitive, specific methods. For Tier-I tests with birds administered a microbial pesticide orally or by inhalation, USEPA (1996k,l) state that reports of findings for gross necropsies and histopathologies should include those for any attempts, using appropriate techniques, to reisolate the infectious micro-organism from examined tissues. For Tier-III tests for *chronic* pathogenic and reproductive effects on birds, USEPA (1996m) specifies that the test report shall contain information on the re-isolation of micro-organisms from selected body tissues, at the end of the test, together with an assessment of the clinical significance of such isolations. For *acute* tests with rodents administered microbial pesticides orally or by inhalation, USEPA (1996o,q) state that the concentration of the micro-organism in certain tissues, organs, and body fluids (including kidney, brain, liver, lung, spleen, blood, and representative lymph nodes) should be determined during and at the end of the test. Similar analyses for infectivity are recommended by USEPA (1996r,u) for other acute tests (USEPA, 1996r), and for chronic tests (USEPA, 1996u) with rodents exposed to microbial pesticides.

In keeping with the USEPA's (1996a–v) Series 885 test guidelines for microbial pesticides, SOPs for private testing laboratories performing such tests frequently recommend or require measurements for infectivity as part of their test *protocols*, although certain SOPs make no mention of this. For instance, SOPs for private testing laboratories performing such tests typically do not address measurements for infectivity as part of their protocols for honey bees or other insects. This is consistent with the absence of guidance in USEPA (1996i,j) on measuring infectivity during tests with nontarget terrestrial invertebrates.

For purposes of the *Canadian Environmental Protection Act*, 1999, micro-organisms or microbial products that exert or are capable of exerting a harmful effect on the environment are classified as "*CEPA-toxic*" whether they cause an adverse effect due to their pathogenicity and/or toxicity (see Section 2). In EC and HC (2001), Section 4.2.7 *"Information in respect of the ecological effects of the micro-organism"* focuses on information and testing requirements for measuring the toxicity and/or pathogenicity, and does not provide guidance on associated procedures for measuring infectivity. This is in keeping with Schedule XV, Part 5(*a*) of the NSN Regulations regarding information with respect to the ecological effects of the micro-organism. However, Schedule XV, Part 1(*f*)(ii) of these Regulations states that information *on "the infectivity, pathogenicity to non-human species, toxicity and toxigenicity"* is also required as part of the information requirements for the micro-organism under investigation. Accordingly, the inclusion of testing for infectivity is not a requirement when performing any of the biological test methods described in Sections 9–14 herein. Notwithstanding, such information obtained during the performance of certain biological test methods recommended herein would prove useful in satisfying the reporting requirements associated with Schedule XV Part 1(*f*)(ii) of the NSN Regulations. Additionally, data on infectivity gleaned during the performance of a biological test method for ecological effects of a new microbial substance would also prove useful when interpreting the findings in terms of the presence or absence of adverse effects that might be due to pathogenic and/or toxic actions associated with the test material.

The inclusion of measurements for infectivity, as part of tests for the pathogenic and/or toxic effects of a new microbial substance on aquatic or terrestrial plants or invertebrates, or aquatic vertebrates, is recommended in instances where the biological test method is able to provide sufficient tissue, organ, or whole-body homogenate for this analysis.[39] A second caveat is that the analytical method used to quantify infectivity during or at the end of the test should be relatively simple, rapid, specific, and sensitive (USEPA, 1996ee,ff); as well as standardized and able to provide meaningful results with an acceptable degree of precision. A third caveat is that the investigator(s) should be aware of the costs of such analyses, and proceed as

[39] This might not be the case for tests involving pelagic microcrustaceans such as daphnids (Section 10) or small terrestrial invertebrates such as springtail *arthropods* (Section 13).

warranted in light of cost-benefit considerations.[40] Analytical techniques permitting, tests involving terrestrial vertebrates (i.e., birds or rodents) must include measurements for infectivity in selected organs, tissues, or body fluids, at the end of the test; additional measurements for infectivity as the test proceeds are optional but encouraged to determine any time-dependent onset of an infection and its progression (or clearance) as the test progresses. The inclusion of additional replicates in the test design might be required for this purpose.

For tests that include measurements for infectivity, these determinations might be restricted to the analysis of tissues, organs, or whole-body homogenates taken from test organisms representing each treatment at the end of the test, following the determination of all observations and biological endpoints for those organisms. For those biological test methods that require dry-weight determinations of test organisms at test end, measurements for infectivity at test end should be based on additional replicates included in the study design for this purpose (in which instance these groups would be analyzed for infectivity rather than dried and weighed). If measurements for infectivity as the test proceeds are desired, additional replicates representing each treatment should be included in the study design, and used specifically for this purpose.[41]

[40] A notifier might already have sufficient information on the infectivity of the micro-organism to satisfy Schedule XV Part 1(*f*)(ii) of the NSN Regulations (Government of Canada, 1997), and might also not be concerned with distinguishing whether any adverse ecological effects of the test material are attributable to its pathogenic and/or toxic properties. In this instance, the inclusion of measurements of infection as part of a biological test method might not be necessary or warranted.

[41] If there is clinical evidence indicating that there might be adverse effects during or following exposure to a particular new microbial substance, sequential assessments for infectivity might be advised or required. In such an instance, representative samples should be taken from selected tissues or organs for microbial isolation during the course of the laboratory test for pathogenic and/or toxic effects.

Principles of Good Laboratory Practice

Key Guidance

- *Any test performed in the laboratory to measure the pathogenicity and/or toxicity of a new microbial substance according to this guidance document should follow and adhere to the OECD Principles of Good Laboratory Practice.*

- *The OECD Principles are summarized under these headings:*
  1. *Organization and Responsibilities of Testing Laboratory*
  2. *Quality Assurance Program*
  3. *Test Facilities*
  4. *Apparatus, Materials, and Reagents*
  5. *Test Systems*
  6. *Test Substances*
  7. *Standard Operating Procedures*
  8. *Performance of the Study*
  9. *Reporting of Study Results*
  10. *Storage and Retention of Records and Materials*

The New Substances Notification (NSN) Regulations (Government of Canada,1997) under the *Canadian Environmental Protection Act*, 1999 state as follows, in subsections 31(1) and 31(2) under the heading "*Testing Requirements*":

31(1) *The conditions and test procedures to be followed in developing test data in respect of a substance in order to comply with the information requirements of section 81 of the Act or requests for information under paragraph 84(1)(c) of the Act shall be consistent with the conditions and procedures set out in the "OECD Test Guidelines", being Annex 1 of the* OECD Guidelines for Testing of Chemicals, *adopted by the Organisation for Economic Co-operation and Development Council on May 12, 1981, that are current at the time the test data are developed.*

31(2) *The laboratory practices to be followed in developing test data referred to in subsection (1) shall be consistent with the practices set out in the "Principles of Good Laboratory Practice", being Annex 2 of the* OECD Guidelines for Testing of Chemicals, *adopted by the Organisation for Economic Co-operation and Development Council on May 12, 1981.*

The test data referred to in these subsections do not include those for live micro-organisms, for which laboratory testing requirements are defined in Part II.1 of the NSN Regulations. Nonetheless, the concept and principles of *Good Laboratory Practice* (GLP) should be applied to tests involving living organisms (including live micro-organisms) until such time that the Act and/or the NSN Regulations include such a requirement for living organisms.

Since the adoption of Annexes 1 and 2 of the OECD Guidelines for Testing of Chemicals by the OECD Council in 1981, member countries of the Organisation for Economic Co-operation and Development (OECD) including Canada considered that there was a need to review and update the OECD Principles of Good Laboratory Practice (GLP). An Expert Group, comprised of representatives of 25 countries including Canada plus the International Organisation for Standardisation, prepared Revised OECD Principles of GLP, which were adopted by the OECD Council on November 26, 1997 and thus formally amended Annex II of the 1981 Council Decision. The revised OECD Principles of Good Laboratory Practice have been published (OECD, 1998a), as have 12 related guidance documents (OECD, 1995a,b,c,d; 1998b; 1999a,b,c,d,e; 2000a; and 2002a).[42]

The OECD Principles of Good Laboratory Practice are intended to be applied to the non-clinical safety

[42] These OECD documents can be downloaded off the Internet at www.oecd.org/ehs/. Printed copies can also be obtained from: CEPA GLP Compliance Monitoring Unit, Environment Canada, Environmental Technology Centre, 335 River Road, Ottawa, Ontario K1A 0H3. Alternatively, copies can be purchased by contacting the OECD Environment Directorate, Environment Health and Safety Division, 2 rue André-Pascal, 75775 Paris Cedex 16 France, fax (33-1) 45 24 16 75, e-mail ehscont@oecd.org.

testing of various substances including industrial chemicals, pesticide products, and substances comprised of or containing living organisms (OECD, 1998a). Health Canada's Pest Management Regulatory Agency has published a Regulatory Directive entitled *"Good Laboratory Practice"* (PMRA, 1998) that is consistent with OECD (1998a; and others of this series) when testing pest control products for safety to human health or the environment. Laboratory tests undertaken to measure the pathogenicity and/or toxicity of new microbial substances as part of the information requirements associated with these Regulations (EC and HC, 2001) should also be performed according to the OECD Principles of Good Laboratory Practice.

The intent of this section is to familiarize the reader and involved parties (e.g., notifiers, regulatory personnel, environmental consultants, testing laboratories) with some of the basic Principles of Good Laboratory Practice established by OECD, that apply when preparing for and undertaking laboratory tests with new microbial substances for their potential ecological effects on aquatic or terrestrial plants and animals. These are laid out in full in Section II of OECD's (1998a) primary (*"Number 1"*) guidance document on Principles of Good Laboratory Practice. Other OECD documents of this series focus on specific aspects of GLP, as follows:

- *Revised Guides for Compliance Monitoring Procedures for Good Laboratory Practice* (No. 2; OECD, 1995a).
- *Revised Guidance for the Conduct of Laboratory Inspections and Study Audits* (No. 3; OECD, 1995b).
- *Quality Assurance and GLP* (No. 4; OECD, 1999a).
- *Compliance of Laboratory Suppliers with GLP Principles* (No. 5; OECD, 1999b).
- *The Application of the GLP Principles to Field Studies* (No. 6; OECD, 1999c).
- *The Application of the GLP Principles to Short Term Studies* (No. 7; OECD, 1999d).
- *The Role and Responsibilities of the Study Director in GLP Studies* (No. 8; OECD, 1999e).
- *Guidance for the Preparation of GLP Inspection Reports* (No. 9; OECD, 1995c).
- *The Application of the Principles of GLP to Computerised Systems* (No. 10; OECD, 1995d).
- *The Role and Responsibilities of the Sponsor in the Application of the Principles of GLP* (No. 11; OECD, 1998b).
- *Requesting and Carrying Out Inspections and Study Audits in Another Country* (No. 12; OECD, 2000a).
- *The Application of the OECD Principles of GLP to the Organisation and Management of Multi-Site Studies* (No. 13; OECD, 2002a).

Some of these guidance documents (i.e., Nos. 6, 12, and 13) do not apply in the present context. Others (e.g., Nos. 2, 3, 5, and 9) are of peripheral interest in that they deal with such issues as compliance monitoring procedures, compliance of laboratory suppliers, or laboratory inspections and study audits associated with GLP. The appropriate OECD guidance documents referred to herein should be reviewed and consulted for definitive guidance.

6.1 Organization and Responsibilities of Testing Facilities

According to OECD (1998a), the management of each testing facility should ensure that the Principles of Good Laboratory Practice are complied with when undertaking each test. These include a statement in the test report identifying the individual(s) within a test facility who fulfil these management functions. Other management responsibilities include the establishment, approval, and use of appropriate and technically valid Standard Operating Procedures for the performance of each test method as well as various other related activities conducted by laboratory personnel (see Section 6.7). Management should also ensure that there is a Quality Assurance Program (see Section 6.2) with designated personnel functioning within the facility responsible for ensuring and monitoring

the performance of each test according to the Principles of GLP. Before each study is initiated, management should designate a *Study Director* who has the appropriate qualifications, training, and experience.

The responsibilities of the Study Director are provided in Section 1.2 of OECD (1998a). A separate OECD document (No. 8, OECD, 1999e) deals solely with the role and responsibilities of the Study Director in GLP studies. As stated in both OECD (1998a) and OECD (1999e), the Study Director represents the single point of study control with ultimate responsibility for the overall scientific conduct of the study. In this regard, the Study Director serves to assure that the scientific, administrative, and regulatory aspects of the study are controlled by coordinating the inputs of management, scientific/technical staff, and the Quality Assurance program. Scientifically, the Study Director is usually the scientist responsible for study plan design and approval, as well as overseeing data collection, analysis, and reporting. The Study Director is also responsible for drawing the final overall conclusions from the study. Compliance with regulations, including ensuring that the study is carried out in accordance with the Principles of GLP, is the responsibility of the Study Director. This person's signature is required on the final study report (OECD, 1998a; 1999e).

The responsibilities of the *Principal Investigator*(s) and all other study personnel involved in the conduct of a study, with respect to the Principles of GLP, are described in Sections 1.3 and 1.4 of OECD (1998a).

6.2 Quality Assurance Program

A Quality Assurance Program is defined in OECD (1998a; 1999a) as *"a defined system, including personnel, which is independent of study conduct and is designed to assure test facility management of compliance with these Principles of Good Laboratory Practice"*. One of the responsibilities of the management of a testing facility is to ensure that there is a documented Quality Assurance Program in place, with designated personnel, and that functions are performed in compliance with the Principles of Good Laboratory Practice (see Section 6.1). Management should also ensure *"that the Study Director has made the approved study plan available to the Quality Assurance personnel"* (OECD, 1998a; 1999a).

The Quality Assurance Program should be carried out by personnel responsible to management, who are familiar with the test procedures and conditions. These Quality Assurance (QA) personnel should not be involved in conducting the study. Their responsibilities include (OECD, 1999a):

(i) maintaining copies of all approved study plans and Standard Operating Procedures in use at the testing facility, including up-to-date copies of any revised SOPs;

(ii) verifying (including documentation) that the study plan contains the information required for compliance with the Principles of GLP;

(iii) conducting inspections to determine if all studies are conducted in compliance with these Principles of GLP;

(iv) inspecting the final reports to confirm that the methods, procedures, and observations are accurately and completed described and that the reported results accurately and completely reflect the *raw data* of the studies;

(v) reporting promptly any inspection results in writing to management, the Study Director, and the Principal Investigator(s), when applicable; and

(vi) preparing and signing a statement, to be included with the final report, that specifies the types of inspections and their dates, including the phase(s) of the study inspected and the date(s) that inspection results were reported to management and the Study Director and Principal Investigator(s), if applicable.

The manager ultimately responsible for GLP should be clearly identified. The person appointed to be responsible for QA must have direct access to the different levels of management, particularly to top-level management of the testing facility. Quality Assurance personnel are not normally involved in drafting SOPs, although it is desirable that they review them to assess clarity and compliance with

GLP Principles. The OECD (1999a) provides guidance on the performance of QA inspections and audits.

The Principles of GLP require a signed Quality Assurance statement to be included in the final report. This statement specifies types and dates of inspections including the phase(s) of inspection and the date(s) that inspection results were reported to management, the Study Director, and the Principal Investigator(s). The format of the QA statement is specific to the nature of the report. The OECD (1999a) recommends that the QA statement only be completed if the Study Director's claim to GLP compliance can be supported.

At small testing facilities, it might not be practicable for management to maintain personnel dedicated solely to QA; however, at least one individual must be given permanent (even if part-time) responsibility for coordination of the QA function. It is acceptable for individuals involved in studies that comply with GLP to perform the QA function for GLP studies conducted in other departments within the testing facility. It is also acceptable for personnel from outside the testing facility to undertake QA functions if the necessary effectiveness required to comply with the GLP Principles can be ensured (OECD, 1999a).

6.3 Test Facilities

Section II(3) in OECD (1998a) describes the Principles of GLP that apply to testing facilities. This includes principles related to the general design and layout of the testing facility, dedicated testing facilities, dedicated handling and storage facilities, archive facilities, and waste disposal facilities.

The design of the testing facilities should provide an adequate degree of separation of the different tests and activities to assure the proper conduct of each study. A sufficient number of rooms or areas should be available to assure the isolation of test systems as well as individual tests, substances, or organisms. Suitable rooms or areas should be available for the diagnosis, treatment, and control of diseases.

Storage rooms/areas for the *test substance*s should be separate from rooms/areas containing the test systems. These rooms/areas should be adequate to preserve identity, concentration, purity, and stability, and to ensure the safe storage of hazardous substances. In preparation for the test, there should also be separate rooms/areas for the receipt of test substances, and for their mixing, handling, and preparation (as test concentrations).

Archive facilities should be provided for the secure storage and retrieval of study plans, raw data, final reports, samples of test substances, and specimens. The design of such facilities should protect their contents from untimely deterioration.

Handling and disposal of wastes should be carried out in such a way as to prevent the integrity of studies from being jeopardized. This includes the provision of appropriate collection, storage, and disposal facilities, as well as necessary decontamination and transportation procedures.

6.4 Apparatus, Materials, and Reagents

Good Laboratory Practice Principles for the apparatus, materials, and reagents used in a study are described in Section II(4) of OECD (1998a). The OECD consensus document No. 5 *"Compliance of Laboratory Suppliers with GLP Principles"* (OECD, 1999b) gives further details, which apply to the:

- standards and accreditation schemes;
- animal feed, bedding and water;
- computer systems and applications software;
- reference substances;
- inspection and calibration of instruments; sterilization of materials;
- accreditation of general reagents;
- choice of detergents and disinfectants; and
- documentation of substances required for microbiological testing.

The OECD consensus document No. 10 *"The Application of the Principles of GLP to Computerised Systems"* (OECD, 1995d) provides detailed guidance on the use and validation of computer software and hardware applied to the generation, measurement, or assessment of data intended for regulatory submission in keeping with GLP Principles.

According to Section II(4) in OECD (1998a), apparatus including validated computerized systems used for the generation, storage, and retrieval of

data, and for controlling environmental factors relevant to the study, should be suitably located, of appropriate design, and of adequate capacity. Apparatus should be periodically inspected, cleaned, maintained, and calibrated according to Standard Operating Procedures. Records of these activities should be maintained. Apparatus and materials should not interfere adversely with the test system(s). Chemicals, reagents, and solutions should be labelled for identification (with concentration if appropriate), expiry date, and specific storage conditions. Information concerning source, preparation date, and stability should be available.

The OECD (1999b) report states that the user of substances required for microbiological testing (i.e., microbial substances) should be responsible for ensuring (by arrangement with the supplier) that all such substances are labelled with at least the following information: source, identity, date of production, shelf life, and storage conditions. The supplier should ensure that documentation is available giving evidence of any accreditation status. Where there is no national accreditation scheme, the supplier should provide the user with a validation document providing evidence that the substance is as described by its label.

Demonstration that a computerized system is suitable for its intended purpose is of fundamental importance (OECD, 1995d). Referred to as computer validation, this should be undertaken by means of a formal validation plan performed before operational use. Considerations to assist in the application of GLP principles to computerized systems are identified and discussed in OECD (1995d) under the following headings:

- responsibilities,
- training,
- facilities and equipment,
- maintenance and disaster recovery,
- data,
- security;
- validation of computerized systems,
- documentation, and
- archives.

6.5 Test Systems

The GLP Principles applicable to test systems are included in Section II(5) of OECD (1998a). Apparatus used for the generation of physical/chemical data should be suitably located, of appropriate design, and of adequate capacity. The integrity of the physical/chemical test systems should be ensured.

Proper conditions for storage, housing, handling, and care of biological test systems should be established and maintained to ensure quality of data. Newly received animals and plants to be used as test organisms (or as cultures for their propagation) should be isolated until their health status has been evaluated. If any unusual mortality or *morbidity* occurs, lots received should not be used in studies and, when appropriate, should be humanely destroyed. Test systems should be free of any disease or conditions at the start of the test, that might interfere with the purpose or conduct of the study. Records of source, date of arrival, and arrival conditions of test organisms (or cultures thereof) should be maintained. Test organisms should be acclimated to the test environment for an adequate period before the first administration of the test substance. All information needed to properly identify biological test systems (e.g., test chambers) should appear on their housing or containers. Housing or containers for biological test systems should be cleaned and sanitized during (or before and following) their use. Any material that comes into contact with the biological test system should be free of contaminants that would interfere with the study.

6.6 Test Substances

Section II(6) in OECD (1998a) provides GLP Principles for the receipt, handling, sampling, storage, and characterization of test substances (including reference substances). Records to be maintained include dates of receipt of each test substance, expiry date, quantities received, and quantities used in studies. Handling, (sub)sampling, and storage procedures should be identified to ensure sample homogeneity and stability to the extent possible, and to preclude sample contamination or mixup. Storage container(s) should carry identification information, expiry date,

and specific storage instructions. Each test substance, including any reference substance, should be appropriately identified (e.g., batch number, purity, composition, percent viability, concentrations, or other characteristics which define each batch). If the test substance is supplied by the sponsor, there should be a mechanism developed in cooperation with the sponsor and the testing facility by which the identity of the test substance can be verified.

The stability of test and reference substances under storage and test conditions should be known, for each study. If the test substance is administered in a vehicle (carrier), the homogeneity, concentration, and stability of the test substance in that vehicle should be determined. For all studies except short-term ones, a (sub)sample should be retained from each batch of a test substance for analytical purposes.

6.7 Standard Operating Procedures

According to Section II(7) in OECD (1998a), a testing facility should have written Standard Operating Procedures (SOPs), approved by the facility's management, that are intended to ensure the quality and integrity of the data generated by that facility. Revisions to SOPs should also be approved by the management of the testing facility. Published text books, analytical methods, articles, and manuals may be used as supplements to these SOPs.

As part of OECD's GLP Principles, Standard Operating Procedures should be available for (but not limited to) the following categories of activities and related items within a testing facility:

- test substances;
- handling and maintaining apparatus, materials, and reagents;
- record-keeping, reporting, storage, and retrieval;
- computerized systems (including their validation, operation, maintenance, security, and back up);
- the test system (including maintenance of the test facility, test chambers, and test organisms); and
- Quality Assurance procedures (OECD, 1998a).

6.8 Performance of the Study

Section II(8) in OECD (1998a) presents GLP Principles related to aspects of the performance of the study. These Principles deal with the study plan, the content of the study plan, and the conduct of the study.

A written plan should exist before the study is initiated. The study plan should be approved by the dated signature of the Study Director and verified for GLP compliance by Quality Assurance personnel. Amendments to the study plan should be justified and approved by the dated signature of the Study Director, and maintained with the study plan. Deviations from the study plan should be described, explained, acknowledged, and dated in a timely manner by the Study Director and/or the Principal Investigator(s), and maintained with the raw data for the study. A general study plan accompanied by a study-specific supplement may be used for short-term studies (OECD, 1999d).

The study plan should contain, but not be limited to, the following information:

- identification of the study and the test substances (including reference substances);
- information concerning the sponsor and the testing facility (names and addresses of the sponsor, testing facility, Study Director, and Principal Investigator);
- dates (including the date of approval of the study plan as signed by the sponsor, laboratory management, and the Study Director; as well as the proposed experimental starting and completion dates);
- test methods (reference to the biological test method or guideline to be used);
- issues (e.g., justification for selection of the test method, characterization of the test system, dose/concentration including frequency and duration of administration, detailed information on the experimental design); and

- records (a list of records to be retained).

6.9 Reporting of Study Results

The OECD's Principles of Good Laboratory Practice include guidance on reporting study results. As indicated in Section II(9) of OECD (1998a), a study report should be prepared for each study. In the case of short-term studies, a standardized final report accompanied by a study-specific extension may be prepared.[43] Reports of Principal Investigators or scientists involved in the study should be signed and dated by them. Additionally, the final report should be signed and dated by the Study Director to indicate acceptance of responsibility for the validity of the data. The extent of compliance with OECD's Principles of Good Laboratory Practice should be indicated. Corrections and additions to a final report should be in the form of amendments specifying the reason for these modifications. Such amendments should be signed and dated by the Study Director. The final report should include an identification of the study and the test substances (including reference substances). Details that characterize the test substance, including its purity, stability, and homogeneity should be provided. Names and addresses of the sponsor, testing facility, Study Director, Principal Investigator(s), and other scientists contributing to the final report should be identified. Experimental starting and completion dates are to be included. A Quality Assurance Program statement listing the types of inspections made and their dates, together with the dates that any inspection results were reported to the laboratory management, Study Director, and Principal Investigator(s), should be included in the final report.[44] This report should also include a description of methods and materials used, a summary of results, all information and data required by the study plan, a presentation of the results including calculations and statistical determinations, an evaluation and discussion of the results, and, where appropriate, conclusions. Additionally, the final report should identify the locations(s) where the study plan, (sub)samples of test and reference substances, specimens, raw data, and the final report are to be stored (OECD, 1998a).

6.10 Storage and Retention of Records and Materials

Principles of Good Laboratory Practice pertaining to the storage and retention of records and materials are listed in Section II(10) of OECD (1998a). Information to be retained in the archives for the period specified by the appropriate authorities includes:

- the study plan, raw data, (sub)samples of test substances (including reference substances), and the final report;
- records of all inspections performed by the Quality Assurance Program;
- records of qualifications, training, experience, and job descriptions of personnel;
- records and reports of the maintenance and calibration of apparatus;
- validation documentation for computerized systems (see OECD, 1995d); and
- the historical file of all Standard Operating Procedures.

It should be justified and documented if (sub)samples of test substances are disposed of, for any reason, before the required retention period

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[43] The OECD (1999d) provides further guidance on the application of GLP Principles to short-term studies. In this context, a short-term study is loosely defined as one that differs significantly from a chronic one, as well as one of short duration. Within this definition is the concept that such a study is one that is routinely performed at the testing facility using a standardized method. Short-term studies include *acute toxicity* studies and acute ecotoxicological studies (OECD, 1999d).

[44] For a short-term study, inspections may be limited to "process-based" ones that are conducted periodically to monitor procedures or processes of a repetitive nature. Such inspections are generally performed randomly, and take place when a process (i.e., the same biological test method) is undertaken at a high frequency within a facility which makes it inefficient or impractical to undertake study-based inspections (OECD, 1999d).

expires. These samples and specimens should be retained only as long as the quality of the preparation permits evaluation. Material retained in the archives should be indexed so as to facilitate its orderly storage and retrieval. Only personnel authorized by the laboratory's management should have access to the archives. Movement of material in and out of the archives should be properly recorded. If a testing facility or an archive contracting facility goes out of business and has no legal successor, the archive should be transferred to the archives of the sponsor of the study (OECD, 1998a).

Laboratory Biosafety and Animal Care and Use

This section focusses on two important considerations when handling and testing infectious micro-organisms in the laboratory: (1) worker safety, and (2) animal care and use. Definitive information and direction in these respects is found in key Canadian guidance documents on these subjects (HC, 2001; CCAC, 1993). These subjects are addressed here in brief, primarily to draw attention to them and the associated guidance documents. Mention is also made of relevant issues when protecting worker safety and testing animals in the laboratory.

7.1 Laboratory Biosafety

Key Guidance

- *Laboratory personnel should be familiar with and follow Health Canada's* Laboratory Biosafety Guidelines *(HC, 2001 or subsequent editions) when handling, testing, and containing infectious micro-organisms.*

- *Guidance therein on the following subjects should be consulted and followed: choosing the appropriate containment level; handling potentially infectious substances using appropriate and designated facilities; requirements for minimum operational practices at each containment level; laboratory design and physical requirements; laboratory commissioning, certification, and re-certification processes; requirements for large-scale production and/or processing of micro-organisms; human risks associated with experimental animals; selected guidelines for work with unique hazards; guidance on decontamination; and the use of biological safety cabinets.*

Health Canada has published two editions of *Laboratory Biosafety Guidelines* to guide laboratories in their development of biosafety policies and programs when handling, testing, and containing infectious micro-organisms. A third edition of this publication, which has been updated to reflect current biosafety and biocontainment principles and practices, has been prepared as a public draft (HC, 2001).[45] Laboratory personnel performing tests for ecological effects of new microbial substances should be familiar with this or subsequent editions of Laboratory Biosafety Guidelines published by Health Canada, and follow the guidance therein when handling, testing, and containing these substances.

It is the responsibility of all laboratory workers as well as their supervisors and directors of the laboratory to be familiar with and apply the biosafety information available in these *Guidelines*, in a cautious and rigorous manner (HC, 2001). *Risks* to humans associated with work involving infectious microbial substances can be minimized through the application of appropriate biosafety and containment principles and practices. Serious consideration to laboratory biosafety must be given at all stages of handling and testing a new microbial substance or test concentrations thereof.[46]

Some of the recommendations or requirements for laboratory biosafety that are addressed in Health Canada's *Laboratory Biosafety Guidelines* (HC, 2001) follow. Subjects addressed therein are also summarized briefly here. This Health Canada guidance document should be consulted for details and definitive advice.

[45] This guidance document (as a third draft, and as any subsequent revisions available to the public including the final edition) may be accessed on the Internet at www.hc-sc.gc.ca by entering a search using the words "Laboratory Biosafety Guidelines".

[46] HC (2001) cites a number of articles that report laboratory-associated infections of workers related to the handling and testing of infectious micro-organisms, some of which resulted in death.

Biological safety (Chapter 2)

- Choose the appropriate containment level required to work with a particular micro-organism.[47]
- A risk assessment should be carried out to select the appropriate containment level.
- The designation of a Biological Safety Officer is recommended. Activities and responsibilities are described.

Handling infectious substances (Chapter 3)

- Means of infection and types of events leading to an infection are reviewed.
- Exposure to aerosols might be the greatest biohazard facing laboratory workers. Techniques to minimize the creation of aerosols are described.
- General practices required for all laboratories handling infectious substances are described.
- Minimum operational practices required for containment level 2 are outlined. These are in addition to the general practices required.
- Minimum operational practices required for containment level 3 are outlined. These are in addition to the general practices required as well as those for containment level 2.
- Minimum operational practices required for containment level 4 are outlined. These are in addition to the general practices required as well as those for containment levels 2 and 3.
- Each laboratory must adopt laboratory security practices to minimize opportunities for unauthorized entry into facilities and storage areas, and the unauthorized removal of infectious materials from the laboratory.

Laboratory design and physical requirements (Chapter 4)

- Guidance on the design and layout required to achieve each of the four containment levels is provided.
- Requirements for each containment level are identified for each of the following: laboratory location and access; surface (i.e., floors, walls, ceilings, sealants) finishes and casework; air handling/heating, ventilation, and conditioning; containment perimeter; and laboratory services (i.e., water, gas, electricity, and safety equipment).

Commissioning, certification, and recertification (Chapter 5)

- *Commissioning* processes are normally undertaken to verify that the facility design meets applicable codes and standards and that it has been constructed in accordance with the design intent.
- To ensure that the physical requirements for the intended containment level and use of the facility have been met, each laboratory must undergo a detailed commissioning regime.
- For certification of level-3 or level-4 facilities, room integrity must be tested; requirements for testing at each level are provided.
- Various components of a containment room's air handling system must be commissioned.
- Integrity testing of HEPA filters installed in level-3 and level-4 facilities must be performed.

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[47] Four containment levels are described. Level-1 facilities require no special design features beyond those suitable for a well-designed and functional laboratory. Level-2 facilities include primary containment devices such as biological safety cabinets, personal protective equipment, and decontamination equipment (autoclaves). Level-3 facilities include additional features appropriate for respiratory protection (i.e., HEPA filtration of exhausted laboratory air and strictly controlled laboratory access). Level-4 facilities represent the maximum containment available, and include an isolated and sealed unit, protection of workers using positive pressure suits or Class III biological safety cabinets, and specialized systems for filtering intake air and decontaminating effluents. Guidance for the selection of appropriate containment levels is found at the following website, maintained by Health Canada's Office of Laboratory Security: www.hc-sc.gc.ca/hpb/lcdc/biosafty.

- Acceptance criteria for the supply and exhaust ductwork must be satisfied.

- Testing and verification of operation of various laboratory equipment and services is described.

Large-scale production of micro-organisms (Chapter 6)

- Large-scale production and processing of agents requiring containment level 3 might result in a serious hazard to the people working at or near the facility, animals in or around the facility, or the environment surrounding the facility.

- Special precautions relating specifically to the handling and testing of large-scale quantities (e.g., in excess of 10 L) of infectious or potentially infectious liquid suspensions are necessary.

- Specific requirements have not been outlined for large-scale research or production of viable organisms requiring containment level 4. These requirements are to be established on a case-by-case basis.

- Specific large-scale operational practices and physical requirements are detailed for containment levels 1, 2, and 3.

- Minimal containment requirements for large-scale process areas are listed. These are to be used in addition to the requirements listed for the corresponding laboratory-scale containment facility.

Laboratory animals (Chapter 7)

- Work with animals poses a variety of unique hazards, including exposure to infectious agents (naturally occurring or experimentally produced), animal bites or scratches, allergies, and physical hazards such as noise and temperature.

- Unnecessary exposure to allergens can be minimized through engineering controls, ventilation, use of containment caging systems, and appropriate use of respiratory and other personal protection.

- Facilities for work with small animals should be designed and operated in accordance with the *Containment Standards for Veterinary Facilities* (CFIA, 2001) and the *Guide to the Care and Use of Experimental Animals* (CCAC, 1993).

- Animals rooms should be separated from other activities in the laboratory and, ideally, should be a physically separated unit.

- Specific entry and exit protocols for scientific staff, animal handlers, animals, test substances and concentrations thereof, equipment, feed, and wastes should be developed for each project.

Selected guidelines for work with unique hazards (Chapter 8)

- Most recombinant-DNA research and genetic manipulation, in itself, does not pose any specific risks to worker safety; however, some genetic manipulation does raise a significant possibility of risk.

- Guidance on how to assess potential risks in recombinant DNA research are available but can only be very general.

- Factors to consider when determining the containment level of a recombinant organism are discussed.

- A detailed assessment must be made as to the level of hazard associated with the maintenance and manipulation of each new primary line (culture) of micro-organisms in the laboratory, to determine the appropriate level of precautions to be taken. Guidance on this is provided. The microbial line is to be handled at the containment level appropriate to the level of risk determined by the assessment.

- Hazards to workers associated with specific types of microbial lines (e.g., viral or fungal, non-mammalian or mammalian) are considered.

- Hazards when working with micro-organisms that produce toxins are addressed, and practices to be followed are described. Decontamination

procedures and solutions for toxins are briefly addressed.

- A section of this chapter is dedicated to working safely with mycobacteria. Stratified containment levels are described for specific manipulations. Special safety precautions related to protective clothing, respiratory protection, and showering are discussed. Guidance on disinfecting when working with mycobacteria is given.

- Containment guidelines for working with arthropods are provided. Risks related to insects transmitting diseases to humans are discussed. Some general guidance and biosafety considerations when working with insects is provided. Subjects addressed include arthropod containment levels, arthropod manipulations, action plans in the event of escapes, and testing the integrity of the system.

Decontamination (Chapter 9)

- Decontamination by sterilization and disinfection is addressed in this chapter.

- Lists of various decontaminants and their effectiveness are referred to.

- It is the responsibility of all laboratory workers to ensure the effective use of decontaminants for waste disposal, removal of materials and equipment from containment zones, sampling handling, laundry, decontaminating surfaces and rooms, and spills of infectious material.

- Specific written protocols must be developed and followed for each process.

- Employees must be trained in all decontamination procedures specific to their activities.

- Guidance on effective decontamination of autoclaves is provided.

- Guidance on the selection and use of chemical disinfectants is provided.

- Guidance on decontamination by gas, liquid effluent treatment systems, radiation, incineration, and new technologies is given.

Biological safety cabinets (Chapter 10)

- The use of biological safety cabinets for primary containment work with pathogens is addressed.

- Applications of biological safety cabinets include procedures with the potential for producing infectious aerosols and for high concentrations or large volumes of infectious material.

- Every employee working in a biological safety cabinet must be trained in its correct use.

- The various classes of biological safety cabinets and their selection, installation, and certification are discussed.

- Start-up procedures, working procedures, and shut-down procedures when performing work in a biological safety cabinet are provided.

7.2 Animal Care and Use

Key Guidance

- *The latest publication on the care and use of experimental animals by the Canadian Council on Animal Care (i.e., CCAC, 1993 or subsequent edition) should be followed as a guide when working with animals in the laboratory.*

- *The focus of this document is on the care and use of mammals held in the laboratory for experimental purposes, although some consideration is given to other vertebrates including birds and fish.*

- *This guide should be consulted for guidance on: the design of animal facilities; cage design for animals; suitable climate control (e.g., for temperature, humidity, ventilation, and lighting); influences of environmental variables such as noise, chemicals, bedding, and population density; transporting and acclimating animals; care of the animal; care of the facility; social and behavioural requirements; care during restraint and manipulations; occupational health and safety in an animal laboratory; control of animal pain; anaesthesia; and euthanasia.*

All experimental care and use of animals in Canada is subject to the requirements of the Canadian Council on Animal Care (CCAC), a national, peer-reviewed organization founded in Ottawa in 1968

(CCAC, 1993). The mandate of this Council is "*to work for the improvement of animal care and use on a Canada-wide basis*".

In 1993, the Canadian Council on Animal Care published the second edition of its guide to the care and use of experimental animals. This publication (CCAC, 1993) provides guidance on animal care and use that applies in the laboratory when testing new microbial substances for ecological effects on animals. Following is a brief summary of some of the subjects, recommendations, or requirements on animal care and use found in CCAC (1993), that apply when caring for and testing animals in the laboratory. This guidance document should be consulted for details and definitive advice on animal care and use.[48] It focusses on the care and use of mammals, although some consideration is given to other vertebrates including birds and fish. Guidance on the laboratory care and use of aquatic and terrestrial invertebrates is not included within the CCAC program at this time.

Laboratory animal facilities (Chapter II)

- Construction guidelines for facilities to house animals are provided.
- Animal facilities should be located so as to minimize public access or through-traffic. Direct access to the outside is desirable. Access to experimental animal facilities must be restricted.
- Animal rooms should be designed for ease of sanitation. They should be separate from experimental rooms, and their size based on the species to be maintained.
- The size of caging chosen to house each species should be appropriate for that species. Solid-bottom cages should be chosen for housing rodents, unless contra-indicated by the nature of the studies.

The environment (Chapter III)

- The design, optimization, and monitoring of climate-control features in the animal facilities, including systems for controlling temperature, humidity, ventilation, and lighting, are discussed.
- Environmental requirements vary with the species and the experimental protocol; thus the design of the animal facility should permit adjustments.
- Noise control in an animal facility should be considered during the design phase, when choosing equipment, and as part of good management practices.
- Monitoring and controlling chemicals to which animals are exposed in various ways (e.g., by water, air, food, bedding, or contact surfaces) is addressed. Particular concerns regarding ammonia buildup due to poor husbandry are discussed.
- The choice of bedding materials and cage flooring is considered.
- Population density and group size during animal holding can profoundly affect experimental results.
- Microbial control within the animal-holding facilities is discussed. Whenever possible, the health status of animals should be ascertained before they are brought to the facility. Regular monitoring of the health status of animals within a facility should be carried out. Personnel must be instructed in the precautions they must take to avoid introducing diseases into the facility.
- Practices to be followed to reduce the spread of infectious agents within a conventional facility, or to prevent this occurrence within a "barrier" facility, are described.
- Biohazard containment is required for animals exposed to known infectious micro-organisms. The design and operation of infectious disease units is considered.

Laboratory animal care (Chapter V)

[48] The table of contents from CCAC (1993) can be found on the Internet at the Canadian Council on Animal Care's Web site www.ccac.ca. Information for ordering CCAC (1993) as well as related publications is also provided there.

- All animal facilities should have in place Standard Operating Procedures for animal care. All animals must be observed at least once daily.

- Procedures for gentle and proper handling of animals are described.

- Recommended practices for receiving and handling new animals arriving at a facility are considered. The use of a conditioning/quarantine period, whereby new animals are held in facilities separated from other animals, is discussed.

- Proper identification and record keeping for each group of animals held and maintained is addressed.

- Advice on food quality and storage, water quality and provision, and exercise of laboratory animals, is provided.

- Care of the animal facility, including cleaning and sanitation, waste disposal, and vermin control, is discussed.

- As a general rule, animals should be moved to freshly cleaned cages at least once a week.

- Weekend and holiday care of laboratory animals is essential. Animal care is a continuous and daily responsibility.

Social and behavioural requirements of rodents and rabbits (Section G of Chapter VI)

- It is desirable to house two or more mice or rats per cage. Sexually mature male mice or rabbits paired together are frequently incompatible.

- Enrichment devices (e.g., empty plastic water bottles for mice, or resting boards for rabbits to hide under) should be considered for inclusion in cages.

- Cage design, floor space per animal, bedding preferences, and the use of solid-bottomed cages are described and recommendations provided.

