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Microbial pesticides
[data requirements for environmental risk assessment]

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PREFACE AND ACKNOWLEDGEMENTS

Drs. B.J.W.G. Mensink (RIVM/CSR) has written this report. It has been peer-reviewed by ir. J.B.H.J. Linders (RIVM/CSR). It has been critically reviewed by dr. A. Sterkenburg (RIVM/ECO), dr. J. van Emden (RIVM/LIO), and dr. C.F. van Kreyl (RIVM/LEO).

This report primarily presents a concise — but not exhaustive — overview of national and international data requirements for the environmental risk assessment of microbial pesticides. The report is primarily meant as a contribution to the further development of a consistent and scientifically valid framework for evaluating biological pesticides in the Netherlands. However, this goal should at all times take note of the current discussions within the European Union (EU). The authors hope that this report will contribute to further discussions on the establishment of a proper evaluating system for microbial pesticides in the Netherlands within a European context.

The emphasis in this study is on those data, that are considered appropriate for a proper environmental risk assessment prior to the regulatory admission of a microbial pesticide. Therefore various related issues — e.g. particular data on Genetically Modified Organisms (GMOs), data dealing with the active ingredient vs. the product, problems with monitoring the "stability" of a microbial pesticide during tests — are only discussed briefly in this study. Also too detailed data on e.g. the requirements for obtaining an Experimental Use Permit — sometimes necessary prior to testing in the field — in the US and Canada are avoided. However, if necessary, the reader is referred to relevant literature for further reading.

Emphasised in this report is also a proposal by the European Commission (EC). It is presented to and discussed with the member states for the first time in 1996. The proposal was amended in some expert meetings at the end of 1996, and distributed again for further comments within the member states. Therefore this proposal is not finally agreed upon — yet — by the member states. Finally, it will have to be discussed by the EC Standing Committee on Plant Health.

ABSTRACT

The market for microbial pesticides is — though slowly — expanding. Therefore more research with these pesticides will be carried out in the near future, not only for agronomical and economical, but also for environmental reasons. As more chemical pesticides are going to be banned, microbial pesticides — with a more environmental friendly image —can be an interesting alternative for farmers.

Regulatory agencies therefore need to build up a consistent framework for the requirement of various data. Such frameworks exist in the US and Canada; they do not exist in Europe, although the European Commission (EC) is elaborating a proposal. All these frameworks deal with data requirements in general, and to a lesser extent with the test protocol (except the US and Canada), the format of the test report, and the quality of the required data. They, also, don't include guidance on how to carry out a proper environmental risk assessment, when all required data are submitted to the regulatory agencies. Therefore, it is recommended to construct a specific Dutch framework for data requirements, and, subsequently, environmental risk assessment, in addition to the current endeavour of the EC.

This report entails a concise — but not exhaustive — overview of the data requirements for the notification of microbial pesticides in the US, Canada, Denmark, The Netherlands, and the United Kingdom. They are compared with an EC-proposal from January 1997. The report also entails various recommendations with respect to the further development of a Dutch framework for data requirements, risk assessment, and risk management.

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ABBREVIATIONS

APHIS	Animal and Plant Health Inspection Service of the USDA
AU	Active Units (of micro-organisms e.g. per gram soil or water)
BW	Body Weight
CA	Competent Authorities
CTB	College voor de Toelating van Bestrijdingsmiddelen (in English: Dutch Board for the Authorisation of Pesticides)
CFU	Colony Forming Units
DR	Data Requirement
EC	European Commision
EPA	Environmental Protection Agency (US)
EU	European Union
FWS	Department of the Interior, Fish & Wildlife Service (US)
GMM	Genetically-Modified-Micro-organism
GMO	Genetically-Modified-Organism
I	Infectivity
Max	Maximum
MCC	Maximum Challenge Concentration
MEC	Maximum Environmental Concentration
MO	Micro-organism
MP	Microbial Pesticide
NBCI	National Biological Control Institute (US)
NTH	Non-Target Host
NTO	Non-Target Organism
OPPTS	Office of Prevention, Pesticides and Toxic Substances (US EPA)
P	Pathogenicity
Sp	Species
T	Toxicity
TO	Target Organism
USDA	US Department of Agriculture

SUMMARY

This report presents a concise overview of national and international environmental data requirements for the regulatory admission of microbial pesticides. The emphasis is on those data that are considered relevant for an adequate environmental risk assessment, prior to the introduction of a new microbial pesticide.

Consistent operational frameworks for such data requirements do exist in the US and Canada, but not in European countries, where data are required on a case-by-case basis. A proposal for such a more consistent framework for European countries is, however, under development. A proposal by the European Commission on the data requirements for micro-organisms with pesticidal activity will finally be discussed within the EC Standing Committee on Plant Health and the OECD within the foreseeable future. This EC proposal refers to the kind of studies that should be submitted, without detailed prescriptions on the test protocol, the test report, and the quality of the data.

This EC framework is two-tiered: the first tier of the EC proposal entails the identity, (micro)biological properties, and data on e.g. the field of use and production. If, subsequently, the potential environmental risk cannot be assessed, a base set of laboratory tests has to be performed (Tier II).

The US/Canada framework is four-tiered and more detailed in its prescriptions on the methodology of the tests, the test reports, and the quality of the data to be submitted. The ecotoxicological and environmental behaviour tests are intermingled in this framework. The first tier is a base set of laboratory tests with *maximum challenge concentrations* (1000 times higher than those concentrations, the organisms would be exposed to, in case of a max. application rate in the field). In case of effects, additional laboratory or field tests with *maximum environmental concentrations* (those — e.g. calculated — concentrations, the organisms would be exposed to, in case of a max. application rate in the field) should be performed (Tier II). The potential exposure should also be assessed. If the Tier II ecotoxicological tests show effects, additional laboratory or field tests must be performed, taking life-cycles, potential exposure, and dose-effect relations into account (Tier III). Finally, small plot field test may be performed to simulate actual use under natural conditions (Tier IV). It should be noted that the Canadian approach is still a regulatory proposal, whereas the US approach is implemented into legislation.

A tiered Dutch data requirement proposal — that never became operational — was not integrated into the EC proposal. This does not necessarily mean that a further elaboration of

this Dutch framework for data requirements is meaningless. It should be envisaged to use it for further development into an environmental risk assessment scheme, without doubling or re-interpreting the EC proposal. A Dutch framework may be vital in coping with the international developments on data requirements and risk assessment. It may be also helpful in "feeding" the discussions in the EU and the OECD within the foreseeable future.

It is recommended to develop a Dutch framework for data requirements and, subsequently, risk assessment, as such a consistent framework does not exist. The required data should be the basis for the environmental risk assessment, consisting generally of the exposure assessment, the effects assessment (incl. dose-effect relations), and the risk characterisation which may include risk estimation (i.e. the quantification of the incidence of effects, likely to occur). It should be envisaged, when developing such a framework, to take into account the specific and variable properties of a micro-organism. The test protocol, the quality of the test report, and the quality of the data should also be taken into account.

A panel of Dutch experts should be called into existence. Panel members should be experts on e.g. mammalian toxicology, ecotoxicology, micro-biology, virology, and environmental behaviour. The experts of relevant institutes and companies should also be involved in these discussions.

SAMENVATTING

Dit rapport geeft een beknopt overzicht van de aan te leveren gegevens in het kader van de milieu-risicobeoordeling voor microbiële pesticiden. Het overzicht omvat zowel nationale als internationale gegevensvereisten. Het betreft die gegevens die in het kader van toelatingsprocedures (kunnen) worden gevraagd.