- High (e.g., 50 to 70 decibels or higher) levels of noise, adverse light intensity and/or *photoperiod*, sudden changes in humidity, inadequate air changes, infrequent cage cleaning, and daily routines, are described as influencing the well-being of rodents or rabbits.

Special practices (Chapter VII)

- Recommended conditions and procedures during animal acquisition are described.

- Laboratory animals must be acquired from a licensed supplier. The supplier, on request, will be required to provide detailed information on health status monitoring, breeding, and husbandry practices followed.

- Regulations and legislation concerning the humane transport of animals are referred to.

- Stressors during animal transportation are described, and advice provided on how to reduce transportation stress.

- It is essential that animals brought to the laboratory be allowed to equilibrate to their new environment. *Acclimation* to that environment, and a stabilization of the animal physiologically and behaviourally following transport, are essential prerequisites before the animal is used in a test.

- Considerations related to breeding animals in the laboratory are briefly discussed.

- Gentle handling and restraint of animals during certain manipulations is described. General guidelines for care of restrained animals are provided. Special surveillance and restraint devices that minimize stress on animals are considered.

- Guidelines for blood removal from laboratory mammals or birds are referred to.

Occupational health and safety (Chapter VIII)

- All personnel working with animals must understand how to handle the species involved, both for their own safety and health, and for that

of the animals.

- Those working with experimental animals risk exposure to physical hazards (e.g., heat, noise), chemical hazards (e.g., disinfectants, cleaning solutions), as well as intestinal parasites, enteric bacteria, pathogenic organisms, and animal bites.

- Biosafety guidelines and associated SOPs should be developed and enforced.

- Certain infectious micro-organisms found in animals can cross species barriers and infect human contacts. Transmission of infections from animals to humans (i.e., *zoonosis*) can generally be avoided through education, proper veterinary care, and adherence to SOPs for the control of infectious agents.

- Work involving exposure to hazardous micro-organisms might require prior immunization of the staff, if a vaccine is available, and the appropriate containment facilities (e.g., Level 4; see footnote 47 in Section 7.1) for the implementation of such studies.

- Allergies to laboratory animals are a significant occupational health concern for people regularly working with common species of laboratory animals. Symptoms are described, along with measures to reduce the degree of exposure to animal allergens.

- Advice is provided on minimizing physical and chemical injuries to laboratory personnel responsible for handling animals in the laboratory.

Control of animal pain (Chapter X)

- The assessment and management of pain and suffering in animals is a challenge that must be faced if the animals are to be treated ethically and humanely. In addition to ethical concerns, pain or distress in animals interjects unwanted variables into studies that can greatly interfere with the interpretation of the results.

- One of the characteristics of pain or distress in animals is a change in behaviour. Animal-care personnel and research investigators must be familiar with the normal behavioural characteristics of the experimental animal, for the success or failure of the study can depend on the expertise of the person(s) observing the animals to minimize pain and distress.

- The presence or absence of stress appears to be the only acceptable indicator of animal well-being.

- Veterinary training and expertise play a vital role in fulfilling an institution's responsibilities to prevent and minimize pain and suffering in all animals used for research, teaching, and testing.

- Some species-specific changes in behaviour and physical appearance, which allow early identification of an animal experiencing pain or distress, are described. Descriptions are included for rodents, rabbits, birds, and fish.

Anaesthesia (Chapter XI)

- Guidance and information on anaesthesia and relief of pain in experimental animals is provided in this chapter.

- Sedatives, *analgesics*, and general *anaesthetic* agents must be used for the control of pain and distress unless contrary to the achievement of the objectives of the study.

- Conditions of handling and fasting, in conjunction with anaesthesia, are described.

- The use of various tranquillizers and sedatives is considered. General anaesthetics suitable for use with mammals or birds are also described. The application of muscle relaxants in conjunction with anaesthetics is discussed briefly. Use of neuromuscular blocking agents on conscious animals is described as prohibited.

- Species considerations associated with anaesthesia are addressed. Anaesthetics and analgesics suitable for use with rabbits, rodents,

birds, and fish are described together with application procedures and precautions.

Euthanasia (Chapter XII)

- Death of animals in the laboratory must be "humane"; that is, it must be painless, must minimize fear and anxiety, be reliable, reproducible, irreversible, simple, safe, and rapid.

- Ten criteria to be met to achieve a humane death are provided.

- No animal should be considered dead until reflex movement as well as cardiac and respiratory movements have ceased.

- Humane procedures for physical methods of *euthanasia* are discussed, including those using stunning, cervical dislocation, electrocution, pithing, decapitation, shooting, maceration, microwave radiation, and exsanguination.

- Humane procedures for causing death by injection of an overdose of an anaesthetic are described.

- Procedures for euthanasia using an overdose of inhaled anaesthetics (e.g., ether, halothane, methoxyflurane) are considered. The past use of chloroform for this purpose is no longer recommended.

- Procedures for euthanasia using non-anaesthetic gases (e.g., carbon monoxide, carbon dioxide, nitrogen, argon, or cyanide) are discussed. The use of carbon monoxide and cyanide are ruled against due to safety problems and, in the case of cyanide, convulsions or seizures of exposed animals.

- Specific guidance for euthanizing various species of animals including fish, amphibians, and reptiles is provided.

Choosing Test Organisms and Biological Test Methods

Key Guidance

- *The NSN Regulations require, under Schedule XV, up to six separate and distinct biological test methods to be applied when measuring the pathogenic and/or toxic effects of a new microbial substance. These include a test using a species of aquatic plant, aquatic invertebrate, aquatic vertebrate, terrestrial plant, terrestrial invertebrate, and terrestrial vertebrate.*

- *In each instance, the use of a standardized biological test method should be applied, where available and appropriate. Each test method to be applied should be adapted as necessary for testing a new microbial substance, and shown to be responsive to pathogenic as well as toxic effects.*

- *When choosing biological test methods using aquatic plants, invertebrates, or vertebrates (i.e., fish), a decision should be made as to whether freshwater and/or estuarine/marine organisms (and associated test methods) are appropriate. This decision should take into account the environmental expression and fate of the micro-organisms within the test material as influenced by salinity, as well as the intended regions of application of a test material and its likelihood of entering inland or coastal waters at concentrations of potential concern. An equally important consideration is whether the new microbial substance is mesophilic or psychrophilic.*

- *The choice of species of terrestrial plant to be used depends on a number of considerations including the findings of earlier field or laboratory studies identifying crops susceptible to the new microbial substance, as well as reported results for similar genera of micro-organisms.*

- *Choosing the appropriate test method(s) to be applied to terrestrial invertebrates depends on: the likelihood of exposure of ecologically relevant species, the availability of standardized test methods for representative species, the nature of the test material, the biology of the micro-organism in the test material, the potential effect of any genetic alterations made to the micro-organism, and the intended manner of dispersal or entry of the test material into the terrestrial environment. Findings of earlier field or laboratory studies involving terrestrial invertebrates exposed to the test material or one containing similar (i.e., same genus of) micro-organisms are also pertinent.*

- *When testing for effects on terrestrial vertebrates, candidate tests involve birds or small mammals. Mallard ducks or northern bobwhite quail are recommended when testing an avian species. Rats or mice are recommended for tests with small mammals. The nature of the new microbial substance, likely field exposures of birds or small mammals during its intended application, and findings of relative susceptibility should be considered when choosing the test(s) to be performed with birds and/or small mammals.*

8.1 Six Categories of Biological Test Methods

Sections 8.2 to 8.7 discuss six separate categories of *biological test methods* that are dependent on organism type (i.e., aquatic plant, aquatic invertebrate, aquatic vertebrate, terrestrial plant, terrestrial invertebrate, or terrestrial vertebrate). Each section lists the test(s) that should be considered by the investigator(s) when selecting biological test methods from each category to measure and evaluate the ecological effects of new microbial substances required under Section XV of the NSN Regulations. Guidance is also included on conditions and procedures to be followed when applying these category-specific biological test methods, which are recommended for measuring the pathogenicity and/or toxicity of a new microbial substance.

For many applications, the New Substances Notification Regulations under CEPA 1999 require a notifier to provide data on the potential ecological effects of a new microbial substance on appropriate plant, invertebrate, and vertebrate species representing the aquatic and terrestrial

environments. This has precipitated the need for six categories of biological test methods which, when performed in the laboratory using standardized conditions and procedures, are capable of detecting and measuring adverse pathogenic and/or toxic effects of new microbial substances (see Section 2). These Regulations have also necessitated that up to six separate and distinct biological test methods be applied to each new microbial substance under investigation.

In some cases, it might not be necessary for a notifier to conduct tests using all six categories of test (host) organisms. For instance, appropriate test data for certain host organisms (e.g., rodents) might already be available for the micro-organism for which notification information is being provided. Appropriate test data for other closely related micro-organisms might also be available. In certain cases, a waiver from providing data on the ecological effects of the new microbial substance might be granted by Environment Canada if the information is not needed to determine the toxicity of the living micro-organism according to CEPA or it's capability of becoming toxic. A waiver might also be granted if it is not practicable or feasible to obtain the test data necessary to generate this information (EC and HC, 2001). However, if relevant historic test data are not available and a waiver is not applicable, the notifier should arrange for an appropriate series of laboratory tests for pathogenic and/or toxic effects on host organisms to be performed and reported as part of the notification process.

The biological endpoints for most standardized single-species test methods used to measure the toxicity of chemicals in the laboratory are primarily growth inhibition, reproduction inhibition, whole-organism alterations in appearance or behaviour, and mortality (acute or chronic). These same biological endpoints can be responsive to disease, provided that the test duration is sufficiently long to enable such responses to disease to be manifested using such test methods. Accordingly, a carefully selected set of standardized biological test methods (modified as necessary to be responsive to pathogenic effects) should serve well in measuring both pathogenic and toxic effects of new microbial substances. As necessary and appropriate[49], certain test methods should incorporate gross and histological examinations of test organisms upon completion of the test, to diagnose damage to organs and tissues and report their frequency of occurrence.

8.2 Recommended and Other Biological Test Methods

The routine use of standardized (modified as necessary) biological test methods to measure the pathogenicity and/or toxicity of new microbial substances as part of the NSN Regulations is advisable to ensure that the tests applied are rigorous, meaningful, and comparable. The use of a "specialized" procedure that does not represent one of these standardized biological test methods, or a suitable modification thereof, might be fraught with problems if the intent is to derive meaningful and comparable results intended for regulatory purposes.[50] Such procedures frequently lack a set of criteria for judging if the results are valid and acceptable. They also commonly lack explicit instructions on certain test conditions associated with the procedure, such as details on environmental conditions and procedures requiring standardization if the results are to be considered valid on a broader

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[49] Certain limitations exist for biological test methods involving a species of test organism for which necropsy procedures are not well defined and/or for which the gross and microscopic appearance of healthy or diseased tissues and organs has not been delineated.

[50] Many research-oriented procedures have been used by various investigators to discern pathological and/or toxic effects of various micro-organisms on a particular species of an aquatic or terrestrial plant, invertebrate, or vertebrate (see Douville, 2001 for a state-of-the-art review of this scientific literature). Such investigations are useful and meaningful in terms of illustrating the varied types of effects and responses that can occur when a particular species and life stage of a host (test) organism is exposed to a specific type of micro-organism, under certain defined laboratory conditions. Without a standardized methodology suitable for broad application in the laboratory, however, such procedures are limited in their worth. For instance, research-oriented procedures reported in the scientific literature typically lack the rigorous steps for method development and validation that are required by regulatory agencies responsible for the publication of proven and accepted biological test methods.

(i.e., regulatory) scale when appraising the ecological effects of a particular test material. Standard Operating Procedures used by certain testing laboratories for measuring the pathogenicity and/or toxicity of a microbial pesticide might also not be suitable for broad application to new microbial substances intended to satisfy the reporting requirements on ecological effects specified as part of the information requirements of the NSN Regulations under Schedule XV.[51]

The guidance in this document focusses on using a series of appropriately selected, standardized biological test methods responsive to both pathogenic and toxic effects of new microbial substances. The use of alternate methods or procedures that are non-routine (and, in some instances non-standardized), might prove counter-productive if they are used instead of ones selected from those recommended herein. However, exceptions occur, in which case the application of other methods or procedures in addition to (or in place of) the use of the biological test methods recommended herein might prove warranted.[52] An example of this would be instances in which there are valid concerns based on other studies showing adverse effects of a similar type of micro-organism on a commercially and/or ecologically relevant aquatic or terrestrial species likely to be exposed to the new microbial substance.

In this guidance document, recommended biological test methods are ones that were judged to be suitable and appropriate for use in meeting the requirements of the NSN Regulations with respect to the ecological effects of new microbial substances (see Section 2 herein, and Section 4.2.7 in EC and HC, 2001). These test methods are ones that have been published by Environment Canada or another recognized regulatory agency (e.g., USEPA) or authority (e.g., ASTM) responsible for standardized testing guidelines. Typically, each has been peer reviewed and subjected to interlaboratory (round-robin) evaluation before being published. Such methods include guidance and instructions on handling and culturing the test (host) organisms, associated health criteria, the required use of a negative control (and, in some instances, other controls), and one or more "test acceptability" criteria used to judge if the results for a particular test are to be considered valid and acceptable for the intended use. Details on category-specific methods identified herein as recommended biological test methods are provided in Sections 9.1.2, 10.1.2, 10.1.3, 10.2.2, 10.2.3, 11.1.2, 12.2, 13.2, 14.1.1, and 14.1.2.

A description of other test methods or procedures that might deserve consideration in addition to (or as a substitute for) the biological test methods recommended herein is provided in Sections 9.2.3, 10.1.4, 10.2.4, 11.1.3, 11.2.3, 12.3, 13.3, 14.1.3, and 14.2.3. In some instances, these are standardized biological test methods that might prove suitable for inclusion as part of a series of tests to be applied with a new microbial substance for the present purposes (see Sections 1.2 and 2). Certain biological test methods identified as "other test methods" in these sections might be more suitable than a recommended test method, such as when performing more labour-intensive and costly tests for delayed or long-term effects of exposure to a particular new microbial substance. Also included in "*Other Methods or Procedures*" are non-standardized methods or procedures, such as ones that are based on reported findings for certain research-oriented tests with a particular type of host organism exposed to a particular type of micro-organism. In-house Standard Operating Procedures

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[51] The absence of a peer review by scientists of a particular in-house SOP used by a certain testing laboratory, as well as the lack of an inter-laboratory evaluation of its performance with one or more test materials using the same set of standardized conditions and procedures defined therein, also limit the widespread acceptance and use of such SOPs as a substitute for standardized biological test methods.

[52] In such instances, the intended alternate (or additional) procedures or non-standard test methods under consideration should be discussed between the notifier and the appropriate representative(s) of Environment Canada's New Substances Branch (NSB) before finalizing the study design. Written details on the rationale for including such non-standard test procedures or methods as part of the data requirements on the potential ecological effects of a particular new microbial substance should be provided, together with specifics on the Standard Operating Procedure to be followed by the testing laboratory when performing these tests. Such details are appropriate for discussion with the NSB during a Pre-Notification Consultation (see Section 1.1).

used by a testing laboratory that are inconsistent with (or unsanctioned modifications of) a standardized biological test method are also considered to be an "other method or procedure". Intentions to use a test method or procedure other than those identified as a recommended biological test method must be considered and reviewed with Environment Canada's New Substances Branch (NSB) as part of the Pre-Notification Consultation process (see Section 1.2).

Certain methods or procedures described under "*Other Methods or Procedures*" are not considered to be suitable alternatives to the recommended biological test methods. As described in these sections, limitations such as their brevity (e.g., acute tests with durations of four days or less), inappropriate biological or statistical endpoint(s), lack of one or more criteria for judging test validity, or other considerations, rule against the use of such methods or procedures. Consideration of such methods or procedures as unacceptable for the testing and notification of new microbial substances is, in some instances, also influenced by the availability of more suitable standardized biological test methods.

A worthwhile approach is to test each new microbial substance under investigation using a series of six (more or less, in certain instances) standardized biological test methods; each of which is adapted as necessary for testing a new microbial substance, and, data permitting, also shown to be responsive to pathogenic as well as toxic effects.[53] Each of the following six category-specific sections on recommended biological test methods provides one or more methods for consideration when designing a testing program intended to measure the ecological effects of a particular new microbial substance under controlled and defined laboratory conditions. Selecting the appropriate test method(s) to be applied to a test material from those listed in each of these six categories depends on a number of considerations including the intended application route(s) for a particular new microbial substance (e.g., by aerial spray, or by applying a solid substance to or on soil or water), the types of organisms of concern in the environment most likely to be exposed, and the similarity of these organisms to those used in one of the recommended biological test methods.

It is noted here that not all of the standardized biological tests recommended for use in Sections 9 to 14 inclusive have been demonstrated to be responsive to pathogenic microbial substances. For instance, little if any evidence is available which demonstrates that earthworms or springtail invertebrates are sensitive to pathogenic micro-organisms using the test methods recommended in Section 13.2. However, the test duration and other conditions described in these sections are considered to be appropriate (and sufficiently long) to enable such effects to be realized and measured as part of these recommended test methods, while providing greater assurance (based on past experience) that these test methods are responsive to toxic substances including those associated with micro-organisms (e.g., endotoxins or toxic metabolites). The introductory section to each of Sections 9 to 14 includes a brief review of past uses and findings when applying the biological test methods now recommended (with appropriate modifications, as described therein) for use in measuring pathogenic and/or toxic effects of new microbial substances. As testing progresses, further information will become available with respect to the responsiveness of each of these biological test methods to pathogenic (as well as toxic) effects. In some instances, other more suitable test methods (or procedural modifications of methods recommended herein), will undoubtedly arise, as testing and research with micro-organisms for pathogenic and/or toxic effects progress.

8.3 Tests with Aquatic Plants, Invertebrates, or Vertebrates

Consideration should be given to the likelihood of freshwater, estuarine, and/or marine organisms being exposed to a new microbial substance according to its intended application route(s), when choosing a suitable biological test method to measure its adverse effects on aquatic plants, invertebrates, or vertebrates. If the intended

[53] Additional non-standardized procedures and methods might also be chosen and included by the notifier along with the performance of six (more or less, in certain instances) standardized biological test methods (see Sections 9 to 14), depending on the nature of the new microbial substance, its mode of application, and possible concerns regarding one or more species of sensitive plants or animals that might be exposed to it.

application route(s) will be restricted to inland locales removed from coastal waters, tests for effects on estuarine or marine organisms need not be performed and only those using freshwater organisms should be considered and chosen. If, however, the intended application route includes estuarine or marine waters, or land nearby and draining to coastal waters, the tests for effects on aquatic life should be restricted to or include those using sensitive estuarine or marine organisms. In instances where a new microbial substance is to be applied in a manner that it could enter either freshwater or estuarine/marine waters in significant concentrations, consideration should be given to testing this substance using freshwater plants, invertebrates, and vertebrates, as well as those representing the estuarine/marine environment. The findings of any previous studies on the *environmental expression* of the micro-organism (see Section 2) should also be taken into account when choosing the appropriate aquatic test methods to be used. For instance, if such studies showed that these micro-organisms did not survive in brackish or full-strength seawater, the use of tests using estuarine or marine organisms might be ruled against in favour of those using freshwater organisms.

Sections 9, 10, and 11 provide guidance on recommended biological test methods for freshwater plants, invertebrates, and vertebrates and, in separate subsections, those for estuarine or marine test organisms. When choosing a test using aquatic plants or animals, the influence of the physical and chemical properties of the receiving water (e.g., salinity, temperature, nutrients) on the environmental expression and fate of the new microbial substance should be known (see Section 3.1) and considered. A key consideration influencing an appropriate choice is whether the new microbial substance is represented by micro-organisms that are *mesophilic* or *psychrophilic*. For example, if the new microbial substance is *mesophilic* (i.e., it requires temperatures of $\geq$15°C to grow and multiply), the aquatic test methods chosen should have test temperatures that are warm enough to not inhibit microbial activity. Similarly, if the new microbial substance is *psychrophilic* (i.e., cold loving, with an optimal temperature for replication of $\leq$15°C), the aquatic test methods chosen should have test temperatures that are cool enough to not inhibit the survival and replication of this micro-organism.

To measure the effect of a new microbial substance on aquatic plants (see Section 9), the choice of test method is dictated primarily by whether the substance is most likely to reach concentrations of potential concern in the freshwater or marine environment, and the environmental fate of the micro-organisms therein as influenced by salinity and temperature. When selecting the test method(s) to be applied to aquatic invertebrates, consideration should be given to the physicochemical characteristics of the test material (e.g., solid or liquid, or suspension rate for fresh water or seawater), and the known partitioning of the micro-organisms therein in water and/or sediment. Findings from previous studies on the environmental fate of the new microbial substance, when entering or mixed in a particular fresh, estuarine, or marine water, should also be considered when choosing the test(s) with aquatic invertebrates. These considerations should assist in choosing a biological test method using pelagic invertebrates which occupy the water column, versus ones that are primarily found on or in the sediment below (see Section 10). When choosing the biological test method(s) to be applied to aquatic vertebrates (i.e., fish), the same considerations on physicochemical characteristics of the new microbial substance (or its associated microbial product) and its environmental expression and fate[54], when mixed in fresh water or estuarine/marine water, should be taken into account. In this instance, the test organisms are fish adapted to fresh water or seawater. The choice of test species might also be influenced by concerns related to a particular commercial or recreational species of fish that could be subjected to the new microbial substance.

Computerized data bases on the biological effects of known microbial pathogens on various species of

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[54] Section 2 discusses associated tests for environmental expression, as well as studies of environmental fate. Section 4.2.6 "*Information in respect of the environmental fate of the micro-organism*" in EC&HC (2001) describes the information on environmental fate that is required to be obtained and reported as part of the overall information requirements associated with the NSN Regulations.

aquatic plants, invertebrates, or vertebrates are available.[55] Consultation of these data bases is advisable as an early step, when choosing the appropriate test method(s) and test (host) species to be used for measuring the pathogenic and/or toxic effects of a particular new microbial substance on aquatic organisms. Data-base results for micro-organisms within the genus of the new microbial substance should be identified and reviewed. Host organisms shown to be affected adversely by these micro-organisms should be identified and considered for tests with the new microbial substance, provided that a suitable biological test method is available. In instances where there is no known species of pathogenic micro-organism within the genus of the new microbial substance, for which pathogenic and/or toxic effects are identified and demonstrable in laboratory tests, the new microbial substance should be subjected to the biological test methods recommended herein (see Sections 9.1.2, 9.2.2, 10.1.2, 10.1.3, 10.2.2, 10.2.3, 11.1.2, 11.2.2) or, if more appropriate, other suitable test methods identified in Sections 9.1.3, 9.2.3, 10.1.4, 10.2.4, 11.1.3, or 11.2.3.

8.4 Tests with Amphibians

Under certain circumstances, particularly where the new microbial substance or its end-use microbial product is likely to have an adverse effect on aquatic animals in quiescent pools or ponds, a biological test method using a selected amphibian species as the host organism might be applied. Edginton (2001) reviewed laboratory test methods available for measuring the effects of aquatic contaminants on amphibians and identified a lack of standardized test methods for anurans (i.e., frogs or toads). Standardized methods by recognized agencies were restricted to an acute (4–8 day) static-renewal (or continuous flow) test with early larvae of *Rana* spp. or *Bufo* spp. published by ASTM (2000a,b), or a (draft) 12-day static-renewal test with larvae of the African toad *Xenopis laevis* by ISO (2001). Shortcomings of these methods include their short test duration and, in the case of ISO (2001), their use of an anuran species not found within Canada. Edginton (2001) recommended that Environment Canada develop a standardized static-renewal test using laboratory-cultured frogs of the species *Rana pipiens*, which is found in all provinces of Canada. A test of ≥46 days using this species, which would include early embryonic life stages as well as actively feeding larvae (tadpoles), was one of three test options proposed by Edginton (2001). This test option, if developed and validated by Environment Canada, could likely be readily adapted as a suitable test method for measuring the pathogenic and/or toxic effects of new microbial substances on a sensitive amphibian species common to Canadian freshwater ponds.[56] Until such time, no standard test method using *Rana pipiens* or another amphibian species is recommended for inclusion in a series of laboratory tests intended to measure the adverse ecological effects of new microbial substances on aquatic or terrestrial organisms.

8.5 Tests with Terrestrial Plants

Guidance on tests for measuring the pathogenic and/or toxic effects of new microbial substances on terrestrial plants is presented in Section 12. The choice of the test method to be applied, including the selection of the species of plant to be used and the manner(s) in which plants are to be exposed to a new microbial substance, depends on various considerations including the species of plants that might be subjected to the test material as part of its intended means of application within the terrestrial environment. For instance, for a test using a sensitive agricultural or grassland species of plant, the selection of a *monocotyledon* or a *dicotyledon* might be influenced by the greater likelihood that one or the other of these types of plants might be

[55] Useful data bases for diseases of aquatic organisms include the following Web sites: http://fisheries.fws.gov/FHC/Handbook.htm — this handbook of standard protocols to be followed when carrying out health inspections on aquatic animals contains chapters on bacteriology, virology and parasitology; http://www.fao.org/DOCREP/003/X9199E/X9199E03.htm — a Food and Agriculture Association of the United Nations Circular that includes a chapter on bacterial and viral pathogens of salmonids and shrimp; and http://www.diplectanum.dsl.pipex.com/purls/host.htm — a World Wide Web Virtual Library on diseases and parasitology of fish and shellfish.

[56] Frogs and other amphibians are known to be susceptible to a variety of bacterial and viral diseases (Hird *et al.*, 1981; Chinchar, 2002; O-Rourke and Schultz, 2002), and are also known to be susceptible to diverse chemical contaminants in water.

exposed during the normal use of the new microbial substance under investigation. If it is anticipated that both monocotyledonous and dicotyledonous species might be exposed to the new microbial substance during its normal use, tests involving plants representing each of these types of agricultural plants (see Section 12) are recommended. If the intended use of a new microbial substance is more likely to expose trees than agricultural crop, market garden, or grassland species of plants, use of a sensitive species of tree seed as the host plant would be favoured (see Section 12).

Results of previous laboratory tests involving terrestrial plants exposed to the new microbial substance or ones having similar characteristics (including potentially similar pathogenic and/or toxic effects) should be taken into consideration when choosing the species of plant to be exposed to a particular new microbial substance. Any available research findings from experimental field studies with the new microbial substance, required as part of the NSN Regulations (see Section 4.2.5 in EC and HC, 2001) should also be reviewed and considered when choosing the appropriate test method(s), test (host) species, and exposure route(s). Other considerations include aspects related to the route(s) of exposing the host plants to the new microbial substance (e.g., in the test water, in the test soil, by wounding and spraying; see Section 12), life stage(s) exposed, and potential routes of entry of the pathogen (e.g., seed, root, leaf). Climatic conditions during testing for pathogenic and/or toxic effects must be rigidly controlled, as most micro-organisms have stringent temperature and humidity requirements under which they induce disease in plants. Similarly, environmental conditions including light, temperature, and fertility have a major influence on the ability of a plant to express symptoms of disease. For instance, some micro-organisms can cause latent infections which become apparent only as the plant reaches maturity or becomes senescent. Certain bacteria and viruses might not induce any symptoms, but yet colonize the entire plant.

Computerized data bases on the biological effects of known microbial pathogens on various species of terrestrial plants are available.[57] It is advisable to consult these data bases as an early step, when choosing the appropriate test method(s) and test (host) species to be used for measuring the pathogenic and/or toxic effects of a particular new microbial substance. Data-base results for micro-organisms within the genus of the new microbial substance should be identified and reviewed. Host plants shown to be affected adversely by these micro-organisms should be identified and considered for tests with the new microbial substance, provided that a suitable biological test method is available. The new microbial substance should be subjected to the biological test method recommended herein (see Section 12.2) or, if more appropriate, another suitable test method identified in Section 12.3, in instances where there is no known species of pathogenic micro-organism within the genus of the new microbial substance for which pathogenic and/or toxic effects are identified and demonstrable in laboratory tests.

8.6 Tests with Terrestrial Invertebrates

Section 13 describes two biological test methods recommended for measuring the pathogenic and/or toxic effects of new microbial substances on a soil-dwelling terrestrial invertebrate (i.e., a species of earthworm or collembolan springtail). However, a series of tests to measure and evaluate the pathogenicity and/or toxicity of a new microbial substance might include a test with honey bees or another species of beneficial, plant-dwelling terrestrial arthropod (e.g., the convergent lady beetle or the green lacewing; see Section 13) for which a recognized and standardized biological test method is presently unavailable.[58] Choosing which

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[57] The University of Bonn provides a searchable Plant Pathology Internet Guide Book (PPIGB) with reference material specific to bacteriology, mycology, and virology (http://www.pk.uni-bonn.de/ppigb/menu.htm).

[58] A decision to use a non-standard biological test method for which a recognized testing protocol is presently unavailable might be predicated on concerns about the exposure of a particular beneficial species (e.g., the honey bee or the convergent lady beetle), recommendations by regulatory agencies that these species be considered for use as test organisms, and the existence of an apparently worthwhile and defensible Standard Operating Procedure for performing such a test

biological test method(s) to use when measuring the potential ecological effects of a particular new microbial substance on terrestrial invertebrates depends once again on a number of considerations related to the most likely and predominant route(s) of entry of the new microbial substance under investigation into the terrestrial environment (e.g., by aerial application as a spray, by dispersal of a solid substance on soil or vegetation, or by mixing in soil or subsoil). For instance, if a new microbial substance is intended to be sprayed near flowers or blossoms, or otherwise come in contact with bees or the petals or foliage that they might frequent, a laboratory test using honey bees might be considered a good choice.[59] For a new microbial substance that is more likely to be deposited on or in soil or subsoil, the use of the recommended biological test method using earthworms (i.e., a soil invertebrate) would be prudent. Another consideration when choosing which biological test method(s) to use for terrestrial invertebrates, might be based on the results for previous field or laboratory studies showing the relative sensitivity of honey bees, earthworms, springtails, or other terrestrial invertebrates to the test material or to other microbial substances similar in type and mode of effect.

In selecting a suitable test (host) organism and associated biological test method, the biology of the new microbial substance should also be considered. For example, are any known pathogens in the same family or genus as that or those representing the test material and, if so, what if any host organisms and test methods were used to demonstrate their pathogenicity? If any terrestrial invertebrates are known to be susceptible to pathogens closely related to the substance, the species of invertebrate selected to be a test organism should be one of these hosts. If none of the recommended (Section 13.2) or alternate (Section 13.3) species of test (host) organisms identified herein are likely to come into contact with the substance, then consideration could be given to using another suitable and sensitive test species (and associated biological test method, if available) that would be as closely related as possible to one of the known hosts affected by the new microbial substance. In addition, any genetic alterations to the new microbial substance should also be considered when selecting the host species.

Computerized data bases on the biological effects of known microbial pathogens on various species of terrestrial invertebrates are available.[60] Consultation of these data bases is advisable as an early step, when choosing the appropriate test method(s) and test (host) species to be used for measuring the pathogenic and/or toxic effects of a particular new microbial substance. Data-base results for micro-organisms within the genus of the new microbial substance should be identified and reviewed. Host invertebrates shown to be affected adversely by these micro-organisms should be identified and considered for tests with the new microbial substance, provided that a suitable biological test method is available. In instances where there is no known species of pathogenic micro-organism within the genus of the new microbial substance, for which pathogenic and/or toxic effects are identified and demonstrable in laboratory tests, the new microbial substance should be subjected to one or both of the biological test methods recommended herein (see Section 13.2) if there are concerns about potential effects on soil-dwelling invertebrates. An appropriate test using bees or foliar insects as identified in Section 13.3.1 should be applied if there are concerns about potential effects on plant-dwelling invertebrates (i.e., pollinating or foliar insects).

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by a private testing laboratory. This should be discussed *a priori* during a PNC with governmental representatives of the New Substances Program.

[59] Such a choice should depend on the existence of a scientifically defensible SOP for a laboratory test using honey bees, with a test duration that is sufficiently long to enable pathogenic as well as toxic effects to be discerned; as well as available baseline data on the routine survival rate for bees in the negative control treatment indicating that survival is sufficiently high (e.g., ≥80%) to warrant the use of this SOP.

[60] The Illinois Natural History Survey provides a useful data base for insect pathogens at the Web site http://cricket.inhs.uiuc.edu/edwip_search.htm. Other data bases for insect pathogens and host species are available, including those at: http://www.atcc.org/Cultures/Products.cfm, http://sis.agr.gc.ca/brd/ccc/ccctitle.html, and http://nrrl.ncaur.usda.gov/.

8.7 Tests with Terrestrial Vertebrates

The first consideration when choosing the biological test method(s) for measuring the pathogenic and/or toxic effects of a particular new microbial substance on terrestrial vertebrates, is whether to use an avian or small mammalian species. This choice should be made in light of the environmental regions within Canada where the new microbial substance under investigation might contact wildlife including birds or small mammals (e.g., farmland, forested land, wetlands). The findings of any known studies on the ecological effects on birds or small mammals determined previously for the new microbial substance should also be considered when making this choice. Available information from experimental field exposures (see Section 4.2.5 in EC and HC, 2001) showing the relative sensitivity of small mammals and birds to the test material should be taken into account as well. The nature of the new microbial substance (e.g., powder, suspension, mist), its particle size if a solid, and its means of application in the environment (e.g., by aerial spraying as a mist, by spreading on the ground, or by mixing in soil or subsoil), should be considered in terms of the likelihood of exposure for nearby birds or small mammals.

The USEPA's Series 885 laboratory tests for effects of microbial pesticides on birds recommends that either mallard ducks (*Anas platyrhynchos*) or bobwhite quail (*Colinus virginianus*) be used as test organisms (USEPA, 1996b,k,l,m). If the intended means of application of a substance within Canada favours greater exposure of a herbivorous species of bird that frequents grasslands, bobwhite quail rather than mallard ducks are recommended as the test species in a 30-day test for pathogenic and/or toxic effects. If, however, the intended application route favours greater exposure of mallard ducks (i.e., a migrant, largely insectivorous species that frequents wetlands), this is the preferred avian species for use in a test for effects on a terrestrial vertebrate. The widespread distribution of mallard ducks across Canada, unlike that for the bobwhite quail (which is most prevalent in North America within the midwestern and eastern US states, but also found in southern Ontario), supports using the mallard duck as an appropriate avian test organism in instances where differing habitats and associated patterns of use and dispersal of a new microbial substance are not a primary consideration.

The USEPA (1996o,q,t,u) indicates that mice or rats are the preferred test organisms when performing Series 885 laboratory tests with small mammals administered a microbial pesticide orally or by inhalation. In keeping with this recommendation and the use of these USEPA biological test methods as guidance when measuring the pathogenicity and/or toxicity of a new microbial substance to small mammals, mice or rats are the preferred test species for measuring the effects of a test material on small mammals (see Section 14). If another species is used (e.g., gerbels, guinea pigs, or rabbits), the reasoning and justification for this alternate selection should be discussed with regulators from EC and/or HC during a PNC (see Section 1.1.6), and this rationale provided as part of the test report. In instances where there is a particular concern for environmental exposure of a certain species of wild mammal that is not considered at risk[61], field specimens of wild animals of this species could be collected and, following their acclimation to laboratory conditions, tested for effects of a new microbial substance using the recommended method described in Section 14.

As with tests for pathogenic and/or toxic effects on other host species, computerized data bases showing biological effects of known microbial pathogens on various species of birds or small mammals should be consulted as an early step when choosing the appropriate biological test method(s) and host species to be applied to terrestrial vertebrates.

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[61] The Government of Canada's Committee on the Status of Endangered Wildlife in Canada (COSEWIC) provides a useful Web site (www.cosewic.gc.ca) on Canadian species of wildlife that are identified as "at risk". A category-specific summary of the number of species classified as being at risk (including those considered to be extinct, extirpated, endangered, threatened, or of special concern) is available at www.cosewic.gc.ca/htmlDocuments/Full_List_Species_e.htm.

Computerized data bases are available[62] for this purpose.

8.8 Questionnaire

In 2002, an informal questionnaire was sent to the USEPA's Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division (Washington, DC), requesting information on available data showing the approximate number of laboratory tests performed to date with MPCAs or their EPs using various categories of test (host) organisms (e.g., aquatic plants, aquatic invertebrates, aquatic vertebrates, terrestrial plants, terrestrial invertebrates, or terrestrial vertebrates). Additional information, such as the number of these tests showing pathogenic and/or toxic effects, the use of various types of controls, etc., was requested as part of the questionnaire. This questionnaire was also sent to three private US laboratories with experience in testing MPCAs or microbial EPs for pathogenic and/or toxic effects on various host organisms, under controlled laboratory conditions. Some of the findings of these informal questionnaires are summarized briefly in the following six "host-specific" sections of this guidance document, under the heading *"Previous Tests with Micro-organisms or Microbial Products"*.

[62] The American Association of Veterinary Laboratory Diagnosticians maintains a Web site (www.aavld.org) that, if contacted, enables access to a comprehensive data base on pathogens affecting avian or mammalian species. A membership fee is required for its access. General veterinarian information on diseases is available at http://netvet.wustl.edu. Another useful site on systemic pathology, which requires registration, is http://vetpath4.afip.org/systemic/index.php. Information on gross pathology is available at http://w3.vet.cornell.edu/nst/nst.asp.

Tests Using Aquatic Plants

9.1 Freshwater Plants

Key Guidance

- *A seven-day test for effects on growth of the freshwater macrophyte* Lemna minor *is recommended as the standard biological test to be used when measuring the pathogenic and/or toxic effects of new microbial substances on a species of freshwater plant. This recommended method represents an adaptation of EC (1999a) for measuring the sublethal toxicity of a substance or material on this test organism.*
- *This biological test method is to be performed as a static-renewal test. The route of exposure is by mixing the test material in laboratory-formulated fresh water.*
- *This 7-day test method might be too short to detect pathogenic effects caused by certain micro-organisms. Extending the test duration (e.g., to 14 days) to achieve this is not recommended without further investigative work and, as necessary, modification to the criterion for test validity implicit in the test method.*
- *OECD (2002b) has a draft guideline for a 7-day growth inhibition test using* Lemna minor *or* L. gibba *similar to EC (1999a); this method could be used as an acceptable alternative. Other suitable biological test methods for measuring adverse effects of new microbial substances on freshwater plants are not available or are unproven for regulatory applications.*

9.1.1 Previous Tests with Micro-organisms or Microbial Products

Douville (2001) reviewed research studies investigating the pathogenic and/or toxic effects of specific micro-organisms or microbial products on various species of aquatic plants, under controlled laboratory conditions. Published reports of such studies are not extensive. A few investigations on measurements of growth for the freshwater macrophyte *Lemna minor* exposed to specific species of bacteria or fungi have been reported. Studies noting pathogenic effects caused by exposure of the Eurasian water milfoil (*Myriophyllum spicatum*) to certain species of fungi are also cited in Douville (2001). This literature review identified only one study reporting the use of tests with algae or diatoms to measure the pathogenic and/or toxic effects of micro-organisms or microbial products.

The responses to an informal questionnaire sent to USEPA's Microbial Pesticide Branch as well as three private US laboratories during the summer of 2002 (see Section 8.8) indicated that available data for freshwater plants exposed to MPCAs or their EPs were restricted to a few (nine) tests. Of these, five involved bacterial pesticides, and four involved fungal pesticides. Host plants were duckweed (*Lemna* sp.), freshwater algae (*S. capricornutum*), or a freshwater diatom. Most of these tests showed positive (i.e., pathogenic and/or toxic) effects.

9.1.2 Recommended Biological Test Method

In its Series 885 test guidelines for microbial pesticides, USEPA (1996c) addresses test methods and associated species for studying the pathogenic and/or toxic effects of micro-organisms on non-target plants. For microbial pesticides that have freshwater uses or might be expected to disseminate to and survive in freshwater ecosystems, aquatic plants including *Lemna gibba* (duckweed), *Selenastrum capricornutum* (a freshwater green alga), and a freshwater diatom are identified as suitable test organisms. However, no test-specific biological test methods are provided for these species in USEPA (1996c) or other Series 885 test guidelines. Additionally, no Standard Operating Procedures used by government or private laboratories for testing the pathogenicity and/or toxicity of micro-organisms or new microbial substances on freshwater plants have been found.

In its guidelines on data to be reported on the ecological effects of a new microbial substance, EC and HC (2001) identified the aquatic duckweed *Lemna* sp. as a species of aquatic plant deserving of consideration as a test organism. In 1999, Environment Canada published a biological test method that uses the freshwater macrophyte *Lemna minor* to measure the inhibition of plant growth caused by toxic chemicals or other test substances (EC, 1999a). Duckweed growth inhibition tests are

commonly applied within North America and abroad to determine sublethal toxic effects of test substances on freshwater plants (USEPA, 1996gg; ASTM, 2000c; OECD, 2002b). In Canada, *L. minor* is one of the most common and widespread of the duckweed species, and is found in still or slightly moving water of freshwater ponds, marshes, lakes, and quiet streams throughout most of the provinces and the territories. Duckweed species have many attributes that make them advantageous for use in laboratory toxicity tests, including their small size, structural simplicity, ease in culturing, rapid growth, and susceptibility to aquatic contaminants. Unlike tests using algae, test concentrations can be renewed during assays with duckweeds, and coloured or turbid concentrations can be tested (EC, 1999a). These features support the use of a duckweed growth inhibition test for measuring adverse ecological effects of new microbial substances on aquatic plants.

An adapted version of Environment Canada's duckweed growth inhibition test (EC, 1999a) is recommended as the biological test method to be applied when studying the adverse ecological effects of new microbial substances on freshwater plants. The adaptations to be used are summarized in Table 1, along with other test specifics that apply. When applying this test method, each test concentration (including control solutions or suspensions) must be renewed at least twice during the 7-day test; more frequent (i.e., daily) renewal should be considered and applied when the microbial concentration(s) of a test material is/are particularly unstable in fresh water. As with other test methods to be applied to new microbial substances, each test using *Lemna minor* must include a negative control (see Section 4.1). In keeping with EC (1999a), the use of a reference toxicant (i.e., a positive chemical control) as part of (or in conjunction with) this test is also required. The use of a non-infectious control (Section 4.4) is strongly recommended, and use of a sterile filtrate control (Section 4.5) is optional. Measurements for infectivity, using whole-body homogenates of plants from each treatment upon completion of the test, are optional and dependent on the study objectives (see Section 5). Test specifics when undertaking either a single-concentration test (Section 3.3.1) or a multi-concentration test (Section 3.3.2) using *L. minor* are summarized in Table 1. The route of exposure of this freshwater plant to the test material is by mixing it in water.

The biological endpoints for this test are growth measurements at test end, determined as both the number of *fronds* in each test chamber and on their dry weight attained. Statistical endpoints for a single-concentration test using the maximum hazard concentration are mean (± SD) number of fronds in each treatment (including the controls) at test end, as well as mean (± SD) dry weight of fronds in each treatment at test end. Growth endpoint values for the MHC and negative control treatments are compared statistically using a *Student's t-test* or other appropriate pairwise comparison (EC, 2004d); values for each control other than the negative control that is included in a test are also compared in this manner with those for the negative control treatment. If a multi-concentration test is performed, these same statistical values are determined for each treatment. As well, the 7-day IC25 for each biological endpoint (i.e., attained number of fronds and frond dry weight, each treatment) is calculated, data permitting, along with its 95% confidence interval (EC, 2004d). The NOEC and LOEC for attained number of fronds and frond dry weight are also calculated, data permitting (EC, 2004d). If one or more controls besides the negative control are included in a multi-concentration test, pairwise comparison of the endpoint statistics for such treatment(s) versus those for the negative control treatment should be performed as previously described. Environment Canada's statistical guidance document for environmental toxicity tests (EC, 2004d) should be consulted and followed when making these calculations and comparisons.

9.1.3 Other Methods or Procedures

Depending on the manner and rate of infectivity of a particular new microbial substance, and on the time required for growth inhibition of *Lemna minor* due to its pathogenicity, a 7-day test using this species of freshwater plant might be too short to detect this effect. Extending the duration of the test seems like a logical solution to this potential weakness of a 7-day test for pathogenic and/or toxic effects using *L. minor*. However, such a modification should not be applied without also changing, as necessary and appropriate, the criterion for test validity (Table 1) that was developed for this test method (EC, 1999a).

Table 1 Recommended Methodology for a 7-Day Pathogenicity/Toxicity Test Using the Freshwater Macrophyte, *Lemna minor*

Universal		
Test method	—	in keeping with EC (1999a) "Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*"
Test type	—	static renewal of each test concentration (including controls) during a 7-day period; renewal of each test concentration (including each control solution or suspension) at least twice, on Days 3 and 5 of the test
Test organism	—	*Lemna minor* from 7- to 10-day old culture, each plant with three fronds
Acclimation	—	acclimated for a minimum of 18 to 24 h to the control/dilution water
Number plants/test chamber	—	2
Volume/test chamber	—	100–150 mL, in a 150-mL beaker or other suitable test chamber
Control/dilution water	—	SIS (Swedish Standard) growth medium (see Section 5.3 in EC, 1999a); DO 90 to 100% saturation when added to test chambers
Water temperature	—	daily mean of 25 ± 2 °C throughout the test
Lighting	—	continuous full spectrum
pH	—	no adjustment if pH of test concentration(s) within range of 6.5 to 9.5; a second (pH-adjusted) test is recommended if pH of any treatment outside that range
Aeration	—	none required during test
Controls	—	each test must include a negative control; sensitivity of test organisms to a reference toxicant (i.e., a positive chemical control) must be determined; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route of exposure	—	test material mixed in control/dilution water
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-body homogenates of *Lemna minor* from each treatment, during and/or at end of test
Measurements	—	temperature measured daily in representative test chambers; pH measured at start and end of test and before and after each renewal, in one or more replicates of each treatment including the control(s); light intensity measured at several locations in the test area once during the test; analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of test and at the beginning and end of at least one of the renewal cycles during the test
Observations	—	number of fronds and appearance at test start and test end; dry weight at test end; optional counting of fronds on two other occasions during the test for growth rate calculation

Biological endpoints	—	growth based on increase in the number of fronds during the test and dry weight at the end of the test
Test validity	—	invalid if increase in the number of fronds in negative control during the 7-day test period is less than eight-fold (i.e., the mean number of fronds for the negative control must be ≥48 at the end of the test, for the test to be valid)

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	minimum of three per concentration (treatment), including each control treatment
Number of plants/treatment	—	minimum of six (2/replicate; 3 replicates/treatment)
Exposure route	—	mixed in fresh water (i.e., SIS control/dilution water), at MHC
Statistical endpoints	—	mean (± SD) number of fronds in each treatment at test end; mean (± SD) dry weight of fronds in each treatment at test end
Statistical comparisons	—	MHC versus negative control, for significant difference in mean number of fronds at test end and mean dry weight of fronds at test end; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; ≥seven plus negative control recommended; additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	minimum of three per concentration (treatment), including each control treatment; must have four per treatment if NOEC/LOEC to be determined
Number of plants/treatment	—	minimum of six (2/replicate; 3 replicates/treatment)
Exposure route	—	mixed in fresh water (i.e., SIS control/dilution water), at MHC and lower concentrations
Statistical endpoints	—	mean (± SD) number of fronds in each treatment at test end, mean (± SD) dry weight of fronds in each treatment at test end; data permitting — 7-day IC25 for attained number of fronds, 7-day IC25 for frond dry weight, NOEC/LOEC for attained number of fronds and frond dry weight
Statistical comparisons	—	test concentrations versus negative control, for significant decrease in mean number of fronds and mean dry weight of fronds at test end; if other control(s), same comparisons with negative control

Further evaluation by Environment Canada, together with interlaboratory validation, seems prudent before any extended test duration is considered and approved as a means of improving the likelihood that the biological test method for freshwater plants recommended herein (see Section 9.1.2) will detect growth inhibition due to various types of pathogenic micro-organisms. Since an extension of the duration of this test to 14 days or longer seems desirable in this respect, it is recommended that use of an extended test duration (i.e., ≥14 days) be considered during the pre-notification consultation with regulators (see Section 1.1), bearing in mind concerns about the associated modification to the criterion for test validity indicated in Table 1. Over time, the increased use of this biological test method with a variety of diverse types of pathogenic micro-organisms will also assist Environment Canada in determining if such modification (or the use of an alternate test method for measuring effects on freshwater plants) is warranted. Based on the proven performance of this test method in detecting toxic effects within the 7-day test period, no such concern exists regarding the ability of the test to measure sublethal effects on *L. minor* caused by toxic metabolites produced by the new microbial substance, and/or those associated with any chemical(s) included while preparing the test material.

The OECD (2002b) has a draft guideline for undertaking a 7-day growth inhibition test using *Lemna minor* or *L. gibba*. The test procedures and conditions described in this guideline are similar to those in the duckweed test method published by EC (1999a), that is identified in Section 9.1.2 as the recommended test method for freshwater plants. A duckweed growth inhibition test performed according to OECD (2002b) could be used as an acceptable alternative to the test method of EC (1999a). If applying this test method, the comments in the preceding paragraph related to extending the test duration apply equally here.

The draft ecological effects test guideline developed by the USEPA for performing an aquatic plant toxicity test using *Lemna* spp. (USEPA, 1996gg), while similar to EC (1996a), is not recommended as an alternative to Environment Canada's test method using *Lemna minor*. One disadvantage of this draft USEPA method is that it does not include a criterion for test validity based on frond growth in the negative control. A second disadvantage is that the "ECX" statistical endpoint (e.g., EC50) used in USEPA (1996gg) for a multi-concentration test is incorrect, in that this endpoint is used for *quantal* data; whereas growth in a multi-concentration test should be expressed using a *quantitative* endpoint (e.g., IC25).

The American Society for Testing and Materials (ASTM) has published a standard guide for conducting *static* toxicity tests using *Lemna gibba* (ASTM, 2000c). This 7-day test for growth inhibition of this species of duckweed could be modified for testing the pathogenicity and/or toxicity of new microbial substances in keeping with the recommended methodology presented in Table 1 for *Lemna minor*.

The ASTM has published a standard guide for conducting a static-renewal toxicity test with freshwater emergent macrophytes growing in sediment (ASTM, 2000d). This standard guide recommends the use of domestic rice (*Oryza sativa*) as the test organism. In this test, a selected freshwater plant species is exposed to various concentrations of a test substance dissolved in a nutrient solution in which the plants (in pots containing negative control sediment) are placed. After a two-week exposure period, the concentration of chlorophyll in extracts of leaf matter is determined for each treatment as a measure of plant growth. This test method is not recommended for measuring the pathogenicity and/or toxicity of new microbial substances to freshwater plants, due to a lack of evidence that plant pathogens typically reduce concentrations of chlorophyll in this test organism and since this is an indirect measurement of plant growth without other endpoints indicative of pathogenicity.

Environment Canada has published a biological test method for measuring the sublethal toxicity of test substances to the freshwater alga *Selenastrum capricornutum*, which also uses growth inhibition as the biological endpoint (EC, 1992b). Neither this test method, nor a related one published by USEPA (2002a; see Section 14 therein), are recommended for measuring the pathogenicity and/or toxicity of new microbial substances to a selected species of freshwater plant. Rather, these test methods are considered unacceptable for the present purposes. In each instance, these test methods are performed

as static tests without any renewal of test concentrations during the test, and the test duration is very brief (72 h, in EC, 1992b; 96 h; in USEPA, 2002a). Also, these test methods do not lend themselves to measurements for infectivity, due to the relatively small amount of tissue available from tests using single-celled algae. A further drawback is that measurements of growth inhibition using *S. capricornutum* are not possible if the test suspensions are cloudy, and microbial suspensions are frequently turbid at the MHC and certain lower concentrations.

The test method for measuring the pathogenic and/or toxic effects of new microbial substances on freshwater plants (Section 9.1.2) is performed at a temperature of 25 ± 2 °C. Such a temperature is suitable for most mesophilic micro-organisms, but too high for psychrophilic ones. At the present time, there is no standardized biological test method suitable for measuring the pathogenic and/or toxic effects of a coldwater (psychrophilic) micro-organism on a species of freshwater plant. Research leading to the development of such a test method should be encouraged.

9.2 Estuarine or Marine Plants

Key Guidance

- *A 9-day test for effects on the survival and reproductive success of the red macroalga* Champia parvula *is recommended as the standard biological test to be used when measuring the pathogenic and/or toxic effects of new microbial substances on a species of marine plant.*

- *The recommended method represents an adaptation of one published by the USEPA as a rapid procedure for measuring the chronic toxicity of samples of effluent or receiving water on this test organism. Test endpoints are based on the 9-day survival of female plants, the number of cystocarps produced in each treatment by sexual reproduction, and the appearance of surviving plants including evidence of lesions, necrotic tissue, or developmental anomalies specific to the cystocarps.*

- *This biological test method is to include a 48-h exposure to each treatment (including the controls) with static renewal of test concentrations after 24 h, followed by transfer of female plants to clean seawater and a subsequent 7-day period of observation for post-exposure effects. The route of exposure is by mixing the test material in seawater adjusted to a salinity of 30 ± 2‰.*

- *This 9-day test method might be too short to detect pathogenic effects caused by certain micro-organisms. Extending the test duration to achieve this is not recommended without further investigative work and, as necessary, modification to the criterion for test validity implicit in the test method. Other suitable biological test methods for measuring adverse effects of new microbial substances on marine or estuarine plants are not available or are unproven for regulatory applications.*

9.2.1 Previous Tests with Micro-organisms or Microbial Products

Douville (2001) reviewed research studies investigating the pathogenic and/or toxic effects of specific micro-organisms or microbial products on various species of aquatic plants, under controlled laboratory conditions. Most research studies cited in Douville (2001) used freshwater plants as test (host) organisms; only one (Kerwin *et al.*, 1988) study addressed the effects of a micro-organism on a species of estuarine or marine plant.

Responses by USEPA's Microbial Pesticide Branch and three private US laboratories to an informal survey sent during 2002 (see Section 8.8) revealed no reports of laboratory tests performed with marine or estuarine plants exposed to MPCAs or their EPs.

9.2.2 Recommended Biological Test Method

There is little regulatory guidance on plant species and methods suitable for measuring the pathogenic and/or toxic effects of micro-organisms or new microbial substances on estuarine or marine plants. The USEPA Series 885 test guidelines (USEPA, 1996c) for microbial pesticides, focusses on testing for effects on nontarget plants. Except for mentioning the possible use of the marine diatom *Skeletonema costatum*, however, no guidance is provided in USEPA (1996c) or other Series 885 test guidelines on test methods appropriate for estuarine or marine plants. Environment Canada and Health

Canada (2001) does not mention any candidate species (or associated test methods) of marine or estuarine plants for measuring effects of new microbial substances. The PMRA (2001) states that tests involving marine plant species are required for evaluating MPCAs if, as a result of their use pattern, a potential exists for their exposure; however, no further guidance is provided in that respect.

An adapted version of the USEPA's sexual reproduction test using the red macroalga *Champia parvula* (Section 16 in USEPA, 2002b) is recommended as a suitable method for measuring the pathogenic and/or toxic effects of new microbial substances on marine plants. This test method is now widely used as a short-term test for measuring the *chronic toxicity* of samples of effluent or receiving water on a marine plant under controlled laboratory conditions. Within Canada, it is required as part of Environmental Effects Monitoring (EEM) Programs for the pulp and paper and metal mining industries discharging to coastal waters (Scroggins *et al.*, 2002a,b). The ASTM (2000e) has also published a standard guide for conducting sexual reproduction tests with seaweeds including procedures and conditions for *C. parvula* similar to those defined in USEPA (2002b), while indicating that this test method may also be used for measuring effects of individual chemicals.

Table 2 provides the adaptations and procedural specifics when applying this test method (an adaptation of USEPA, 2002b) to new microbial substances. As adapted, this test method involves a 48-h exposure of sexually mature female and male branches of this marine plant to replicates of the MHC (and, if a multi-concentration test, lower concentrations) and control groups, followed by a 7-day period of recovery in seawater for observations and for development of *cystocarps* resulting from fertilization during the exposure period. During the 48-h exposure period, a renewal of each test concentration (including the controls) is required at 24 h. Biological endpoints for the test are the percent survival of female plants and, as a measure of reproductive success, the number of cystocarps per female plant produced during the test. Test observations also include the presence of necrotic tissue and evidence of morphological changes (ASTM, 2000e).

As with other test methods to be applied to new microbial substances, each test using *C. parvula* must include a negative control (see Section 4.1). In keeping with USEPA (2002b), the use of a reference toxicant (i.e., a positive chemical control) as part of (or in conjunction with) this test is also required. The use of a non-infectious control (Section 4.4) is strongly recommended, and use of a sterile filtrate control (Section 4.5) is optional. Measurements for infectivity, using whole-body homogenates of plants from each treatment upon completion of the test, are optional and dependent on the study objectives (see Section 5). Test specifics when performing either a single-concentration test (Section 3.3.1) or a multi-concentration test (Section 3.3.2) using *C. parvula* are summarized in Table 2. The route of exposure of this marine plant to the test material is by mixing it in water.

The endpoints for this test are, for each replicate and each treatment, percent survival of female plants at test end, percentage of female plants showing any signs of atypical appearance (e.g., lesions, necrotic tissue, or abnormally developed cystocarps), and numbers of cystocarps per female plant produced during the test. For a single-concentration test, the endpoint values for the MHC and negative control treatments are compared statistically using a *Student's t-test* or other appropriate pairwise comparison (EC, 2004d); values for each control besides the negative control included in a test are also compared in this manner with those for the negative control treatment. If a multi-concentration test is performed, the 9-day LC50 for female plants is calculated together with its slope and 95% confidence limits, data permitting. Additionally, the 9-day IC25 for attained number of cystocarps per female plant is calculated together with its 95% confidence limits, data permitting. The NOEC and LOEC for both attained number of cystocarps per female plant, and percentage of female plants showing an atypical appearance, are also calculated, data permitting (EC, 2004d). If one or more controls other than the negative control are included in a multi-concentration test, pairwise comparison of the endpoint statistics for such treatment(s) versus those for the negative control treatment should be performed as previously described. Environment Canada's statistical guidance document for environmental toxicity tests (EC, 2004d) should be consulted and followed when making these calculations and comparisons.

Table 2 Recommended Methodology for a 9-Day Test Measuring Pathogenic and/or Toxic Effects on Marine Plants Using the Red Macroalga *Champia parvula*

Universal

Test method	—	in keeping with Section 16 of USEPA (2002b) "Test Method — Red Macroalga, *Champia parvula*, Sexual Reproduction Test Method 1009.0"
Test type	—	48-h exposure to each test concentration (including controls), with static renewal of each test concentration after 24 h exposure; transfer of plants to control/dilution water after 48 h for recovery period of 7 days
Test organism	—	red macroalga, *Champia parvula*
Acclimation	—	unialgal stock cultures of males and females maintained in separate 1000-mL Erlenmeyer flasks containing aerated culture medium
Volume/test chamber	—	≥100 mL, in a 200-mL polystyrene cup or a 250-mL Erlenmeyer flask
Plants/ test chamber	—	5 female plants, comprised of branch tips 7–10 mm in length; and 1 male plant, comprised of a branch 2–3 cm in length
Control/dilution water	—	natural seawater at 30‰, or a mixture of ≥50% natural seawater and ≤50% artificial seawater adjusted to a salinity of 30‰; DO 90 to 100% saturation when added to test chambers
Salinity	—	30 ± 2‰
Water temperature	—	daily mean of 23 ± 1 °C throughout the test
Lighting	—	full spectrum (e.g., cool-white fluorescent lights); photoperiod, 16-h light : 8-h dark
pH	—	no adjustment if pH of test concentration(s) ranging within 7.0–8.5; a second (pH-adjusted) test is recommended if pH of any treatment outside that range
Aeration	—	gently in each test chamber, throughout the test; DO should not fall below 4.0 mg/L
Controls	—	each test must include a negative control; sensitivity of test organisms to a reference toxicant (i.e., a positive chemical control) must be determined; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route of exposure	—	test material mixed in control/dilution water
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-body homogenates of *Champia parvula* from each treatment, during and/or at end of test
Measurements	—	during initial 48 h, temperature, pH, DO, and salinity measured for one replicate of each treatment, for fresh and aged suspensions or solutions; daily in representative test chambers; daily measurements thereafter until test end; analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of each renewal period during initial 48 h of test

Observations	—	daily, each test chamber, for appearance of test solutions or suspensions, aeration rates, and appearance of plants; appearance of each plant and number of cystocarps per female, at test end, for each test chamber; signs of necrotic tissue and/or morphological changes
Biological endpoints	—	survival and appearance (including lesions or atypically developed cystocarps) of female plants, and number of cystocarps per plant
Test validity	—	invalid if <80% survival, or if mean number of cystocarps in negative control <10/plant

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	four per concentration (treatment), including each control treatment
Number of plants/treatment	—	20 female branch tips and four male plants
Exposure route	—	mixed in 30 ± 2‰ seawater, at MHC
Statistical endpoints	—	percent survival of female branch tips in each treatment at test end; mean (± SD) number of cystocarps per plant in each treatment at test end; percentage of plants in each test chamber and for each treatment, showing atypical appearance including lesions or abnormally developed cystocarps at test end
Statistical comparisons	—	MHC versus negative control at test end, for significant difference in percent survival of female plants, mean number of cystocarps per plant at test end, and percentage of female plants showing atypical appearance; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; ≥seven plus negative control recommended; additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	minimum of three per concentration (treatment), including each control treatment; must have four per treatment if NOEC/LOEC to be determined
Number of plants/treatment	—	20 female branch tips and four male plants
Exposure route	—	mixed in 30 ± 2‰ seawater, at MHC and lower concentrations
Statistical endpoints	—	each replicate and each treatment at test end, for percent survival of female branch tips, mean (± SD) number of cystocarps per plant, and percentage of female plants showing atypical appearance including lesions or abnormally developed cystocarps; data permitting — 9-day LC50 for female plants, 9-day EC50 for percentage of female plants in each treatment showing atypical appearance, 9-day IC25 for attained number of cystocarps per female, NOEC/LOEC for attained number of cystocarps per female
Statistical comparisons	—	test concentrations versus negative control, for significant decrease in percent survival of female plants, percentage of female plants showing atypical appearance, and mean number of cystocarps per female at test end; if other control(s), same comparisons with negative control

As indicated in Table 2, this 9-day test is performed at a required salinity of 30 ± 2‰. *Champia parvula* is not able to tolerate brackish seawater; thus this test method is more representative of the marine environment than of estuarine conditions.

9.2.3 Other Methods or Procedures

The chronic test method for measuring potential ecological effects of new microbial substances on a species of marine plant (i.e., *Champia parvula*) recommended in Section 9.2.2 has been adapted herein to enable detection of pathogenic as well as toxic effects. Further modifications, such as extending the exposure and/or post-exposure periods for this test, might enhance the ability to discern pathogenic effects for certain micro-organisms that show a significant delay in infectivity or subsequent pathogenicity. However, alterations such as these are not appropriate without adequate experimentation and interlaboratory validation of the influence of these modifications on test sensitivity and the criteria for test validity established for the test method (see Table 2). Application of this biological test method to a variety of new microbial substances will provide Environment Canada with data for various types of micro-organisms (i.e., bacteria, viruses, fungi, protozoans). This will assist in determining the ability of the test method, performed according to Section 9.2.2, to discern pathogenic effects as well as toxic ones. As necessary, further procedural modifications could be made thereafter to enhance test performance using new microbial substances.

The USEPA has published a short-term method for measuring the chronic toxicity of effluents or receiving waters on marine plants, which uses the giant kelp *Macrocystis pyrifera* as the test organism and success of spore *germination* as well as growth as biological endpoints (Section 17 in USEPA, 1995). Although applicable to another species of marine plant, this test method is not recommended here for testing new microbial substances due to certain limitations. Once again, this test method is restricted to full-strength seawater (34 ± 2‰ salinity), and cannot be applied to estuarine conditions requiring more brackish water as the *control/dilution water*. The duration of this test method is very brief (only 48 h), and, as specified in USEPA (1995), the test is performed without any renewal of test concentrations during this period. Accordingly, this (i.e., Section 17 in USEPA, 1995) test method is unacceptable for the present purposes.

Various test methods are available and have been applied for measuring the toxicity of test substances to planktonic marine or estuarine microalgae in the laboratory (HC and USEPA, 2001). However, such test methods, using an estuarine or marine diatom (e.g., *Skeletonema costatum* or *Thalassiosira pseudonana*) or species of dinoflagellate (e.g., *Gonyaulax polyedra* or *Pyrocystis lunula*) are typically of brief duration (e.g., 96 h), and are performed as static tests (ASTM, 2000f,g). No particular worth of these methods for measuring pathogenic effects of new microbial substances is evident, although such tests would be suitable for measuring toxic effects.

The test method recommended in Section 9.2.2 for measuring the pathogenic and/or toxic effects of new microbial substances on estuarine or marine plants using the red macroalga *Champia parvula* is performed at a temperature of 23 ± 1 °C. Such a temperature is suitable for most mesophilic micro-organisms, but too high for psychrophilic ones. At the present time, there is no standardized biological test method suitable for measuring the pathogenic and/or toxic effects of a coldwater (psychrophilic) micro-organism on a species of estuarine or marine plant. Research leading to the development of such a test method should be encouraged.

Tests Using Aquatic Invertebrates

10.1 Freshwater Invertebrates

Key Guidance

- *A 21-day test using the freshwater cladoceran* Daphnia magna, *performed according to OECD (1998c), is recommended as the standard biological test method to be used when measuring the pathogenic and/or toxic effects of new microbial substances on a pelagic freshwater invertebrate. This test is performed as a static-renewal one, whereby the test material is mixed in fresh water. Endpoints include 21-day survival of parental daphnids, and number of live young produced per parental daphnid.*

- *Adaptations of Environment Canada's survival-and-growth tests (EC, 1997a,b) using either midge larvae (*Chironomus tentans *or* C. riparius*) or the amphipod* Hyalella azteca, *are recommended as standard methods for measuring the pathogenicity and/or toxicity of new microbial substances to benthic freshwater invertebrates. Each test method is performed with the static renewal of water overlying sediment in test chambers. For a single-concentration test, the test material is mixed in both the sediment and the overlying water. For a multi-concentration test, the test material is mixed in either sediment or overlying water.*

- *Environment Canada's test method using* H. azteca *(EC, 1997b) is also suitable for tests performed under brackish-water (≤15‰) conditions.*

10.1.1 Previous Tests with Micro-organisms or Microbial Products

Douville (2001) reviewed research studies investigating the pathogenic and/or toxic effects of various types of micro-organisms (i.e., bacteria, viruses, protozoans, and fungi) on selected species of freshwater invertebrates. Of the pelagic species, tests using daphnids (water fleas) were the most common, and researchers typically used either *Daphnia magna* or *Ceriodaphnia dubia* as test (host) organisms. Research studies with benthic freshwater invertebrates exposed to micro-organisms used a number of differing species of insect larvae or infaunal amphipods as test organisms, with no consistent testing methodology or standardized approach evident.

The USEPA's Series 885 test guidelines for microbial pesticides using freshwater aquatic invertebrates as test (host) organisms (USEPA, 1996d) do not specify any particular species or biological test methods to be used. Rather, USEPA (1996d) states

> "*where direct aquatic exposure is anticipated, testing shall be performed on two aquatic invertebrate species, one of which is planktonic and the other benthic*".

For these freshwater tests, USEPA (1996d) specifies that the test material is to be administered as a suspension in the water, larval stages should be used whenever possible, and the test duration should be at least 21 days. The report by EC and HC (2001) indicated that a test using daphnids (*Daphnia* sp. or *Ceriodaphnia* sp.) should be considered when choosing species and test methods for measuring the potential ecological effects of a new microbial substance on freshwater invertebrates. The PMRA (2001) also supported the use of pelagic freshwater daphnids (e.g., *Daphnia magna*) for this purpose.

Private US laboratories testing microbial agents or products for pathogenic and/or toxic effects have developed in-house Standard Operating Procedures for performing a 21-day static-renewal test using *Daphnia magna*, that are compatible with the test guidelines in USEPA (1996d) for testing the effects of MPCAs on freshwater invertebrates. The results of an informal questionnaire sent to three of these laboratories during 2002 (see Section 8.8) showed that 24 such tests had been performed with MPCAs or microbial EPs; 19 with bacteria, three with viruses, and two with fungi. Of these 24 tests, most (92%) were multi-concentration tests with 79% including a non-infectious control, and 46% using a sterile filtrate control. None included a positive microbal control or a positive chemical control, and none attempted to test for infectivity of the host organisms (Section 5). Most (71%) of these 21-day tests with *D. magna* measured the concentration of micro-organisms in the test suspensions during the tests.

The response to the informal questionnaire by USEPA's Microbial Pesticides Branch indicated that data were available for ~87 separate tests involving 21-day tests using *D. magna* exposed to MPCAs or their EPs. Of these tests, ~60 involved bacteria, ~6 involved viruses, ~20 involved fungal pesticides, and one involved a protozoan MPCA. For this series of tests, ~91% were single-concentration tests using the *maximum hazard concentration* (Section 3.3.1) only. Sterile filtrate controls (see Section 4.5) were only included in ~3% of these tests. Approximately 45% included a non-infectious control (Section 4.4), none included a positive chemical control (Section 4.2), and none used a positive microbial control (Section 4.3). Microbial concentrations in the test suspension(s) (see Section 3.5) were measured for ~61% of these tests; none attempted to discern infectivity (see Section 5) during the test.

One private US testing laboratory has developed an in-house Standard Operating Procedure for measuring the pathogenicity and/or toxicity of MPCAs to benthic midge larvae (*Chironomus tentans)*. This is a 21-day test, whereby the test material is administered in the water overlying sediment in each test chamber. A static renewal of each test concentration in the overlying water takes place three times per week, for each treatment including the controls. At the end of the test, the number and percentage of midge larvae surviving each 21-day exposure is determined, and each is examined for gross signs of atypical appearance (e.g., lesions or discolouration) as well as possible histopathologies. Dry-weight measurements at test end are used to indicate if adverse effects on growth occurred during the test. The results of the 2002 survey (see Section 8.8) indicated that this SOP had received limited application with samples of new microbial substances.

10.1.2 Recommended Biological Test Method for Pelagic Invertebrates

The use of a 21-day pathogenicity/toxicity test involving the freshwater cladoceran *Daphnia magna* is recommended for testing the potential ecological effects of a new microbial substance in fresh water on pelagic freshwater invertebrates. This recommendation is in keeping with the widespread use by US investigators of a 21-day test with *D. magna* exposed to MPCAs or other microbial substances, when measuring the pathogenic and/or toxic effects of these test materials on a suitable species of pelagic freshwater invertebrate. The international Organisation for Economic Co-operation and Development has published a standard guide for conducting a 21-day static-renewal test for measuring the effects of toxic substances on the survival and reproduction of *D. magna* (OECD, 1998c). This biological test method, with appropriate adaptations herein for tests with new microbial substances, should serve well for this purpose. Table 3 provides a summary of the adaptations as well as key procedures and conditions associated with the performance of a 21-day test for pathogenic and/or toxic effects of new microbial substances on the survival and reproduction of *D. magna*.

Daphnids are broadly distributed in bodies of fresh water (e.g., ponds, lakes, or quiescent streams) in many Canadian provinces. These organisms, which swim actively within the water column, are an important link in many aquatic food chains and a significant source of food for juvenile stages of salmonid and other fish species. Daphnids have a relatively short life cycle, and are easy to culture in the laboratory. They are sensitive to a broad range of aquatic contaminants and are widely used as test organisms for measuring the toxicity of samples of chemical, effluent, or receiving water. Their small size requires only small volumes of test water when performing a single-concentration test or a multi-concentration test.

As indicated in OECD (1998c), this biological test method begins with groups of *neonate* daphnids that are less than 24-h old at the start of the test, and proceeds for 21 days during which time each test concentration (including the controls) is renewed three times per week on non-consecutive days (e.g., on each Monday, Wednesday, and Friday). Acclimation and test conditions are summarized in Table 3. Each test requires a negative control (see Section 4.1). The use of a non-infectious control (Section 4.4) is strongly recommended, and use of a sterile filtrate control (Section 4.5) is optional. Testing for infectivity is also optional (see Section 5). Test specifics when performing either a single-concentration test (see Section 3.3.1) or a multi-concentration test (Section 3.3.2) are summarized in Table 3. The route of exposure of test organisms to a new microbial substance is by mixing a measured quantity in the control/dilution water, which

Table 3 Recommended Methodology for a 21-Day Pathogenicity/Toxicity Test Using the Freshwater Cladoceran *Daphnia magna*

Universal		
Test method	—	in keeping with OECD (1998c) "Guidelines for the Testing of Chemicals — *Daphnia magna* Reproduction Test"
Test type	—	static renewal of each test concentration (including controls) throughout a 21-day period; three times per week on non-consecutive days (e.g., Mon, Wed, Fri)
Test organism	—	*D. magna* neonates, <24-h old at start of test
Acclimation	—	test organisms obtained from a single, healthy culture acclimated for at least two generations to the test conditions (i.e., those for test temperature, test photoperiod, food, and the control/dilution water) before the start of the test
Test chamber/volume	—	glass beaker, 50–100 mL capacity; 50–100 mL test solution/suspension in each
Number dapnids/test chamber	—	1 neonate, at start of test
Control/dilution water	—	Elendt M4 or M5 media (Annex 2 in OECD 1998c); other media acceptable provided criteria for test validity are met; recommended DO 90–100% saturation when added to test chambers
Water temperature	—	ranging within 18–22 °C; variation within these limits, ≤2 °C
Lighting	—	cool-white fluorescent or full spectrum, intensity not exceeding 15–20 μEinsteins/($m^2 \cdot s$); preferably, gradual transition from light to dark and dark to light
pH	—	no adjustment if pH of test concentration(s) within range of 6.0 to 8.5; a second (pH-adjusted) test is recommended if pH of any treatment outside that range
Dissolved oxygen	—	≥3 mg/L in each test chamber throughout test, must not aerate during test
Feeding	—	preferably once/day and at least three times/week, throughout test; use concentrated suspension of living algal cells (as per OECD, 1998c)
Controls	—	each test must include a negative control; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route of exposure	—	mixed in fresh water
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-body homogenate of parental daphnids during and/or at end of test

Measurements	—	temperature, pH, hardness, and DO measured at least once/week in fresh and old test suspensions/solutions of each control and the highest test concentration; analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of test and at the beginning and end of at least one of the renewal cycles during each week of the test
Observations	—	daily throughout test, for survival of parental daphnid in each test chamber; number of live young in each test chamber counted daily, followed by their removal
Biological endpoints	—	survival of parental daphnids, and cumulative number of live young produced per parental daphnid; for any test chamber wherein parental daphnid dies during test, exclude this replicate from the analysis
Test validity	—	invalid if either of the following occurs: <80% survival of parental daphnids in negative control at test end; mean of <60 live young per adult in negative control

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	at least 10 per concentration (treatment), including each control treatment
Number of daphnids/treatment	—	≥10 neonates (one in each replicate test chamber), at start of test
Statistical endpoints	—	percent survival of parental daphnids in each treatment, at test end; mean (± SD) number of young produced per parental daphnid, for each treatment
Statistical comparisons	—	MHC versus negative control, for significant difference in percent survival and decreased number of young produced; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	at least 10 per concentration (treatment), including each control treatment
Number of daphnids/treatment	—	≥10 neonates (one in each replicate test chamber), at start of test
Statistical endpoints	—	percent survival of parental daphnids in each treatment, at test end; mean (± SD) number of live young produced per parental daphnid, for each treatment; data permitting — 21-day LC50 for parental daphnids, 21-day IC25 for number of live young produced per parental daphnid, NOEC/LOEC
Statistical comparisons	—	test concentrations versus negative control, for significant difference in percent survival of parental daphnids and number of live young produced per parental adult; if other control(s), same comparisons with negative control

represents the maximum hazard concentration or, for a multi-concentration test, the MHC plus a series of lower concentrations.

The biological endpoints for this test are based on the 21-day survival of the parental daphnids used to start the test, and their reproductive success during the test.

For a single-concentration test, statistical endpoints to be determined following completion of the 21-day test are based on the percent survival of parental daphnids exposed to each treatment, and on the mean (± SD) number of live young produced per parental daphnid in each treatment. For a multi-concentration test, the two statistical endpoints described in the preceding paragraph apply. Data permitting (see Section 3.3.2), the 21-day LC50 for parental daphnids exposed to a range of concentrations of the test material should be calculated together with its slope and 95% confidence limits. The 21-day IC25 for number of live young produced per parental adult should also be calculated, data permitting. The NOEC and LOEC for attained number of young produced in each treatment during the 21-day test should also be calculated and reported, data permitting. If one or more controls other than the negative control are included in a multi-concentration test, pairwise comparison of the endpoint statistics for such treatment(s) versus those for the negative control treatment should be performed using a *Student's t-test* or other appropriate statistical test.

Statistical guidance provided in EC (2004d) should be consulted and followed when determining each of these statistical endpoints.

10.1.3 Recommended Biological Test Methods for Infaunal Invertebrates

Laboratory tests measuring the toxicity of samples of freshwater sediment are now commonly used in Canada and elsewhere. Environment Canada has published two biological test methods for measuring the toxicity of samples of freshwater sediment using either midge larvae (*Chironomus tentans* or *C. riparius*) or a species of freshwater amphipod (*Hyalella azteca*) as test organisms, and survival and growth as endpoints (EC, 1997a,b). These test methods, which use sensitive species of freshwater invertebrates common to many provinces of Canada, include detailed instructions on measuring the toxicity of test substances mixed in *clean* sediment, as well as guidance for performing single-concentration tests or multi-concentration tests. Numerous Canadian and US laboratories maintain cultures of *Chironomus* sp. or *H. azteca* for use in sublethal toxicity tests with samples of freshwater sediment, and are experienced in performing such tests using specific test substances. With appropriate adaptations, these biological test methods should also serve to measure the pathogenic and/or toxic effects of new microbial substances on these species of benthic (infaunal) freshwater invertebrates.

Tables 4 and 5 outline recommended methodologies for measuring the pathogenic and/or toxic effects of a new microbial substance using either midge larvae or the freshwater amphipod *Hyalella azteca* as test organisms. The procedures, conditions, and apparatus required when undertaking each of these biological test methods are similar, as are the biological endpoints (i.e., long-term survival and growth of the test organisms). As indicated in these tables, either method may be performed as a single-concentration test or a multi-concentration test. Guidance for testing a new microbial substance, including the route(s) of exposure, determining the MHC for the exposure route(s), measurements of concentrations of new microbial substance in the sediment or overlying water within test chambers, testing for infectivity, appropriate biological and statistical endpoints, and suitable statistical comparisons for endpoint data, is also summarized. Further guidance on these aspects is found in other sections of this document.

As indicated in Tables 4 and 5, single-concentration tests using midge larvae or freshwater amphipods as test (host) organisms should use two routes of exposure to the test material (i.e., by mixing it in the sediment as well as the overlying water). Maximum hazard concentrations for each of these exposure routes are described in Section 3.3.1 and summarized in these tables. Guidance for mixing and administering the test material in the (overlying) water and the sediment is provided in Sections 3.4.1 and 3.4.2, with specifics on rates of static renewal for the overlying water identified in Tables 4 and 5. For multi-concentration tests, only one exposure route should be used in each test (see Section 3.2).