Een consistent en operationeel raamwerk met daarin de bovengenoemde gegevensvereisten bestaat in zowel de Verenigde Staten als Canada. Het bestaat niet in Europa, waar het vaststellen van de vereiste gegevens voor microbiële pesticiden op *ad hoc* basis plaatsvindt. Er is echter een voorstel van de Europese Commissie (EC) in ontwikkeling, waarin getracht is een dergelijk meer consistent raamwerk te formuleren. Dit voorstel wordt uiteindelijk besproken in zowel het Permanente Comité voor Gewasbescherming (EU) als de OECD. Het EC voorstel verwijst vooral naar het type studie dat zou moeten worden uitgevoerd zonder veel details over het testprotocol, de rapportage en de kwaliteit van de aan te leveren gegevens.

Het voorgestelde EC raamwerk is twee-traps. De eerste trap betreft gegevens over de identiteit, (micro)biologische eigenschappen en gegevens over de wijze van toepassing en productie. Als op grond hiervan geen adequate milieu-risicobeoordeling kan worden uitgevoerd, dient een basis set van testen te worden uitgevoerd (tweede trap).

Het VS/Canadese raamwerk is daarentegen vier-traps en meer gedetailleerd wat betreft het testprotocol, de wijze van rapportage en de kwaliteit van de aan te leveren gegevens. De ecotoxicologische testen en die om het gedrag in het milieu vast te stellen staan in een duidelijk onderling verband. De eerste trap bestaat uit een basisset van testen met concentraties, die tot een factor 1000 hoger liggen dan die (bijvoorbeeld berekende) concentraties, waaraan het organisme zou zijn blootgesteld bij de hoogste dosering, zoals die in de praktijk op het gewas zal kunnen worden toegepast. Indien hieruit effecten blijken, dienen additionele laboratorium- of veldtesten te worden uitgevoerd, met (berekende) concentraties, waaraan het organisme zou zijn blootgesteld bij de hoogste praktijkdosering (tweede trap). Tevens dient de potentiële blootstelling te worden vastgesteld met laboratorium- of veldtesten. Indien uit de ecotoxicologische testen van de tweede trap effecten blijken, dienen aanvullende testen te worden uitgevoerd die rekening houden met de levenscyclus van het organisme, de potentiële blootstelling en de dosis-effect relaties (derde trap). Tenslotte kunnen veldtesten worden uitgevoerd, indien de drie voorafgaande trappen geen uitsluitsel geven over de te verwachten blootstelling en effecten. Het Canadese raamwerk is (nog) niet geimplementeerd in de wetgeving en het Amerikaanse raamwerk wel.

Een voorstel voor een getrapt Nederlands raamwerk is nooit operationeel geworden. Het is ook niet meegenomen in het bovengenoemde EC voorstel. Dit betekent niet dat het verder uitwerken van dit voorstel een nutteloze actie is. Het kan immers als basis fungeren om het raamwerk verder uit te werken tot het niveau van de individuele testprotocollen, de testrapportage en de kwaliteit van de aan te leveren gegevens. Vervolgens kan hierop een consistente milieu-risicobeoordeling gebaseerd worden. Vanzelfsprekend moeten doublures met het Europese raamwerk worden vermeden. Bovendien is de ontwikkeling van een Nederlands raamwerk nuttig om aan de hand van weloverwogen standpunten de discussies in internationaal verband (EU, OECD) te "voeren". Dergelijke internationale discussies lopen al, of worden op korte termijn gestart.

Het wordt aanbevolen een Nederlands raamwerk op te zetten met de vereiste data en vervolgens met de daarop gebaseerde risico-beoordeling. De gegevens die aangeleverd moeten worden dienen primair om de risico-beoordeling adequaat uit te kunnen voeren. Deze bestaat uit het schatten van de blootstelling en de effecten (incl. dosis effect relaties) én de risico-karakterisatie. Laatstgenoemde kan de risico-schatting (i.e. het quantificeren van de kans op effecten) omvatten. Wanneer een dergelijk raamwerk wordt opgezet dient rekening te worden gehouden met de specifieke en variabele eigenschappen van micro-organismen. Bovendien moet het raamwerk betrekking hebben op het test protocol, de kwaliteit van de rapportage en de kwaliteit van de aangeleverde gegevens.

Een panel van Nederlandse experts op het gebied van bijv. toxicologie (zoogdieren), ecotoxicologie, micro-biologie, virologie en het gedrag in milieu moet in het leven geroepen worden. Onderzoekers van concerns dienen ook bij deze discussies betrokken te worden.

1. INTRODUCTION

The science of biological control is expanding due to commercial and environmental interests. The regulatory institutions, however, have difficulties in coping with these changes, primarily as they are used to authorise chemical rather than biological pesticides. Therefore, there is in the EU, in general, more expertise on the data requirements for the authorisation of chemical than of biological pesticides. There is a trend to introduce more biological pesticides in view of the adverse environmental impact of various chemical pesticides. It was estimated that the mondial turnover of biological pesticides in 1991 equalled US $ 120 mln. of which 83% was on account of *Bacillus thuringiensis* (Scheepens & Lotz, 1994; see cadre below). This turnover is only a small fraction of the total turnover of chemical pesticides. However, the mondial turnover of chemical pesticides does not increase, whereas the turnover of biological pesticides yearly increases with 15-25%. In view of the expanding market for biological pesticides, data requirements on this issue should be developed and implemented in the national legislation.

As stated above, ***Bacillus thuringiensis*** is the current market dominating Microbial Pesticide. It is used as an insecticide all over the world, and it exerts its activity after ingestion by the larvae, when it releases a toxin that degrades the midgut epithelium. The arthropod stops feeding and dies within two to six days. ***Bacillus*** is a soil bacterium, primarily used for controlling lepidopterae.

The emphasis of expertise on chemical rather than on biological pesticides probably has a second reason: contrary to chemical pesticides, biological pesticides have generally a long, global history of safe release. This is one of the reasons why the Dutch governmental policies on the control of pesticides aim a.o. at promoting the development, availability, and applicability of biological pesticides: the multi-year crop protection plan (Kant, 1996). An interdepartmental project "Guidance on the Registration of Biological Pesticides" was started in January 1991 in the Netherlands with two goals:

- stating explicitly the environmental quality objectives and criteria from the government in the evaluation of biological pesticides for registration;
- differentiating the guidelines to connect the data requirements properly with the variable properties of biological pesticides.

Currently, most countries in the EU are updating and improving the data requirements for biological pesticides. There is, however, an interaction between the national and the

international efforts: to be more precise, between the EU countries and the European Commision. The goal of the EC is — of course — harmonisation of the authorisation procedures and their scientific back up for the biological pesticides.

The aim of this study is to list the data requirements as currently proposed by the EC, to compare these with the current national data requirements of individual countries, not only within the EU, but also within the OECD. The emphasis is on those data necessary for environmental risk assessment.

Within the context of this study, data requirements refer to all data that may be required by the Competent Authorities to evaluate the environmental risks of micro-organisms as biological pesticides. These data requirements include the extent and quality of the data that must be submitted by the applicant to the CA. As soon as data requirements are authorised by national or international institutions, they become guidelines (i.e. officially authorised for the protocol and the report of tests).