Table 4 Recommended Methodology for a 10-Day Pathogenicity/Toxicity Test Using the Larvae of Freshwater Midges (*Chironomus tentans* or *C. riparius*)

Universal		
Test method	—	in keeping with EC (1997a) "Biological Test Method: Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges (*Chironomus tentans* or *Chironomus riparius*)"
Test type	—	static renewal of the overlying water in each test chamber four times during the test, on non-consecutive days (e.g., Mon, Wed, Fri)
Test organisms	—	third instar *C. tentans* or first instar *C. riparius* (≤48 h post-hatch) to start test; all obtained from the same laboratory culture
Test chamber and volumes of sediment and water	—	300-mL high-form glass beaker, 100 mL wet sediment and 175 mL overlying water
Number of larvae/test chamber	—	10
Control/dilution water	—	natural or artificial fresh water; DO 90 to 100% saturation when added to test chambers
Sediment	—	natural or artificial (laboratory formulated) sediment
Water temperature	—	daily mean of 23 ± 1 °C throughout test
Lighting	—	overhead full spectrum (fluorescent or equivalent); 500 to 1000 lux at water surface; 16 ± 1 h light : 8 ± 1 h dark
Dissolved oxygen	—	≥40% DO saturation in each test chamber throughout test, aerate gently in all test chambers
Feeding	—	four times only, on non-consecutive days (e.g., Mon, Wed, Fri), with ground tropical fish food flakes; 15.0 mg dry solids in a 3.75-mL suspension added to each test chamber
Controls	—	each test must include a negative control comprised of *clean* sediment and *clean* overlying water; sensitivity of test organisms to a reference toxicant (i.e., a positive chemical control) must be determined; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route(s) of exposure	—	mixed in both fresh water and sediment, if a single-concentration test; mixed in fresh water or sediment, if a multi-concentration test
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
MHC for sediment	—	10^6 microbial units/g sediment (dry wt), or 1000 times the expected micro-organisms concentration in the aqueous environment, whichever is greater and attainable (see Section 3.3.1.2)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-body homogenate of *Chironomus* sp. during and/or at end of test

Measurements of overlying water in test chambers	—	≥3 times/week, each treatment, for DO and temperature; start and end of test and just before renewal, each treatment, for pH, conductivity, and ammonia; analyses permitting, concentration of new microbial substance in each treatment including the control(s) at the beginning and end of the test and at the beginning and end of at least one of the renewal cycles during each week of the test
Measurements of sediment in test chambers	—	analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of test
Observations	—	during static renewal of overlying water, for numbers of midge larvae on sediment surface and their behaviour, appearance, and survival; gross appearance of each test organism at test end
Biological endpoints	—	survival and gross appearance of each test organism; mean dry weight per organism, determined for group of surviving midge larvae recovered from each test chamber at test end
Test validity	—	invalid if mean 10-day survival in negative control treatment <70%; invalid if individual mean dry weight for negative controls at test end is <0.6 mg (*C. tentans*) or <0.5 mg (*C. riparius*)

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	five per concentration (treatment), including each control treatment
Number of larvae/treatment	—	50, at start of test (5 replicates of 10 larvae/chamber)
Statistical endpoints	—	percent survival of midge larvae in each test chamber and for each treatment, at test end; percentage of organisms in each test chamber and for each treatment, showing atypical appearance at test end; mean (± SD) dry weight of surviving midge larvae in each treatment, at test end
Statistical comparisons	—	MHC versus negative control, for significant difference in percent survival, percent atypical appearance, and mean dry weight of survivors at test end; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	five per concentration (treatment), including each control treatment
Number of larvae/treatment	—	50, at start of test (5 replicates of 10 larvae/chamber)
Statistical endpoints	—	percent survival of midge larvae in each test chamber and for each treatment, at test end; percentage of organisms in each test chamber and for each treatment, showing atypical appearance at test end; mean (± SD) dry weight of surviving midge larvae in each treatment, at test end; data permitting — 10-day LC50, 10-day EC50 for atypical appearance of surviving larvae, 10-day IC25 for weight of surviving larvae, NOEC/LOEC
Statistical comparisons	—	test concentrations versus negative control, for significant difference in percent survival, percent atypical appearance, and mean dry weight of survivors at test end; if other control(s), same comparisons with negative control

Table 5 Recommended Methodology for a 14-Day Pathogenicity/Toxicity Test Using the Freshwater Amphipod *Hyalella azteca*

Universal		
Test method	—	in keeping with EC (1997b) "Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*"
Test type	—	static renewal of the overlying water in each test chamber three times per week on non-consecutive days (e.g., Mon, Wed, Fri)
Test organisms	—	amphipods 2–9-day old at start of test; all obtained from the same laboratory culture
Test chamber and volumes of sediment and water	—	300-mL high-form glass beaker, 100 mL wet sediment and 175 mL overlying water
Number animals/test chamber	—	10
Control/dilution water	—	natural or artificial fresh water; DO 90 to 100% saturation when added to test chambers
Sediment	—	natural or artificial (laboratory formulated) sediment
Water temperature	—	daily mean of 23 ± 1 °C throughout test
Lighting	—	overhead full spectrum (fluorescent or equivalent); 500 to 1000 lux at water surface; 16 ± 1 h light : 8 ± 1 h dark
Dissolved oxygen	—	≥40% DO saturation in each test chamber throughout test; aerate gently in all test chambers
Feeding	—	three times per week on non-consecutive days (e.g., Mon, Wed, Fri), with ground tropical fish-food flakes; ~6.3 mg dry solids in a 3.5-mL suspension added to each test chamber during each feeding
Controls	—	each test must include a negative control comprised of *clean* sediment and *clean* overlying water; sensitivity of test organisms to a reference toxicant (i.e., a positive chemical control) must be determined; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route(s) of exposure	—	mixed in both fresh water and sediment, if a single-concentration test; mixed in fresh water or sediment, if a multi-concentration test
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
MHC for sediment	—	10^6 microbial units/g sediment (dry wt), or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.2)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-body homogenate of *H. azteca* during and/or at end of test

Measurements of overlying water in test chambers	—	≥3 times/week, each treatment, for DO and temperature; start and end of test and just before renewal, each treatment, for pH, conductivity, and ammonia; analyses permitting, concentration of new microbial substance in each treatment including the control(s)at the beginning and end of the test and at the beginning and end of at least one of the renewal cycles during each week of the test
Measurements of sediment in test chambers	—	analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of test
Observations	—	during static renewal of overlying water, for numbers of amphipods on sediment surface and their behaviour, appearance, and survival; gross appearance of each test organism at test end
Biological endpoints	—	survival and gross appearance of each test organism; mean dry weight per organism, determined for group of surviving amphipods recovered from each test chamber at test end
Test validity	—	invalid if mean 14-day survival in negative control treatment <80%; invalid if individual mean dry weight for negative controls at test end is <0.1 mg

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	five per concentration (treatment), including each control treatment
Number of animals/treatment	—	50, at start of test (five replicates of 10 amphipods/chamber)
Statistical endpoints	—	percent survival of amphipods in each test chamber and for each treatment, at test end; percentage of organisms in each test chamber and for each treatment, showing atypical appearance at test end; mean (± SD) dry weight of surviving amphipods in each treatment, at test end
Statistical comparisons	—	MHC versus negative control, for significant difference in percent survival, percent atypical appearance, and mean dry weight of survivors at test end; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	five per concentration (treatment), including each control treatment
Number of animals/treatment	—	50, at start of test (five replicates of 10 amphipods/chamber)
Statistical endpoints	—	percent survival of amphipods in each test chamber and for each treatment, at test end; percentage of organisms in each test chamber and for each treatment, showing atypical appearance at test end; mean (± SD) dry weight of surviving amphipods in each treatment, at test end; data permitting —14-day LC50, 14-day EC50 for atypical appearance of surviving amphipods, 14-day IC25 for weight of surviving amphipods, NOEC/LOEC
Statistical comparisons	—	test concentrations versus negative control, for significant difference in percent survival, percent atypical appearance, and mean dry weight of survivors at test end; if other control(s), same comparisons with negative control

As with other test methods to be applied to new microbial substances, each test using midge larvae or freshwater amphipods must include a negative control (see Section 4.1). In keeping with EC (1997a,b), the use of a reference toxicant (i.e., a positive chemical control) as part of (or in conjunction with) each of these tests is also required.[63] The use of a non-infectious control (Section 4.4) is strongly recommended, and use of a sterile filtrate control (Section 4.5) is optional. Measurements for infectivity, using whole-body homogenates of midge larvae or amphipods from each treatment upon completion of the test, are optional and dependent on the study objectives (see Section 5).

Each of these recommended biological test methods includes careful inspection of test organisms recovered from each test chamber at the end of the test, for signs of atypical appearance (e.g., overt lesions, or discoloured or sloughed tissue). A stereomicroscope (e.g., at ~240× magnification) should be used for this purpose. The percentage of animals in each test chamber and for each treatment showing any overt signs of disease and/or toxic effects (i.e., an atypical appearance) are to be calculated. These data are to be used along with those representing percent survival and dry weights of test organisms, when making statistical comparisons of differences due to treatment (see Tables 4 and 5 "Statistical comparisons").

Pairwise comparisons, using *Student's t-test* or other appropriate statistics, should be used to determine if differences in statistical endpoints derived for each treatment (e.g., MHC versus negative control; negative control versus another control) are significant. Guidance on determining and comparing statistical endpoints for laboratory toxicity tests provided in EC (2004d) should be consulted and applied as appropriate.

10.1.4 Other Methods or Procedures

10.1.4.1 Alternative tests with pelagic invertebrates. The ISO (1999a) and ASTM (2000h) have published standardized test methods for measuring the long-term toxicity of substances to *Daphnia magna*. Each of these test methods can be performed as a 21-day static-renewal assay. The method by the ISO (1999a) is very similar to that of the OECD (1998c). The method by the ASTM (2000h) includes growth (dry weight and/or length of each surviving parental daphnid, at test end) as an endpoint along with 21-day survival and number of young produced per parental daphnid. Each of these test methods may be used as a suitable alternative to the OECD's (1998c) 21-day test using *D. magna* recommended in Section 10.1.2.

Standardized test methods for measuring the acute lethality of test substances to *Daphnia magna* or *D. pulex* have been published by Environment Canada (1990a, 2000a). These test methods are performed as static 48-h tests, and do not include measurements of sublethal effects on growth or reproduction, nor effects on long-term survival. Given these limitations, such acute toxicity tests are not amenable to testing new microbial substances for pathogenic effects or for toxic effects that might result from a more prolonged exposure. The use of an acute lethality test with *D. magna* could, however, serve to identify if a particular test material can cause acute toxic effects and might also serve in selecting concentrations to be used in a definitive 21-day multi-concentration test performed with this daphnid species to measure pathogenic and/or toxic effects.

Both Environment Canada (EC, 1992c) and the USEPA (Section 13 in USEPA, (2002a) have published a short-term method for measuring the chronic toxicity of samples of test substances to the freshwater daphnid *Ceriodaphnia dubia*. In each instance, the test duration is only 7 ± 1 days. It uses survival and reproductive success as its endpoints. While this test method serves well as a rapid means of measuring the chronic toxicity of various types of test substances, it is not recommended here for measuring the pathogenicity and/or toxicity of new microbial substances to daphnids in fresh water, and should not be used for this purpose. The 21-day test method using *Daphnia magna* recommended herein (see Section 10.1.2) offers a number of advantages over a 7±1-day test using *Ceriodaphnia dubia*, including a greater likelihood of detecting pathogenic effects (due to the longer test duration).

[63] The tests with a reference toxicant may be performed as static *water only* tests of 96-h duration, for cost-effectiveness (see EC, 1997a,b for test specifics).

10.1.4.2 Alternative tests with infaunal invertebrates. The USEPA has published a second edition of methods for measuring the toxicity of sediment-associated contaminants to freshwater invertebrates (USEPA, 2000). This edition includes the following four biological test methods for samples of sediment:

(1) a *Chironomus tentans* 10-day survival-and-growth test;
(2) a life-cycle test for *C. tentans*, which includes reproduction endpoints as well as those for survival and growth;
(3) a *Hyalella azteca* 10-day survival-and-growth test; and
(4) a 42-day test using *H. azteca* which measures effects on survival, growth, and reproduction.

The reproductive endpoints for the longer-duration test methods with *C. tentans* or *H. azteca* typically showed more variable results than those for survival or growth (USEPA, 2000). Nonetheless, certain new microbial substances might not cause pathogenic effects in midge larvae or the freshwater amphipod *Hyalella azteca* within an exposure period of 10 to 14 days. In this instance, the longer-duration test methods prescribed by USEPA (2000) for these test organisms might prove advantageous, with appropriate modifications for testing new microbial substances consistent with those defined in Section 10.1.3.

10.1.4.3 Tests using mesophilic or psychrophilic micro-organisms. The three biological test methods for freshwater invertebrates recommended in Sections 10.1.2 and 10.1.3 are performed at warm temperatures (i.e., 18–22 °C if *D. magna*, and 23 ± 1 °C if using *Chironomus* sp. or *H. azteca*). These test temperatures are suitable when measuring pathogenic and/or toxic effects of mesophilic micro-organisms, but are unsuitable for psychrophilic ones. No standardized test methods are presently available for measuring the ecological effects of micro-organisms in the laboratory under coldwater conditions suitable for psychrophilic micro-organisms. Research leading to the development of such methods should be encouraged, to satisfy this gap in testing methodologies when measuring the pathogenic and/or toxic effects of psychrophilic micro-organisms on freshwater invertebrates. One approach might be to conduct a 21-day (or longer) test with *Daphnia magna* at a temperature that does not exceed 15 °C, following acclimation to cool-water conditions. However, preliminary tests would be required to determine the typical reproductive rates for parental daphnids in negative control water, to ensure that reproduction proceeded at an acceptable rate using the chosen test temperature. When modifying and adapting the 21-day test for *D. magna* recommended in Section 10.1.2, the criterion for test validity based on the minimal number of live young produced per parental daphnid would need to be re-evaluated and modified as necessary, before such a method could be considered standardized and suitable for cool-water conditions and psychrophilic micro-organisms.

10.2 Estuarine or Marine Invertebrates

Key Guidance

- *In keeping with USEPA (1996e), a 30-day static-renewal test using the euryhaline shrimp* Paleomonetes vulgaris *is recommended as the standard biological test method to be used when measuring the pathogenic and/or toxic effects of new microbial substances on an epibenthic invertebrate within the estuarine or marine environment. This test, performed with commercially available adult grass shrimp, is conducted at a chosen salinity ranging within 10–35‰, and at a chosen temperature ranging within 5–25 °C. The test measures effects on the survival, appearance, and behaviour of grass shrimp as observed over the 30-day exposure. This recommended test method includes the necropsy of shrimp at test end, for overt changes in the gross appearance of tissues or organs and, as necessary, for histological effects.*

- *An adapted version of the 28-day test for measuring the bioaccumulation of contaminants using the euryhaline infaunal mollusc* Macoma balthica, *published by USEPA (1993), is recommended as a standard method for measuring the pathogenicity and/or toxicity of new microbial substances to benthic (infaunal)invertebrates in the estuarine or marine environment. This test method is performed with the static renewal of water overlying sediment in test chambers. The test is conducted at a chosen salinity ranging within 10–35‰., and at a selected temperature ranging within 5–25 °C (± 2 °C) . For a single-concentration test, the test material is mixed in both the sediment and the overlying water.*

- *A 28-day static-renewal test using blue mussels (*Mytilus edulis*), oysters (*Crassostrea *spp.), or scallops (*Pecten *spp.), adapted from a standard guide by ASTM (2000i), deserves consideration as an alternative test using a species of epibenthic estuarine (if* M. edulis*) or marine invertebrate. Using adults of these species, this test offers flexibility in terms of the test temperature and, if using* M. edulis*, may be applied to psychrophilic or mesophilic micro-organisms. An adaptation of USEPA's (2002b) 7-day static-renewal test using mysids (*Mysidopsis bahia*) is a second alternative, although this test must be performed at a warm (26 ± 1 °C) temperature and thus is only suitable for mesophilic micro-organisms.*

- *A 14-day static-renewal test using the euryhaline infaunal polychaete worm* Polydora cornuta, *adapted from a standard test method published by EC (2001a), deserves consideration as an alternative to the test method recommended herein for the infaunal bivalve mollusc* M. balthica . *This test can only be performed at a temperature of 23 ± 1 °C, and is thus unsuitable for testing psychrophilic micro-organisms. Another alternative is a 28-day static-renewal test using the euryhaline infaunal amphipod* Leptocheirus plumulosus, *adapted from USEPA (2001); this test must be performed at 25 ± 2 °C and is thus also restricted to testing mesophilic micro-organisms.*

10.2.1 Previous Tests with Micro-organisms or Microbial Products

Researchers have exposed a number of species and life stages of estuarine or marine invertebrates to micro-organisms or microbial products in the laboratory. Test organisms and approaches used in studies reviewed by Douville (2001) included:

- static 96-h LC50s with fungal insecticides, performed at a salinity of 20‰ using ≤24-h old mysids *(Mysidopsis bahia)* as test organisms;

- static 48-h LC50s with fungal, viral, and bacterial insecticides, conducted at salinities of 24–28‰ using larval coot clams *(Mulinia lateralis)*;

- a 27-day test for infectivity and pathogenicity of bacterial spores to adult American oysters *(Crassostrea virginica)*;

- a test for infectivity and pathogenicity of bacterial or fungal spores to adult Eastern oysters exposed to these pathogens for 3–14 days at a salinity of 15‰;

- static 6–9 day LC50s with fungal spores, using embryonic grass shrimp *(Palaemonetes pugio)*;

- static 15-day tests with pathogenic fungi, for effects on survival and development of embryonic grass shrimp *(P. pugio)* at a salinity of 20‰;

- a 23-day static-renewal test with a fungal insecticide at a salinity of 20‰, for survival and developmental anomalies of larval grass shrimp *(P. pugio)*;

- 7-day tests with a fungal insecticide at a salinity of 20‰, for survival of adult grass shrimp *(P. pugio)*;

- a 30-day test with an insect virus fed to adult grass shrimp *(P. vulgaris)* at a salinity of 25‰, for survival and histopathologies.

The last approach listed was endorsed by the USEPA (1996e) in its test guidelines for pathogenic and/or toxic effects of microbial pesticides on estuarine or marine invertebrates. At least one private US testing laboratory has developed an in-house Standard Operating Procedure for measuring the infectivity and pathogenicity and/or toxicity of MPCAs to adult grass shrimp *(P. vulgaris)* according to the test guidelines provided in USEPA (1996e). The response by the USEPA's Microbial Pesticide Branch to the 2002 questionnaire (see Section 8.8) showed records for five single-concentration (MHC) 30-day tests with *P. vulgaris* exposed to bacterial (2), viral (2), or fungal (1) MPCAs. One of these five tests detected a pathogenic and/or toxic response. None of these five tests included a sterile filtrate control or a positive microbial control, although two included a non-infectious control and each of the five also included testing for infectivity. Three of these five tests measured microbial concentrations within each test treatment.

Private laboratories responding to the 2002 questionnaire reported a total of six tests involving adult grass shrimp (*P. vulgaris*) exposed to bacterial

MPCAs or their EPs. Each of these used a single concentration (i.e., the MHC) only, and none demonstrated pathogenic and/or toxic effects. All of these six tests included a non-infectious control, two included a sterile filtrate control, none included a positive microbial control or a positive chemical control, one tested for infectivity of shrimp, and all measured the bacterial concentration to which the shrimp in each treatment were exposed during the test.

In responding to the 2002 questionnaire, the USEPA's Microbial Pesticide Branch did not identify any records for tests involving infaunal marine or estuarine invertebrates exposed to MPCAs or microbial products under laboratory conditions. Responses by the participating private US laboratories showed data for only one such test, which was performed using adult oysters *(Crassostrea virginica)* exposed to a bacterial MPCA for four days. A pathogenic and/or toxic effect was noted for this multi-concentration test.

10.2.2 Recommended Biological Test Method for Epibenthic Invertebrates

A 30-day test with euryhaline grass shrimp (*Paleomonetes vulgaris*) is recommended to measure the pathogenic and/or toxic effects of new microbial substances on a species of pelagic or epibenthic invertebrate found in the estuarine or marine environment. The recommended methodology for this test is consistent with the test guideline for shrimp described in USEPA (1996e). This species of shrimp is found in estuarine waters along the Atlantic and Gulf coasts. Although grass shrimp have no commercial or recreational importance as food for humans, they are an important species from an ecological perspective by serving as a link for energy transfer between trophic levels in the coastal food web. They are consumed in large quantities by commercially important species of fish. Grass shrimp feed on detritus, algae, and dead plant and animal matter.

Field and laboratory studies have indicated that adult *P. vulgaris* can tolerate salinities ranging from <5‰ to >40‰ (Anderson, 1985). Since this species of shrimp is euryhaline, the test may be performed at a salinity ranging from 10 to 35‰ (± 2‰ of the selected test salinity) to reflect either estuarine or marine conditions.

Grass shrimp are eurythermal. Adult *P. vulgaris* can tolerate temperatures ranging from 5–35 °C (Anderson, 1985). Given this broad range of thermal tolerance, a 30-day test for pathogenic and/or toxic effects of a new microbial substance on *P. vulgaris* may be performed at a temperature ranging from 5–25 °C.[64] Such a temperature range will enable the evaluation of psychrophilic as well as mesophilic micro-organisms. For a given test, the test temperature should remain within 5–25 °C at all times, and should not vary from the mean test temperature by more than ± 2 °C. Before the test is started, the test organisms should be adjusted gradually (≤3 °C/day) to the selected test temperature and acclimated to it for at least 14 days.

Table 6 provides a summary of the recommended methodology for performing a 30-day test using grass shrimp, including procedures and conditions to be applied when using this test for measuring the pathogenicity and/or toxicity of new microbial substances. The routes of exposure of shrimp to the test material for a single-concentration test, are by mixing it in both the test water (initially and upon each renewal) and the food, at the respective maximum hazard concentrations for these substrates (see Section 3.3.1 and Table 6). For a multi-concentration test, the effects of one or both of these means of exposure should be investigated separately (see Section 3.2). Guidance provided in Sections 3.4.1 (for water) and 3.4.4 (for food) should be consulted and followed when mixing the test material in water or food (i.e., commercial fish-food flakes or pellets).

Test organisms are obtained as adults from a commercial supplier. Those animals used in the test should be of approximately the same size, i.e., the length of the largest shrimp included in the test should not exceed the length of the smallest by more than two-fold. Following their acclimation to laboratory conditions (see Table 6), 10 shrimp (30/treatment) are placed in each test chamber at the start of the test. The test is performed using clear glass aquaria of ≥20-L capacity, with 15 L of test solution/suspension in each chamber. Depth of water in each test chamber must be ≥14 cm, and loading density at all times must be ≤0.8 g/L. The

[64] Temperatures higher than 25 °C are unrealistic for Canadian coastal waters.

Table 6 Recommended Methodology for a 30-Day Pathogenicity/Toxicity Test Using the Euryhaline Grass Shrimp *Paleomonetes vulgaris*

Universal		
Test method	—	in keeping with USEPA (1996e) "Microbial Pesticide Test Guidelines — Estuarine and Marine Animal Testing, Tier I"
Test type	—	static renewal of each test concentration (including controls), at least twice weekly throughout a 30-day period
Test organism	—	adult grass shrimp (*Palaemonetes vulgaris*) from a commercial supplier; all of similar size (length of largest shrimp should not exceed length of smallest by more than two-fold)
Acclimation	—	test organisms acclimated gradually to test conditions (salinity, temperature, lighting) and then held for at least 14 days before testing; mortality of population not to exceed 10% during final two days preceding test
Test chamber/volume	—	clear glass aquaria, ≥20-L capacity; 15 L test solution/suspension in each
Depth, loading density	—	≥14 cm in each test chamber; loading density, ≤0.8 g/L
Number of shrimp/test chamber	—	10
Control/dilution water	—	natural or artificial seawater; salinity, 10–35‰; DO 90–100% saturation when added to test chambers
Water temperature	—	ranging within 5–25 °C; acceptable variation within these limits, ± 2 °C of mean
Lighting	—	cool-white fluorescent or full spectrum, 300–1000 lux at water surface; normally 16 ± 1 h light : 8 ± 1 h dark; preferably, gradual transition from light to dark and dark to light
pH	—	no adjustment if pH of test concentration(s) within range of 7.0 to 8.5; a second (pH-adjusted) test is recommended if pH of any treatment outside that range
Dissolved oxygen	—	≥60% of saturation in each test chamber throughout test, aerate gently in all test chambers
Feeding	—	feed *ad libidum* once daily during acclimation and test, using a commercially prepared fish food diet; during testing, each test concentration to receive a measured quantity of the test material mixed in food
Controls	—	each test must include a negative control; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route(s) of exposure	—	mixed in both seawater and food, if a single-concentration test; mixed in seawater or food, if a multi-concentration test
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
MHC for food	—	100 times the expected microbial concentration in the aquatic environment (see Section 3.3.1.3)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-body homogenate of adult shrimp during and/or at end of test

Measurements	—	temperature, pH, salinity, and DO at the beginning and end of each water renewal, for at least one replicate of each treatment; analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of the test and at the beginning and end of at least one of the renewal cycles during each week of the test
Observations	—	daily throughout test and at test end, for percent survival of shrimp in each test chamber; necropsy upon death of each shrimp during test and at test end, for gross external and internal appearance; histology of selected tissues and organs as warranted
Biological endpoints	—	survival, appearance (including that from necropsy at test end), and behaviour in each test chamber and for each treatment, during and at test end
Test validity	—	invalid if <80% survival in negative control at test end

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, inclusion of non-infectious control strongly recommended; inclusion of sterile filtrate control optional
Number of replicates	—	three per concentration (treatment), including each control treatment
Number of shrimp/treatment	—	30 (10 per replicate), at start of test
Statistical endpoints	—	percent survival of shrimp in each test chamber and for each treatment, at test end; percentage of surviving shrimp in each test chamber and for each treatment, showing atypical behaviour and/or atypical appearance of organs and/or tissues at test end
Statistical comparisons	—	MHC versus negative control, for significant difference in percent survival and percentage of surviving shrimp showing atypical behaviour and/or atypical appearance of organs and/or tissues; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; additionally, inclusion of non-infectious control strongly recommended; inclusion of sterile filtrate control optional
Number of replicates	—	three per concentration (treatment), including each control treatment
Number of shrimp/treatment	—	30 (10 per replicate), at start of test
Statistical endpoints	—	percent survival of shrimp in each test chamber and for each treatment, at test end; percentage of surviving shrimp in each test chamber and for each treatment, showing atypical behaviour and/or atypical appearance of organs and/or tissues at test end; data permitting — 30-day LC50, 30-day EC50 based on percentage of surviving shrimp in each treatment showing atypical appearance and/or atypical behaviour, NOEC/LOEC
Statistical comparisons	—	test concentrations versus negative control, for significant difference in percent survival and percentage of surviving shrimp showing atypical behaviour and/or atypical appearance of organs and/or tissues; if other control(s), same comparisons with negative control

test is conducted with static renewal of each test suspension or solution at least twice weekly throughout the 30-day test period. The survival, appearance, and behaviour of shrimp in each test chamber should be observed daily throughout the test, and at its end.

Three replicate aquaria (test chambers) are required for each treatment, including each test concentration, the negative control, and any additional controls included in the test. The test must include a negative control (see Section 4.1). Use of a non-infectious control (Section 4.4) is strongly recommended, and use of a sterile filtrate control (Section 4.5) is optional. Testing for infectivity (see Section 5) is also optional.

The biological endpoints for this test are based on 30-day survival, and on the appearance and behaviour of shrimp in each test chamber during the test and at its end. During the daily observations, any shrimp observed to have died must be removed from its test chamber, and a detailed examination made of the gross appearance of its external and (following dissection) internal tissues and organs. On Day 30, each of the surviving shrimp exposed to each treatment (including the negative control treatment) must also be killed and necropsied in this manner. It is recommended that the investigator(s) be versed in accepted and standardized necropsy procedures for shrimp (e.g., see Bell and Lightner, 1988); these should be followed when performing each necropsy. If signs of pathology are evident, the affected tissue(s) or organ(s) should be dissected, preserved, and stored for future microscopic examination as necessary and appropriate. Additionally, the affected tissue or organ should be homogenized, and an attempt made to culture the micro-organism and confirm its infectivity.

For a single-concentration test, statistical endpoints to be determined following completion of the 30-day test include:

(1) percent survival of shrimp in each test chamber and for each treatment;

(2) percentage of shrimp in each test chamber and for each treatment, showing atypical behaviour (e.g., surfacing, erratic swimming, loss of equilibrium); and

(3) percentage of shrimp in each test chamber and for each treatment, showing an abnormal appearance of one or more organs or tissues (e.g., external or internal lesions, opaque or hemorrhaged eye, discoloured or otherwise atypical hepatopancreas).

Respective values for these endpoints determined for the maximum hazard concentration and any control treatments other than the negative control should be compared against those for the negative control, using an appropriate statistical test for pairwise comparisons such as *Student's t-test*. Environment Canada's guidance document on statistical methods to determine endpoints of toxicity tests (EC, 2004d) should be consulted when choosing and applying the appropriate statistics.

For a multi-concentration test, the statistical endpoints described in the preceding paragraph also apply. Data permitting (see Section 3.3.2), the 30-day LC50 for the test material should be calculated together with its slope and 95% confidence limits. A 30-day EC50 based on atypical appearance and/or atypical behaviour of individual shrimp exposed to each treatment should also be calculated together with its slope and 95% limits, if the data enable this. Data permitting, the LOEC and NOEC for effects on 30-day survival as well as data showing atypical appearance and/or atypical behaviour should be calculated and reported as well. Guidance in EC (2004d) on appropriate software programs to use (and their application) when determining an LC50 or EC50, or calculating LOEC and NOEC, should be followed. Environment Canada (2004d) should also be consulted when choosing the appropriate statistics to be applied to the data derived from the study on shrimp appearance (including that at necropsy) and behaviour.

10.2.3 Recommended Biological Test Method for Benthic (Infaunal) Invertebrates

A 28-day test using the euryhaline bivalve mollusc *Macoma balthica* is recommended to measure the pathogenic and/or toxic effects of new microbial substances on a species of infaunal invertebrate representing the benthic estuarine or marine environment. The recommended test method is an adaptation of a 28-day test for measuring the bioaccumulation of contaminants in samples of bedded sediment, that was published by USEPA (1993) and included *M. balthica* as a recommended

test species. The procedures and conditions for this test method, as summarized herein (see Table 7), are similar to those presented in Section 10.1.3 for biological test methods recommended for measuring the pathogenicity and/or toxicity of new microbial substances to benthic invertebrates in fresh water (i.e., the midge larvae *Chironomus tentans* or *C. riparius*, or the amphipod *Hyalella azteca*).

M. balthica frequents the estuarine and marine environments around Canada's Atlantic, Pacific, and Arctic coasts. This bivalve mollusc lives a few centimetres below the surface of sand, mud, and muddy sand. It is found from the upper regions of the intertidal into the sublittoral, particularly in estuaries and tidal flats (Budd and Rayment, 2001). *M. balthica* provides an important food source for birds, fish, crustaceans, and polychaete worms. Males and females become sexually mature at a small size (≤6 mm), although they can reach shell widths up to 25 mm. As both surface deposit feeders and infaunal suspension feeders, adult *M. balthic* feed actively on diatoms, deposited plankton, suspended phytoplankton, and detritus. Within the laboratory, high (≥90%) 28-day survival rates occur without feeding, if an adequate supply of uncontaminated natural seawater is provided (USEPA, 1993). Adults of this species adapt well to a wide range of salinity and temperature, and have proven useful in laboratory tests using samples of contaminated (or potentially contaminated) sediment.

M. balthica is a small, contaminant-sensitive, euryhaline bivalve clam that is ideal for measuring the pathogenicity and/or toxicity of new microbial substances that might contact and multiply in sediment within the estuarine or marine environment. Using the recommended methodology described in Table 7, this test may be performed at a selected salinity ranging within 10–35‰ and a selected temperature ranging within 5–25 °C. Accordingly, this 28-day test is suitable for measuring the potential ecological effects of both psychrophilic and mesophilic micro-organisms within the estuarine or marine environment. The test is performed in large (2-L) glass beakers or aquaria, with replicate groups of molluscs (ten per test chamber) held in sediment with overlying seawater. The microbial test material is mixed in the seawater and/or the sediment (see Sections 3.2, 3.3, and 3.4). This test is conducted as a static-renewal one (USEPA, 1993), with replacement of the overlying seawater (with or without a specific concentration of the test material) three times per week on non-consecutive days throughout the test's duration.

Endpoints for this test are based on the 28-day survival of adult *M. balthica* exposed to each treatment, and on the appearance of their tissues and organs upon necropsy at test end using recognized procedures for bivalve molluscs (e.g., Shuster and Eble, 1961; Elston *et al.*, 1987). Using a 28-day test with adult *M. balthica*, a quantity of whole-body tissue sufficient for measuring infectivity during or/at the end of the test (see Section 5) can be made available.

This test is performed using a population of adult *M. balthica* from a single source (collected from an uncontaminated site), that consists of organisms similar in size and all from the same year class (Table 7). When received at the laboratory, the organisms to be used in the test should be adjusted gradually (temperature change, no more than 3 °C /day; salinity change, no more than 5‰ /day), and acclimated thereafter to the selected test temperature and test salinity for a minimum period of seven days (USEPA, 1993). Then, 10 molluscs are transferred randomly to replicate test chambers containing the same quantity of sediment and overlying (aerated) seawater (see Table 7). The biological endpoints for this test are based on the 28-day survival of molluscs exposed to each treatment, as well as on the appearance of the tissues and organs of each surviving test organism upon necropsy (e.g., as per Shuster and Eble, 1961 or Elston *et al.*, 1987). Observations during the necropsies of surviving test organisms are scored as numbers and percentage of molluscs in each test chamber and for each treatment, showing any signs of an abnormal appearance.

This recommended test method is undertaken as either a single-concentration test (involving the MHC, a negative control and, if included in the design, one or more additional controls), or a multi-concentration test. For a single-concentration test, the test material is mixed in both the test water used for each renewal, and in the sediment added to each test chamber. Maximum hazard concentrations for each of these exposure routes are given in Section 3.3.1 and summarized in Table 7. Guidance for

Table 7 Recommended Methodology for a 28-Day Pathogenicity/Toxicity Test Using the Euryhaline Bivalve Mollusc *Macoma balthica*

Universal

Test method	—	adapted from USEPA (1993) "Guidance Manual: Bedded Sediment Bioaccumulation Tests"
Test type	—	static renewal of the overlying water in each test chamber three times per week on non-consecutive days (e.g., Mon, Wed, Fri)
Test organisms	—	adults measuring 6 to 20 mm; all from same year class and similar in size; for group used in test, the distance from the tip of the umbo to the distal valve edge of the largest mollusc should be no more than 1.5 times that of the smallest mollusc
Test chamber and depth of sediment and water	—	2-L glass beaker or (larger) glass aquarium; depth of sediment, minimum of 3 cm; depth of overlying seawater, minimum of 3× that of sediment depth
Number of animals/test chamber	—	10
Control/dilution water	—	natural seawater (preferably unfiltered); salinity must range within 10–35‰ and within 5‰ of the porewater salinity of the test sediment; held within ± 2‰ of mean salinity throughout the test; adjusted to test temperature before use; dissolved oxygen, 90–100% saturation when added to test chambers
Sediment	—	natural or artificial (laboratory formulated) sediment
Water temperature	—	acceptable range, 5 to 25 °C; held within ± 2 °C of mean temperature throughout test
Dissolved oxygen	—	60–100% DO saturation in each test chamber throughout test, aerate gently in all test chambers
Dissolved ammonia	—	concentration of un-ionized ammonia in overlying seawater in each test chamber should not exceed 20 µg/L at any time
Lighting	—	overhead full spectrum (fluorescent or equivalent); 400 to 1000 lux at water surface; normally 16 ± 1 h light : 8 ± 1 h dark
Feeding	—	none
Controls	—	each test must include a negative control comprised of *clean* sediment and *clean* overlying water; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route(s) of exposure	—	mixed in both seawater and sediment, if a single-concentration test; mixed in seawater or sediment, if a multi-concentration test
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
MHC for sediment	—	10^6 microbial units/g sediment (dry-wt basis), or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.2)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-body homogenate of *M. balthica* during and/or at end of test

Measurements of overlying water in test chambers	—	≥3 times/week, each treatment, for temperature; start and end of test and just before renewal, each treatment, for DO, pH, salinity, and ammonia; analyses permitting; concentration of new microbial substance in each treatment including the control(s) at the beginning and end of the test and at the beginning and end of at least one of the renewal cycles during each week of the test
Measurements of sediment in test chambers	—	analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of test
Observations	—	during static renewal of overlying water, for number of organisms on sediment surface and their survival; necropsy upon death of each organism during test and at test end, for gross appearance of organs and tissues; histology of selected organs and tissues as warranted
Biological endpoints	—	survival; gross appearance of each test organism upon necropsy
Test validity	—	invalid if mean 28-day survival in negative control treatment <90%

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, inclusion of non-infectious control strongly recommended; inclusion of sterile filtrate control optional
Number of replicates	—	three per concentration (treatment), including each control treatment
Number of molluscs/treatment	—	30 (10 per replicate), at start of test
Statistical endpoints	—	percent survival of organisms in each test chamber and for each treatment, at test end; percentage of surviving molluscs in each test chamber and for each treatment, showing atypical appearance at test end
Statistical comparisons	—	MHC versus negative control, for significant difference in percent survival, and percentage of surviving molluscs showing atypical appearance of any organs and/or tissues at test end; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; additionally, inclusion of non-infectious control strongly recommended; inclusion of sterile filtrate control optional
Number of replicates	—	three per concentration (treatment), including each control treatment
Number of molluscs/treatment	—	30 (10 per replicate), at start of test
Statistical endpoints	—	percent survival of organisms in each test chamber and for each treatment, at test end; percentage of surviving molluscs in each test chamber and for each treatment, showing atypical appearance of any organs and/or tissues at test end; data permitting —28-day LC50, 28-day EC50 for atypical appearance of any organs and/or tissues in surviving molluscs at test end; NOEC/LOEC
Statistical comparisons	—	test concentrations versus negative control, for significant difference in percent survival and percentage of surviving molluscs showing atypical appearance of any organs and/or tissues at test end; if other control(s), same comparisons with negative control

mixing and administering the test material in the (overlying) water and the sediment is provided in Sections 3.4.1 and 3.4.2, with specifics on the rate of static renewal for the overlying water identified in Table 7. For multi-concentration tests, only one exposure route should be used in each test (see Section 3.2).

Each 28-day test using *M. balthica* must include a negative control (see Section 4.1). The use of a non-infectious control (Section 4.4) is strongly recommended, and use of a sterile filtrate control (Section 4.5) is optional. Measurements for infectivity, using whole-body homogenates of test organisms from each treatment upon completion of the test, are optional and dependent on the study objectives (see Section 5).

The statistical endpoints for either a single-concentration test or a multi-concentration test using adult *M. balthica* are summarized in Table 7. Guidance on statistical comparisons (e.g., for endpoint data derived for the negative control versus those for the MHC or one or more control treatments) is also provided in summary (Table 7). Environment Canada's guidance document on statistics appropriate for results of environmental toxicity tests performed in the laboratory (i.e., EC, 2004d) should be consulted and followed when calculating and comparing statistical endpoints.

10.2.4 Other Methods or Procedures

10.2.4.1 Alternative tests with epibenthic invertebrates. ASTM (2000i) provides a suitable alternative to the 30-day test using grass shrimp recommended in Section 10.2.2, when testing for effects on an epibenthic estuarine or marine invertebrate. A standard guide for performing a 28-day laboratory test with a number of species of bivalve molluscs including the filter-feeding blue mussel *Mytilus edulis* is described (ASTM, 2000i). This bivalve species is both euryhaline and eurythermal; therefore, it can be used to test the pathogenic and/or toxic effects of either mesophilic or psychrophilic micro-organisms, throughout a wide range of test salinities. *M. edulis* is frequently used in laboratory and field studies concerned with measuring and monitoring the effects of environmental contaminants. The blue mussel is known to be susceptible to many types of pathogenic micro-organisms[65] as well as diverse chemical contaminants in seawater.

ASTM (2000i) should be consulted for a description of suitable acclimation and test conditions and procedures when performing a 28-day test with the blue mussel. Many of the test specifics described in Section 10.2.2 (including Table 6) for a 30-day test with grass shrimp, could be applied to a 28-day (or longer) test with *M. edulis*. Although ASTM (2000i) describes a continuous-flow test, this test could also be performed using static renewal with replacement of the test water 2–3 times each week throughout the test. Natural seawater (unfiltered and unsterilized) is recommended for this test, to provide as much natural planktonic food as possible (ASTM, 2000i). Exposure to the test material is restricted to mixing it in the water used for each renewal. Biological endpoints for this test would be based on 28-day survival and on the appearance of the mussels in each test chamber upon necropsy at test end. Recognized procedures for performing necropsies on bivalves (e.g., Shuster and Eble, 1961; Elston *et al.*, 1987) should be followed at this time.

Other species of epibenthic molluscs recommended by ASTM (2000i) for use in 28-day laboratory tests with aquatic contaminants include oysters (*Crassostrea gigas*) and scallops (*Pecten* spp.). The use of selected species of adult oysters or scallops common to Canadian coastal waters, in 28-day tests as described for *Mytilus edulis*, provides additional alternatives for testing the pathogenic and/or toxic effects of new microbial substances on a sensitive species of epibenthic marine invertebrate. Oysters and mussels are known to be susceptible to a variety of microbial pathogens.[66] The salinity tolerance (unlike *M. edulis*, oysters and scallops are not euryhaline) and temperature tolerance (oysters and scallops are warm-water species, although adults can tolerate cool waters) of species of adult oysters

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[65] Fisheries and Oceans Canada provides a useful description of known diseases and pathogens of molluscs including mussels (www.pac.dfo-mpo.gc.ca/sci/sealane/aquac/pages/toc.htm).

[66] Fisheries and Oceans Canada provides a useful description of known diseases and pathogens of molluscs including mussels and oysters at (www.pac.dfo-mpo.gc.ca/sci/sealane/aquac/pages/toc.htm).

or scallops should be reviewed when considering them for use as test organisms in a 28-day test (ASTM, 2000i), as a possible alternative to the test method with grass shrimp recommended in Section 10.2.2.

The USEPA has published a number of short-term methods for estimating the chronic toxicity of effluents or receiving waters to estuarine or marine invertebrates, using laboratory tests performed with a selected test organism and life stage. These include:

- A 7-day static-renewal test for effects on the survival, growth, and fecundity (females) of mysids (*Mysidopsis bahia*), performed at a salinity of 20 ± 2 to 30 ± 2‰ (Section 14; USEPA, 2002b).

- A 7-day static renewal test for effects on the survival and growth of the mysid crustacean *Holmesimysis costata*, carried out at a salinity of 34 ± 2‰ salinity (Section 12; USEPA, 1995).

- A 72-h static (non-renewal) test for effects on developing embryos of the sea urchin *Strongylocentrotus purpuratus* or the sand dollar *Dendraster excentricus*, undertaken at a salinity of 34 ± 2‰ (Section 15; USEPA, 1995).

- A 48-h static test for effects on developing embryos and larvae of the Pacific oyster *Crassostrea gigas* or the mussel *Mytilus* spp., performed at a salinity of 30 ± 2‰ (Section 13; USEPA, 1995).

- A 48-h static test for effects on the development of larval red abalone (mollusc; *Haliotus rufescens*), conducted at a salinity of 34 ± 2‰ (Section 14; USEPA, 1995).

- An 80-min static test for effects on fertilization success using eggs and sperm of the sea urchin *Arbacia punctulata*, performed at a salinity of 30 ± 2‰ (Section 15; USEPA, 2002b).

- A 40-min static test for effects on fertilization success using eggs and sperm of either the sea urchin *Strongylocentrotus purpuratus* or the sand dollar *Dendraster excentricus*, conducted at a salinity of 34 ± 2‰ (Section 16; USEPA, 1995).[67]

Of these biological test methods, only a 7-day static renewal test with mysids is suitable for testing the pathogenic and/or toxic effects of new microbial substances, because the other tests have very brief durations (i.e., 40 minutes to 48 hours). Another limitation for each of these test methods (including the one using the mysid *H. costata*), with the exception of a 7-day test using the moderately euryhaline mysid *Mysidopsis bahia*, is that they are restricted to high-salinity conditions (i.e., ≥28‰ salinity) and are unsuitable for application to estuarine conditions.

The 7-day static renewal test for effects on the survival and growth (or survival, growth, and fecundity) of the moderately euryhaline mysid *M. bahia* (Section 14; USEPA, 2002b) could be adapted as a test for pathogenic and/or toxic effects of mesophilic micro-organisms. This test method, however, is unsuitable for psychrophilic micro-organisms, since the specified test temperature is 26 ± 1 °C (USEPA, 2002b). If applied as an acceptable alternative to a 30-day test with grass shrimp (see Section 10.2.2), this test must include both a non-infectious control and a sterile filtrate control. A 7-day test with mysids should not be used as an alternative to a 30-day test with grass shrimp in instances where it is known or suspected that the pathogenic and/or toxic effects of the test material might require an exposure period of more than 7 days before they become evident.

The ASTM has published a standard guide for conducting life-cycle toxicity tests with saltwater mysids including *Mysidopsis bahia* (ASTM, 2000j). This biological test method is performed as a continuous-flow (flow-through) test. It begins with laboratory-cultured individuals that are <24-h post release from the *brood sac* and continues until at least seven days past the median time of first brood release in the negative control. Biological endpoints measure effects on survival, growth, and reproduction. The level of effort required to

[67] Environment Canada has published similar test methods for measuring the sublethal effects of test substances on the fertilization success of echinoids (sea urchins or sand dollars) at a test salinity of 30 ± 2‰ (EC, 1992d).

perform this biological test method is greater than that for the 7-day static-renewal test using *Mysidopsis bahia* described in Section 14 of USEPA (2002b). A life-cycle test with saltwater mysids, with appropriate adaptations, might be considered a suitable or preferred alternative to the one recommended in Section 10.2.2, if there are particular concerns about the potential adverse effects of a test material on the reproduction, growth, and development of pelagic or epibenthic invertebrates in the estuarine or marine environment.

10.2.4.2 Alternative tests with infaunal invertebrates. The ASTM has published two standard guides for conducting sediment toxicity tests using estuarine or marine polychaete worms (ASTM, 2000k,l). Neither of these test methods uses spionid polychaete worms common to Canadian coastal waters (e.g., *Polydora cornuta*). *Neanthes arenaceodentata* is the species of worm used most commonly by laboratories performing either of these tests. It is a *stenohaline* marine polychaete that must be held and tested at salinities ≥28‰. One of these guides describes the procedures and conditions for a static test of 20–28-day duration that measures survival and growth (dry weight at test end) as the biological endpoints (ASTM, 2000k). The other describes procedures and conditions for a static-renewal life-cycle test of up to a 3-month duration (if using *N. arenaceodentata*), which begins with juveniles, continues until they lay eggs, and determines the number of embryos laid per female as the biological endpoint (ASTM, 2000k).[68] Either of these test methods deserves consideration as an alternative to the 28-day test using the infaunal estuarine mollusc *M. balthica* that is recommended in Section 10.2.3. If applied, adaptations similar to those described in Section 10.2.3 for *M. balthica* would be necessary when testing for the pathogenic and/or toxic effects of a new microbial substance. It should be noted, when considering a test method using a species of polychaete worm, that these tests are performed at warm temperatures (i.e., 23 ± 1 °C if using *P. cornuta* according to EC, 2001a; or 17–20 °C if *Neanthes arenaceodentata* or other species of polychaetes if tested according to ASTM 2000k). Thus, tests with marine or estuarine polychaete worms, unlike a 28-day test using *M. balthica*, are only suitable for measuring the pathogenic and/or toxic effects of mesophilic (warm-water) microbial substances.

Environment Canada (EC, 2001a) has published a 14-day laboratory test using the polychaete worm *Polydora cornuta*, as a standard test method for measuring the chronic toxicity of samples of sediment to an infaunal estuarine or marine invertebrate species. This test might, in certain instances, serve as a suitable alternative to the 28-day test using the euryhaline infaunal mollusc *M. balthica* that is recommended in Section 10.2.3. Endpoints for this test are based on 14-day survival, growth inhibition, and the appearance of the tissues and organs of surviving worms upon necropsy at test end. Disadvantages of this test method, compared to that for infaunal invertebrates recommended in Section 10.2.3, include a shorter test duration (which might be inadequate for pathogenic effects to be realized), the restriction of the test to a warm temperature (i.e., 23 ± 1 °C) suitable only for investigating the adverse effects of mesophilic (not psychrophilic) micro-organisms, and the minimal and restrictive quantities of whole-body tissue available for investigating infectivity.

The USEPA (2001) has published a 28-day test for measuring the chronic toxicity of samples of contaminated sediment using the estuarine amphipod *Leptocheirus plumulosus*.[69] It is performed at 25 ± 2 °C, with a fixed salinity (± 2‰) that may range from 5 to 35‰. This test deserves consideration as an alternative to the 28-day test method for the euryhaline infaunal mollusc *M. balthica* recommended in Section 10.2.3, when studying the pathogenic and/or toxic effects of a

[68] The use of other species of polychaete worms for life-cycle tests is described in ASTM (2000k). Choices include the estuarine species *Capitella capitata* (in which instance the test duration is five weeks), the minute (adults, ≤5 mm in length) species *Ophryotrocha diadema* (for a four-week test), and the minute (adults, ≤1 mm in length) species *Dinophilus gyrociliatus* (for a 10-day test).

[69] *L. plumulosus* is a contaminant-sensitive infaunal amphipod found in subtidal portions of Atlantic Coast brackish estuaries. It can be readily cultured in the laboratory (USEPA, 2001).

mesophilic micro-organism on a species of infaunal estuarine or marine invertebrate. Unlike the test method with *M. balthica*, a test using *L. plumulosus* is restricted to warm-water conditions and thus can only be applied to mesophilic micro-organisms. The test method for *L. plumulosus* published by USEPA (2001) is a static-renewal one, whereby the seawater overlying sediment in each 1-L test chamber is renewed three times per week (on non-consecutive days) at which time supplemental food (commercial fish-food flakes) is provided. Endpoints are based on survival, growth, and reproduction. As with the test using *M. balthica* (Section 10.2.3), this test method could be applied to a new microbial substance by mixing the test material in the sediment and/or the seawater used for each renewal.

Section 10.1.3 includes a recommended methodology for a 14-day pathogenicity and/or toxicity test using the freshwater amphipod *Hyalella azteca*. Since this sensitive infaunal species can tolerate salinities of ≤15‰, this methodology may also be applied to measure the adverse effects on an estuarine species of infaunal invertebrate caused by a new microbial substance entering or within an estuarine environment, provided that the test salinity does not exceed 15‰.

Environment Canada has published two biological test methods for measuring the acute toxicity of samples of sediment to a selected species of estuarine or marine amphipod. Environment Canada (1992e) provides a static test that exposes replicate groups of one of the following species of infaunal amphipods, as juveniles or adults, to one or more samples of chemical-spiked sediment or field-collected sediment for 10 days: *Amphiporeia virginiana, Corophium volutator, Eohaustorius estuarius, Eohaustorius washingtonianus, Foxiphalus xiximeus, Leptocheirus pinguis,* or *Rhepoxynius abronius*. The biological endpoint for this test is percent mortality at Day 10; the use of sublethal endpoints including numbers of survivors emerged from the sediment and/or numbers of survivors not reburying in negative control sediment at test end is optional. Environment Canada (1998a) is a related *reference method* for determining the acute lethality of samples of sediment to marine or estuarine amphipods (*A. virginiana; E. estuarius; E. washingtonianus;* or *R. abronius*) in a 10-day static test. The biological test method described in EC (1992e), which can be applied as a single-concentration test or a multi-concentration one using a test material mixed in sediment, could be modified and applied to measure the acute pathogenicity and/or toxicity (lethal and/or sublethal) of new microbial substances using adaptations similar to those summarized in Table 7 for the recommended test method with the infaunal mollusc *M. balthica*. Adaptations could include the static renewal of the overlying water and the mixing of the MHC in this water as well as in the sediment if a single-concentration test, or mixing of the MHC and lower concentrations in either the overlying water or the sediment if a multi-concentration test (see Section 10.2.3). Although typically performed at a test temperature of 15 ± 2 °C (EC, 1992e; 1998a), an acute test with marine or estuarine amphipods could be conducted at warmer (for mesophilic micro-organisms) or cooler (for psychrophilic ones) temperatures provided that the amphipods were adjusted to and held at the intended test temperature during their acclimation period preceding the test.

Tests Using Aquatic Vertebrates

11.1 Freshwater Fish

Key Guidance

- *A 28-day test for effects on survival, growth, appearance, and behaviour of juvenile freshwater-acclimated fish, performed as an adaptation of OECD (2000b) and largely in keeping with USEPA (1996g), is recommended as the standard biological test to be used when measuring the pathogenic and/or toxic effects of new microbial substances on fish in fresh water. For a psychrophilic micro-organism, the use of rainbow trout held at a selected temperature ranging within 5–16 °C is recommended. For a mesophilic micro-organism, the use of bluegill sunfish held at a selected temperature ranging within 17–25 °C is recommended. This biological test method includes the necropsy of fish at test end, for overt changes in the gross appearance of tissues or organs and, as necessary, for histological effects.*

- *Alternative (standardized) test methods include a test involving early life stages of rainbow trout (including embryos and alevins) (EC, 1998b) that is available when testing for effects of a psychrophilic micro-organism on fish in fresh water, and a 7-day test for effects of a mesophilic micro-organism on the survival and growth of fathead minnows. These methods might be applied in certain instances. The 28-day test recommended herein for psychrophilic micro-organisms and rainbow trout could also be applied to a species of underyearling salmon in fresh water.*

11.1.1 Previous Tests with Micro-organisms or Microbial Products

Douville (2001) provides a review of research studies investigating the pathogenic and/or toxic effects of new microbial substances or specific micro-organisms on various species of freshwater fish, under controlled laboratory conditions. Rainbow trout (*Oncorhynchus mykiss*) and other species of salmonid fish acclimated to fresh water have frequently been used in these investigations. Tests with salmonids have demonstrated their susceptibility and sensitivity to bacterial, viral, fungal, and protozoan pathogens for various routes of exposure including direct contact through the water or orally by infected food or by gavage (Douville, 2001). In tests with salmonid or other species of fish, biological endpoints responsive to these pathogens include acute or chronic mortality, impaired growth, changes in hematological and biochemical indices of stress or disease, and gross or microscopic symptoms of pathology evident during *necropsy* of exposed fish.

As part of its Series 885 test guidelines for microbial pesticides, the USEPA published a 30-day test for effects on the survival and apparent wellbeing of freshwater fish (USEPA, 1996g). This biological test method recommended underyearling rainbow trout *swimup fry* or *fingerlings* as a preferred test species and life stage. Biological endpoints included 30-day survival as well as observations for infectivity and symptoms of pathogenicity.

Private US laboratories testing microbial agents or products for pathogenic and/or toxic effects on fish have developed in-house Standard Operating Procedures for performing a 30-day test using rainbow trout, that are in keeping with the test guidelines described in USEPA (1996g). Responses received from three laboratories applying these tests showed that a total of 28 tests had been performed with MPCAs or microbial products. Of these, 22 involved bacteria, three used viruses, and three tested fungi. Most (57%) were multi-concentration tests. Approximately one third of these tests demonstrated pathogenic and/or toxic effects on rainbow trout. Non-infectious controls were included in 57% of these tests, 32% used sterile filtrate controls, and only a few (7%) measured infectivity of fish during the test or at its end. None of the 28 tests included a positive microbial control or a positive chemical control.

Response to the questionnaire by the USEPA's Microbial Pesticide Branch in 2002 (see Section 8.8), indicated a data base for approximately 87, 30-day tests with rainbow trout or bluegill sunfish (*Lepomis macrochirus*) exposed to MPCAs or their EPs. Of these 87 microbial substances evaluated using fish in fresh water, ~60 were bacterial, six were viral, ~20 were fungal, and one was protozoan. In each instance, the adverse effects of these

MPCAs on fish in fresh water were measured using a single-concentration test. Few (<3%) included a sterile filtrate control or a non-infectious control, none included a positive microbial control or a positive chemical control, and <3% included testing for infectivity of exposed fish. Approximately half of these tests measured the concentration of micro-organisms in the test water to which fish were exposed.

11.1.2 Recommended Biological Test Method

The use of a 28-day (or longer) test for pathogenic and/or toxic effects on juvenile fish acclimated to fresh water is recommended, to measure the potential adverse ecological effects of a new microbial substance on a selected species and life stage of fish. The method recommended is an adaptation of the juvenile growth test for fish published by OECD (2000b), and includes endpoints related to the observed behaviour and appearance (including that for necropsies at test end) of fish in each treatment. This test method is consistent with USEPA (1996g), and with certain procedures and conditions described in ASTM's 28-day test method (ASTM, 2000i).

Juvenile (underyearling) rainbow trout *(O. mykiss)* are recommended as test organisms when testing for the effects of a psychrophilic micro-organism on a species of freshwater fish (USEPA, 1996g; OECD, 2000b). Using this species, a test temperature should be selected ranging within 5–16 °C. Juvenile bluegill sunfish are recommended as test organisms when testing for the effects of a mesophilic micro-organism on a species of freshwater fish (USEPA, 1996g; ASTM, 2000i). Using this species, a test temperature ranging from 17–25 °C should be chosen. In each instance, the test organisms should be adjusted to the test temperature gradually (≤3 °C/day), and then acclimated to that temperature for a minimum of two weeks before the 28-day test is started.

Table 8 outlines the procedures and conditions to be followed when performing a 28-day test with juvenile, freshwater-acclimated rainbow trout or bluegill sunfish. Additional guidance on performing laboratory tests with rainbow trout, which also applies here, is found in other Environment Canada publications. Environment Canada (1990b) details procedures and conditions for holding and acclimating underyearling rainbow trout in preparation for acute lethality tests, and provides guidance on the test organism, appropriate test facilities (including test apparatus), and universal test procedures (or those for testing chemicals) that pertain here as well. A *reference method* for determining the acute lethality of effluents to rainbow trout is also available (EC, 2000b). Environment Canada has also published two editions of biological test methods for performing toxicity tests using early life stages of rainbow trout (EC, 1992g; 1998b). The standard guidance for culturing and testing bluegill sunfish found in Appendix X6 of ASTM (2000m) should be consulted and followed when performing tests with this species.

As indicated in Table 8, this 28-day test requires the static renewal of each test concentration on non-consecutive days (e.g., on each Monday, Wednesday, and Friday), three times per week throughout the duration of the test. Acclimation and test conditions and procedures are defined in Table 8. Each test requires a negative control (see Section 4.1). As well, the use of a non-infectious control (Section 4.4) is strongly recommended, and use of a sterile filtrate control (Section 4.5) is optional. Measurements for infectivity, using selected organ(s) (e.g., liver, kidney, brain, muscle), tissue(s), body fluid(s) (e.g., blood or urine), or whole-body homogenate[70], are optional and dependent on the study objectives (see Section 5).

Test specifics when performing either a single-concentration test (see Section 3.3.1) or a multi-concentration test (Section 3.3.2) are summarized in Table 8. Two concurrent routes of exposure of test fish to the test material are accomplished by mixing the test material in both the water and the food when performing a single-concentration test. The effects of one or both of these means of exposure should be investigated separately for a multi-concentration test (see Section 3.2). Guidance provided in Sections 3.4.1 (for water) and 3.4.4 (for food) should be consulted and followed when mixing the test material in water or food.

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[70] If information on time-dependent infectivity is desired, additional replicates could be included in the study for this sole purpose (e.g., for sampling at weekly intervals and subsequent analyses of fish tissues and organs for the presence or absence of the test micro-organism).

Table 8 Recommended Methodology for a 28-Day Pathogenicity/Toxicity Test Using Juvenile Freshwater-Acclimated Fish

Universal		
Test method	—	adapted from OECD (2000b) "OECD Guideline for the Testing of Chemicals — Fish, Juvenile Growth Test"; with additional guidance from USEPA (1996g) and ASTM (2000i)
Test type	—	static renewal of each test concentration (including controls) throughout a 28-day (or longer) period; three times per week on non-consecutive days (e.g., Mon, Wed, Fri)
Test fish	—	juveniles in exponential growth phase; all from the same population; length of longest fish no more than twice that of shortest fish; individual wet weights should be within ±10% of mean wet weight, and must be within 25% of mean wet weight; recommend rainbow trout (*Oncorhynchus mykiss)* for cool-water tests with pyschrophilic microbial substances and bluegill sunfish (*Lepomis macrochirus*) for warm-water tests with mesophilic micro-organisms
Water temperature	—	acceptable range, 5–16 °C if rainbow trout or 17–25 °C if bluegill sunfish; held within ± 2 °C of mean temperature throughout test
Lighting	—	full spectrum, 100–500 lux at surface; normally 16 ± 1 h light : 8 ± 1 h dark; preferably, gradual transition from light to dark and dark to light
pH	—	no adjustment if pH of test concentration(s) within range of 6.5 to 8.5; a second (pH-adjusted) test is recommended if pH of any treatment outside that range
Dissolved oxygen	—	≥60% DO saturation in each test chamber throughout test; aerate gently in all test chambers only if necessary
Acclimation	—	minimum of two weeks, to test conditions (i.e., test temperature, test photoperiod, and the control/dilution water)
Control/dilution water	—	natural or artificial fresh water; DO 90 to 100% saturation when added to test chambers
Controls	—	each test must include a negative control; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Number fish/test chamber	—	10
Depth, loading density	—	≥15 cm in each test chamber; fish loading density ≤0.5 g/L
Feeding	—	at least once per day, throughout test, with commercial fish food pellets; daily ration, 4% of wet body weight; withhold feeding 24 h prior to weighing
Route(s) of exposure	—	mixed in both fresh water and food, if a single-concentration test; mixed in fresh water or food, if a multi-concentration test
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
MHC for food	—	100 times the expected microbial concentration in the aquatic environment (see Section 3.3.1.3)
Testing for infectivity	—	optional; based on measured concentrations of test micro-organism in tissue(s), organ(s), body fluids (e.g., blood or urine), or whole-body homogenate of fish from each treatment, during and/or at end of test

Measurements	—	temperature, pH, and DO at the beginning and end of each renewal, for at least one replicate of each treatment; wet weight of individual fish in each test chamber at start and end of test (and, optionally, on Day 14); analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of the test and at the beginning and end of at least one of the renewal cycles during each week of the test
Observations	—	daily throughout test, for fish survival, appearance, and behaviour in each test chamber; necropsy upon death of each fish during test and at test end, for gross external and internal appearance, histology of selected tissues and organs as warranted
Biological endpoints	—	survival, growth (mean ± SD wet weight of individual fish), appearance (including that from necropsy at test end), and behaviour in each test chamber and for each treatment, during and at test end
Test validity	—	invalid if <80% survival in negative control at test end

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	three per concentration (treatment), including each control treatment
Number of fish per treatment	—	30
Exposure routes	—	mixed in fresh water and food, at MHC for each
Statistical endpoints	—	percent survival in each test chamber and for each treatment, at test end; mean (± SD) individual wet weight of surviving fish in each test chamber and for each treatment, at test end; percentage of surviving fish in each test chamber and for each treatment, showing atypical behaviour and/or atypical appearance of organs and/or tissues at test end
Statistical comparisons	—	MHC versus negative control, for significant difference in percent survival, individual wet weight for surviving fish at test end, and percentage of surviving fish showing atypical behaviour and/or atypical appearance of organ(s) and/or tissue(s); if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	one per concentration (treatment), including each control treatment
Number of fish/treatment	—	10
Exposure route	—	mixed in fresh water or food (one exposure route per test)
Statistical endpoints	—	percent survival for each treatment, at test end; mean (± SD) individual wet weight of surviving fish in each treatment, at test end; percentage of fish in each treatment showing atypical behaviour and/or atypical appearance of organs and/or tissues at test end; data permitting — 28-day LC50, 28-day IC25 for individual wet weights of surviving fish, 28-day EC50 based on percentage of surviving fish in each treatment showing atypical appearance and/or atypical behaviour, NOEC/LOEC
Statistical comparisons	—	test concentrations versus negative control, for significant difference in percent survival, percent atypical appearance of organs and/or tissues, percent atypical behaviour, and mean wet weight of survivors at test end; if other control(s), same comparisons with negative control

Juvenile fish added to each test chamber must be in their exponential growth phase. Individuals chosen should be of similar length; i.e., the length of the longest fish should be no more than twice that of the shortest fish. Additionally, the individual wet weight of each fish selected should be within ± 10% of the mean wet weight of all fish used in the study, and must be within 25% of that weight (OECD, 2000b). The weight of each fish added to each test chamber is determined and recorded at the start of the test. A food ration equivalent to ~4% of the mean weight of all fish used in the study (OECD, 2000b) should be provided at that time. If the fish are re-weighed on Day 14, the ration should be recalculated (OECD, 2000b).[71] At the end of the test, the wet weight of each surviving fish is measured and recorded. Food should be withheld from fish for 24 hours before each weighing.

The biological endpoints for this test are based on 28-day survival and growth (i.e., wet weight of individual fish in each test chamber and for each treatment, at test end), and on the appearance and behaviour of fish in each test chamber during the test and at its end. Appendix E in EC (1990b) describes the type of observations on the appearance and behaviour of underyearling rainbow trout during the test, that are useful and apply here as part of the daily observations of each group of fish (whether rainbow trout or bluegill sunfish) in each test chamber. During these daily observations, any fish observed to have died must be removed, and a detailed examination made of the gross appearance of its external and (following dissection) internal tissues and organs (e.g., epithelium, eyes, gills, fins, peritoneal cavity, swim bladder, liver, spleen, fat deposits, kidney, and head kidney). On Day 28, each of the surviving fish exposed to each treatment (including the negative control treatment) must also be killed and weighed, followed by a necropsy performed in this manner. It is recommended that the investigator(s) be versed in accepted and standardized necropsy procedures for fish (e.g., Fisher and Myers, 2000); these should be followed when performing each necropsy. If signs of pathology are evident, the affected tissue(s) or organ(s) should be dissected, preserved, and stored for future microscopic examination as necessary and appropriate.

For a single-concentration test, the statistical endpoints to be determined for each test chamber and each treatment after completing the 28-day test include:

(1) percent survival of fish;

(2) percentage of fish showing atypical behaviour (e.g., surfacing, erratic swimming, loss of equilibrium);

(3) percentage of fish showing an abnormal appearance of one or more organs or tissues (e.g., lesions on the epithelium or elsewhere, opaque or hemorrhaged eye, discoloured or otherwise atypical liver); and

(4) mean (± SD) wet weight of each surviving fish.

Respective values for these endpoints determined for the maximum hazard concentration and any control treatments other than the negative control should be compared against those for the negative control, using an appropriate statistical test for pairwise comparisons such as *Student's t-test*. Environment Canada's guidance document on statistical methods to determine endpoints of toxicity tests (EC, 2004d) should be consulted when choosing and applying the appropriate statistics.

For a multi-concentration test, the statistical endpoints described in the preceding paragraph apply. Data permitting (see Section 3.3.2), the 28-day LC50 for the test material should be calculated together with its slope and 95% confidence limits. If data permit, a 28-day IC25 for growth inhibition should be determined, based on the mean (± SD) wet weight of fish in each treatment (including the negative control) at test end. If possible, a 28-day EC50 based on atypical appearance and/or atypical behaviour of individual fish exposed to each

[71] A useful alternative is to include additional replicates of the negative control in each test, which are used solely for the purpose of monitoring weight gains (e.g., at weekly intervals) and for readjusting ration levels based on these values. For each test chamber used in the study, the daily ration (~4% body weight) should also be readjusted in consideration of fish mortalities.

treatment should also be calculated together with its slope and 95% limits. Data permitting, the LOEC and NOEC for endpoint measurements (i.e., 28-day survival and growth, as well as data showing atypical appearance and/or atypical behaviour), should be calculated and reported as well. Guidance in EC (2004d) on appropriate software programs to use (and their application) when determining an LC50, IC25, or EC50, or calculating LOEC and NOEC, should be followed. Environment Canada (2004d) should also be consulted when choosing the appropriate statistics to be applied to the data derived from the study on fish appearance (including that at necropsy) and behaviour.

11.1.3 Other Methods or Procedures

Spacie (1992) reviewed methods and procedures for measuring the pathogenic and/or toxic effects of microbial agents on various species of freshwater, estuarine, or marine fish or crustaceans. Useful recommendations on test species (including rainbow trout), test designs, routes of exposure, test durations, and statistical analyses, are included in that report (Spacie, 1992).

The 28-day test recommended here (see Section 11.1.2) could be applied to salmon fry or fingerlings. For instance, coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), or Atlantic salmon (*Salmo salar*) could be used as the test species, while following the procedures and conditions outlined in Table 8 for rainbow trout. Such use (rather than rainbow trout) might be favoured in instances where the intended application of a new microbial substance raises particular concerns regarding its potential adverse effects on salmon fry in fresh water. However, the results for a 28-day test using rainbow trout will serve as a good surrogate in this respect, and for the most part should be adequate in terms of measuring potential adverse effects of a particular new microbial substance on all species of salmon or trout in fresh water.

Environment Canada's standardized biological test methods for measuring the toxicity of chemicals, effluents, or receiving waters to early life stages of rainbow trout (EC, 1998b) could be applied to a psychrophilic micro-organism. Each of the following is included as a test option:

(a) a 7-day test using embryos (*E* test), for which exposure to a test material starts upon fertilization and ends seven days thereafter;

(b) a test using embryos and *alevins*, which includes exposure from fertilization up until the alevins have absorbed their yolk and become swimup fry (*EA* test); and

(c) an embryo/alevin/fry (*E/A/F*) test, which tests for effects of exposure from fertilization up to and including 30 days of feeding by the surviving swimup fry.

One of these test options might be considered and applied when there are particular concerns about the adverse effects of a psychrophilic micro-organism on rainbow trout embryos or alevins. The E test is likely too brief in many instances when investigating for potential pathogenic effects, and the options involving a longer exposure period from fertilization onwards (i.e., EA or EAF options) would likely be more revealing.

A freshwater biological test method that measures the growth and survival of larval fathead minnows (*Pimephales promelas*) has been published by both EC (1992f) and USEPA (2002a). This test has been widely applied when measuring and monitoring the toxicity of effluents and receiving waters, as well as chemicals. This is a seven-day static-renewal test, which includes growth retardation as a sublethal endpoint. It has not been widely applied when testing micro-organisms for potential pathogenic and/or toxic effects, although it could prove useful in this respect. The short duration of exposure for this test method is limiting in terms of measuring pathogenic effects, compared to a 28-day test with bluegill sunfish (see Section 11.1.2). However, the use of this test method, which is performed at a temperature of 25 ± 1 °C (EC, 1992f), might be considered as an alternative to a 28-day test with bluegill sunfish if the new microbial substance is mesophilic and it is known or likely to exert its pathogenic and/or toxic effects on fish within a relatively short period of time (e.g., 7 days).

11.2 Estuarine or Marine Fish

Key Guidance

- *A 28-day test for effects on survival, growth, appearance, and behaviour of juvenile seawater-acclimated fish is recommended as the standard biological test to be used when measuring the pathogenic and/or toxic effects of new microbial substances on fish in estuarine or marine water. For a psychrophilic micro-organism, the use of seawater-acclimated rainbow trout, pink salmon, or chum salmon at a selected temperature ranging within 5–16 °C is recommended. For a mesophilic micro-organism, the use of the inland silverside, topsmelt, threespine stickleback, or sheepshead minnow at a selected temperature ranging within 17–25 °C is recommended. This biological test method is performed as an adaptation (for seawater-acclimated fish) of OECD (2000b) and incorporates guidance in USEPA (1996g) and ASTM (2000i). It includes the necropsy of fish at test end, for overt changes in the gross appearance of tissues or organs and, as necessary, for histological effects.*

- *Suitable test salinities are species dependent. The acceptable range of salinity for each species within which a test is performed is recommended as: rainbow trout, ≤14‰; pink or chum salmon, 10–32‰; inland silverside, topsmelt, or threespine stickleback, 5–32‰; and sheepshead minnow, 20–32‰. For a given test, variation should be within ± 2‰ of the mean salinity throughout the test.*

- *One of four short-term methods for measuring the chronic effects of aquatic contaminants on seawater-acclimated fish published by USEPA (2002b; 1995) might be suitable as an alternative to the 28-day test recommended here. Caution in such applications is advised, though, due to the shorter duration (typically, 7 days) of these tests.*

11.2.1 Previous Tests with Micro-organisms or Microbial Products

Numerous researchers have investigated the pathogenic and/or toxic effects of certain bacteria, viruses, or other micro-organisms on salmonid or other species of fish in seawater. Douville (2001) reviewed some of this literature, including information on the type of micro-organism tested (bacterium, virus, protozoan, fungus), the mode of exposure, and the nature of the effects observed. Many of these studies have involved various species of salmon held in seawater while being subjected to a particular pathogen. Laboratory studies reporting effects of a particular pathogen on the survival, behaviour, blood chemistry, physiology, or appearance (including effects on histology) of other species of fish (e.g., inland silversides, halibut, cod, English sole, sea bass, turbot) held in estuarine or full-strength seawater are widely available in the scientific literature. Published reports of pathogenic and/or toxic effects of new microbial substances on fish in seawater are less common, although some exist (Douville, 2001).

In responding to the 2002 questionnaire regarding tests for pathogenic and/or toxic effects performed with MPCAs or microbial products (see Section 8.8), USEPAs Microbial Pesticide Branch indicated that no data were available for tests using fish in seawater as the host organisms. Responses to this survey by three private US laboratories showed that such studies were restricted to six tests involving the exposure of seawater-acclimated sheepshead minnows (*Cyprinodon variegatus*) to bacterial pest control products. Each of these tests, which was performed at the MHC only, found no evidence of pathogenic or toxic effects. None of these six tests included a positive microbial control or a positive chemical control; and only one test included a sterile filtrate control as well as a non-infectious control. Each of the six tests with seawater-acclimated sheepshead minnow measured the concentration of bacteria to which fish were exposed during the test. Additionally, each of these tests included testing for infectivity of the exposed fish.

11.2.2 Recommended Biological Test Method

A 28-day test with a selected species of juvenile fish acclimated to brackish or full-strength seawater is recommended, when measuring the potential pathogenic and/or toxic effects of a new microbial substance on fish in seawater. The recommended test method is an adaptation of a testing guideline published by OECD (2000b), and incorporates additional guidance found in USEPA (1996g) and ASTM (2000i). The biological test method to be followed is much the same as that described in

Section 11.1.2 for juvenile freshwater-acclimated fish. Test endpoints are based on the survival and growth of juvenile fish during the 28-day test, as well as on their behaviour and appearance including that at necropsy. Table 9 provides a summary of the procedures and conditions to be applied when undertaking this test.

The use of seawater-acclimated rainbow trout (*O. mykiss*), chum salmon (*Oncorhynchus keta*), or pink salmon (*Oncorhynchus gorbuscha*) is recommended[72] when testing for the effects of a psychrophilic microbial substance on a species of juvenile fish in seawater, together with a test temperature ranging within 5–16 °C (see Table 9). Juvenile rainbow trout are a suitable choice if the test salinity is ≤14‰, whereas juvenile chum or pink salmon may be used at salinities ranging from 10 to 32‰. If using rainbow trout, fingerlings weighing 1–5 g should be adjusted gradually (e.g., ≤3‰ salinity increase per day) to ≤14‰ seawater, and tested at the same salinity following an acclimation period of two weeks or more.[73] If using chum or pink salmon, fry or fingerlings weighing between 0.5 and 5 g should be adjusted gradually to a selected salinity (± 2‰) ranging within 10–32‰, and tested at the same salinity once they have been acclimated to it for a minimum of two weeks. These species of salmon are considered to be suitable for 28-day tests using seawater-acclimated fish exposed to a psychrophilic micro-organism, since they naturally migrate to seawater shortly after they start feeding. The use of other species of salmon, including Atlantic salmon, coho salmon, chinook salmon, or sockeye salmon, is not recommended for this test method since each of these species normally spends one or more years in fresh water before smolting and migrating to the sea. It is desirable to use small-sized fish in the test to minimize water-volume requirements that must be met in keeping with the maximum fish-loading density in each test chamber (see Table 9). Underyearling rainbow trout, chum salmon, or pink salmon acclimated to an appropriate temperature and salinity within their range of tolerance (see Table 9) are therefore suitable and recommended.

One of the following four species is recommended, together with a selected test temperature ranging within 17–25 °C (see Table 9), when testing for the effects of a mesophilic micro-organism on a species of juvenile fish in seawater: the inland silverside (*Menidia beryllina*), the sheepshead minnow (*C. variegatus*), the topsmelt (*Atherinops affinis*), or the threespine stickleback (*Gasterosteus aculeatus*). The inland silverside, topsmelt, and threespine stickleback are all contaminant-sensitive species of fish that are amenable to laboratory conditions and can tolerate a wide range of both temperature and salinity. The USEPA (2002b) has published a survival-and-growth test for samples of effluent or receiving water using the inland silverside. This test is performed at a selected salinity ranging within 5–32‰ and at a temperature of 25 ± 1 °C. Similarly, the USEPA (1995) has published a survival-and-growth test for contaminants in seawater using the topsmelt, carried out at a selected salinity ranging within 5–34‰ at a temperature of 20 ± 1 °C. The threespine stickleback is also used in laboratory tests with aquatic contaminants (EC, 1990c), and is one of several fish species recommended by ASTM (2000i) for use in 28-day seawater tests.[74] If a 28-day test using the threespine

[72] The use of an appropriate species and life stage of seawater-acclimated salmonid fish is ideal for measuring the pathogenicity and/or toxicity of a psychrophilic micro-organism in a 28-day test, for several reasons. As with the rationale for using underyearling rainbow trout in freshwater tests, the use of this species or chum or pink salmon in seawater tests is supported by: (i) the recreational and/or commercial value of these species; (ii) their known susceptibility to a variety of microbial pathogens in seawater; (iii) their sensitivity to toxic chemicals in seawater as well as fresh water; (iv) the widespread use of these and other salmonid species in laboratory studies of environmental effects; (v) available information on the growth rates of these and other species of juvenile salmonid fish in seawater; (vi) a wealth of information on their gross and microscopic anatomy; and (vii) a good understanding of their husbandry and behaviour under laboratory conditions.

[73] Young rainbow trout fingerlings can readily adapt to up to 14‰ salinity; higher salinities might prove stressful or intolerable.

[74] This species has a number of desirable features including its small size, its ready adaptability to various salinities, its widespread distribution in Canadian coastal waters, its known biology and life history, and its sensitivity to aquatic contaminants.

Table 9 Recommended Methodology for a 28-Day Pathogenicity/Toxicity Test Using Juvenile Seawater-Acclimated Fish

Universal		
Test method	—	adapted from OECD (2000b) "Guideline for the Testing of Chemicals — Fish, Juvenile Growth Test", with additional guidance from USEPA (1995; 1996g; 2002b) and ASTM (2000i)
Test type	—	static renewal of each test concentration (including controls) throughout a 28-day (or longer) period; three times per week on non-consecutive days (e.g., Mon, Wed, Fri)
Test fish	—	juveniles in exponential growth phase; all from the same population; length of longest fish no more than twice that of shortest fish; individual wet weights should be within ± 10% of mean wet weight, and must be within 25% of mean wet weight; recommend seawater-acclimated rainbow trout (test salinity, ≤14‰), chum salmon, or pink salmon for cool-water tests with pyschrophilic microbial substances; recommend seawater-acclimated inland silverside, sheepshead minnow, topsmelt, or threespine stickleback for warm-water tests with mesophilic micro-organisms
Water temperature	—	acceptable range, 5–16 °C if salmonid fish, or 19–25 °C if inland silversides, sheepshead minnow, topsmelt, or threespine stickleback; held within ± 2 °C of mean temperature throughout test
Salinity	—	acceptable range:≤14‰ if rainbow trout; 10–32‰ if chum or pink salmon; 5–32‰ if inland silverside, topsmelt, or stickleback; 20–32‰ if sheepshead minnow ; held within ± 2‰ of mean salinity throughout the test
Lighting	—	full spectrum, 100–500 lux at surface; normally 16 ± 1 h light : 8 ± 1 h dark; preferably, gradual transition from light to dark and dark to light
pH	—	no adjustment if pH of test concentration(s) within range of 7.0–8.5; a second (pH-adjusted) test is recommended if pH of any treatment outside that range
Dissolved oxygen	—	≥60% DO saturation in each test chamber throughout test; aerate gently in all test chambers only if necessary
Acclimation	—	minimum of two weeks, to test conditions (i.e., test temperature, test photoperiod, and marine or estuarine control/dilution water at the test salinity)
Control/dilution water	—	natural or artificial seawater at the test salinity; DO 90 to 100% saturation when added to test chambers
Controls	—	each test must include a negative control; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Number of fish/test chamber	—	10
Depth, loading density	—	≥15 cm in each test chamber; fish loading density ≤0.5 g/L
Feeding	—	at least once per day, throughout test, with commercial fish food pellets; daily ration, 4% of wet body weight; withhold feeding 24 h before weighing
Route(s) of exposure	—	mixed in both seawater and food, if a single-concentration test; mixed in seawater or food, if a multi-concentration test
MHC for water	—	10^6 microbial units/mL water, or 1000 times the expected microbial concentration in the aqueous environment, whichever is greater and readily attainable (see Section 3.3.1.1)
MHC for food	—	100 times the expected microbial concentration in the aquatic environment (see Section 3.3.1.3)
Testing for infectivity	—	optional; based on measured concentrations of test micro-organism in tissue(s), organ(s), body fluids (e.g., blood or urine), or whole-body homogenate of fish from each treatment, during and/or at end of test

Measurements	—	temperature, pH, salinity, and DO at the beginning and end of each renewal, for at least one replicate of each treatment; wet weight of individual fish in each test chamber at start and end of test (and, optionally, on Day 14); analyses permitting, concentration of new microbial substance in each treatment including the control(s), at beginning and end of the test and at the beginning and end of at least one of the renewal cycles during each week of the test
Observations	—	daily throughout test, for fish survival, appearance, and behaviour in each test chamber; necropsy upon death of each fish during test and at test end, for gross external and internal appearance; histology of selected tissues and organs as warranted
Biological endpoints	—	survival, growth (mean ± SD wet weight of individual fish), appearance (including that from necropsy at test end), and behaviour in each test chamber and for each treatment, during and at test end
Test validity	—	invalid if <80% survival in negative control at test end

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	three per concentration (treatment), including each control treatment
Number of fish/treatment	—	30
Exposure routes	—	mixed in seawater and food, at MHC for each
Statistical endpoints	—	percent survival in each test chamber and for each treatment, at test end; mean (± SD) individual wet weight of surviving fish in each test chamber and for each treatment, at test end; percentage of surviving fish in each test chamber and for each treatment, showing atypical behaviour and/or atypical appearance of organs and/or tissues at test end
Statistical comparisons	—	MHC versus negative control, for significant difference in percent survival, individual wet weight for surviving fish at test end, and percentage of surviving fish showing atypical behaviour and/or atypical appearance of organ(s) and/or tissue(s); if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHC, plus negative control; additionally, non-infectious control strongly recommended; sterile filtrate control optional
Number of replicates	—	one per concentration (treatment), including each control treatment
Number of fish/treatment	—	10
Exposure route	—	mixed in seawater or food (one exposure route per test)
Statistical endpoints	—	percent survival for each treatment, at test end; mean (± SD) individual wet weight of surviving fish in each treatment, at test end; percentage of fish in each treatment showing atypical behaviour and/or atypical appearance of organs and/or tissues at test end, data permitting — 28-day LC50, 28-day IC25 for individual wet weights of surviving fish, 28-day EC50 based on percentage of surviving fish in each treatment showing atypical appearance and/or atypical behaviour, NOEC/LOEC
Statistical comparisons	—	test concentrations versus negative control, for significant difference in percent survival, percent atypical appearance of organs and/or tissues, percent atypical behaviour, and mean wet weight of survivors at test end; if other control(s), same comparisons with negative control

stickleback is considered, preliminary tests to assure an acceptable survival rate for control fish during an exposure period as long as 28 days or more might be warranted before undertaking the study.[75] For each of these three fish species, a selected test salinity ranging within 5–32‰ is suitable and recommended.