Data requirements should never be seen independently. They should be useful for particular goals. Such goals with respect to environmental risk assessment for biological pesticides can be as presented in Table 1. In this table the term *biological* pesticides is restricted to *microbial* pesticides. Such pesticides are particular micro-organisms: any microbial entity, cellular or non-cellular, capable of replication or of transferring genetic material (Anon., 1997a). Micro-organisms — defined as such — are e.g. bacteria[1], fungi, protozoa, viruses, and viroids[2]. Generally, nematodes are not considered as micro-organisms. Generally, any strain of a species shall be regarded as an independent active living micro-organism (conforming to DEPA, 1994). It should be noted that the definition of a *biological* pesticide may differ per country. In the Netherlands, a biological pesticide is a pesticide with a micro-organism (incl. viruses, viroids, mycoplasmae, protozoa, bacteria, yeasts, and fungi) as active ingredient (CTB, 1995b), whereas in Sweden a biological pesticide is the whole set of micro-organisms, viruses, nematodes, insects, and arachnids, developed particularly to prevent or counteract sanitary nuisance or damage to property from animals, plants, micro-organisms or viruses (pers. communication KEMI to RIVM, 1997). The Dutch definition includes indigenous[3] and non-indigenous micro-organisms. It also includes e.g. "upgraded" and genetically engineered micro-organisms. The Swedish definition includes all active organisms which have been changed with the aid of gene technology, in such a manner that is not naturally occurring.

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1 Including mycoplasmae — particular bacteria without a rigid cell wall — and rickettsiae — intracellular parasitic bacteria.

2 Viroids are defect, small viruses.

3 Indigenous is native to a particular area, not introduced.

The EPA distinguishes conventional and biorational pesticides. The former include the chemical pesticides (generally synthetic), whereas the latter include the biochemical and microbial pesticides (generally natural). The biochemical pesticides entail e.g. pheromones, insect or plant growth regulators, and enzymes, the microbial pesticides entail e.g. micro-organisms. Chemicals similar to biorationals are classified by the EPA as biorational or conventional, dependent on the chemical structure and the mode of action in the target species.

In this study the term *biological pesticide* is synonymous with a micro-organism with pesticidal action. These microbial pesticides contain a micro-organism (incl. any associated metabolite) as active ingredient. Other biological pesticides will not be discussed in this report. In case the pesticidal action is due to a toxin, the micro-organism — producing the toxin — is defined as a microbial pesticide, if the product contains the living toxin-producing micro-organism (conforming to DEPA, 1994). If the product contains the toxin only, the pesticide is defined as a chemical pesticide.

Table 1. Goals of environmental data requirements for microbial pesticides. Risk assessment is the proces that contains some of or all these three elements (Anon., 1997a, adapted from Van Leeuwen & Hermens, 1995)

RISK ASSESSMENT	
Exposure assessment:	predicting the distribution and fate of microbial pesticides in the environment to estimate concentrations to which non-target species or populations may be exposed;
Effects assessment:	identification of adverse effects of microbial pesticides to various non-target species or populations; estimating the relationships between doses or concentrations of microbial pesticides, and the incidence and severity of adverse effects;
Risk characterisation:	estimating the incidence and severity of the adverse effects likely to occur in an environmmental compartment due to predicted exposure to a microbial pesticide (e.g. the risk of killing fish, terrestrial predators or large vertebrates).

Besides for risk assessment, the data requirements should be used for labelling the packages with hazard symbols, for formulating the environmental risk and safety phrases, and for determining special precautions on the protection of flora and fauna, if necessary (Anon., 1997a).

In most countries the data requirements for MPs are grafted upon those for chemical pesticides. Currently, efforts are made to finetune and improve these requirements, thus detaching them from the chemical pesticides. This is a valid development from a scientific point

of view, for, contrary to chemical pesticides, microbial pesticides may survive and reproduce in the environment, to subsequently infect or cause diseases in non-target organisms.

Data requirements are defined within the context of this report as data in a more broader sense, including the dossier requirements, as the criteria for the dossiers should result into appropriate data that subsequently can be used for risk assessment. Therefore the term "data requirements" has been preferred above "dossier requirements".

Microbial pesticides may be genetically modified or engineered. Therefore a framework for evaluating microbial pesticides should include a section dealing with genetically-modified or engineered micro-organisms. Genetically-modified micro-organisms are those modified through alteration of genetic material (incl. genetically-engineered micro-organisms); genetically-engineered organisms are those modified through *in vitro* alteration of genetic material. It can be expected that especially the latter group will be used increasingly in the near future.

2. METHODOLOGY

The OECD (1996) survey on data requirements for the registration of biological pesticides was the starting point for this present report. Various experts dealing with this issue were contacted a.o. for hard copies with (inter)national data requirements. However, a selection was made with respect to the countries and institutions "to be interviewed", as the time schedule for this study was limited. Therefore those countries and institutions were approached that were considered to be "key countries", this inevitably with a touch of arbitrariness. The result of this approach is that the inventory in this report is not exhaustive, but adequate enough to comprise the whole mondial spectrum of current registration requirements.

As data requirements should never be seen independently, it is attempted to link these requirements with the particular goal of risk assessment.

Consulted experts:

- M. Lans (CTB, Wageningen);
- E. Müller (Plant Protection Service, Wageningen);
- J. van Emden (RIVM/LIO);
- L. Smeets (European Commission, Bruxelles);
- D. Dinkins (EPA/OPP, Washington);
- N. Grandy (OECD, Paris);
- H. Pederson (Danish EPA, Copenhagen);
- B. Schreider (KEMI, Solna);
- G. Lewis (Pest Management Regulatory Agency, Ontario);
- A. Wilkening (BBA, Braunschweig).

3. RESULTS

All countries that require experimental data on the environmental fate and behaviour and on ecotoxicological effects of MPs require adequate research facilities, if this research has to be performed within their borders. Laboratory and greenhouse studies with non-indigenous MPs or GMMs in e.g. Canada should be in accordance with the Laboratory Biosafety Guidelines (Health Canada and Medical Research Council of Canada, Cat.No.MR 21-1/1990E) to meet the demands on proper containment and disposal (Anon., 1993a).

The environmental data requirements in four countries (US, Canada, Denmark, The Netherlands) and those proposed by the EC are listed in Table 3. This "key"table rather reflects the different point of views, than pretending to be exhaustive. The listed elements will be further elucidated below. An empty cell in Table 3 does not necessarily mean that no data on this issue are required at all.

There is no consistent framework on how the assess the (potential) environmental risks, prior to the regulatory admission of a microbial pesticide. Therefore there is no consistent view within the countries and institutions that were investigated, on what to do with the required data. These risk assessments are primarily performed on a case-by-case basis.

3.1 Non-genetically-modified microbial pesticides

EC

The current discussions on the environmental data requirements for microbial pesticides are focused on the proposals of the European Commision. A group of experts in Brussels in November and December 1996 has agreed on an EC proposal (Anon., 1997a,b) that will be discussed by the Stranding Committee on Plant Health after consultation of all EU countries individually. In advance of these discussions, the national efforts to establish guidelines on this issue stagnate. The EC proposal will also be discussed within the OECD. The OECD has taken up the discussions since 1992 (OECD, 1994, 1996).

The EC intends to propose data requirements as summarised briefly in Table 3. These requirements refer to both Annex II and III of the EC Directive 91/414/EEC. They give a frame of reference for the CA in the EU countries. It is intended that the latter will determine the format of the summaries and evaluations. The EC proposal recommends — though not strictly prescribed — a two-tiered data requirement framework: first, data on the *identity* (name and species description, composition), *biology* (target specificity, infectivity, dispersal, colonisation ability, similarity with other pathogens, genetic stability, and the presence of toxins), and *data on the production and the intended use* (e.g. type, natural occurrence, safety measures); second, various basic laboratory tests are required, if no appropriate risk assessment can be performed

on the basis of the first group. All these elements of the EC proposal have been summarised in Table 3.

The environmental risk assessment for the product should preferably be performed in accordance with the EPPO/Council of Europe decision-making schemes (Anon., 1997b). These schemes were published by EPPO (1993, 1994). Remarkably, this preference is stated in the EC proposal related to the evaluation of the product (Annex III; Anon., 1997b), and not in the proposal for the active ingredient (Annex II; Anon., 1997a). Environmental risk assessment is not necessary according to the EC proposal, if exposure for organisms is unlikely.