The sheepshead minnow is less euryhaline than the inland silverside or the threespine stickleback. The USEPA (2002b) has published a survival-and-growth test using the sheepshead minnow, which must be performed with samples of effluent or test water at a selected salinity ranging within 20–32‰.[76] Accordingly, this salinity range is recommended for a 28-day test conducted with mesophilic micro-organisms using the sheepshead minnow and warm-water conditions. The ASTM (2000i) lists the sheesphead minnow as a suitable fish species for performing a 28-day test with one or more contaminants in seawater; ASTM (2000m; see Appendix X8) provides useful guidance for culturing this species and for performing a 28-day test with its early life stages.

The appropriate salinity to use when applying this 28-day test to a selected species of juvenile, seawater-acclimated fish depends on the intended use of a particular new microbial substance, and whether it might enter estuarine or marine waters at strengths that could prove harmful. For environmental concerns regarding the entry of a new microbial substance to estuaries, a test salinity of 12 ± 2‰ is recommended. For concerns regarding the entry of a test material to marine waters, a test salinity of 28 ± 2‰ is recommended. As with temperature, the choice of test salinity influences the test species to be used in this test. Environment Canada has provided guidance for preparing seawater at differing salinities for use in toxicity tests with estuarine or marine organisms (EC, 2001b), which should be consulted and applied for the present purpose; USEPA (2002b) provides similar guidance.

11.2.3 Other Methods or Procedures

There are other standardized biological test methods that might be suitable (following modification, as necessary) for measuring the pathogenicity and/or toxicity of a new microbial substance. The USEPA has published short-term methods for measuring the chronic toxicity of effluents or receiving waters to estuarine or marine fish, that could be used to study the pathogenic and/or toxic effects of a new microbial substance under laboratory conditions. These include the following test methods:

- a seven-day static-renewal test measuring the survival and growth of larval inland silversides, at 25 ± 1 °C (Section 13; USEPA, 2002b).

- a seven-day static-renewal test measuring the survival and growth of larval sheepshead minnow, at 25 ± 1 °C (Section 11; USEPA, 2002b).

- a seven-day static-renewal test measuring the survival and growth of larval topsmelt, at 20 ± 1 °C (Section 11; USEPA, 1995).

- a static-renewal test using freshly fertilized sheepshead minnows, measuring embryo mortality and gross morphological deformities at 25 ± 1 °C for 9 days or until 4 days post-hatch, whichever is earlier (Section 12; USEPA, 2002b).

One of these test methods might be considered as a possible alternative to the recommended 28-day test using a selected species of juvenile seawater-acclimated fish described in Section 11.2.2, if the new microbial substance is *mesophilic*. The tests using the inland silverside or the topsmelt can be performed at any chosen salinity ranging from 5–32‰ (USEPA,2002b); whereas that using the sheepshead minnow should be conducted at a salinity of 20–32‰ (USEPA, 1995). In each instance, the test suspension or solutions are replaced daily. For the seven-day tests using larval inland silversides, sheepshead minnows, or topsmelts, fish are fed daily during the test; no feeding is required for the test using embryos of sheepshead minnows. Each of these test methods

[75] Threespine stickleback used in laboratory toxicity tests are typically captured from uncontaminated coastal waters and adapted to laboratory conditions (including a diet of commercial fish-food pellets) before testing begins.

[76] According to USEPA (2002b), the growth of sheepshead minnow might be impaired at salinities below 20‰.

was designed for measuring the chronic toxicity of samples of effluent or receiving waters. Their application as a test for measuring the pathogenic and/or toxic effects of a mesophilic new microbial substance assumes that the test material will have sufficient time to cause an infection and display resulting pathogenic effects on the test organism within the limited timespan (typically, seven days) of these tests. Without supportive data, no assurance is offered that this is the case; thus caution in the application of one or more of these short-term tests is advised.

No reports have been identified showing the use of any of these tests to measure the pathogenicity and/or toxicity of a particular micro-organism. In its Series 885 test guidelines for Tier-I testing of estuarine or marine animals, the USEPA (1996e) does not recommend any species of marine or estuarine fish, or an associated test method. The USEPA (1996h) refers to the use of either sheepshead minnow or inland silversides when testing for Tier-III effects of microbial pesticides on the life cycle of fish, without providing a definitive test protocol for either species. No guidance on species of fish to use when testing for effects of a new microbial substance in seawater is provided in EC and HC (2001). The PMRA (2001) mentions that sheepshead minnow or other estuarine or marine fish species should be used in cases where estuarine or marine waters are likely to be affected by a microbial pesticide.

Tests Using Terrestrial Plants

Key Guidance

- *A test for effects on emergence, growth, and appearance of a selected species of agricultural crop, market garden, or grassland plant is recommended as the standard biological test to be used when measuring the pathogenic and/or toxic effects of new microbial substances on terrestrial plants.*
- *This biological test method, which is adapted from EC (2004a), begins with seeds of a chosen plant species. Endpoints include percent emergence of seedlings during the test, measurements of the length and dry weight of shoots and roots, and the percentage of surviving plants at test end showing pathologies including lesions, necroses, and chlorosis.*
- *For a single-concentration test, the test material is mixed and applied in both the test soil in which plants are growing (at start of test, only), as well as in the test water sprayed on emerged plants and the soil surface at regular intervals throughout the test. For a multi-concentration test, the test* material *is mixed in either soil or water.*

12.1 Previous Tests with Micro-organisms or Microbial Products

Douville (2001) reviewed the scientific literature describing procedures for performing tests for pathogenic and/or toxic effects of micro-organisms or microbial products on selected species of terrestrial plants. Of the 148 publications reviewed, 68 dealt with plant exposures to bacteria, 39 used viruses, and 39 used fungi. Procedures used in these studies were diverse, and no standard biological test methods were evident. Routes of exposure varied widely from study to study, and included spraying, mixing in soil, dipping roots or tubers, soil surface inoculation, injecting various plant parts, wounding by diverse means, and using insects (e.g., aphids) as vectors. Exposure durations were typically brief (i.e., from a few seconds up to 24 h), whereas test durations ranged from a few hours up to two years post-exposure. Test endpoints were also diverse, but frequently were based on observations of *necrosis*, lesions, or *chlorosis* and in some instances included measurements of growth. For tests starting with seeds, observations and endpoints were based on germination as well as *seedling emergence*, growth, and survival (Douville, 2001).

One of the USEPA's Series 885 test guidelines for MPCAs or their products is a guideline for performing laboratory tests with non-target plants (USEPA, 1996c). This guidance document indicates that the species of host (test) plants used in tests should be chosen from those with the most important commercial value, and should include species of dicotyledons and monocotyledons. According to USEPA (1996c), the exposure route(s) should be that or those which are expected by the proposed use pattern, for the end-use product of the MPCA to be applied in the environment. Various means of exposure, including foliar spraying, seed treatment, root or soil application, direct application to water, or, in some cases, wounding of plants or the use of insect vectors, might be the most appropriate procedure. The duration of exposure should be sufficient to allow for the manifestation of a delayed pathogenic response (USEPA, 1996b), and might continue until normal harvest or death (USEPA, 1996c). Observations and endpoints are only described as those related to "obvious adverse effects".

According to EC and HC (2001), representative Canadian species of ecological or economic importance should be considered for tests involving terrestrial plants. These include important agricultural or forestry species generally found across Canada. Further specifics for tests involving terrestrial plants, however, are not found in this (EC and HC, 2001) guidance document.

Health Canada's Pest Management Regulatory Agency provides guidance similar to that found in USEPA (1996c), when testing MPCAs and EPs for effects on terrestrial plants (see Part 9.8 in PMRA, 2001). The PMRA (2001) has adopted the route(s) of exposure stated in USEPA (1996c). Test (host) plants should be selected from a list of 12 families of terrestrial plants with environmental or economic importance in Canada. For MPCAs and EPs

intended for forestry use, PMRA (2001) states that species of Pinaceae and Salicaceae should be selected for testing. Microbial pest control agents that do not resemble any known plant pathogen would require little if any testing using terrestrial plants, as is also the case for those microbial pesticides intended to control an aquatic plant (PMRA, 2001).

The response by USEPAs Microbial Pesticide Branch to an informal survey (see Section 8.8) identified records showing that ~20 MPCAs (or their EPs) had been evaluated for effects on terrestrial plants using the USEPA (1996c) test guideline. Of these 30-day (or longer) tests, ~10 involved bacterial MPCAs and ~10 involved fungal MPCAs. Each of these tests was performed at the MHC only. Signs of pathogenic and/or toxic effects were evident in 10% of the tests. None of the tests included a positive microbial control or a sterile filtrate control, whereas 10% of these tests included a positive chemical control and 10% used a non-infectious control. No measurements were made of the microbial concentrations to which terrestrial plants were exposed during these tests, and none of the tests included testing for infectivity.

12.2 Recommended Biological Test Method

A modified version of Environment Canada's biological test method for determining the toxicity of test substances to terrestrial plants (EC, 2004a) is recommended herein for measuring the pathogenicity and/or toxicity of a new microbial substance to this category of host (test) organisms. This adapted test method (summarized in Table 10) is largely in keeping with USEPA (1996c). One or more of the following species of agricultural crop, market garden, or grassland plants are recommended by Environment Canada as host (test) organisms:

- **the monocotyledons**
    - barley (*Hordeum vulgare* var. Chapais),
    - blue grama grass (*Bouteloua gracilis*),
    - northern wheatgrass (*Elymus lanceolatus*; formerly identified as *Agropyron dasystachyum*),
    - red fescue (*Festuca rubra* var. creeping), or
    - Durum wheat (*Triticum durum* var. Durum); and
- **the dicotyledons**
    - alfalfa (*Medicago sativa* var. greencrop),
    - carrot (*Daucus carota* var. Royal Chantenay),
    - cucumber (*Cucumis sativa* var. Marketmore76),
    - lettuce (*Lactuca sativa* var. Buttercrunch),
    - radish (*Raphanus sativus* var. Champion or Cherry Belle),
    - red clover (*Trifolium pratense* var. greencrop), or
    - tomato (*Lycopersicon escuentum* var. Heinz 1439).

The test duration is either 14 days or 21 days, depending on the species of plant chosen. The test begins with seeds, and measures adverse effects on developing plants in soil. Routes of exposure are in the test soil as well as via the test water used during the wetting of soil and the watering of plants, if a single-concentration test; and by one of these means only, if a multi-concentration test. Environment Canada (2004a) should be consulted for specifics on the performance of this biological test method other than those described and summarized here.

The choice of species of test plant to be used with this biological test method might be influenced by the intended manner in which the test material might enter or be dispersed in the terrestrial environment and the likelihood of it contacting species identical or similar to those recommended as host (test) organisms (see Table 10). Known information on the sensitivity of one or more species of terrestrial plants to the test material, and their similarity to the choice of test species presented herein, should be considered when choosing one or more species of plants to be used with this test method. Section 8.5 provides additional guidance when choosing the species of plant to be included in a test according to this biological test method.

For a single-concentration test, the maximum hazard concentration (MHC) applicable to soil and water should be applied to the test organisms using both of these exposure routes (Section 3.2). Guidance on mixing and administering the test material in water or soil is provided in Sections 3.4.1 and 3.4.3, respectively. The portion of the test material to be administered in the soil is that mixed in a sample of artificial or field-collected *clean* soil at the MHC for

Table 10 Recommended Methodology for a Pathogenicity/Toxicity Test Using Various Species of Terrestrial Plants

Universal	
Test method	— in keeping with EC (2004a) "Biological Test Method: Tests for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil"
Test type	— test for adverse effects on seeds and developing plants in soil
Test duration	— 14 days for barley, durum wheat, alfalfa, cucumber, lettuce, radish, red clover, or tomato; 21 days for carrot, blue grama grass, northern wheatgrass, or red fescue
Test species	— if a monocotyledon, choose barley (*Hordeum vulgare* var. Chapais), blue grama grass (*Bouteloua gracilis*), northern wheatgrass (*Elymus lanceolatus;* formerly identified as *Agropyron dasystachyum*), red fescue (*Festuca rubra* var. creeping), or Durum wheat (*Triticum durum* var. Durum); if a dicotyledon, choose alfalfa (*Medicago sativa* var. greencrop), carrot (*Daucus carota* var. Royal Chantenay), cucumber (*Cucumis sativa* var. Marketmore76), lettuce (*Lactuca sativa* var. Buttercrunch), radish (*Raphanus sativus* var. Champion or Cherry Belle), red clover (*Trifolium pratense* var. greencrop) or tomato (*Lycopersicon escuentum* var. Heinz 1439)
Soil	— natural or artificial (laboratory formulated) soil
Test chamber	— 1-L polystyrene cup, covered until plants reach top of container
Amount of soil/ test chamber	— 500 g (wet wt)
Moisture content, test soil	— if field-collected soil, hydrate and mix if and as necessary until a homogeneous crumbly texture is achieved; if artificial soil, hydrate to ~70% of water holding capacity
Number of seeds/ test chamber	— five for barley, northern wheatgrass, red fescue, Durum wheat, cucumber, lettuce, radish, red clover, or tomato; 10 for blue grama grass, alfalfa, or carrot
Temperature	— daily mean of 24 ± 2 °C during the day and 15 ± 2 °C during the night, throughout the test
Lighting	— full spectrum (fluorescent or equivalent); 16 ± 1 h light : 8 ± 1 h dark; intensity, ~400 µmol/(m2 · s)
Watering	— test water sprayed over plants and soil surface until saturation of soil, every 3 days when test chambers are covered and 1–2 times per day once covers are removed
Controls	— each test must include a negative control; sensitivity of test organisms to a reference toxicant (i.e., a positive chemical control) must be determined; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional but recommended
Route(s) of exposure	— mixed in both water and soil, if a single-concentration test; mixed in water or soil, if a multi-concentration test
MHC for water	— microbial concentration equivalent to (or no less than) the maximum concentration specified by the notifier for the final tank mix of a microbial product, when it is applied at the "maximum label rate" (see Section 3.3.1.4)
MHC for soil	— 10^6 microbial units/g soil (dry wt), or 1000 times the expected microbial concentration in soil within the terrestrial environment, whichever is greater and readily attainable (see Section 3.3.1.5)
Testing for infectivity	— optional; based on measured concentrations of new microbial substance in whole-organism homogenate of plants from each treatment, during and/or at end of test
Measurements	— temperature in test facility, daily max/min or continuously; moisture content (%) , conductivity, and pH, at the beginning and end of the test for at least one replicate of each

		treatment; light intensity in test facility at least once during test; analyses permitting, concentration of new microbial substance in the soil for each treatment including the control(s), at beginning and end of the test as a minimum
Observations	—	number of emerged seedlings on Day 7 and at test end, in each test chamber; shoot/root length and shoot/root dry weight at test end; number of surviving plants at test end showing an atypical appearance (e.g., chlorosis, lesions)
Biological endpoints	—	emergence of seedlings during test; length of shoot and longest root; dry weight of shoot and root; appearance of surviving plants at test end
Test validity	—	invalid if, for plants in negative control soil, any of the following occurs at test end: mean emergence rate: <60% if carrot; <70% if alfalfa, cucumber, blue grama grass, lettuce, red fescue, or tomato; <80% if barley, northern wheatgrass, Durum wheat, or red clover; <90% if radish mean root length: <40 mm if carrot or tomato; <70 mm if blue grama grass or red fescue; <100 mm if lettuce or red clover; <120 mm if radish, northern wheatgrass, alfalfa, or cucumber; <200 mm if barley or Durum wheat mean shoot length: <20 mm if lettuce or red clover; <40 mm if carrot; <50 mm if radish, alfalfa, cucumber, blue grama grass, or tomato; <80 mm if northern wheatgrass or red fescue; <130 mm if barley or Durum wheat

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	six per concentration (treatment), including each control treatment
Number of seeds/treatment	—	30, if barley, northern wheatgrass, red fescue, Durum wheat, cucumber, lettuce, radish, red clover, or tomato; 60, if blue grama grass, alfalfa, or carrot
Statistical endpoints	—	for each test chamber and each treatment: percent emergence on Day 7 and at test end; percentage of surviving (emerged) plants showing atypical appearance at test end; mean (± SD) length of shoots and longest roots at test end; mean (± SD) dry weight of shoots and roots at test end
Statistical comparisons	—	MHC versus negative control at test end, for significant difference in percent emergence, percentage of plants with atypical appearance, mean length of shoots and longest roots, and mean dry weight of shoots and roots; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of nine including MHC, plus negative control; additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	six per treatment, for each negative and other control; four replicates for each of lowest three concentrations, three replicates for each of middle three concentrations, and two replicates for each of three highest concentrations
Number of seeds/treatment	—	30 per negative or other control, if barley, northern wheatgrass, red fescue, Durum wheat, cucumber, lettuce, radish, red clover, or tomato; 60 per negative or other control, if blue grama grass, alfalfa, or carrot
Statistical endpoints	—	for each test chamber and each treatment: percent emergence on Day 7 and at test end, percentage of emerged plants showing atypical appearance at test end, mean (± SD) length of shoots and longest roots at test end, mean (± SD) dry weight of shoots and roots at test end; data permitting — 7-day and 21-day EC50 for emergence, 21-day EC50 for appearance; 21-day IC25 for length and weight of shoots and roots of surviving plants
Statistical comparisons	—	test concentrations versus negative control at test end, for significant difference in percent emergence, percentage of plants with atypical appearance, mean length of shoots and longest roots, and mean dry weight of shoots and roots; if other control(s), same comparisons with negative control

a new microbial substance in soil (see Section 3.3.1). This in-soil route of exposure is applied only once, just before setting up the test chambers and adding the seeds to the soil therein. The portion of the test material to be administered in the water, at the MHC for water (Section 3.3.1), is that mixed in a sample of deionized water used to hydrate the test soil before and during the test. This mixture of test material in water at the MHC is applied every three days during the first part of the test when each test chamber is covered, and is also applied once or twice per day after the chamber covers are removed (Table 10). For each of these applications, a fresh mixture of the test material in water should be prepared just before its use, and sprayed onto the surface of the soil in the test chamber as well as the foliage of any plants therein, until the surface of the soil is saturated with water (EC, 2004a). The negative control treatment and any other controls must be treated in the same manner, using an identical quantity of deionized water only (if the negative control) or an identical quantity and concentration (i.e., the MHC) of the modified test material as required for a sterile filtrate control or a non-infectious control.

For a multi-concentration test, the effects of one or both of these means of exposure should be investigated separately (see Section 3.2). A decision as to which exposure route to use in a multi-concentration test depends on the intended means of application of the new microbial substance in the environment (e.g., as an aerial spray or as a solid deposited onto or mixed in the soil) and the most likely route(s) of the substance contacting plants. If it is likely that a particular microbial substance might contact plants via water (e.g., aerial spray) as well as by uptake from soil, a separate multi-concentration test should be performed using each of these exposure routes. In the event that a multi-concentration test using one of these exposure routes causes no discernible adverse effect at the MHC and lower concentrations, and a preceding single-concentration test using two exposure routes did, the investigator(s) must perform a second multi-concentration test using the other exposure route applied simultaneously in that single-concentration test (Section 3.2). Each multi-concentration test includes the MHC for the route of exposure used in that test as well as a series of lower concentrations, plus a negative control and, depending on the study design, one or more other controls (see Sections 3.3.2 and 4). For this biological test method, a minimum of nine test concentrations plus the control(s) is required (EC, 2004a) to increase the likelihood of obtaining each of the multi-concentration statistical endpoints described (see Table 10).

As with other biological test methods to be applied as a series of tests to each test material, each test using one of the recommended species of terrestrial plants (see Table 10) must include a negative control. Consistent with EC (2000b), the use of a reference toxicant (i.e., a positive chemical control) as part of (or in conjunction with) this test is also required (see Section 4.2), in keeping with the specifications in EC (2004a). The use of a non-infectious control (Section 4.4) is strongly recommended, and the use of a sterile filtrate control (Section 4.5) is optional but recommended. Measurements for infectivity, using whole-organism homogenates of plants from each treatment upon completion of the test[77], are optional and dependent on the study objectives (see Section 5).

The biological and statistical endpoints using this test method, as determined for each test chamber and each treatment, are based on the following observations:

(i) numbers of emerged seedlings in each test chamber and treatment, on Day 7 and at test end;

(ii) lengths of shoots and longest roots;

(iii) dry weights of shoots and roots; and

(iv) number of surviving plants showing an atypical appearance (e.g., lesions or chlorosis).

[77] If desired and included in the experimental design, testing for infectivity could be measured and monitored as the test progresses as well as at its end. Such “during test” measurements would require the establishment of additional test chambers that are destructively sampled on the intended occasions (e.g., on Days 7 and 14) for determinations of microbial concentrations in whole-organism homogenates representing each treatment.

Table 10 lists the appropriate statistical endpoints for single-concentration and multi-concentration tests, together with the appropriate statistical comparisons for differing treatments.

For a single-concentration test, the statistical endpoints to be determined for each test chamber and treatment (including those for the control treatment or treatments) following completion of the test are:

(a) percent emergence on Days 7 and at test end;

(b) percentage of emerged plants showing an atypical appearance at test end;

(c) mean (± SD) length of shoots at test end;

(d) mean (± SD) length of longest roots at test end;

(e) mean (± SD) dry weight of shoots at test end; and

(f) mean (± SD) dry weight of roots at test end.

These calculated endpoints are used for statistical comparisons of data derived for the MHC versus the negative control, as well as for comparisons of data for the negative control versus any other controls included in a test (Table 10). For each data set, an appropriate statistical test for pairwise comparisons (such as *Student's t-test*) should be used. Environment Canada's guidance document on statistical methods to determine endpoints of toxicity tests (EC, 2004d) should be consulted when choosing and applying the appropriate statistics.

The statistical endpoints described in the preceding paragraph apply when performing a multi-concentration test. Data permitting (see Section 3.3.2), EC50s on Day 7 and at test end should be calculated for reduced emergence, together with their 95% confidence limits. An EC50 for appearance of surviving plants at test end should also be determined, data permitting, using the observations at that time of the percentage of plants in each treatment showing signs of pathogenic and/or toxic effects (e.g., lesions, necroses, or chlorosis). An attempt should be made to determine the IC25 (together with its 95% confidence limits) at test end (i.e., on Day 14 or 21, depending on species of host plant) for each of the following four measurements of plant growth:

- length of shoots at test end;
- length of longest roots at test end;
- dry weight of shoots at test end; and
- dry weight of roots at test end.

Environment Canada's statistical guidance document for environmental toxicity tests performed in the laboratory using single species of test organisms (EC, 2004d) should be consulted when choosing and applying the appropriate statistical tests.

12.3 Other Methods or Procedures

Campbell and Sands (1992) reviewed methods and procedures for testing the effects of microbial agents on terrestrial plants. They indicated that there was no standard methodology or set of accepted protocols for performing tests to determine the effects of MPCAs on terrestrial plants. Worthwhile recommendations regarding suitable test species, test conditions, routes of plant exposure, test endpoints, test statistics, and experimental designs, were included in their review (Campbell and Sands, 1992).

The ASTM has published a standard guide for conducting toxicity tests with terrestrial plants (ASTM, 2000n). This guide describes a number of differing test methods designed to determine the effects of test substances on plant growth and development including:

- short-term tests using physiological endpoints (i.e., *biomarkers*);

- short-term tests during the early stages of plant growth, with several endpoints related to survival, growth, and development;

- a test for determining the inhibitory effects of test substances on the growth and development of woody plant species under laboratory conditions; and

- life-cycle tests that emphasize reproductive success.

Useful guidance on test methods and procedures, that are similar to or differ from those recommended herein (see Section 12.2), is also provided.

Depending on their adaptability to tests for measuring the pathogenic and/or toxic effects of microbial substances, the life-cycle tests (or others, based on growth and development of plants) that are described in ASTM (2000n), may be used as an acceptable alternative to that method recommended in Section 12.2 when testing for the pathogenic and/or toxic effects of a new microbial substance on one or more species of terrestrial plants.

Depending on the intended use pattern of a new microbial substance, it might be appropriate to use a forest tree species as the host (test) organism. In keeping with PMRA (2001), a forestry species of Pinaceae or Salicaceae is proposed. Many of the procedural specifics and endpoints identified in Section 12.2 for the recommended biological test method using agricultural species could be applied when testing a forest tree species. Adaptations including the use of an extended test duration might be necessary, however. The duration of the test should be sufficient to allow for manifestation of effects on development, growth, and the appearance of pathologies (e.g., lesions or necroses) attributable to disease. At the present time, no recognized and standardized laboratory test methods demonstrated to be suitable for measuring the ecological effects of new microbial substances on one or more tree seedlings common to Canadian forested regions are available. Such methodology development and standardization is warranted.

Certain researchers have wounded plant parts (e.g., leaves, stems, or roots) by cutting or slashing, as a means of exposing test plants to a particular micro-organism or microbial product (Douville, 2001). Dhingra and Sinclair (1995) provide a useful text on basic plant pathology methods which includes various procedures for wounding and inoculating agricultural or forest species with micro-organisms, as well as subsequent tests for their disease resistance. Wounds are known to be a primary means of entry of many viruses and bacteria into plants. Accordingly, for a new microbial substance known or anticipated to be "wound opportunistic", any test method of sufficient duration[78] should include a procedure that wounds the emerged plants in each treatment. Wounding can be achieved by manually rubbing a mixture containing the *test water* and a fine abrasive such as 500 to 600 mesh Carborundum™ (silicon carbide) or Celite™ (diatomaceous earth), onto a selected leaf of each plant. Alternatively, the abrasive can be mixed in a portion of the *test water* (i.e., that containing either the MHC, a lower concentration if a multi-concentration test, or *clean* water alone for the negative control treatment) used to hydrate the plants during the test (see Section 12.2), followed by spraying the mixture onto the plants using a specially designed compressed air gun. In each instance, a mixture containing 50 to 100 mg abrasive per mL of test water is recommended. Either of these two procedures for wounding and inoculation need only be applied once to each treatment, when plants representing each treatment have reached the 5–7 leaf stage. The procedure and timing for wounding emerged plants must be identical for each treatment including the negative and other control groups.

For manual wounding and inoculation, a drop of the mixture containing abrasive and *test water* (i.e., clean water or that containing the MHC or other concentration of the new microbial substance) should be applied as a 100-µL inoculum onto one leaf of each plant in the test chamber. Primary or very small leaves should be avoided when selecting a leaf to be wounded and inoculated. Immediately following the application, the drop should be smeared on the leaf with a gloved finger or a sterile cotton swab, without applying pressure. Gloves or cotton swabs must be changed for each treatment. Two to three minutes following the wounding and inoculation of each plant in a test chamber, all plants

[78] The duration of the recommended biological test method for terrestrial plants presented in Section 12.2 is only 14 or 21 days (depending on plant species being tested). This test duration is insufficient to enable the plants to develop to a life stage suitable for wounding, and for any subsequent adverse effects caused by a microbial substance to become evident. A modified test method, which involves a sufficiently long test duration (e.g., 6 to 8 weeks, or longer), should include the wounding of plants within each treatment as part of the experimental design.

should be rinsed with the designated test water for that treatment.

For wounding and inoculation using a pressurized spray, the mixture of abrasive and test water for each treatment should be sprayed directly onto the leaves of each plant in a test chamber using a suitable compressed air gun designed for this purpose (e.g., Laidlaw, 1986; 1987). Research has shown greater rates of infection of plants by viruses, using this procedure rather than manual applications (Laidlaw, 1986; 1987). Spray applications, however, increase the risk of contaminating the applicator as well as the surrounding area (and cross-contamination of neighbouring treatments). Safety considerations include wearing a suitable protective mask while applying mixtures of abrasive and inoculum by spraying. All plants within a test chamber should be rinsed with the respective test water for that treatment after 2–3 minutes following the application of the mixture of abrasive and inoculum by spraying.

Tests Using Terrestrial Invertebrates

Key Guidance

- *No standard test for pathogenicity and/or toxicity using the honey bee or another species of plant-dwelling (i.e., pollinating or foliar) terrestrial insect can be recommended at this time, due to the absence of standardized methodologies. The standardization of a suitable test method using the honey bee or other beneficial species of insect is desirable. A test using a beneficial insect is particularly relevant for new microbial substances dispersed in the terrestrial environment by aerial spraying.*

- *An 8-week (56-day) test with the earthworm* Eisenia andrei *is recommended for measuring the pathogenic and/or toxic effects of new microbial substances on soil-dwelling terrestrial invertebrates. This test method is particularly relevant for substances that contact or are mixed in soil frequented by earthworms. It measures the effects of a 28-day exposure on the survival, behaviour, and appearance of adult earthworms, as well as their reproductive success and the survival, growth, behaviour, and appearance of their progeny during a subsequent 28-day period. Exposure routes are by mixing the test material in both soil and food if a single-concentration test, and by one of these routes only in a multi-concentration test.*

- *A 28-day test using the collembolan springtail* Folsomia candida *is recommended as an additional or alternative test method for measuring the adverse effects of new microbial substances on soil-dwelling invertebrates. This test method is relevant for substances that contact or are mixed in soil frequented by collembolan invertebrates. It measure the effects of a 28-day exposure on the survival of first-generation springtails, and on the reproduction rate of their progeny. The exposure route is by mixing the test material in the soil.*

13.1 Previous Tests with Micro-organisms or Microbial Products

As reviewed in Douville (2001), numerous research studies have investigated the pathogenic and/or toxic effects of micro-organisms or microbial products on single species of terrestrial invertebrates under controlled laboratory conditions. The species and life stages chosen have been diverse, as have the route(s) of exposure, test duration, and study-specific procedures and endpoints. Of 154 publications considered by Douville (2001), 71 involved bacteria, 36 involved viruses, and 24 involved fungi.

Host species treated with micro-organisms or microbial products have typically been insects of the Order Lepidoptera; and most have been pests for agricultural crops (Douville, 2001). Some of the studies reviewed by Douville (2001) used honey bees (*Apis mellifera*) or convergent lady beetles (*Hippodamia convergens*) as beneficial nontarget species. Others have used the domestic cricket (*Acheta domesticus*), the green lacewing (*Chrysoperla carnea*), or parasitic wasps (*Nasonia vitripennis* or *Aphidus colemani*) of the Order Hymenoptera. Besides these species of insects, a limited number of studies with microbial substances reviewed by Douville (2001) used earthworms (*Lumbricus terrestris* or *Eisenia fetida*) or nematodes as host species of soil-dwelling invertebrates.

Routes of exposure of host invertebrates to microbial substances have most commonly been by their incorporation in the diet (Douville, 2001). Other exposure routes have included the topical application of test materials on insects, dipping of various life stages of insects in the test material, injection, spraying, and, for terrestrial invertebrates that reside in or on the soil, mixing in soil. Administration via the food was considered to be an appropriate exposure route by many researchers, since bacteria, viruses, and protozoans typically get entry into terrestrial invertebrates through the gut (Douville, 2001).

For the studies reviewed, test duration varied depending on the host species and life stage chosen. Frequently, exposure (e.g., using topical application or by injection) to the test material was a single event at the start of the test. Observations proceeded over a few hours to several days, or until

a specific stage of development had been reached (Douville, 2001). Mortality was the most common test endpoint. On occasion, measurements of growth, development, adult longevity, reproduction, and symptoms of disease were used as biological endpoints.

As part of its Series 885 *"Microbial Pesticide Test Guidelines"*, the USEPA published guidelines for testing MPCAs for their end-use products (EPs) using nontarget insects as host organisms (USEPA, 1996i). Additionally, a separate guidance document for performing tests for pathogenic and/or toxic effects of MPCAs or EPs on honey bees was published as part of this series (USEPA, 1996j).

The USEPA (1996i) states that, for tests intended to measure the pathogenic and/or toxic effects of MPCAs or their EPs on nontarget insects, testing should be performed using three species of insects, chosen from at least two identified groups (i.e., parasitic dipterans, predaceous hemipterans, predaceous coleopterans, predaceous mites, predaceous neouropterans, and parasitic hymenopterans). The exposure route should be consistent with the most likely route of exposure under natural environmental conditions; exposure in the diet is a preferred route (USEPA, 1996i). Test duration should be 8 to $\geq$30 days, depending on the host species and life stage and on the type of micro-organism under investigation. The biological endpoints for the test should include survival (mortality) as well as symptoms of pathologies.

Only limited guidance is provided in USEPA (1996j) on procedures and conditions to use when performing a test with a microbial pest control agent using honey bees. Regarding age of test organisms, this is not specified other than a statement that the test should include larval bees if it might be expected that the MPCA will affect this life stage. The USEPA (1996j) states that honey bees must be exposed orally to the MPCA when it is anticipated that the MPCA might act by a dietary route of exposure or have particles of such a size that they could be carried back to the hive like pollen. It is also stated that testing in the hive might be necessary. Control and treated bees should be observed for at least 30 days after dosing (USEPA, 1996j). Biological endpoints for a test using honey bees are not indicated in USEPA (1996j).

Health Canada's Pest Management Regulatory Agency provides guidance similar to that in USEPA (1996c), when testing MPCAs and EPs for effects on terrestrial insects (see Part 9.5 in PMRA, 2001). Additionally, PMRA (2001) refers briefly in Part 9.6 to tests with non-arthropod invertebrates (e.g., earthworms). According to PMRA (2001), particular consideration should be given to insects established in the ecozone(s) of intended use, and to those "beneficial" species with broad environmental or economic importance such as honey bees. The PMRA (2001) supported the position of USEPA (1996i) that host organisms should be exposed in a manner that is consistent with the most likely route of exposure under natural environmental conditions, with diet used as a major exposure route. Additionally, PMRA (2001) recommended the use of life stages that are the most likely to be exposed or that are the most susceptible. Test endpoints should be based on mortality and signs of pathologies.

Private US testing laboratories have performed a considerable number of tests for effects of microbial substances (typically, MPCAs or their EPs) on terrestrial invertebrates, using the USEPA's Series 885 test guidelines for nontarget insects (USEPA, 1996i) or honey bees (USEPA, 1996j) as general guides. Standard Operating Procedures for performing tests using various species of insects including honey bees, parasitic wasps, convergent lady beetles, green lacewing larvae, and crickets have been developed and applied by these laboratories.

An informal survey of tests conducted by three US laboratories (see Section 8.8) identified a total of 22 tests performed using adult honey bees exposed to MPCAs or microbial products during laboratory tests of 2–30-day duration. Of these 22 tests, 20 involved bacteria and 2 involved fungi. Most (68%) of these tests with honey bees were performed as multi-concentration tests. Pathogenic and/or toxic effects were identified in 36% of the tests. Only 5% of these tests included a positive microbial control, 9% included a sterile filtrate control, 59% included a non-infectious control, and 18% included a positive chemical control. None of the tests measured the microbial concentration(s) to which the honey bees were exposed during the test, and none included testing for infectivity.

The response by the USEPA's Microbial Pesticide Branch to the 2002 survey (Section 8.8) indicated data available for ~87 tests with honey bees for effects of MPCAs or EPs, that were performed in accordance with the USEPA (1996j) test guideline. Of these tests, ~60 involved bacteria, ~6 were with viruses, ~20 involved fungi, and one involved a protozoan. Test duration was 15 days or until 20% mortality of negative controls occurred. All of these tests were performed as single-concentration tests. Pathogenic and/or toxic effects were found in only ~2% of the tests involving honey bees. Only ~2% of these tests included a sterile filtrate control, ~2% used a non-infectious control, ~3% included a positive chemical control, and 0% involved a positive microbial control. None of the tests involved testing for infectivity. Microbial concentrations to which the bees were exposed were measured in <23% of these tests.

Records reviewed by USEPA's Microbial Pesticide Branch in response to the 2002 questionnaire indicated that no data were available for tests performed with earthworms exposed to MPCAs or their EPs. Responses by the participating US private laboratories indicated that a total of 15 tests (12 with bacteria, one with a virus, and two with fungi) had been conducted using earthworms exposed to microbial substances. Most (87%) of these laboratory studies, which typically had a 14-day duration, involved multi-concentration tests. None of them showed any pathogenic or toxic effects. Both a sterile filtrate control and a non-infectious control were included in 7% of these tests; none included a positive microbial control whereas 40% involved a positive chemical control. None of these studies included testing for infectivity, and none measured the microbial concentration(s) to which the earthworms were exposed during the test.

13.2 Recommended Biological Test Methods

13.2.1 Honey Bees

No test for measuring the pathogenicity and/or toxicity of new microbial substances to the honey bee *(A. mellifera)* or another species of plant-dwelling (i.e., pollinating or foliar) insect can presently be identified as a recommended biological test method, as there is no standardized and proven biological test method suitable for this purpose. Although USEPA (1996j) has published guidelines for a laboratory test (≥30-day duration) intended to measure the pathogenic and/or toxic effects of MPCAs on the honey bee, these guidelines lack definitive guidance on the appropriate test procedures and conditions. The USEPA, however, is currently undertaking research studies with the intent of developing a standardized laboratory test method for measuring the ecological effects of microbial substances on honey bees (Vaituzis, Z. and R. Rose, personal communication, Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division, USEPA, Washington, DC, 2003). The honey bee is a preferred test organism due to the ecological and economic importance of this beneficial insect, its widespread distribution within Canada and elsewhere, its known sensitivity to infectious micro-organisms and toxic chemicals, and its recommended use for measuring the pathogenicity and/or toxicity of new microbial substances to plant-dwelling terrestrial invertebrates (USEPA, 1996j; EC and HC, 2001; PMRA, 2001). Accordingly, any efforts to develop a standardized biological test method suitable for measuring the pathogenic and/or toxic effects of microbial substances on the honey bee, under controlled laboratory conditions, are deserving. Investigators should consider the application of any such new test method(s) for measuring adverse effects of new microbial substances on honey bees, if and when they become available.

13.2.2 Earthworms

A 56-day (8-week) test using the earthworm *Eisenia andrei* has been chosen as a recommended biological test method for soil-dwelling terrestrial invertebrates. This test method, which has been standardized and published by Environment Canada (2004b) for various uses, was selected because of the ecological importance of earthworms in maintaining soil structure and nutrient cycling, their sensitivity to contaminants in soil, and the widespread (international) use of this species or its sibling *E. fetida* as a recommended test organism in laboratory tests designed to measure the effects of the long-term exposure of earthworms to contaminated soil on their survival, reproduction, development, and growth (USEPA, 1996hh; ISO, 1998; ASTM, 2000o; OECD, 2000c; EC, 2004b).

Table 11 summarizes the procedures and conditions that apply when performing a 56-day test for the pathogenic and/or toxic effects of a new microbial substance on the earthworm *E. andrei.* This biological test method represents an adaptation of the test developed by Environment Canada (2004b) for measuring the long-term effects of contaminated soil on the survival and reproduction of adult earthworms as well as those effects on the development, growth, and survival of their progeny. This test, which uses laboratory-cultured earthworms, starts by placing two adult worms in each of a series of 500-mL glass jars (10 replicate jars/treatment) containing a measured wet weight equivalent to ~350 mL of test or clean (negative control) soil.

The survival rate for the replicate groups of adult worms in each treatment is determined following a 28-day exposure. Each of these surviving worms is examined carefully for signs of atypical appearance and/or atypical behaviour, before they are discarded. The test is continued for an additional 28 days with their progeny. At the end of the 56-day test period, the number of live juvenile worms produced in each replicate and treatment is determined and the treatment means compared. Surviving worms are also examined carefully for signs of atypical appearance and/or atypical behaviour. Additionally, the dry weight of individual juvenile worms surviving at test end is determined for each replicate, and the treatment means compared.