OECD

The OECD surveyed data requirements from various countries for registration of biological pesticides (OECD, 1996). It was shown that the EU and most non-European countries require basic acute toxicity, infectivity, and pathogenicity tests (birds, freshwater invertebrates, fish, honey bees). Eight of the 16 responding OECD countries — incl. Hungary as a prospective member — reported that these basic acute tests were frequently (i.e. 80-99% of the notifications), rather than always required. Acute tests on other organisms — e.g. algae, estuarine or marine animals, earthworms, wild mammals — were seldom or never required. Long-term studies and field tests were also seldom or never required. The EU and six other countries said they might require tests on the fate and behaviour of MPs in the environment. However, only seven of all responding countries stated that they *always* required these environmental tests.

US

In the US each variety of a species of a microbial pest control agent should be tested. The EPA is responsible for the registration of the "natural" microbial pesticides. The registration of non-indigenous or biotechnologically engineered pesticides, is the responsibility of APHIS of the USDA (Copping, 1996). Delfosse (1996) of the National Biological Control Institute of the USDA proposes that one should distinghuish between *precedented* and *unprecedented* organisms. A precedented organism is an organism for which a permit has been granted in the past by APHIS and/or by one or more State Departments on Agriculture for importation, interstate movement, or release into the environment. NBCI proposes that a notification for the release of a precedented organism into the environment should contain a certificate of the applicant "that no catastrophic population-level non-target effects are known to have been caused following past releases of the precedented organism, or evidence indicating no likely potential non-target hosts occur in the new location, or that such potential effects have been evaluated for possible negative effects" (Delfosse, 1996). APHIS distinguishes two types of potential risk due to the introduction of an unprecedented biological pesticide. First, a plant pest risk, especially by plant-feeding/attacking organisms, including, for convenience, plant pathogens. Second, a non-target species risk, e.g. for Threatened and Endangered species

(T&E). A US proposal for a two-tiered system with data requirements for the release of an unprecedented organism into the environment is shown in Appendix 1 (conforming to Delfosse, 1996). Customers (e.g. farmers, agronomists) of APHIS propose some exclusions from regulatory oversight: e.g. precedented organisms for which there is no "documented damage at the population level of non-target species". Guidelines from the Office of Prevention, Pesticides and Toxic Substances (OPPTS, 1996) of the US EPA state that the ecotoxicology requirements are processed into a four tiers system. The purpose of this system is primarily "to develop data necessary to assess the potential hazard of MPs to terrestrial wildlife, aquatic animals, plants, and beneficial insects" (EPA, 1989). The EPA wants a high level of confidence that no unreasonable adverse environmental effects will result from the actual use of MPs. Tier I includes short-term, single — non-target — species testing with a maximum dose. If adverse effects are ascertained in these tests, Tier II tests are required to estimate the potential exposure for populations in their environments. If, then, a significant potential exposure is ascertained, Tier III tests are required to establish dose-effect relations — also chronically, if appropriate (e.g. with fish and wildlife). Then, it can be concluded whether the minimum effective dose is less then the expected concentration, or whether there are factors in the environment that might decrease the observed effects. Tier IV tests will follow on a case-by-case basis under simulated or actual environmental conditions, if the lower tiers testing indicate potentially adverse effects on the environment.

NTOs in the acute ecotoxicity tests of Tier I should be tested at the "maximum hazard dosage level"(EPA, 1989). This highest daily oral dose for birds is the number of active units (AU) in the product *times* the amount of MP per kg body weight *times* the weight of the test bird (e.g. an MP with 1×10^9 AU/ml and an application rate of 5 ml/kg body weight, that is fed to a 25 gram quail, results in a max. daily dose of 1.25×10^8 AU). This "maximum hazard dosage level" for fish — in case of aqueous exposure — is at least 10^6 AU/ml water, or at least 1000 times the concentration max. calculated in a 15 cm water layer, immediately following application.

Waivers — resulting in reduced data requirements — are granted by the EPA in case of (Copping, 1996):

- low exposure pesticide formulation (e.g. traps, controlled release formulations),
- low rates of application (20 gram or less AU per 4000 m^2),
- non-aquatic use sites (applied directly to land),
- high volatility (reduces likelihood of residues in water, sediment, soil, or vegetation)

Canada The framework for evaluating environmental effects of microbial pesticides in Canada can be compared with this framework in the United States (Anon., 1993a,b). Contrary to other countries, the US and Canada have more detailed requirements with respect to both the dossier format of the evaluative summaries — that have to be submitted by the applicant to the CA — and the scientific test data needed for a proper evaluation, listed in fairly detailed test protocols. It should be noted that the Canadian framework, as presented in Table 3, is still part of a so-called Regulatory Proposal, and as such not implemented in the Canadian legislation.

There are, however, some differences between Canada and the US with respect to the requirements. The final goals of the evaluation process are most clearly defined by Canada (Anon., 1993a,b). The purposes of environmental fate and ecotoxicological testing as required by the Canadian Plant Industry Directorate are stated in Table 2.

Table 2. Purposes of environmental and ecotoxicological testing in Canada (conforming to Anon. 1993a)

	PURPOSES
	the growth characteristics of an MP
	the ability of an MP to propagate or persist in a niche or host after it has been introduced into the environment
	the ability of an MP to disperse beyond the area of application
	the ability for genetic information introduced into an MP to be transferred to other organisms or persist in the environment
Ecotoxicology	the infectivity, toxicity, and pathogenicity of an MP to non-target organisms

The Canadian tiered system for testing the environmental fate and the ecotoxicology of MPs is represented in Figure 1. Environmental fate testing will be required for all indigenous MPs showing either poor host specificity or significant effects on non-target organisms of environmental and/or economic importance. First, — if laboratory tests are insufficient for proper risk assessment — small-plot field studies will be required. In view of the results of these studies and the results of the ecotoxicological studies — Tier I studies for non-indigenous and Tier II studies for indigenous MPs (see Figure 1) —, it can be decided by the CA to require large-plot field studies on environmental fate and behaviour.

The Canadian approach contains four tiers in which the ecotoxicology and environmental behaviour tests are intermingled. The first is a base set of laboratory tests with *Max. Challenge Concentrations* (MCCs) (Tier I). If results of these are positive, additional laboratory or field tests with *Max. Environmental Concentrations* (MECs) should be performed (Tier II). If these are positive, additional laboratory or field tests must be performed, taking life-cycles and dose-effect relations into account (Tier III). Finally, small

plot-field tests may be performed to simulate actual use under natural conditions (Tier IV). It should be noted that the Canadian approach is still a regulatory proposal, whereas the US approach is implemented into legislation.

All these field studies should be performed in the ecozone[4] where the MP is intended to be used. The Tier II and III ecotoxicity tests should be performed with both the microbial organism (active ingredient) and the product in which the MP is formulated. Ecotoxicity tests should be done with MCCs in Tier I and with MECs in Tier II. The MCC is at least 1000 times higher than a calculated concentration — e.g. with a model — that represents a worst-case scenario. In an acute oral toxicity test with birds, the test dose — single dose in Tier I — should be at least 1000 times greater than the maximum daily intake expected in the field under a worst-case scenario. However, if it is known that concentrations of an MP significantly increase in the environment, — e.g. viruses in insects — the dose should be at least the highest possible dose in the field. In the toxicity tests with fish, terrestrial and aquatic arthropods (freshwater/estuarine/marine), micro-organisms, aquatic and terrestrial plants the single doses should in general be $\geq 10^6$ AU per gram (fish feed, soil, water) *or* $\geq$ 1000 times the expected MEC in a 15 cm layer (water, soil).

In general, Tier IV field ecotoxicity tests should be conducted at maximum application rate, and under those conditions optimal for disease development. Only those non-target species need to be tested that may potentially be susceptible for the MP according to Tier II testing.

The absence of experimental studies on the potential toxic, infectious, or pathogenic effects of MPs on NTHs should be supported by sound scientific evidence.

Two Tier I short-term toxicity tests with juvenile fish should always be performed: one with application of the MP to the fish feed; the other with application of the MP to water in which the fish swim. Tier II tests should also be executed with actively feeding juvenile fish.

If the application of the MP — intended for controlling arthropods — is directly to soil or water, initial toxicity tests with arthropods should be performed. Terrestrial arthropods should be exposed topically, dietary or a combination of these. An observation period of 30 days, or until mortality in the control increases significantly, is recommended. Aquatic arthropods should be exposed via the water for at least 48 hours — or the time necessary for an effect, whichever is

4 Large and very generalised ecologically distinctive area based on the interaction between biotic and abiotic factors.

Table 3. Data requirements for environmental risk ass[illegible]ent of microbial pesticides as proposed by the EC [illegible] those in various countries (partly not yet implemented in legislation)

type of data	EC	US	CAN	DK	NL
name & species description	natural type? (non)indigenous? mutant? stock variant? manipulated? GMM? method of identification?	product identity? information on ingredients? morphological type? genetically altered?		for viruses: taxonomic designation of the agent, serotype, strain or mutant?	criteria for nomenclature [*morphological, biochemica serological*]?
composition test material/formulation	micro-biological purity [*e.g. as number of active units per volume or weight*]? impurities? additives? extraneous micro-organisms? toxins? development phase [*e.g. spores*]? type of formulation and ingredients? appearance , storage stability, and physicochemical data of formulation?	recognised units of potency? MP units per unit weight or volume of product? unintentional ingredients [*e.g. human, non-target animals pathogens*]? [*manufacturing process and purification steps should be reported fully*]	active ingredients [*CFUs, spores, conidia*] per unit weight or volume?	contaminants [*i.e. microbial contaminants and their products*]?	biologically active ingredie per unit weight or volume? other ingredients [*gram/litre*]? other pesticid [*gram/litre*]? purpose of ine ingredients?
use & natural occurrence	data on application dosage [*gram, kg or litre for the product, and appropriate units for the MO*], type? country, region? ecological context of e.g. the product vs. the natural type [*type of ecosystem, e.g. soil from which the MO was isolated*]?	natural occurrence?		known distribution & habitat? previous use? function [*e.g repellant, insecticide, growth regulator*]?	
target organism, infectivity, dispersal & colonisation ability	mode of entry of MO or toxin into TO, fate and distribution in host, mode of action [*e.g. fungitoxic or fungistatic*]? infectivity? transmissibility? persistence in host?, environmental requirements for survival, reproduction, and colonisation? temperature range for growing?	mode of action?	amount of inoculum necessary for infection?	is the micro-organism pathogenic or antagonistic? is it growth regulating? does it induce resistence of a plant against target species? infective dose? transmission to other targets/non-targets by the micro-organism itself, via a vector, via abiotic factors? does it invade e.g. immunosuppressed animals?	system for distribution [*e.g sporulation, vectors, precipitation, via air*]? persistence in target host [*dependent on biotic and abiotic factors, e.g. temp., humidity, light*]? infectivity *vivo*?
host specificity	effects on non-target organisms nearby target organisms?	pest host range?	specificity for close phenetic and/or phylogenetic species?		which — other — organisr may be affected?
biology micro-organism	life-cycle? occurrence of resting stages? virulence?	life-cycle?	optimum and range for growth and persistence MP [*see below*]?	effect of temperature, light, humidity, pH and the concentrations of certain substances? methods to prevent virulence?	
relationships with known pathogens	how to distinguish active and extraneous micro-organisms from known plant, animal, or human pathogens?				
genetic stability	mutation rates? reversion tendency? capacity to transfer genetic material to closely related species? plasmid stability?			mutation rate? plasmid stability? genetic material transfer by conjugation, transduction?	does genetic instability changes the biological properties, host specificity

Table 3. Continued

type of data	EC	US	CAN	DK	NL
toxins	structure? (eco)toxicological, environmental effects? mode of action? effects on non-target species [*human, animals*]? stability of toxins?			stability? structure & function? mode of action? effects on non-target species?	
persistance and multiplication in environmental compartments [*e.g. soil, leaf litter, water, sediment*] *laboratory data* *field data*	population dynamics in seasonally or regionally extreme climates? competitiveness in the agricultural field after use? viability in four aerobic and one anaerobic soil? viability/partitioning in sterile/natural, dark/illuminated water/sediment systems?	is an MP able to survive or replicate in the environment, therebyindicating which NTHs may be exposed [*these data are "Tier II environmental expression data", and are required, if Tier I tests with NTOs show adverse effects*]? [*Tier II tests*] [*Tier III tests*]	optimum and range of factors required for growth and subsistence [*e.g. light, pH, humidity, osmotic pressure, carbon, nitrogen, response to antagonists*]? ability of transferring genetic information into other organisms? fate in soil, vegetation, leaf litter, water, sediment? results ecotoxicology tests [*see Fig.1 for interaction environmental fate and ecotoxicology studies*]? physical and chemical data comparable with the ecozone of application? pure culture vs. microcosm tests? disturbance of the media minimised? relevant data on growth, reduction to environmentally acceptable levels, persistence, dispersion, transfer of introduced genetic information?	possible fate in food chains? is a toxin expected to occur in the environment [*stability, occurrence independent of the micro-organism*]?	non-indigenous MOs: persistence and potential multiplication in soil [*particularly important for GMOs*]? fate, persistence, and multiplication in water/sedime systems [*always required, unles no emissions to water*]?
mobility in the environment	adsorption potential in four soils? mobility of micro-organisms, degradation products and particular life-cycle stages? ability to colonise adjacent areas?		airborne dispersal MP?		mobility in various soils?