Unless stated otherwise, the culturing, handling, and testing procedures and conditions to be followed when performing this test are those detailed in EC (2004b). In keeping with EC (2004b), each test requires a negative control. Use of a reference toxicant (i.e., a positive chemical control) as part of (or in conjunction with) this test is also required. Use of a non-infectious control is strongly recommended, and use of a sterile filtrate control is optional (see Section 4) but recommended. Testing for infectivity is optional but also recommended (see Section 5). Test specifics when performing either a single-concentration test (see Section 3.3.1) or a multi-concentration test (Section 3.3.2) are summarized in Table 11. Routes of exposure of earthworms to the test material are by mixing it in both the test soil and the test food when performing a single-concentration test; for a multi-concentration test, the effects of one or both of these means of exposure should be investigated separately (see Section 3.2). Guidance in Sections 3.4.3 and 3.4.4 for mixing and administering the test material in soil or food should be followed. The portion of the test material to be administered in the soil is that mixed in a sample of artificial or field-collected *clean* soil at the MHC for a new microbial substance in soil (see Section 3.3.1). This in-soil route of exposure is applied only once, just before setting up the test chambers and adding the earthworms to the soil therein. The portion of the test material to be administered in the food, at the MHC for food (Section 3.3.1), is that mixed in each batch of freshly-prepared food immediately before it is dispensed to the appropriate test chambers representing that treatment.

Food is added to each test chamber on Days 0, 14, 28, and 42 only (see Table 11 and EC, 2004b). The negative control treatment and any other controls must be treated in the same manner, using an identical quantity of uncontaminated food only (if the negative control) or an identical quantity and concentration (i.e., the MHC) of the modified test material as required for a sterile filtrate control or a non-infectious control (see Section 4).

For a multi-concentration test, the effects of one or both of the routes of exposure indicated in Table 11 (i.e., in the soil and/or food) should be investigated separately (see Section 3.2). A decision as to which exposure route(s) to use in a multi-concentration test depends on the intended means of application of the new microbial substance in the environment (e.g., as an aerial spray or as a solid deposited onto or mixed in the soil) and the most likely route(s) of the substance contacting soil and earthworms therein. Each multi-concentration test includes the MHC for the route of exposure used in that test as well as a series of lower concentrations, plus a negative control and, depending on the study design, one or more other controls (see Sections 3.3.2 and 4). For this biological test method, a minimum of seven test concentrations plus the control(s) are required, and 10 concentrations plus the control(s) should be considered (EC, 2004b) to increase the likelihood of obtaining each of the multi-concentration statistical endpoints described (see Table 11).

Table 11 Recommended Methodology for a 56-Day Pathogenicity/Toxicity Test Using Earthworms (*Eisenia andrei*)

Universal

Test method	—	in keeping with the test for effects of prolonged exposure of *Eisenia andrei* to contaminated soil on their survival, reproduction, and growth, described in EC (2004b) "Biological Test Methods: Tests for Toxicity of Contaminated Soil to Earthworms"
Test type	—	test for adverse effects on survival, reproduction, and growth of earthworms in soil
Test duration	—	56 days (8 weeks)
Test organisms	—	cultured *E. andrei*; sexually mature adults with clitellum; individual wet weight, 250–600 mg; choose worms as similar in wet weights as possible
Soil	—	natural or artificial (laboratory formulated) soil
Test chamber	—	500-mL glass jar; perforated aluminum foil secured with screw-top ring recommended as cover
Amount of soil/ test chamber	—	identical wet weight, equivalent to a volume of ~350 mL; ~200 g dry weight, if artificial soil
Moisture content, test soil	—	if field-collected soil, hydrate if and as necessary until a homogeneous crumbly texture is achieved; if artificial soil, hydrate to ~70% of water holding capacity
Number of worms/ test chamber	—	2
Temperature	—	daily mean of 20 ± 2 °C; instantaneous, 20 ± 3 °C
Lighting	—	incandescent or fluorescent; intensity, 400 to 800 lux at surface of soil in test chamber; fixed photoperiod (e.g., 16 L : 8 D or 12L : 12D)
Feeding	—	cooked oatmeal; 5 mL (= 1 teaspoonful) per test chamber each feeding; placed in a shallow depression in the center of the soil surface in each test chamber on Days 0, 14, 28, and 42 only
Controls	—	each test must include a negative control; sensitivity of test organisms to a reference toxicant (i.e., a positive chemical control) must be determined; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional but recommended
Route(s) of exposure	—	mixed in both soil and food, if a single-concentration test; mixed in soil or food, if a multi-concentration test
MHC for soil	—	10^6 microbial units/g soil (dry wt), or 1000 times the expected microbial concentration in soil within the terrestrial environment, whichever is greater and readily attainable (see Section 3.3.1.5)
MHC for food	—	microbial concentration equivalent to 100 times that in the maximum concentration of micro-organisms specified by the notifier for the final tank mix of a microbial product (see Section 3.3.1.7)
Testing for infectivity	—	optional; based on measured concentrations of new microbial substance in whole-organism homogenate of earthworms from each treatment, during and/or at end of test
Measurements	—	temperature in test facility, daily max/min or continuously; moisture content (%), conductivity, and pH, at the beginning and end of the test for at least one replicate of

		each treatment; analyses permitting, concentration of new microbial substance in the soil for each treatment including the control(s), at beginning and end of the test as a minimum
Observations	—	total number of live adult worms in each test chamber on Days 0 and 28; number of live juvenile worms in each test chamber on Day 56; obvious pathological symptoms (e.g., open wounds) or distinct behavioural abnormalities (e.g., lethargy) for worms in each test chamber
Biological endpoints	—	for each replicate (test chamber): total number of live survival of adult worms on Day 28; total dry weight and number of live juvenile worms on Day 56; number of surviving adult worms showing atypical appearance and/or behaviour on Day 28; number of surviving juvenile worms showing atypical appearance and/or behaviour on Day 56
Test validity	—	invalid if mean 28-day survival of adults in negative control soil <90%; invalid if mean reproduction rate for adults in negative control soil <3 live juveniles/adult; invalid if mean dry weight of individual live juveniles in negative control soil at test end <2.0 mg

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	10 per concentration (treatment), including each control treatment
Number of worms/ treatment	—	20
Statistical endpoints	—	for each treatment: percent survival of adults on Day 28; percentage of live adults showing atypical appearance and/or behaviour on Day 28; mean (± SD) number of live juveniles on Day 56; mean (± SD) dry weight of live juveniles on Day 56; percentage of live juveniles showing atypical appearance and/or behaviour on Day 56
Statistical comparisons	—	MHC versus negative control at test end, for significant difference in percent survival and atypical appearance and/or behaviour of adults on Day 28, and significant differences in number, atypical appearance/behaviour, and dry weight of live juveniles on Day 56; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of seven including MHC, plus negative control; additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	10 per concentration (treatment), including each control treatment
Number of worms/ treatment	—	20
Statistical endpoints	—	for each treatment: percent survival of adults on Day 28, percentage of live adults showing atypical appearance and/or behaviour on Day 28, mean (± SD) number of live juveniles on Day 56, mean (± SD) dry weight of live juveniles on Day 56, percentage of live juveniles showing atypical appearance and/or behaviour on Day 56, data permitting — 28-day LC50 for adults, 28-day EC50 for atypical appearance/behaviour of adults, 56-day EC50 for atypical appearance/behaviour of juveniles, 56-day IC25 for number of juveniles, 56-day IC25 for dry weight of juveniles
Statistical comparisons	—	test concentrations versus negative control at test end, for significant difference in percent survival and atypical appearance and/or behaviour of adults on Day 28, and significant differences in number, atypical appearance/behaviour, and dry weight of live juveniles on Day 56; if other control(s), same comparisons with negative control

The biological endpoints for this test method, as determined for each treatment, are based on the following observations:

(i) number of live adult worms in each test chamber on Day 28;

(ii) number of live adult worms in each test chamber showing signs of an atypical appearance and/or an atypical behaviour, on Day 28;

(iii) number of live juvenile worms in each test chamber on Day 56;

(iv) number of live juvenile worms in each test chamber showing signs of an atypical appearance and/or an atypical behaviour, on Day 56; and

(v) dry weights of surviving juveniles in each test chamber.

Table 11 lists the statistical endpoints for single-concentration and multi-concentration tests, together with the statistical comparisons for differing treatments.

For a single-concentration test, statistical endpoints to be determined for each treatment following completion of the 56-day test include:

(a) percent survival of adult earthworms on Day 28;

(b) percentage of adult earthworms showing an atypical appearance and/or an atypical behaviour on Day 28;

(c) number of live juveniles on Day 56;

(d) percentage of live juveniles showing an atypical appearance and/or an atypical behaviour on Day 56; and

(e) mean dry weight of surviving juvenile worms on Day 56.

Respective values for these endpoints determined for the maximum hazard concentration and any other control treatments should be compared against those for the negative control, using an appropriate statistical test for pairwise comparisons such as *Student's t-test*. Environment Canada's guidance document on statistical methods to determine endpoints of toxicity tests (EC, 2004d) should be consulted when choosing and applying the appropriate statistics.

The statistical endpoints described in the preceding paragraph apply when performing a multi-concentration test. Data permitting (see Section 3.3.2), the 28-day LC50 for adult earthworms exposed to the test material should be calculated together with its slope and 95% confidence limits. Twenty-eight-day and 56-day EC50s for adult earthworms and their progeny, respectively, should also be calculated (data permitting) together with their 95% confidence limits, based on numbers in each treatment showing signs of atypical appearance and/or atypical behaviour. Additionally, an attempt should be made to calculate the 56-day IC25 (together with its 95% confidence limits) for the number of juvenile worms generated in each treatment as well as that for the dry weight of these juveniles. Guidance in EC (2004d) on appropriate software programs to use (and their application) when determining each of these statistical endpoints should be consulted and followed.

13.2.3 Springtails

A 28-day test using the collembolan springtail *Folsomia candida*[79] has been chosen as a recommended biological test method for soil-dwelling terrestrial invertebrates. This test method, which has been standardized by Environment Canada (2004c) for various uses, was selected because of the key position of collembola in the soil food web as consumers of fungi, detritus, and nematodes and as an important prey organism, the abundance of *F. candida* in southern Canadian soils, the ability to culture this species in the laboratory, its relatively short life cycle, its sensitivity to contaminants in soil, and the widespread (international) use of this species in laboratory tests for effects of environmental contaminants on its survival and reproductive success (ISO, 1999b; Becker-van Slooten *et al.*, 2003; EC, 2004c).

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[79] *F. candida* is a wingless soil invertebrate belonging to the order Collembola (Arthorpoda, Hexapoda). It is a parthenogenic, unpigmented, and eyeless species of springtail invertebrate, that frequents forest and agricultural soils in temperate (southern) regions of Canada and elsewhere. This species reproduces quickly and readily in the laboratory. Adults reach a size of ~2 mm (Becker-van Slooten *et al.*, 2003).

Table 12 summarizes the procedures and conditions that apply when performing a 28-day test for the pathogenic and/or toxic effects of a new microbial substance on the springtail *F. candida*. This biological test method represents an adaptation of the test developed by Environment Canada (2004c) for measuring the long-term effects of contaminated soil on the survival and reproduction of soil-dwelling springtail invertebrates. This test, which uses laboratory-cultured *F. candida*, starts by placing ten juvenile (10–12-day old) springtails in each of a series of 100-mL glass beakers (five replicate beakers/treatment) containing a measured wet weight (30 g) of test or clean (negative control) soil. Following a 28-day exposure, the survival rate for the replicate groups of the first-generation (now adult) springtails in each each treatment is determined and compared, as is the number of their progeny (i.e., second-generation juveniles).

Unless stated otherwise, the culturing, handling, and testing procedures and conditions to be followed when performing this test are those detailed in EC (2004c). Each test requires a negative control. In keeping with EC (2004c), the use of a reference toxicant (i.e., a positive chemical control) as part of (or in conjunction with) this test is also required. Use of a non-infectious control is strongly recommended, and the use of a sterile filtrate control is optional (see Section 4) but recommended. Testing for infectivity is optional but also recommended (see Section 5). Test specifics when performing either a single-concentration test (see Section 3.3.1) or a multi-concentration test (Section 3.3.2) are summarized in Table 12. The route of exposure of springtails to the test material is by mixing it in the test soil. Guidance in Section 3.4.3 for mixing and administering the test material in soil should be followed. The test material administered in the soil is mixed in a sample of artificial or field-collected *clean* soil at the MHC for a new microbial substance in soil (see Section 3.3.1) and, if a multi-concentration test, at this and lower concentrations (see Section 3.3.2). This in-soil route of exposure is applied only once, just before setting up the test chambers and adding the springtails to the soil therein.

Food is added to each test chamber on Days 0 and 14 only (see Table 12 and EC, 2004c). The negative control treatment and any other controls must be treated in the same manner, using an identical quantity of uncontaminated food only (if the negative control) or an identical quantity and concentration (i.e., the MHC) of the modified test material as required for a sterile filtrate control or a non-infectious control (see Section 4).

Each multi-concentration test includes the MHC as well as a series of lower concentrations, plus a negative control and, depending on the study design, one or more other controls (see Sections 3.3.2 and 4). A minimum of seven test concentrations plus the control(s) is required for this biological test method, and as many as ten concentrations plus the control(s) should be considered (EC, 2004c) to increase the likelihood of obtaining each of the multi-concentration statistical endpoints described (see Table 12).

The biological endpoints for this test method, as determined for each treatment, are based on the following observations:

(i) number of live first-generation springtails (now adults) in each test chamber on Day 28; and

(ii) number of second-generation springtails (juveniles) in each test chamber on Day 28.

The latter is a measure of the rate of reproduction for the first-generation springtails, under the defined test conditions. Table 12 lists the appropriate statistical endpoints for single-concentration and multi-concentration tests, together with the appropriate statistical comparisons for differing treatments.

For a single-concentration test, statistical endpoints to be determined for each treatment following completion of the 28-day test include:

(a) percent survival of first-generation springtails on Day 28; and

(b) mean number of juvenile, second-generation springtails on Day 28.

Respective values for these endpoints determined for the maximum hazard concentration and any other control treatments should be compared against those for the negative control, using an appropriate

Table 12 Recommended Methodology for a 28-Day Pathogenicity/Toxicity Test Using Springtails (*Folsomia candida*)

Universal

Test method	— in keeping with the 28-day test for effects of contaminated soil on the survival and reproduction of collembolan springtails (*Folsomia candida*), described in EC (2004c) "Biological Test Method: Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil"
Test type	— test for adverse effects on survival and reproduction of *F. candida* in soil
Test duration	— 28 days
Test organisms	— cultured juveniles, 10–12-day old
Soil	— natural or artificial (laboratory formulated) soil
Test chamber	— glass beaker or jar, 100-mL capacity, diameter ~5 cm, covered with suitable lid
Amount of soil/ test chamber	— 30 g wet wt
Moisture content, test soil	— if field-collected soil, hydrate and mix if and as necessary until a homogeneous crumbly texture is achieved; if artificial soil, hydrate to ~70% of water holding capacity
Number of organisms/ test chamber	— 10
Temperature	— daily mean of 20 ± 2 °C
Lighting	— incandescent or fluorescent; intensity, 400 to 800 lux at surface of soil in test chamber; fixed photoperiod (e.g., 16 L : 8 D or 12L : 12D)
Feeding	— dry yeast, ~2 mg per test chamber each feeding; feed on Days 0 and 14 only
Controls	— each test must include a negative control; sensitivity of test organisms to a reference toxicant (i.e., a positive chemical control) must be determined; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional but recommended
Route of exposure	— mixed in soil
MHC for soil	— 10^6 microbial units/g soil (dry-wt basis), or 1000 times the expected microbial concentration in soil within the terrestrial environment, whichever is greater and readily attainable (see Section 3.3.1.5)
Testing for infectivity	— none (impractical due to limited biomass of test organisms)
Measurements	— temperature in test facility, daily max/min or continuously; moisture content (%), conductivity, and pH, at the beginning and end of the test for at least one replicate of each treatment; analyses permitting, concentration of new microbial substance in the soil for each treatment including the control(s), at beginning and end of the test as a minimum

Observations	—	total number of live adult (first generation) springtails in each test chamber on Days 0 and 28; number of live juvenile (second generation) springtails in each test chamber on Day 28
Biological endpoints	—	for each replicate (test chamber): total number of surviving adult (first generation) springtails on Day 28; total number of second-generation juvenile springtails on Day 28
Test validity	—	invalid if mean 28-day survival of adult (first generation) springtails in negative control soil <70%; also invalid if mean reproduction rate for adults in negative control soil <10 juveniles/adult

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHC and negative control); additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	five per concentration (treatment), including each control treatment
Number of springtails/ treatment	—	50
Statistical endpoints	—	for each treatment: percent survival of first-generation adults on Day 28; mean (± SD) number of second-generation juveniles on Day 28
Statistical comparisons	—	MHC versus negative control at test end, for significant difference in percent survival of first- generation adults on Day 28; significant differences in number of second-generation juveniles on Day 28; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of seven including MHC, plus negative control; additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	five per concentration (treatment), including each control treatment
Number of springtails/ treatment	—	50
Statistical endpoints	—	for each treatment: percent survival of adults on Day 28, mean (± SD) number of juveniles on Day 28, data permitting — 28-day LC50 for adults; 28-day IC25 for number of juveniles generated
Statistical comparisons	—	test concentrations versus negative control at test end, for significant difference in percent survival of adults on Day 28, and significant differences in number of juveniles on Day 28; if other control(s), same comparisons with negative control

statistical test for pairwise comparisons such as *Student's t-test*. Environment Canada's guidance document on statistical methods to determine endpoints of toxicity tests (EC, 2004d) should be consulted when choosing and applying the appropriate statistics.

The statistical endpoints described in the preceding paragraph apply when performing a multi-concentration test. Data permitting (see Section 3.3.2), the 28-day LC50 for the first-generation springtails exposed to the test material should be calculated together with its slope and 95% confidence limits. Additionally, an attempt should be made to calculate the 28-day IC25 (together with its 95% confidence limits) for the number of juvenile springtails generated in each treatment during the test period. Guidance in EC (2004d) on appropriate software programs to use (and their application) when determining each of these statistical endpoints should be consulted and followed.

13.3 Other Methods or Procedures

13.3.1 Tests for Plant-Dwelling Invertebrates

During 1989 to 1992, the USEPA's Environmental Research Laboratory in Corvallis, Oregon prepared a series of protocols for testing the effects of microbial pathogens on non-target, beneficial insects and mites. These reports include methods for testing the pathogenicity and *virulence* of fungi on the predatory mite *Metaseiulus occidentalis* (Sewall and Lighthart, 1989), the parasitic wasp *Trichogramma pretiosum* (Sewall and Lighthart, 1990), the green lacewing *Chrysoperla carnea* (Donegan and Lighthart, 1991), and the convergent lady beetle *Hippodamia convergens* (James and Lighthart, 1992). Tests for pathogenicity and virulence of bacteria were also developed for the convergent lady beetle (James and Lighthart, 1990). The procedure for exposure in these tests is generally by dipping the insects in different concentrations of test material (immersion application), followed by observations for periods of 6 to 10 days and (data permitting) calculation of LC50s. These guidelines are not cited in the Series 885 test guidelines for non-target insects by USEPA (1996i). Nonetheless, they provide useful guidance and approaches for tests intended to measure the effects of microbial pathogens on these species of insects and mites. One or more of these protocols should be considered for use when testing for the pathogenicity and/or toxicity of a new microbial substance on plant-dwelling invertebrates.

Fisher and Briggs (1992) reviewed a number of considerations when testing the effects of microbial pest control agents on nontarget insects in the laboratory. They considered a variety of subjects including choices of test (host) organisms, various routes of exposure, quantifying the test concentration, test duration, and endpoints. Research approaches and (non-standard) test methods for measuring effects of micro-organisms on honey bees and other non-target insects were described briefly (Fisher and Briggs, 1992). This publication might be helpful when choosing the test method(s) for terrestrial invertebrates to be applied to a particular new microbial substance (see Section 8.6).

The scientific literature includes reports on laboratory tests performed with groups of adult honey bees exposed to microbial pathogens and subsequently monitored for mortality rates over a test period of 12 to 14 days. Ball *et al.* (1994) acclimated groups of young adult honey bees (25/cage) to laboratory conditions in cages for 1 week, followed by their exposure to a mycopesticide administered by spray application. The negative control groups (six replicates of 25 bees/cage) showed a mortality rate of only 7% during a subsequent 12-day period of observation. Butt and Goettel (2000) used a similar experimental design to that of Ball *et al.* (1994). These researchers did not report the mortality rate for control groups, although a 14-day mortality of only 11% was found for groups of adult bees subjected to the lowest microbial concentration tested, with higher mortality rates (up to 87%) for higher concentrations (Butt and Goettel, 2000). The research studies by Ball *et al.* (1994) and Butt and Goettel (2000) indicate that acceptably low (e.g., $\leq$10%) mortality rates can be achieved for negative control groups of adult honey bees, in 12–14-day laboratory tests[80] using this experimental design. Although this approach has not been applied as a standardized biological test

[80] Newly emerged adult bees (24-48 h post-emergence) should be used.

method, it appears promising as an acceptable laboratory test for measuring the pathogenic and/or toxic effects of a new microbial substance on young adult honey bees. Investigators wishing to apply this test approach should conduct preliminary tests of 14 days duration to ensure that an acceptable control mortality rate of ≤10% can be achieved. Alternatives for dosing the test groups by feeding them a diet containing the new microbial substance (e.g., by mixing it in a 50% sucrose solution, as per OECD, 1998d) or by spray application (e.g., Ball *et al.* 1994; Butt and Goettel, 2000) should also be considered and experimented with in preliminary trials.

As part of its Series 850 *"Ecological Effects Test Guidelines"* published as "public drafts" by the USEPA's Office of Prevention, Pesticides, and Toxic Substances, two test guidelines (USEPA, 1996ii; USEPA, 1996jj) were prepared describing methods and procedures for measuring the toxicity of pesticides or other toxic substances to honey bees. Neither of these test guidelines is useful when testing for pathogenic effects, since the test durations are too short to enable this (i.e., 24 h, for an acute contact test for toxicity of test material to honey bees; or 48 h, for an acute test for toxic effects of foliage residues on honey bees). The exposure routes identified in these test guidelines are also less appropriate than, and inconsistent with, the oral route of dosing recommended by USEPA (1996j) for a ≥30-day test with honey bees exposed to a microbial pest control agent. Thus neither of the draft test guidelines by USEPA (1996ii; 1996jj) is acceptable for measuring the pathogenicity and/or toxicity of new microbial substances for notification purposes.

The OECD has published two standard guidelines for testing the effects of chemicals on honey bees. One of them (OECD, 1998d) involves the exposure of adult worker bees to a range of concentrations of the test material dispersed in a 50% sucrose solution for 3–4 h, followed by feeding with the sucrose solution alone for the duration of the test. The other test guideline (OECD, 1998e) involves the direct application of the test material (as droplets) to the thorax of the bee. In each instance, the test duration is 48–96 h (OECD, 1998d; 1998e), which is too brief for a test with honey bees intended to measure pathogenic as well as toxic effects. Accordingly, these test methods are unacceptable for measuring the pathogenic and/or toxic effects of a new microbial substance for notification purposes.

Hanley *et al.* (2003) have published a research article on a laboratory test used to demonstrate the potential adverse effects of dietary pollen contaminated with microbial or chemical pesticides, on larval or pupal life stages of honey bees. According to Vaituzis, Z., personal communication, Microbial Pesticides Branch, Biopesticides and Pollution Prevention division, USEPA, Washington, DC (2003), certain aspects of this test design, which included larval and pupal mortality rates as well as reduced pupal weights as biological endpoints, are being considered by the USEPA for possible use when developing a standardized protocol suitable for measuring the pathogenic and/or toxic effects of MPCAs on honey bees in keeping with USEPA (1996j).

13.3.2 Tests for Soil-Dwelling Invertebrates

The test method using earthworms recommended in Section 13.2 is similar to that by ISO (1998) and OECD (2000c). Each of these test methods has a test duration of eight weeks (56 days) and includes observations and measurements for effects on reproduction and the survival, growth, appearance, and behaviour of progeny as biological endpoints. The ASTM has published a standard guide for conducting soil toxicity tests with *Eisenia* sp. (ASTM, 2000o); this test method is more short term (i.e., 7 to 28 days) and does not measure effects on reproduction or the development and survival of exposed progeny. Given these limitations, the test method using earthworms recommended in Section 13.2 is preferred over the one described in ASTM (2000o), when measuring the effects of new microbial substances on earthworms.

Tests Using Terrestrial Vertebrates

14.1 Birds

Key Guidance

- *A 30-day test for effects on survival, appearance, and behaviour of young mallard ducks or northern bobwhite quail is recommended as the standard biological test to be used when measuring the pathogenic and/or toxic effects of new microbial substances on birds. The differing habitats and diets of these two species of birds should be considered when choosing the one to be used in a 30-day test.*
- *This test is performed in keeping with USEPA (1996k) if a new microbial substance is administered orally by gavage, or in keeping with USEPA (1996l) if the test material is administered by inhalation.*
- *This biological test method includes the necropsy of birds at test end, for overt changes in the appearance of tissues or organs and, as necessary, for histological effects. Measurements for infectivity are required at the end of the test, methodology permitting.*
- *Guidance for performing a test for chronic (including reproductive) effects of new microbial substances on birds is available (USEPA, 1996m) and, as necessary, could be applied in instances where a particular concern exists regarding the delayed or long-term effects on birds attributable to a particular new microbial substance.*

14.1.1 Previous Tests with Micro-organisms or Microbial Products

Douville (2001) reviewed published research studies on the exposure of various species of birds to micro-organisms (bacteria, viruses, fungi, and yeast). Host species included the mallard duck (*Anas platyrhynchos*), northern bobwhite quail (*Colinus virginianus*), Japanese quail, pigeons, house finch, red-winged blackbird, and mourning dove. Test procedures and conditions, including life stage exposed, exposure route, test duration, and biological endpoints measured, were diverse and study-specific. Exposure routes included orally (by gavage), by inhalation, and by injection (intravenous, subcutaneous, intraperitoneal, or intramuscular). Test durations ranged from 1 to 30 days. Biological endpoints included mortality, body weight, gross pathology, and histopathology of selected organs and tissues.

In 1996, the USEPA published three guidelines for testing for pathogenic and/or toxic effects of microbial pesticides on birds (USEPA, 1996k,l,m). These three test methods involve:

- a 30-day test whereby groups of young (14–24-day old) birds of the same species (the herbivorous bobwhite quail, and separately mallard ducks or another insectivorous species) receive oral (by gavage) doses of the test material daily for five consecutive days, followed by observations for mortality, weekly body weight, atypical behaviour, and gross and microscopic pathologies at test end (USEPA, 1996k);
- a 30-day test whereby groups of young (14–28-day old) birds of a single species (preferably bobwhite quail) receive one or more doses of the test material by inhalation (i.e., intranasal or intratracheal instillation) daily for five consecutive days, followed by observations for mortality, atypical behaviour, weekly body weight, and gross and microscopic pathologies at test end (USEPA, 1996l); and
- a chronic test for pathogenicity and reproductive effects, starting with one species (preferably the mallard duck or bobwhite quail) exposed to treated diets for $\geq$10 weeks before egg-laying and throughout the egg laying season, followed by observations (mortality, behaviour, gross necropsy, number of eggs laid, hatching success) for $\geq$14 days after last hatchling leaves the shell (USEPA, 1996m).

The guidelines for the registration of MPCAs or their EPs, published by Health Canada's Pest Management and Regulatory Agency (PMRA, 2001), include testing requirements for birds. Both an avian oral test and an avian pulmonary test is required, for all microbial pesticides. Testing should be conducted on one species, preferably the

mallard duck or bobwhite quail. The test should be started with young birds (~14 days old). The MPCA is to be administered to the gut by oral gavage or intubation, and to the respiratory tract by intranasal or intratracheal instillation.[81] Test procedures are in keeping with USEPA (1996k,l). Additional testing (e.g., as per USEPA, 1996m) might be required, depending on the findings of these and other (acute) tests (PMRA, 2001).

In response to a 2002 questionnaire (see Section 8.8), the USEPA's Microbial Pesticide Branch indicated that data were on hand for ~87 tests of 15–30-day duration involving bobwhite quail or mallard ducks exposed to MPCAs or their EPs according to USEPA (1996k,l). Of these tests, ~60 involved bacteria, ~6 involved viruses, ~20 involved fungi, and 1 involved a protozoan. None of these tests, which were performed as single-concentration (MHC) assays, detected any pathogenic or toxic effects attributable to the MPCA or EP studied. No positive microbial control, positive chemical control, or sterile filtrate control was included in any of these tests, whereas ~28% of the tests included a non-infectious control. Although none of these tests included testing for infectivity, some (<23%) measured the microbial concentration to which birds were exposed during the test.

Responses by three private US laboratories to the 2002 questionnaire (Section 8.8) indicated that a total of 41 tests (34 involving bacteria, five with viruses, and two with fungi) had been performed using birds (mallard ducks, bobwhite quail, or domestic chicken) exposed to MPCAs or microbial products. These 28–30-day tests were performed according to USEPA (1996k,l). Most (98%) were single-concentration tests. Pathogenic and/or toxic effects were identified for only 5% of the test materials. None of the tests included a positive microbial control or a positive chemical control, 7% included a sterile filtrate control, and 73% included a non-infectious control. Only 7% of these tests measured the microbial concentration(s) to which the birds were exposed during the test. A few tests (7%) also included testing for infectivity.

14.1.2 Recommended Biological Test Method

A 30-day test for pathogenic and/or toxic effects on groups of young (14–28-day old) mallard ducks *(A. platyrhynchos)* or northern bobwhite quail *(C. virginianus)* is recommended for measuring the potential adverse ecological effects of a new microbial substance on birds. This recommendation is consistent with the test guidelines published by USEPA (1996k,l) for 30-day tests to determine the pathogenic and/or toxic effects of MPCAs or their end-use products on these or other avian species, under controlled laboratory conditions. Environment Canada and Health Canada (2001) identified the mallard duck as a species of bird to be considered when selecting a suitable host organism to measure the effects of a new microbial substance on terrestrial vertebrates. The PMRA (2001) recommended that tests for effects of MPCAs on an avian species be performed using either mallard ducks or bobwhite quail. The use of one of these avian species in a 30-day test is supported by their known sensitivity to microbial pathogens and toxic chemicals, and the acceptance and application of a 30-day test for adverse effects of new microbial substances on mallard ducks or bobwhite quail by government regulators and testing laboratories alike (see Section 14.1.1). Section 8.7 provides the rationale for choosing one of these species of birds rather than (or in addition to) a small mammal, when meeting the requirements for testing for effects on terrestrial vertebrates.

Table 13 outlines the procedures and conditions to be followed when performing a 30-day test with young mallard ducks or bobwhite quail. The basic study design is consistent with test guidelines published by USEPA (1996k) when performing a 30-day test with birds using an oral route of exposure (i.e., by gavage; see Section 3.4.5) to the test material, as well as with those guidelines in USEPA (1996l) when conducting a 30-day test with birds using a respiratory (i.e., by inhalation; see Section 3.4.6) route of exposure. Guidance herein on procedural specifics when determining the degree of acceptable crowding in test cages, as well as suitable lighting, temperature, and humidity

[81] Exposure by injection (intravenous or intraperitoneal) may be used instead of pulmonary exposure if the test material is sufficiently free from exogenous protein and other contaminating substances that would otherwise confound the test. Although this route is environmentally unrealistic, it provides a maximum hazard challenge by bypassing the bird's primary defence mechanisms (PMRA, 2001).

Table 13 Recommended Methodology for a 30-Day Pathogenicity/Toxicity Test Using the Mallard Duck (*Anas platyrhynchos*) or the Northern Bobwhite Quail (*Colinus virginianus*)

Universal		
Test method	—	in keeping with USEPA (1996k) "Microbial Pesticide Test Guidelines — Avian Oral, Tier I" and USEPA (1996l)"Microbial Pesticide Test Guidelines — Avian Inhalation Test, Tier I"
Test type	—	test for adverse effects on survival, behaviour, and appearance (including gross and, as necessary and appropriate, microscopic examination of tissues and organs) of birds exposed orally and/or by inhalation (i.e., using intranasal or intratracheal instillation)
Dosing regime	—	daily, for initial five days of test only
Test duration	—	30 days
Test organisms	—	young birds, 14–28 days old at start of test; acclimated to test chambers and test conditions for ≥7 days before start of test
Test chamber	—	cages (e.g., commercial brooder pens) with a floor area of ≥800 cm^2/bird if ducks, or ≥600 cm^2/bird if quail
Number of birds/test chamber	—	10
Temperature	—	daily mean of 25 ± 5 °C
Relative humidity	—	45 to 70%
Lighting	—	incandescent or fluorescent; intensity in test chamber, 500–1000 lux; photoperiod, 14 ± 1 h light : 10 ± 1 h dark; gradual transition from light to dark and dark to light
Feeding	—	commercial bird food of a suitable size (e.g., starter mash), fed *ad libidum*
Controls	—	each test must include a negative control; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional but recommended
Route of exposure	—	orally (by gavage) or by inhalation, if either a single-concentration test or a multi-concentration test
MHD for oral route	—	expected concentration of micro-organisms in test material or aqueous suspension thereof (units/mL) × 5 mL/kg body weight × weight of bird (kg) (see Section 3.3.1.8)
MHD for inhalation route	—	expected concentration of micro-organisms in test material or aqueous suspension thereof (units/mL) × 0.2 mL/kg body weight × weight of bird (kg) (see Section 3.3.1.9)
Testing for infectivity	—	required at test end, analytical techniques permitting; based on measured concentrations of new microbial substance in selected organs (e.g., heart, brain, kidney, liver), tissues, or body fluids (e.g., blood or urine) of birds from each treatment, at end of test; optional testing for infectivity during the test
Measurements	—	temperature in test facility, daily max/min or continuously; relative humidity in test facility, at least once per week; individual body weights of birds in each test cage and

		treatment at start of test and weekly thereafter; analyses permitting, concentration of new microbial substance in the aqueous suspension administered to each treatment (including the controls) daily for five days
Observations	—	daily for survival, abnormal behaviour (e.g., lethargy, excessive aggression) and appearance (including external lesions) of birds in each test cage; necropsies performed on each bird dying during test as well as those surviving until the end of the test period; animals examined for lesions evident grossly, and selected tissues collected for processing and future microscopic examination where deemed necessary
Biological endpoints	—	survival, appearance (including that from necropsy at test end), and behaviour in each test chamber and for each treatment, during and at test end
Test validity	—	invalid if <90% survival in negative control at test end

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHD and negative control); additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	three per concentration (treatment), including each control treatment
Number of birds/treatment	—	30
Exposure route	—	orally (by gavage) or by inhalation (one exposure route per test)
Statistical endpoints	—	for each test chamber and each treatment: percent survival at test end; percentage of surviving birds showing atypical appearance (based on necropsy) and/or atypical behaviour at test end
Statistical comparisons	—	MHD versus negative control at test end, for significant difference in percent survival and percentage of surviving birds showing atypical behaviour and/or atypical appearance of organs or tissues on Day 30; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHD, plus negative control; additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	one per concentration (treatment), including each control treatment
Number of birds/treatment	—	10
Exposure route	—	orally (by gavage) or by inhalation (one exposure route per test)
Statistical endpoints	—	for each test chamber and each treatment: percent survival at test end; percentage of surviving birds showing atypical appearance (based on necropsy) and/or atypical behaviour at test end; data permitting — 30-day LD50, 30-day ED50 for atypical appearance and/or atypical behaviour, NOED/LOED
Statistical comparisons	—	test concentrations versus negative control at test end, for significant difference in percent survival and percentage of surviving birds showing atypical behaviour and/or atypical appearance of organs or tissues on Day 30; if other control(s), same comparisons with negative control

conditions, was derived in consideration of that described in the interim Ecological Effects Test Guidelines for performing oral toxicity tests with young mallard ducks or bobwhite quail published by the USEPA (1996mm).

Before beginning this test, groups of ten young (e.g., 7–21 days old) mallard ducks or bobwhite quail are held in each of a series of identical test cages for a minimum period of seven days, under conditions (including diet) identical to those to be applied during the definitive test. The test begins with birds that are 14–28 days old and as similar in age and weight as possible. Birds exposed to one (i.e., the MHD) or more (if a multi-concentration test) concentrations of the test material are subjected to daily applications of measured quantities of a new microbial substance throughout the first five days of the 30-day test period. The body weight of live birds is determined for each test chamber and treatment at the beginning of the test as well as weekly until test completion, and these weights are used to determine the treatment-specific doses (see Sections 3.3.1, 3.3.2, 3.4.5, and 3.4.6). Thereafter, exposure to the test material is discontinued, while continuing to observe (and feed) the surviving birds in each test cage daily for their behaviour and appearance (including external lesions) until the test is terminated on Day 30 (USEPA, 1996k,l). Birds in each test chamber are offered an excess ration of the same food[82] *ad libidum* during each day of the acclimation period and daily thereafter until test completion. Necropsies are performed on each bird that dies during the test, as well as on those that survive to test end. Each bird is examined closely for evidence of overt external or internal abnormalities or lesions. Organs or tissues that appear atypical should be dissected, preserved, and examined subsequently for histopathology.

Each test requires a negative control, and the use of a non-infectious control is strongly recommended. The use of a sterile filtrate control is optional (see Section 4), but recommended. Measurements for infectivity, using selected organ(s), tissue(s), or body fluid(s) (e.g., blood or urine) from birds exposed to each treatment, are required upon completion of the test, analytical techniques permitting (see Section 5). Additional measurements for infectivity and clearance, as the test progresses (e.g., at weekly intervals), are optional but worthwhile to monitor the initiation and progression of any infectivity during the test period. Test specifics when performing either a single-concentration test (see Section 3.3.1) or a multi-concentration test (Section 3.3.2) are summarized in Table 13. Guidance in Sections 3.3.1, 3.3.2, 3.4.5, and 3.4.6 should be consulted and followed when mixing and administering the test material orally or by inhalation. The daily dose of new microbial substance administered to birds in each treatment during the first five days of the test should be quantified, if possible (see Section 3.5).

For a single-concentration test, the test birds are subjected to the MHD of the test material by only one route of exposure (i.e., either orally by gavage, or by inhalation). For a multi-concentration test, the effects of one or both of these means of exposure should also be investigated separately (see Section 3.2). A decision as to which exposure route(s) to use in a multi-concentration test depends on the intended means of application of the new microbial substance in the environment (e.g., as an aerial spray or as a solid deposited onto water or soil) and on the most likely route(s) of the substance contacting birds. Each multi-concentration test includes the MHD for the route of exposure used in that test as well as a series of lower concentrations, plus a negative control and, depending on the study design, one or more other controls (see Section 4). For this biological test method, a minimum of five test concentrations plus the control(s) is required for a multi-concentration test.

The biological endpoints for this test are based on 30-day survival, and on the behaviour and appearance (including findings at necropsy and for any subsequent histopathologies) of the group(s) of birds subjected to each treatment. Table 13 summarizes the type of observations on the behaviour and appearance of birds in each treatment

[82] Dietary requirements vary according to the species and age of the test birds. Any unmedicated commercial diet that meets the minimum nutritional standards of the test species is acceptable (ASTM, 2000p). The same uncontaminated commercial bird food (starter mash or larger) should be fed to all animals in each treatment, based on the average bird size (all treatments) and the food manufacturer's recommendations.

(including the negative and other controls), monitored daily throughout the 30-day test period. During these daily observations, any bird observed to have died must be removed, and a detailed examination made of the gross appearance of its external and (following dissection) internal tissues and organs (e.g., plumage, epithelium, eyes, bill, feet, oral cavity, esophagus, stomach, crop, intestines, trachea, lungs, brain, liver, gall bladder, heart, spleen, kidney). On Day 30, each of the surviving birds exposed to each treatment (including the negative control treatment) must also be killed and necropsied in this manner. If lesions or abnormalities are evident at necropsy, representative samples should be collected and fixed in formalin (or another suitable preservative) for future histopathological examination as necessary and warranted. The useful and concise guide on performing avian necropsies by Butcher and Miles (1996), which is available on the Internet (http://edis.ifas.ufl.edu/BODY_VM009), is recommended when preparing for and undertaking these postmortem examinations. The selection of tissues for histopathology might vary depending on the nature of the test material being investigated and past records of its pathogenic and/or toxic effects (see Section 3.1). Representative specimens from organs such as liver, kidney, brain, lung, gastrointestinal tract, spleen, and reproductive tract are recommended. Even in the absence of lesions at necropsy, selected tissues (including brain) should be collected for possible histopathological examination if there was clinical evidence of disease (e.g., poor weight gains, or neurological signs).