Table 3.Continued

type of data	EC	US	CAN	DK	NL
organisms in general *laboratory* *field*	[*MOs may induce not only toxic , but also pathogenic and/or infectious effects*] toxicity? pathogenicity/infectivity? [*the choice of appropriate NTOs should be primarily based on the MO identity and its biology (incl. host specificity, mode of action, ecological niche*]	**hazard to terrestrial wildlife, aquatic animals, plants, and benefecial insects?** [*Tier I, II, III, IV*] [*if relevant*]	**effects of additives?** **effects of max. challenge concentration** [*Tier I*]**? effects of max. concentration to be expected in the environment** [*Tier II*]**? effects on life-cycle stages of NTH** [*Tier III*]**? ability to transmit MP to descendants of NTH** [*Tier III*]**?** LC_{50}**,** LD_{50}**,** EC_{50} **due to toxins** [*Tier III*]**?** **effects in small-plot field studies?**	**effects on non-target animals via secondary poisoning?** [*Tier I, II and III*] **effects: infective/pathogenic or toxic? choise for test animals** [*case-by-case*]**: is the micro-organism non-indigenous, is it distributed directly to water, is it to be used in big or small quantities, does it produce toxins?**	
mammals	is the MO able to infect, to cause illness, allergy, genetic changes, or health effects? the relative hazard due to different routes of exposure?	**pathogenicity? infectivity/unusual persistence? toxicity of MP, microbial contaminants, preparation bye-products?** [*preferably rats or mice for acute oral toxicity/pathogenicity tests*]	**effects indicated by tests with small rodents performed for assessing human toxicology?**		infectivity *in vivo* [*body temperature*]? allergenic potential? known pathogen to mammals [*active ingredient, contaminant, mutant*]? [*minimally, acute tests with sm mammals on oral and intraperitoneal toxicity are required*]
birds [*short term exposure*]	acute infectivity/toxicity [LD_{50}]/pathogenicity?	**pathogenicity/toxicity after single maximum hazard dosage level?** [*at least 10 birds per treatment; one insectivorous, one herbivorous species*]	**effects after oral and respiratoy exposure** [*two species: Colinus v. and Anas p.*]**? oral P/T? intraperitoneal** [*fungi/protozoa*] **or intravenous** [*viruses/bacteria*]**? effects MCC** [*Tier I*]**? histopathology? gross necropsy? organ weights?**	[*use test conforming to EPA (1989), 154A-16, Tier I; at least one free-living species in the area of application*]	is exposure likely [*then, acute oral toxicity test with at least tv species*]?
fish [*short term exposure*]	acute infectivity/toxicity [LC_{50}]/pathogenicity to freshwater fish?	**pathogenicity/toxicity after single maximum hazard dosage level? initial MP concentration maintained during test?** [*two species in case of direct aqueous exposure; ten young fish per treatment; observation for at least 30 days*]	**effects on young juvenile fish over 30 days** [*I/P/T and/or hypersensitivity*]**? effects due to intake feed? effects due to contaminated water? results Tier I tests** [≥ *6 replicates during exposure;* ≥ *3 replicates during recovery*]**, results** LC_{50} **tests** [*3 replicates per concentration*]**?**	[*use test conforming to EPA (1989), 154A-19, Tier I; at least one free-living species in the area of application*]	infectivity for fish?

Table 3. Continued

type of data	EC	US	CAN	DK	NL
aquatic invertebrates [*short term exposure*]	acute toxicity/pathogenicity/infectivity to freshwater invertebrates?	**pathogenicity/toxicity after single max. hazard dosage level? effects on freshwater/estuarine/marine species?** [*observation at least 21 days after application*]	effects on freshwater or estuarine/marine arthropods *[if the MP is against aquatic arthropods, 20 species in freshwater or 10 species in estuarine/marine water; 21 days of observation]*? effects due to diet? effects due to aqueous exposure?	[*use test conforming to EPA (1989), 154A-20, Tier I*]	infectivity for water fleas?
algae [*long term exposure*]	effects on growth and growth rate?	**effects on *Selenastrum capricornutum*, *Skeletonema costatum*, *Anabaena flos-aquae*, and a freshwater diatom [*in case of aquatic use*]?**		[*use test conforming to EPA (1989), 154A-22, Tier I; at least one freshwater algal species existing in the area of application*]	infectivity for algae?
bees [*short term exposure*]	infectivity and pathogenicity? toxicity, if relevant [*oral and contact*]?	**toxicity/pathogenicity to honey bees [*if bees may be exposed*]?**		[*use test conforming to EPA (1989), 154A-24, Tier I*]	toxicity to bees [*in case of application on flowering plants*
terrestrial arthropods [*excl bees*] [*short term exposure*]	infectivity and pathogenicity? toxicity [*mortality and sublethal effects*]?	**toxicity/pathogenicity to e.g. parasitic dipterans, predaceous hemipterans, and predacious mites [*if insect predators and parasites may be exposed*]?**	effects on terrestrial arthropods *[20 different species, if the MP is against arthropods; at least one species from Araneae, Acari, Crustacea, and two species from Insecta; observation period for 30 days]*? effects due to diet? effects due to topical exposure?	*[if the non-target organisms — e.g. parasites/predators — are arthropods other than bees, use test conforming to EPA (1989), 154A-23]*	toxicity to beneficial arthropo [*in case of application in integrated pest management*]?
non-arthropod invertebrates [*short term exposure*]	acute toxicity, infectivity, pathogenicity [*preferably, the highest concentration causing 100% mortality, and the lowest causing no mortality*]?		effects on e.g. earthworms, molluscs?		toxicity to earthworms *[in cas of soil persistence]*?
plants		[*the number of plants tested depends on the MP similarity to known plant pathogens*]	effects on aquatic/terrestrial plants? phytotoxicity/phytopathogenicity?		
micro-organisms soil/water			effects on biogeochemical processes in a microcosm, relevant to the area of application?		

greater — tests. In case of aquatic arthropods, an observation time of at least 21 days — or until mortality in the control increases significantly — is recommended. The actual concentration and infectivity potential should be monitored during the tests.

If intended for the control of micro-organisms, the testing of MPs on environmentally and/or economically important micro-organisms, and on particular microbial biogeochemical processes should be performed. The use of microcosm studies is recommended to investigate relevant biogeochemical processes as carbon and nitrogen cycles and the degradation of cellulose (rather than qualitative or quantitative changes in the microbial populations). Recommended observation times are at least 30 days *or* the time needed to reach pre-treatment levels, whichever is greater.

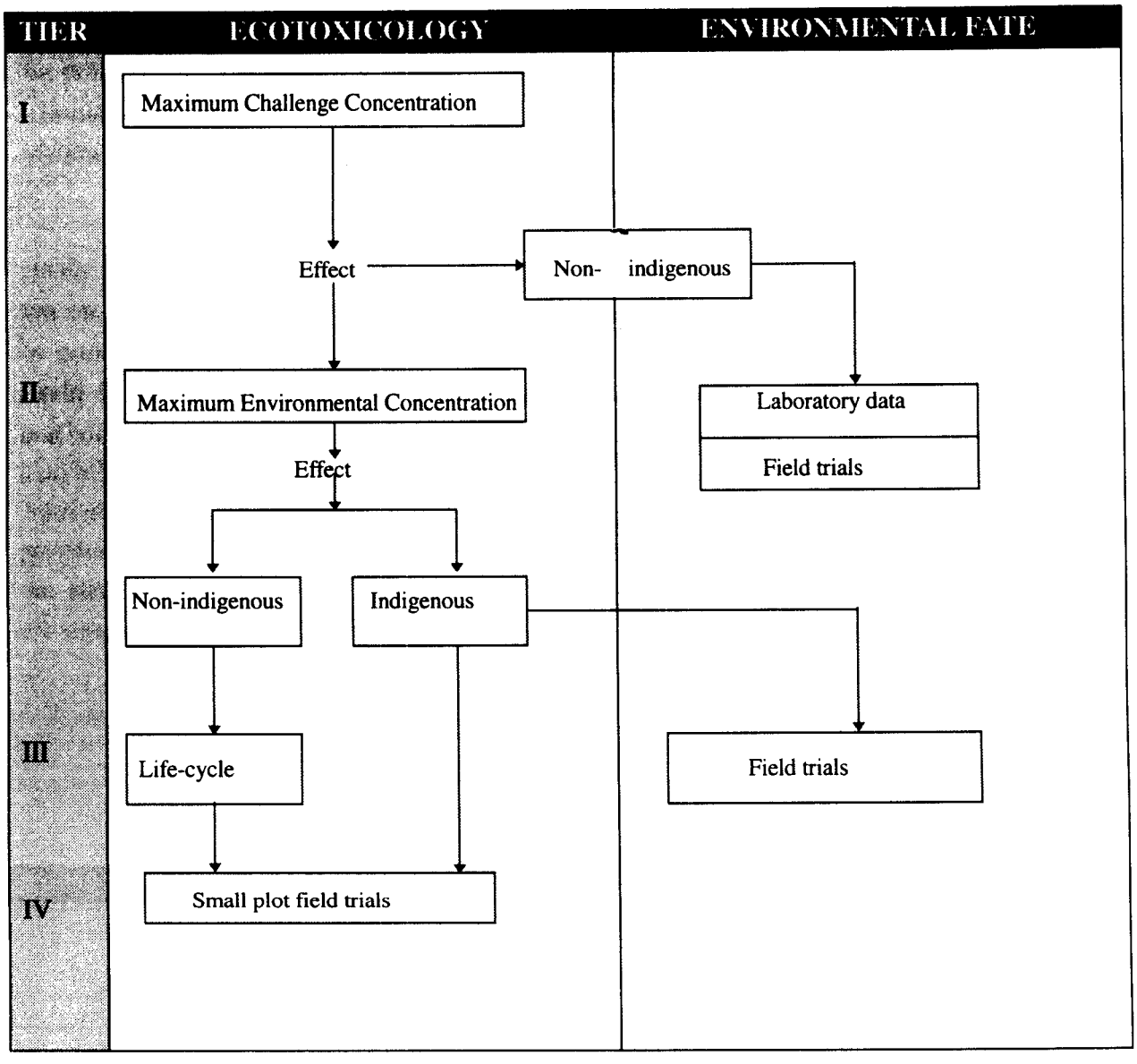
Tier II toxicity tests with non-target plant species should be executed with those plants that show an effect or an infection in viable tissues in Tier I testing. Senescent leaves are not considered relevant to trigger Tier II testing. Tier I testing focuses on the determination of infections or effects on viable tissues, possibly leading to a diminished vitality of the plant. Different parts of the life-cycle may be studied: e.g. germinating seed (incl. seed emergence and root elongation) and vegetative growth stage. It is proposed by the Canadian CA to require data on the effects on one species of algae per family (*Chlorophyceae*, *Cyanophyceae*, *Bacillariophyceae*), and on one species of freshwater plants per family (e.g. *Lemnaceae*, *Potomogetonaceae*, *Juncaceae*, and *Typhaceae*). Guidelines for the size of field trials are represented in Table 4. Further details on a Canadian proposal on specific data requirements for small-, medium-, or large-scale field studies are described in Appendix 2.

Table 4. Guidelines for field studies (Canadian proposal, Anon., 1993b)

	Terrestrial (ha)	Aquatic (ha)	Forestry (ha)
SMALL	up to 10	up to 1	-
MEDIUM	10-1000[5]	≥ 1	up to 3000
LARGE	1000-5000[5]	-	-

[5] Or ten % of the total national crop, whichever is smaller.

Figure 1. Tiered test system for the environmental fate and ecotoxicology of MPs in Canada (conforming to Anon., 1993a)



Environmental risks are theoretically possible when a large quantity and/or a non-indigenous MP is introduced into the environment (Anon., 1993a). Canada suggests a *centrifugal taxonomic approach* for selecting potential non-target hosts. This implies that initially those hosts are selected that are closely related to the target host in a phenetic (i.e. pheno- and genotypic) and/or phylogenetic way. Thereafter less closely related organisms are selected. Other NTHs that may be selected are those:

- known or suspected to be infected by the MP;
- known or suspected to be infected by micro-organisms closely related to the MP;
- morphologically, physiologically, or biochemically related to the target host.

Environmental risks of non-indigenous MPs may be cogitated as unacceptable, if the tests show poor host specificity and/or significant effects on environmentally or economically important NTHs.

Denmark Environmental risks potentially related with the use of microbial pesticides — strictly defined as those MPs with *living* organisms — hinge on the type of organism, the use pattern of the MP, and the environment in which the MP will be used (DEPA, 1994). The Danish approach of environmental risk evaluation on the basis of submitted dossiers consist of three tiers, revealing basic and additional information, subsequently. Conforming to the Canadian and US approach the ecotoxicological tests should preferably be performed in a tiered way: the first tests as a worst-case scenario — for qualitative, screening purposes with respect to infectivity/pathogenicity/toxicity — the second, if necessary, as a realistic scenario, for quantitative data to establish e.g. dose-effect relations or infective doses. Field and microcosm studies need only to be performed, if "experimentally substantiated theories on specific environmental effects shall be confirmed under field or realistic conditions". It is considered very important, that non-indigenous micro-organisms are tested on "spread, survival, establishment, and horizontal gene-transmission". Particularly those MPs should be focused upon, that can form steady populations after release in a new niche. An inventory of endpoints that could be focused upon, is listed in Table 5.

The Danish EPA recommends the following guidelines, if testing microbial pesticides for their behaviour in the environment and their ecotoxicology: OECD — if applicable —, EPA (1989), the EPPO guidelines for efficacy evaluation of plant protection products, the Annex of Commission Directive 92/69/EEC, or Commission Directive 87/302/EEC.

Table 5. Possible endpoints to be studied (DEPA, 1994)

Organisation level	Endpoint
Ecosystem	energy pools and energy conversion, substance pools and substance cycling, nutrient cycling,
Community	community structure and composition, diversity, productivity,
Population	genotypic and phenotypic variation, biomass, fecundity,
Organism	respiration rate, behaviour, growth, death.

Acute ecotoxicological tests with birds have to be performed, if the micro-organism is a built-in part of a product used outdoor (see also Table 3; DEPA, 1994). Acute ecotoxicological tests with water fleas or fish have to be performed, if the micro-organism will be applied by direct release to water outdoor, *or* if the micro-organism will be used outdoor, is non-indigenous to the area of application, and large quantities are expected to be transmitted to aquatic environments (e.g. in forests, and on widely distributed crops). If the micro-organism is a built-in part of a product used outdoor, an acute study with bees is obligatory. If it — besides the intention of outdoor use — is suspected to affect earthworms, an acute study with these is obligatory. Additional data may be required, if there are indications of possible harmful effects on species (flora or fauna) closely related to the target species. Additional data may also be required, in case of toxin production by the notified micro-organism, if such toxins are assumed to have potentially adverse effects, in combination with a considerable persistency.

In addition to the data required for the regulatory admission of the micro-organism as the active ingredient, extra data may be required with respect to the admission of the whole product (DEPA, 1994). Testing the potential adverse effects of a *product* for beneficial organisms — other than bees — is e.g. required, when this is indicated by both ecotoxicological studies and the studies for toxicity and pathogenicity to test mammals, and by knowledge on the dispersion into, and the persistence in the relevant environmental compartment. Test species, then, may be

crayfish, shrimps, other pollinators than honeybees, other vertebrates than those studied, and pest predators as ladybirds. Tests with the product on soil microflora are also obligatory "(1) if use implies direct application to the soil or if the product is intended to protect plant parts in the soil *or* (2) if the product is non-indigenous to Denmark, and if it has shown ability to multiply in the soil".

The Danish Ministry of Environment published clear "biographies" of seven micro-organisms with the emphasis on human health effects (Christiansen et al., 1991). Although these micro-organisms were no agricultural pesticides, the biographies may serve as examples of a proper evaluation, also pertaining to environmental effects, as many basic data — e.g. identification, general biology, survival strategy, pathogenicity — are also required for an environmental evaluation. The micro-organisms were *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Escherichia coli* K12, *Lactococcus lactis*, *Aspergillus oryzae*, and *Saccharomyces cerevisiae*.

Nether-lands

The data requirements for the registration of microbial pesticides are less formalised than for chemical pesticides (CTB, 1995a), primarily due to the larger variety in properties. If the mode of action of an MP is by means of a toxin, the data requirements are in accordance with those for chemical pesticides. Generally, micro-organisms that are closely related to vertebrate — incl. men — pathogens, are not registered. The required data as stated in the particular application form for the regulatory admission of a biological pesticide by the Dutch CA (CTB, 1995a) are listed in Table 3.