Under certain circumstances, if there is evidence at necropsy or clinical evidence of possible adverse effects on the *hematopoietic* system, the collection of a blood sample at necropsy for analysis of variables such as the differential white blood cell (WBC) count, packed cell volume, and plasma protein might prove worthwhile in identifying effects on the immune system. Collection of <1 mL of blood in a microhematocrit tube is sufficient for both of the latter two analyses (Feldman *et al.*, 2000), and a small drop of blood smeared on a glass slide is adequate for the differential WBC count (i.e., percent small lymphocytes, percent large lymphocytes, percent neutrophils, percent eosinophils, percent monocytes, percent macrophages).

For a single-concentration test, statistical endpoints to be determined following completion of the 30-day test include:

(1) percent survival of birds in each test chamber and for each treatment;

(2) percentage of birds in each test chamber and for each treatment, showing atypical behaviour (e.g., increased aggression, lethargy, toe picking); and

(3) percentage of birds in each test chamber and for each treatment, showing an abnormal appearance of one or more organs or tissues (e.g., external or internal lesions, opaque or hemorrhaged eye, swollen or discoloured liver).

Respective values for these endpoints determined for the maximum hazard dose and any other control treatments should be compared against those for the negative control, using an appropriate statistical test for pairwise comparisons such as *Student's t-test.* Environment Canada's guidance document on statistical methods to determine endpoints of toxicity tests (EC, 2004d) should be consulted when choosing and applying the appropriate statistics.

The statistical endpoints described in the preceding paragraph apply when performing a multi-concentration test. Data permitting (see Section 3.3.2), the 30-day LD50 for the test material should be calculated together with its slope and 95% confidence limits. A 30-day ED50 based on atypical appearance and/or atypical behaviour of individual birds exposed to each treatment should also be calculated together with its slope and 95% limits, if possible. Data permitting, the LOED and NOED for effects on 30-day survival as well as data showing atypical appearance and/or atypical behaviour should be calculated and reported as well. Guidance in EC (2004d) on appropriate software programs to use (and their application) when determining an LD50 or ED50 as well as NOED/LOED should be followed. Environment Canada (2004d) should also be consulted when choosing the appropriate statistics to be applied to the data derived from the study on bird appearance (including that at necropsy) and behaviour.

14.1.3 Other Methods or Procedures

Kerwin (1992) reviewed the test methods and procedures available at that time for testing the effects of chemicals and micro-organisms on birds, under controlled laboratory conditions. Useful information on historic perspectives of avian microbial safety tests is included along with some useful guidance on choice of test species, routes of exposure, and statistical considerations.

In addition to the guidelines published by USEPA (1996k,l) for measuring pathogenic and/or toxic effects of microbial pesticides on birds using 30-day tests, USEPA (1996m) gives test guidelines for performing a chronic test for measuring effects of MPCAs on the long-term survival of adult birds (preferably mallard ducks or bobwhite quail) and on their reproductive success and subsequent survival and development of offspring. This test (see Section 14.1.1), using mallard ducks or bobwhite quail as host organisms, should be considered if definitive information is required on the chronic effects of prolonged exposure of birds to low levels of a new microbial substance in the diet. USEPA (1996ll) should be consulted for additional test specifics that apply here. If this test for chronic effects of a new microbial substance on mallard ducks or bobwhite quail is undertaken, much of the recommended procedures and conditions provided in Table 13 (e.g., those on testing for infectivity, measurements, observations, biological endpoints, statistical endpoints, statistical comparisons) apply and should be incorporated in the experimental design. Additional statistics for this test, using quantitative (IDp) endpoints for the reproductive data, would pertain, data permitting, for a multi-concentration test for chronic effects on birds.

Some micro-organisms are known to be *carcinogenic* (or contribute to *carcinogenicity*). Randomly selected test and control animals may be held for a longer observation period (e.g., for one year or longer) if a new microbial substance under investigation is considered to have carcinogenic potential. These animals would then be killed and a complete necropsy performed. Organs would be examined carefully for gross evidence of abnormalities, and any suspect tissues would be collected for microscopic examination. For substances suspected to have the potential to produce adverse effects on the reproductive system, randomly selected adult animals (both test and controls) might be mated to evaluate fertility and conception rates. The offspring resulting from such test matings would be necropsied at birth and examined for abnormalities.

Besides the draft guidelines for an avian reproduction test prepared by USEPA (1996ll), two other “Series 850" interim Ecological Effects Test Guidelines for measuring the effects of test materials on birds were released by the USEPA (USEPA, 1996kk,mm). An avian dietary toxicity test, whereby young (5–10-day old) mallard ducks or bobwhite quails are exposed to a diet containing the test material for five days, followed by a post-exposure period that is typically three days, is described in USEPA (1996kk). The ASTM (2000p) describes a similar test method for these and other species of birds (i.e., exposure to one or more concentrations of a test material in the diet, followed by a post-exposure period of $\geq$3 days). A second acute test, whereby one of these species of birds is exposed as young adults to a single oral dose of the test material administered by gavage followed by observations for $\geq$14 days, is described in USEPA (1996mm). None of these test methods should be considered as an acceptable substitute for the 30-day test recommended in Section 14.1.2, due to their shorter test durations and, in the case of USEPA (1996mm), the use of a single oral dose.

14.2 Small Mammals

Key Guidance

- *A $\geq$21-day test, for effects on survival, appearance, and behaviour of young adult rats or mice, is recommended as the standard biological test to be used when measuring the pathogenic and/or toxic effects of new microbial substances on small mammals.*
- *This test is performed in keeping with USEPA (1996o) if a new microbial substance is administered orally by gavage, or in keeping with USEPA (1996p) if the test material is administered by inhalation.*

- *This biological test method includes the necropsy of rodents at test end, for overt changes in the appearance of tissues or organs and, as necessary and appropriate, for histological effects. Measurements for infectivity are required at the end of the test, methodology permitting.*
- *Guidance for performing a test for subchronic (i.e., 90-day) or chronic (i.e., 1-year) effects of new microbial substances on rodents is available (USEPA, 1996t,u) and, as necessary, could be applied in instances where a specific concern exists regarding the delayed or long-term effects on small mammals attributable to a particular new microbial substance.*
- *The ≥21-day test with rats or mice could also be applied to field-collected specimens of small wild mammals, following their application to laboratory conditions. In most instances, however, a ≥21-day test using domestic rodents (i.e., laboratory-reared rats or mice) will suffice when studying the potential adverse effects of new microbial substances on small mammals.*

14.2.1 Previous Tests with Micro-organisms or Microbial Products

Douville's review of test methods and procedures for assessing the pathogenicity and/or toxicity of micro-organisms to aquatic and terrestrial plants or animals considered past studies and existing methods for testing effects on wild mammals (Douville, 2001). This review identified only two studies involving the exposure of wild mammals (mink, flying squirrel, short-tailed shrew, white-footed mouse, opossum, raccoon) to viral or fungal pathogens under controlled conditions. The findings of tests with domestic mammals (e.g., mice, rats, rabbits) exposed in the laboratory to new microbial substances were not considered (Douville, 2001).

The USEPA (1996b) states, in this background document describing various guidelines for testing the pathogenic and/or toxic effects of MPCAs on various nontarget species of host organisms, that its Series 885 test guidelines for evaluating the hazard of MPCAs to humans (i.e., USEPA, 1996n-u), using domestic small mammals as host (test) organisms, are normally adequate to indicate hazard to wild mammals. An exception is when there is considerable variation in the sensitivity of different mammalian species to the effects of an MPCA or evidence that wild mammals will be heavily exposed to the MPCA, in which case wild mammal testing might be appropriate according to the guidance in USEPA (1996v). The route of exposure to the MPCA, for tests involving wild mammals, is by gavage (acute oral dose) or by inhalation (intranasal instillation). The procedure for dosing should reflect the most likely exposure route (USEPA, 1996v).

The USEPA Series 885 guidelines using domestic small mammals as host organisms include the following tests:

- a ≥21-day test, using young adult rats or mice administered a single high dose of an MPCA orally (by gavage), followed by observations for mortality, weekly body weight, atypical behaviour, infectivity and clearance of the MPCA, and gross and microscopic pathologies at test end (USEPA, 1996o);
- a ≥21-day test, with young adult rats or mice administered a single high dose of an MPCA by inhalation (i.e., by intranasal or intratracheal instillation), followed by observations for mortality, weekly body weight, atypical behaviour, infectivity and clearance of the MPCA, and gross and microscopic pathologies at test end (USEPA, 1996p);
- a ≥21-day test, with young adult rats or mice administered a single high dose of an MPCA by intravenous or intraperitoneal injection, followed by observations for mortality, weekly body weight, atypical behaviour, infectivity and clearance of the MPCA, and gross and microscopic pathologies at test end (USEPA, 1996q);
- a ≥14-day test, with young adult albino rabbits administered a single high dose of an MPCA topically to the skin for 24 h, followed by observations for mortality, skin irritation, weekly body weight, atypical behaviour, and, if toxic effects are evident, gross necropsy at test end (USEPA, 1996r);
- a 14-day test with young adult rats, mice, or (in the case of a test for dermal effects) rabbits,

whereby groups are given a single exposure to multiple concentrations of an MPCA via an oral, pulmonary, or dermal route, followed by observations for post-exposure effects on behaviour and appearance during and at the end of the test (USEPA, 1996s);

- a ≥90-day test, with young adult rats or mice administered a single high dose of an MPCA daily by the oral or inhalation route; with daily observations for signs of toxicity and/or pathogenicity, mortality, weekly body weight, atypical behaviour, infectivity, and gross pathologies at test end (USEPA, 1996t); and

- a long-term test for effects of a single high dose of an MPCA administered orally each day to young (6–8 week) rats or mice on their fertility/reproduction and on the subsequent fetal development of their offspring to term; including daily observations for mortality, atypical behaviour, clinical abnormalities, infectivity, and pathologies at test end (USEPA, 1996u).

The ≥21-day tests, using oral, pulmonary, or injection routes of exposure to an MPCA (USEPA, 1996o,m,n) are designed and intended to provide a toxicological evaluation of a test material with respect to its pathogenicity, infectivity, and toxicity. It is believed that the data from these three tests will provide "*a fairly clear evaluation of the potential risks in most cases*" (USEPA, 1996n). An acute dermal toxicity study performed according to USEPA (1996r) is appropriate primarily to evaluate the toxicity of an MPCA (USEPA, 1996n). A 14-day multi-concentration test conducted according to USEPA (1996s) is intended primarily to determine the median lethal dose. The ≥90-day test described in USEPA (1996t) is intended to provide information on health hazards associated with a subchronic exposure. The long-term test for effects on reproduction and fetal development is, according to USEPA (1996u), designed to provide an estimate of potential human hazard from an MPCA if significant infectivity is observed without signs of toxicity or pathogenicity in a subchronic test performed according to USEPA (1996t), and the MPCA is either a virus or is parasitic to mammalian cells.

In keeping with USEPA (1996b), Health Canada's Pest Management and Regulatory Agency states in Part 9.3 "*Wild Mammals*" of its guidelines for the registration of MPCAs and their end-use products (PMRA, 2001), that the *toxicology* data required to evaluate hazard to human health and safety are usually adequate to indicate hazard to wild mammals. Accordingly, PMRA (2001) states that laboratory tests with small mammals exposed to an MPCA or EP should be performed according to its Part 4 "*Human Health and Safety Testing*". When testing for pathogenic and/or toxic effects as well as infectivity, a single high dose of the test material is administered orally (by gavage) or by intranasal or intratracheal instillation to each animal, followed by observations for at least 21 days and gross and, as appropriate, microscopic necropsy at test end. Guidance on testing domestic small mammals for infectivity of an MPCA or EP is also provided in PMRA (2001). Additional laboratory tests with wild mammals might be necessary if certain species are expected to be heavily exposed to the MPCA under operational conditions of use. In this instance, tests should be performed on representative species from the ecozone(s) of intended use that are most likely to be affected by the use pattern of the MPCA (PMRA, 2001).

The response by USEPAs Microbial Pesticide Branch to a 2002 survey (see Section 8.8) indicated data on hand for ~91 laboratory tests for effects of MPCAs or their EPs on rodents (rats or mice). These tests, which were performed according to Series 885 guidelines defined in USEPA (1996o,p,t), were all conducted as single-concentration (MHC), 21–90-day assays. Few (<4%) of these tests found any demonstrable pathogenic or toxic effects. None of the tests included a sterile filtrate control, positive microbial control, or positive chemical control; whereas ~50% of the tests included a non-infectious control and 100% of the tests included testing for infectivity. Microbial concentrations to which rodents were exposed were not measured in any of these tests.

14.2.2 Recommended Biological Test Method

A ≥21-day pathogenicity/toxicity test with young adult rats or mice is recommended to measure the potential adverse ecological effects of new microbial substances on small mammals. The procedures and conditions to be followed when

performing this biological test method are much the same as those shown in Table 13 for tests with birds, except that the test duration might be shorter and the dose is administered once only (at the start of the test) rather than daily during the first five days of the test. This approach is consistent with the Series 885 test guidelines published by the USEPA for determining the acute (≥21-day) toxicity and/or pathogenicity of an MPCA to young adult rats or mice when administered orally (USEPA, 1996o) or by inhalation (USEPA, 1996p). Certain test conditions defined in ASTM (2000q,r) for toxicity tests with rats[83] were also considered and adapted as appropriate when designing this recommended test method for rodents. These test guidelines and biological test methods should be consulted and followed for test specifics not addressed herein.

The experimental design for the biological test method for rats or mice recommended herein (see Table 14), including the route of administration of the test material, is largely consistent with the guidelines for measuring pathogenic and/or toxic effects of MPCAs or EPs on domestic small mammals published by PMRA (2001). Section 8.7 provides some of the rationale for choosing a rodent (i.e., rats or mice) when performing a test with a new microbial substance for pathogenic and/or toxic effects. That section also provides guidance when choosing to use a species of domestic small mammal rather than an avian species (Section 14.1) for evaluating the potential adverse ecological effects of a particular new microbial substance on terrestrial vertebrates.

Strains of mice or rats commonly used in testing should be used for this test method. The recommended strains are either CD-1 or B6C3F-1 mice, and Sprague-Dawley or Wistar rats. Young adults should be chosen with equal numbers of males and females for each treatment (USEPA, 1996o,p). For a given test, all animals must come from one source and be of the same strain, and they should be as similar in age and size as possible (ASTM, 2000q). At the beginning of the test, the weight variation of animals used in the test should not exceed ± 20% of the mean weight for each sex (USEPA, 1996o,p; ASTM, 2000q). The females should be *nulliparous* and nonpregnant. Before beginning the test, the test organisms should be acclimated to test conditions for a minimum of seven days.[84] During this time, it is recommended that each test animal be isolated in a cage identical to that used in the study.[85] This is the usual arrangement for mice and rats in acute studies. Mice are gregarious by nature, and group housing for the adult females is recommended for chronic studies. Since post-pubertal male mice are likely to fight, it is unlikely that they can be housed together. In the case of rats, both males and females are unlikely to show aggression when housed together with animals of the same sex; however, singly caged animals is the usual arrangement for rats under test. Each test cage used in a study must be identical, and should have a floor space that is ≥250 cm^2 for singly caged rats, and ≥100 cm^2 for singly caged mice or 160 cm^2 per group if group housed. A maximum of four or five mice per cage is allowed if group housed. Mice and rats can be identified by tattooing or subcutaneous microchip implantation. Ear notching is also sometimes used to identify laboratory mice (CCAC, 1993).

The test begins by choosing test animals that are as similar in size as possible, weighing each and determining its sex, and transferring a minimum of five females and five males per treatment (including the negative control) to individual cages. Rodents exposed to one (i.e., the MHD) or more (if a multi-concentration test) concentrations of the test material are subjected to a single dose administered by gavage (see Section 3.4.5) or by inhalation (see Section 3.4.6) at the start of the test period. The body weight of live animals is determined for each test chamber and treatment at the beginning of the test (Day 0) as well as weekly until test completion,

[83] These include the acceptable range for temperature and relative humidity in the test facility (ASTM, 2000q,r), photoperiod during testing (ASTM, 2000q), and minimum floor area in test cages (ASTM, 2000q).

[84] According to ASTM (2000q), rodents to be used in a test should be acclimated to the food, water, temperature, humidity, and lighting of the test facility for a 14-day period before test initiation.

[85] For the acclimation period and throughout the test, each test animal should be held in a separate cage to prevent aggressive interactions (USEPA, 1996o,p; ASTM, 2000q,r).

Table 14 Recommended Methodology for a ≥21-Day Pathogenicity/Toxicity Test Using Rats or Mice

Universal

Test method	—	modification of USEPA (1996o) "Microbial Pesticide Test Guidelines — Acute Oral Toxicity/Pathogenicity" and USEPA (1996p)"Microbial Pesticide Test Guidelines — Acute Pulmonary Toxicity/Pathogenicity"
Test type	—	test for adverse effects on survival, behaviour, and appearance (including gross and, as necessary and appropriate, microscopic examination of tissues and organs) of rats or mice exposed orally and/or by inhalation (i.e., using intranasal or intratracheal instillation)
Dosing regime	—	once only, at the start of the test
Test duration	—	≥21 days
Test organisms	—	young adults of a common laboratory strain; equal numbers of males and non-pregnant females of similar age and size in each treatment; acclimated to test chambers and test conditions for ≥7 days before start of test
Test chamber	—	all-metal cages, with a floor area of ≥250 cm^2 for singly caged rats, and ≥100 cm^2 for singly-caged mice
Number of rodents/ test chamber	—	1
Temperature	—	daily mean of 22 ± 2 °C
Relative humidity	—	40–70%
Lighting	—	incandescent or fluorescent; intensity in test chamber, 500–1000 lux; photoperiod, 12 ± 1 h light : 12 ± 1 h dark; gradual transition from light to dark and dark to light
Feeding	—	commercial rodent food of a suitable size, fed *ad libidum*
Controls	—	each test must include a negative control; use of a non-infectious control is strongly recommended; use of a sterile filtrate control is optional
Route of exposure	—	orally (by gavage) or by inhalation, if either a single-concentration test or a multi-concentration test
MHD for oral route	—	10^8 units of the new microbial substance, administered as a single dose at the start of the test (see Section 3.3.1.10)
MHD for inhalation route	—	10^8 units of the new microbial substance, administered as a single dose at the start of the test (see Section 3.3.1.11)
Testing for infectivity	—	required at test end, analytical techniques permitting; based on measured concentrations of new microbial substance in selected organs (e.g., heart, brain, kidney, liver), tissues, or body fluids (e.g., blood or urine) of rodents from each treatment, at end of test; optional testing for infectivity during the test
Measurements	—	temperature in test facility, daily max/min or continuously; relative humidity in test facility, at least once per week; individual body weights of rodents in each test cage and

		treatment at start of test and weekly thereafter; analyses permitting, concentration of new microbial substance in the aqueous suspension administered to each treatment (including the controls)
Observations	—	daily, for survival, behaviour (e.g., lethargy, tremors, convulsions, coma, atypical sleeping pattern) and appearance (e.g., external lesions) of each test organism; all animals dying during test as well as those surviving at test end to be necropsied; animals examined for evidence of lesions and abnormalities at post mortem (changes might include necrosis of tissues or organs, hemorrhage, etc.); selected tissues to be collected for future microscopic examination where deemed necessary
Biological endpoints	—	survival, appearance (including that from necropsy at test end), and behaviour in each test chamber and for each treatment, during and at test end
Test validity	—	invalid if <90% survival in negative control at test end

Single-Concentration Test

Number of treatments	—	minimum of two (i.e., MHD and negative control); additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	at least ten cages per concentration (treatment), including each control treatment
Number of rodents/treatment	—	at least ten; equal numbers of males and females for each treatment
Exposure route	—	orally (by gavage) or by inhalation (one exposure route per test)
Statistical endpoints	—	for each treatment: percent survival at test end; percentage of surviving rodents showing atypical appearance (based on necropsy) and/or atypical behaviour at test end
Statistical comparisons	—	MHD versus negative control at test end, for difference in percent survival and percentage of surviving rodents showing atypical behaviour and/or atypical appearance of organs or tissues at test end; if other control(s), same comparisons with negative control

Multi-Concentration Test

Number of concentrations (i.e., number of treatments)	—	minimum of five including MHD, plus negative control; additionally, non-infectious control strongly recommended; use of sterile filtrate control optional but recommended
Number of replicates	—	10 cages per concentration (treatment), including each control treatment
Number of rodents/treatment	—	10; five males and five females for each treatment
Exposure route	—	orally (by gavage) or by inhalation (one exposure route per test)
Statistical endpoints	—	for each treatment: percent survival at test end; percentage of surviving rodents showing atypical appearance (based on necropsy) and/or atypical behaviour at test end; data permitting — ≥21-day LD50, ≥21-day ED50 for atypical appearance and/or atypical behaviour, NOED/LOED
Evaluation of results	—	test concentrations versus negative control at test end, for difference in percent survival and percentage of surviving rodents showing atypical behaviour and/or atypical appearance of organs or tissues at test end; if other control(s), same comparisons with negative control

and these weights are used to determine the treatment-specific dose or doses (see Sections 3.3.1, 3.3.2, 3.4.5, and 3.4.6) administered on Day 0. Thereafter, the surviving rodents in each test cage are observed daily for their behaviour and appearance until the test is terminated (USEPA, 1996k,l). Animals in each test chamber are offered an excess ration of the same rodent food [86] during each day of the acclimation period and daily thereafter until test completion. A detailed necrospy of each rodent that dies during the test, as well as those that survive to the test end, is performed upon completion of the test. Each animal is examined closely and methodically for overt external and internal signs of any gross pathologies. Organs or tissues that appear atypical should be dissected, preserved, and stored for future microscopic examination as necessary and appropriate. Even in the absence of lesions at necropsy, selected tissues (including brain) should be collected for possible histopathological examination if there was clinical evidence of disease (e.g., poor weight gains, or neurological signs).

Under certain circumstances, if there is evidence at necropsy or clinical evidence of possible adverse effects on the hematopoieitic system, the collection of a blood sample at necropsy for analysis of variables such as the differential white blood cell (WBC) count, packed cell volume, and plasma protein might prove worthwhile in identifying effects on the immune system. Collection of <1 mL of blood in a microhematocrit tube is sufficient for both of the latter two analyses (Feldman *et al.*, 2000), and a small drop of blood smeared on a glass slide is adequate for the differential WBC count (i.e., percent small lymphocytes, percent large lymphocytes, percent neutrophils, percent eosinophils, percent monocytes, percent macrophages).

Each test requires a negative control, and the use of a non-infectious control is strongly recommended. Use of a sterile filtrate control is optional (see Section 4), but is recommended. Measurements for infectivity, using selected organ(s), tissue(s), or body fluid(s) (e.g., blood or urine) from rodents exposed to each treatment, are required upon completion of the test, analytical techniques permitting (see Section 5). Additional measurements for infectivity and clearance, as the test progresses (e.g., at weekly intervals), are optional but worthwhile to monitor the initiation and progression of any infectivity during the test period. Test specifics when performing either a single-concentration test (see Section 3.3.1) or a multi-concentration test (Section 3.3.2) are summarized in Table 14. Guidance in Sections 3.3.1, 3.3.2, 3.4.5, and 3.4.6 should be consulted and followed when mixing and administering the test material orally and/or by inhalation. The single dose of new microbial substance administered to rodents in each treatment at the start of the test should be quantified, if possible (see Section 3.5).

For a single-concentration test, the test animals are subjected to the MHD of the test material by only one route of exposure (i.e., either orally by gavage, or by inhalation; see Sections 3.4.5 and 3.4.6). The test animals are housed in separate cages and a minimum of 10 rodents is exposed to each treatment.[87] For a multi-concentration test, the effects of one or both of these means of exposure should also be investigated separately (see Section 3.2). A decision as to which exposure route(s) to use in a multi-concentration test depends on the intended means of application of the new microbial substance in the environment (e.g., as an aerial spray or as a solid deposited onto water or soil) and on the most likely route(s) of the substance contacting small mammals. Each multi-concentration test includes the MHD for the route of exposure used in

[86] Diets must be formulated in accordance with the nutrient requirements of the test species. Any unmedicated commercial diet that meets the minimum nutritional standards of the test species is acceptable (ASTM, 2000q).

[87] More than ten animals (e.g., three sets of ten animals per treatment) is desirable to enable statistical comparisons of endpoint data (e.g., for each treatment: percent survival, percentage of surviving rodents showing atypical behaviour, and percentage of surviving rodents with an atypical appearance). However, in keeping with USEPA (1996o,p), this is not a prerequisite.

that test as well as a series of lower concentrations, plus a negative control and, depending on the study design, one or more other controls (see Section 4). For this biological test method, a minimum of five test concentrations plus the control(s) is required for a multi-concentration test.

The biological endpoints for this test are based on survival, and on the behaviour and appearance (including findings at necropsy and for any subsequent lesions identified on microscopic examination) of the group(s) of rodents subjected to each treatment. Table 14 summarizes the type of observations on the behaviour and appearance of animals in each treatment (including the negative and other controls), monitored daily throughout the test period. Particular attention should be directed at observing for signs of tremors, convulsions, lethargy, other neurological changes, poor weight gains, diarrhea, etc. (USEPA, 1996o,p). During these daily observations, any rodent observed to have died must be submitted for post mortem, and a detailed examination made of the gross appearance of the external and internal tissues and organs (e.g., skin, mucous membranes, external ears, oral cavity, respiratory tract, gastrointestinal tract, liver, heart, spleen, and urinary system). At the end of the test, each of the surviving rodents exposed to each treatment (including the negative controls) must also be killed and necropsied in this manner. If lesions are identified at necropsy, or if clinical signs of disease were observed during the test period or at its end, representative samples of selected tissue(s) or organ(s) should be collected and preserved in 10% neutral buffered formalin for processing and future microscopic examination where deemed necessary. Routine histopathological procedures normally include sections of lung, liver, spleen, kidney, small and large intestine, and heart. If any neurological and/or behavioural signs were noted, sections of brain should also be collected for future histopathology where deemed necessary. Investigators should consult standard guides on necropsy procedures for rodents (e.g., Greaves and Faccini, 1984; Feldman and Seely, 1988) when preparing for and undertaking these postmortem examinations.

An evaluation of test results for a single-concentration test should include a consideration of the apparent relationship, if any, between exposure to the test material and the incidence and severity of all abnormalities observed for that treatment (i.e., the MHD) versus the negative control. Such comparisons should consider all observations including behavioural changes, atypical appearance during the test, body weight, mortality, gross changes seen at necropsy, and histopathology (USEPA, 1996o,p).

If replicate sets (e.g., three sets of ten rodents per treatment) are included in a single-concentration study, statistical endpoints such as the following should be determined and compared following completion of the test[88]:

(1) percent survival of rodents exposed to each treatment;

(2) percentage of animals exposed to each treatment, showing atypical behaviour (e.g., tremors, convulsion, lethargy); and

(3) percentage of rodents in each treatment, showing an abnormal appearance of one or more organs or tissues (e.g., external or internal lesions, opaque or hemorrhaged eye, swollen or discoloured liver).

Respective values for these endpoints determined for the maximum hazard dose and any control treatments other than the negative control should be compared against those for the negative control, using an appropriate statistical test for pairwise comparisons such as *Student's t-test*. Environment Canada's guidance document on statistical methods to determine endpoints of toxicity tests (EC, 2004d) should be consulted when choosing and applying the appropriate statistics.

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[88] If the experimental design for a single-concentration test is restricted to a single set of 10 rodents per treatment (with each animal housed separately), in keeping with USEPA (1996o,p), test data are not amenable to statistical comparisons. However, the use of multiple sets of animals per treatment (e.g., three sets of rodents per treatment) enables statistical comparisons among treatments.

The statistical endpoints described in the preceding paragraph apply when performing a multi-concentration test. Data permitting (see Section 3.3.2), the ≥21-day LD50 for the test material should be calculated together with its slope and 95% confidence limits. A ≥21-day ED50 based on atypical appearance and/or atypical behaviour of individual rodents exposed to each treatment should also be calculated together with its slope and 95% limits, if possible. Guidance in EC (2004d) on appropriate software programs to use (and their application) when determining an LD50 or ED50 should be followed. Data permitting, the LOED and NOED for effects on ≥21-day survival as well as data showing atypical appearance and/or atypical behaviour should be calculated and reported as well. Environment Canada (2004d) should also be consulted when choosing the appropriate statistics to be applied to the data derived from the study on rodent survival, appearance (including that at necropsy), and behaviour.

14.2.3 Other Methods or Procedures

Siegel and Shadduck (1992) provide useful background information on past methods and procedures for undertaking laboratory tests designed to measure the effects of microbial pest control agents on mammals. This publication focusses primarily on tests for infection and acute toxicity, while addressing specific mammalian safety tests and considerations when procuring and housing rodents and other small mammals.

The United States Food and Drug Administration (USFDA) includes a Center for Biologics Evaluation and Research (CBER; see Web site www.fda.gov/cber/about.htm) as well as a Center for Drug Evaluation and Research (CDER; see Web site www.fda.gov/cder). The CBER regulates biological substances and, together with the CDER, provides guidance on methods and procedures for testing their environmental effects on rodents or other test organisms. Accordingly, these Web sites might provide useful information on the testing of certain new microbial substances for pathogenic and/or toxic effects on rodents or other small mammals.

As part of its Series 885 "*Microbial Pesticide Test Guidelines*", the USEPA published guidelines for performing two biological test methods for measuring the long-term adverse effects of MPCAs on rodents. The USEPA (1996t) describes a ≥90-day test, that involves the daily administration (orally or by inhalation) of the test material to young adult rats or mice with observations for pathogenic and/or toxic effects as well as infectivity. The USEPA (1996u) describes a test for effects of the daily oral administration of an MPCA on fertility and reproduction of rats or mice and on the development of their offspring to term. Each of these test methods deserves consideration if testing with domestic small mammals additional to that recommended in Section 14.2.2 seems warranted. This might be the case when testing a new microbial substance with a known or suspected long incubation period before pathogenic effects are realized. If micro-organisms similar to those in the test material are known to cause reproductive or developmental effects on small mammals under certain circumstances, and initial testing of that substance according to Section 14.2.2 indicates that further evaluation of its environmental safety is warranted, the test method for reproductive and developmental effects on rodents described by USEPA (1996u) could be applied as either a single-concentration test or a multi-concentration one.

The biological test method for assessing developmental toxicity in rats and rabbits published by ASTM (2000s) would be useful for measuring the pathogenic and/or toxic effects of a new microbial substance on developing fetuses of small mammals. This test method offers the advantage of a relatively short test duration (20 days for rats; 29 days for rabbits), while studying the effects of a test material on pregnant mammals from the time of embryonic implantation through the period during which major organ systems are formed. The ASTM (2000s) describes procedures and conditions for performing this test for developmental effects, as a multi-concentration assay using rats and/or rabbits.

The ASTM has published standard test methods for conducting 90-day toxicity studies with domestic rats to measure effects of chemicals on their survival, behaviour, and appearance (gross and microscopic, at necropsy on Day 90). These test methods administer the test material either orally (ASTM, 2000q) or by inhalation (ASTM, 2000r),

are designed as multi-concentration tests, and include the NOED as an endpoint. Useful procedural specifics described in each of these test methods can be applied to the ≥21-day test method for rats or mice recommended in Section 14.2.2. With appropriate design modifications for new microbial substances (in keeping with those described in Section 14.2.2), either of these 90-day test methods could also be applied as an alternative to the test method recommended in Section 14.2.2, in instances where information is sought on the effects of an extended exposure of small mammals to a new microbial substance.

American Society for Testing and Materials (2000t) is a standard method for determining the chronic oral toxicity of test materials to rats. This multi-concentration test, which begins with young rats that are post-weaned by three weeks, involves the daily (or five days per week) administration of the test material orally (either by gavage or in the diet) throughout a one-year period. Thereafter, urine and blood samples are collected and necropsies are performed for gross and microscopic pathologies. This test method is intended to detect adverse effects that require a long latency period or that are cumulative before they are manifested (ASTM, 2000t). If information is required on the chronic pathogenic and/or toxic effects of a particular new microbial substance, the standard test method for chronic oral toxicity in rats could readily be adapted for this purpose.

In instances where information on the potential ecological effects of a new microbial substance on a particular species of small wild mammal is desired (see Section 14.2.1), consideration could be given to capturing field specimens and transporting them to a laboratory for testing. Both USEPA (1996v) and USEPA (1996nn) provide guidance for wild mammal testing. Once field-collected mammals are acclimated to laboratory conditions and in good health, testing for pathogenic and/or toxic effects of a new microbial substance could be performed using the recommended methodology described in Section 14.2.2.

Guidance on Reporting Requirements

Key Guidance

- *The information requirements specified in EC and HC (2001) for laboratory tests on pathogenic and/or toxic effects of new microbial substances must be satisfied and reported when providing test-specific reports to Environment Canada.*
- *The guidance on reporting study results specified in OECD's (1998a) Principles of Good Laboratory Practice should be followed when preparing test-specific reports for submission to Environment Canada.*
- *Additional to the above guidance, each test-specific report should satisfy each of the pertinent reporting requirements specified in the standard biological test method or test guideline followed when performing a test for pathogenic and/or toxic effects with a particular species of test organisms.*

Part 4.2.7.1 of EC and HC (2001) *"Guidelines for the Notification and Testing of New Substances: Organisms"* includes brief guidance on reporting the findings of laboratory tests conducted to determine the adverse effects of new microbial substances on aquatic and terrestrial plants and animals, as follows: *"Notifiers should provide all information necessary for a complete and accurate description of the test procedures and all data, information, and analysis necessary for Environment Canada to reach an independent conclusion. This should include justification for choosing a particular test species and test method and a statistical analysis of differences between the maximum hazard group and control groups."* As a minimum, these information requirements must be met when providing test-specific reports of studies performed within the context of the present guidance document, to measure the pathogenicity and/or toxicity of a new microbial substance.

The Principles of Good Laboratory Practice published by the Organisation for Economic Co-operation and Development include guidance on reporting study results for laboratory tests applied to the non-clinical safety testing of various substances including industrial chemicals, pesticide products, and substances that are comprised of or contain living organisms (OECD, 1998a). The requirement for GLP within the the New Substances Notification (NSN) Regulations (Government of Canada, 1997) only applies to chemicals, polymers, and biopolymers, and does not apply to living organisms. Nonetheless, OECD's Principles of Good Laboratory Practice should be applied to laboratory tests involving living organisms (including micro-organisms) until such time that the Act and/or the NSN Regulations include such a requirement for living organisms. Section 6.9 *"Reporting of Study Results"* herein summarizes the particulars that should be included in each final report of a test for pathogenic and/or toxic effects of a new microbial substance on a particular test (host) organism, to meet OECD's Principles of Good Laboratory Practice when reporting study results.

Guidance on the reporting requirements to be met for each of the USEPA's Series 885 Test Guidelines is included in USEPA (1996a). According to this Guideline (OPPTS 885.0001), each test report submitted as part of this series shall satisfy the reporting requirements that are specified under the following subheadings (USEPA, 1996a):

(1) general requirements;
(2) format and content;
(3) summary of test results;
(4) description of the test procedure; and
(5) statistical procedures.

Each of the Series 885 Test Guidelines for a particular biological test method includes a section on reporting, that details those test-specific details on procedures, conditions, and results that should or must be included in a final report along with the general reporting requirements described in OPPTS 885.0001 (USEPA, 1996a). Each final report on a test performed according to one of these Series 885 Test Guidelines (or some modification thereof; see Sections 9 to 14) should meet the reporting requirements identified in USEPA (1996a) and in the specific test guideline (USEPA, 1996c-v).

Each of the biological test methods by Environment Canada referred to herein includes a section entitled *"Reporting Requirements"*. Included there is a listing of the minimum reporting requirements for a test-specific report and, in a separate listing, additional reporting requirements or specified data to be held on file.[89] These reporting requirements refer to those test conditions, procedures, and results that should or must be included in either a test-specific report or a general report applicable to all tests performed according to a particular biological test method, or, in some instances (e.g., laboratory benchsheets for data entries as a test progresses), simply held on file for a minimum of five years. The reporting requirements identified in each biological test method by Environment Canada that is performed (with suitable adaptations) when measuring the pathogenic and/or toxic effects of a new microbial substance using a selected species of aquatic or terrestrial plant or animals (see Sections 9 to 14) should be followed when preparing and finalizing the test report.

Certain biological test methods that are recommended or considered in Sections 9 to 14 are standard guides/methods by ASTM (2000c-t), ISO (1998, 1999a,b), OECD (1998c,d,e, 2000b,c, 2002b), or USEPA (1995; 1996c-m; 1996o-v; 1996gg-nn; 2000; 2002a,b). Each of these standard methods or test guidelines includes reporting requirements related to the specifics of a particular test's procedures, conditions, and results. These reporting requirements should be included in each final report that pertains to these methods or guidelines.

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[89] The listing of additional reporting requirements identifies those items that must be either included in the test-specific report or a general report, or held on file for a minimum of five years. An exception to this "two-category" listing of reporting requirements occurs in the early (i.e., 1990 and 1992) publications of biological test methods by Environment Canada, in which instance all items to be reported were identified under a single heading "Reporting Requirements".

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Biological Test Methods and Supporting Guidance Documents Published by Environment Canada's Method Development and Applications Section[1]

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods			
Acute Lethality Test Using Rainbow Trout	EPS 1/RM/9	July 1990	May 1996
Acute Lethality Test Using Threespine Stickleback (*Gasterosteus aculeatus)*	EPS 1/RM/10	July 1990	March 2000
Acute Lethality Test Using *Daphnia* spp.	EPS 1/RM/11	July 1990	May 1996
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	EPS 1/RM/21	February 1992	November 1997
Test of Larval Growth and Survival Using Fathead Minnows	EPS 1/RM/22	February 1992	November 1997
Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)	EPS 1/RM/24	November 1992	—
Growth Inhibition Test Using the Freshwater Alga *Selenastrum capricornutum*	EPS 1/RM/25	November 1992	November 1997
Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	EPS 1/RM/26	December 1992	October 1998
Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)	EPS 1/RM/27	December 1992	November 1997
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon)	EPS 1/RM/28 1st Edition	December 1992	January 1995
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)	EPS 1/RM/28 2nd Edition	July 1998	—
Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges (*Chironomus tentans* or *Chironomus riparius*)	EPS 1/RM/32	December 1997	—

[1] These documents are available for purchase from Environmental Protection Publications, Environmental Protection Service, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada. For further information or comments, contact the Manager, Method Development and Applications Section, Environmental Technology Centre, Environment Canada, Ottawa, Ontario K1A 0H3.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (cont'd.)			
Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33	December 1997	—
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37	March 1999	—
Test for Survival and Growth in Sediment Using Spionid Polychaete Worms (*Polydora cornuta*)	EPS 1/RM/41	December 2001	—
Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei, Eisenia fetida*, or *Lumbricus terrestris*)	EPS 1/RM/43	April 2004	—
Tests for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil	EPS 1/RM/45	September 2004?	—
Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil	EPS 1/RM/47	2005	—
B. Reference Methods[2]			
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods	EPS 1/RM/35	December 1998	—
Reference Method for Determining the Toxicity of Sediment Using Luminescent Bacteria in a Solid-Phase Test	EPS 1/RM/42	April 2002	—

[2] For this series of documents, a *reference method* is defined as a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of test instructions and conditions which are described precisely in a written document. Unlike other generic (multi-purpose or "universal") biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
C. Supporting Guidance Documents			
Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants	EPS 1/RM/12	August 1990	—
Guidance Document on Collection and Preparation of Sediment for Physicochemical Characterization and Biological Testing	EPS 1/RM/29	December 1994	—
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Guidance Document on Statistical Methods to Determine Endpoints of Toxicity Tests	EPS 1/RM/46	October 2004?	—

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Appendix D

Logarithmic Series of Concentrations Suitable for Pathogenicity and/or Toxicity Tests*

Column (Number of concentrations between 10.0 and 1.00, or between 1.00 and 0.10)**

1	2	3	4	5	6	7
10.0	10.0	10.0	10.0	10.0	10.0	10.0
3.2	4.6	5.6	6.3	6.8	7.2	7.5
1.00	2.2	3.2	4.0	4.6	5.2	5.6
0.32	1.00	1.8	2.5	3.2	3.7	4.2
0.10	0.46	1.00	1.6	2.2	2.7	3.2
	0.22	0.56	1.00	1.5	1.9	2.4
	0.10	0.32	0.63	1.00	1.4	1.8
		0.18	0.40	0.68	1.00	1.3
		0.10	0.25	0.46	0.72	1.00
			0.16	0.32	0.52	0.75
			0.10	0.22	0.37	0.56
				0.15	0.27	0.42
				0.10	0.19	0.32
					0.14	0.24
					0.10	0.18
						0.13
						0.10

* Modified from Rocchini *et al.* (1982).

** A series of five (or more) successive concentrations should be chosen from a column. Midpoints between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by weight (e.g., mg/kg) or weight-to-volume (e.g., mg/L). As necessary, values cans be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations should not be used, since such will provide poor resolution regarding the confidence limits surrounding any threshold-effect value calculated.