Analyses on the purity and composition of the product with the MP should be performed max. one year prior to the application for registration (CTB, 1995a). It is particularly important that the technical grade (i.e. non-formulated) active ingredient is checked for the occurrence on pathogens for men, animals or plants. It also may contain components of e.g. the nutrimental medium, or remnants of cells in which a virus was cultured.

Juvenile or young adult small rodents should be tested orally with a single, high dose, that equals the ingestion, if exposed to the recommended amount of MP per hectare, and does not exceed 5000 mg/kg b.w. (CTB, 1995a). As infectivity may occur, body temperature should be monitored. After an observation period of 28 days, urine, faeces, blood, spleen, kidney and liver should be tested for culturing micro-organisms *in vitro*. Besides histopathology and macroscopy, some blood parameters should be monitored regularly, as they are considered important indicators: blood sedimentation, the amount of hemoglobine, the number of leucocytes, erythrocytes, thrombocytes, and a hemogram.

Acute intraperitoneal tests with small rodents should be performed with a single dose of minimally 10^7 bacteria per rat or 10^6 per mouse (equivalent amounts for other micro-organisms and viruses) (CTB, 1995a). Histopathology of the kidneys, liver and spleen is important, even if these organs are not affected.

In case an acute inhalation toxicity test is required, small rodents — preferably rats — should be exposed for four hours to a concentration as high as achievable (CTB, 1995a). Histopathology and macroscopy of the lungs are particularly important.

In vitro infectivity tests with culture media of mammalian tissues may be particularly important for protozoa and viruses (CTB, 1995a). Generally, these *in vitro* tests are more sensitive than *in vivo* infectivity tests. If viruses are tested such, these cultures may provide material to test the potential incorporation of viral DNA into the genome of the host cell.

In case an MP is a micro-organism that occurs naturally in the Netherlands no data have to be submitted to the CA concerning the fate and behaviour in the environment, and the toxicological and ecotoxicological effects (CTB, 1995a). If the micro-organism is non-indigenous, it is particularly important to investigate the biological properties.

The Dutch Board for the Authorisation of Pesticides published in 1992 a proposal for the further development of the evaluation framework for agricultural, microbial pesticides (Wäckers, 1992). Up till 1997, this proposal has had no follow-up. The relevant items in the proposal are listed below:

I. type of micro-organism (fungi, bacteria, virus);
II. pathogenicity for vertebrates;
III. results from toxicological research;
IV. exposure (human, environmental);
V. field of application (crops? closed space?);
VI. application techniques;
VII. indigenous occurrence (on the crop?, in the Dutch environment? in a specific environmental compartment?);
VIII. multiplication in the environment;
IX. persistence in the environment.

The part of this proposal dealing with the data requirements on the environmental fate and behaviour of microbial pesticides is represented in Appendix 3. Ecotoxicological data requirements are represented in Appendix 4.

United Kingdom

The main goals of the environmental evaluation by the UK CA are (PSD/HSE, 1995):

- predicting distribution and fate in the environment, and the time course involved;
- identifying non-target-species and populations at risk, and predicting the extent of potential exposure;
- evaluation of the short and long term risks for non-target species — populations, communities, and processes — as appropriate;
- evaluation of the risk of fish kills, and fatalities in large vertebrates, or terrestrial predators, regardless of effects at population or community level;
- identification of precautions necessary to avoid or minimise contamination of the environment, and for the protection of non-target-species.

As the UK data requirements for microbial pesticides have been reported in a very concise way (almost no details on the type and quality of required data, the test protocol, and the test report; see PSD/HSE, 1995), these are not included in Table 3.

Within the evaluation framework of the UK, the infectivity of the MP to non-pest invertebrates that are closely related with the target organism should be linked with the target specificity of the MP (PSD/HSE, 1995). The acute dietary effects on two species of birds should be investigated. At least one of these species should accept insects in its diet (*Gallus* sp., *Coturnix* sp, or *Colinus* sp.). The effects on fish need to be studied in only one — indigenous — species. With viruses, these *in vivo* tests with birds and fish are only required, if primary bird or fish cell cultures are infected.

3.2 Genetically-modified microbial pesticides

In an OECD survey on data requirements for the registration of biological pesticides, seven of the 16 responding countries stated that they required gene transfer rates and data on competitiveness in the environment, when a GMO is to be registered as an MP.

The urge for an environmental evaluation framework is dependent on the particular type of GMO, as some will be developed and operational within a relatively short time, whereas others won't. Genetic modification of an MP — e.g. a mycoherbicide — can be helpful in increasing the virulence of a pathogen. Genetic modification of e.g. mycoherbicides is considered as technically readily achievable (Scheepens & Lotz, 1994). However, the knowledge on the gene(s) responsible for pathogenicity of these MPs — necessary for increasing the virulence — is lacking. Therefore, one may assume that at least with these MPs, it will take some time prior to operational use in the field, therefore decreasing the urge for an evaluation framework within a short time.

In a Regulatory Proposal (Anon., 1993a), it is stated that in Canada genetically-engineered MPs should be tested and evaluated on environmental fate and behaviour as non-indigenous MPs on the basis of Tier I ecotoxicity studies (see Figure 1; Anon., 1993a).

In the Netherlands, besides the approval of the Dutch Board for the Authorisation of Pesticides — additional approval from the Ministry of Housing, Spatial Planning, and the Environment is necessary, in case the MP is a GMO (CTB, 1995a).

4. DISCUSSION

Consistent frameworks with environmental data requirements for microbial pesticides are scarce. There is, however, a clear tendency in developing these frameworks, instead of relying on case-by-case evaluations. The US and Canada appear to have the most detailed approach, up till now. They require adequately reported test data down to the level of the individual tests (e.g. with fish, with terrestrial arthropods). Other countries refer to these requirements — e.g. Denmark and Sweden — or have a more general framework, sufficiently apt for a case-by-case approach — e.g. The Netherlands and the UK. The OECD and the EU will discuss a proposal by the European Commission within the foreseeable future. This EC proposal has been laid before small groups of experts and will be finally discussed by the Standing Committee on Plant Health. It emphasises the kind of required data, without stating explicitly e.g. particular test conditions which have to be fulfilled. It clearly sums up the aims of all required tests (in other words: the kind of conclusions that ought to be drawn). The proposal deals to a lesser extent with the requirements on how and what to report explicitly. It does not deal with the quality of the data. The EC proposal is a two-tiered framework, contrary to the four-tiered framework in the US and Canada, although it does not exclude additional studies based on expert judgment. It also does not clearly discriminate — with respect to the data requirements — between non-genetically- and genetically-modified micro-organisms. Nevertheless, it is a fruitful attempt for a more consistent approach within the EU. It should be noted that it was outside the scope of this study to investigate how the data requirements frameworks of the US and Canada, as formulated on paper, function in the "daily risk assessment practice". It should also be noted that — mondially — there appears to be no expertise on environmental risk assessment (incl. risk characterisation) in a more consistent way, rather than on a case-by-case basis.

Crucial elements in adapting the test battery are the specific taxonomical classification of the MP — as studies with related species may suffice — and the degree of species specificity — as this may determine the ecological range of impact (EPA, 1996). The importance of taxonomy should be taken in mind, as e.g. human and plant pathogenic bacteria have been best classified due to their health and economic importance, whereas particularly protozoa and fungi are generally less well studied (EPA, 1996). This may hamper the set up of a test battery for the "less well studied" group.

For a proper risk characterisation it is essential to know the life-cycle phase of the MP, that is the "active ingredient", e.g. a vegetative cell, (telio)spore[6], cyst, or virion[7]. Another essential item is that the adverse effects in a study have to be due to toxicity or pathogenicity caused by the test compound, rather than to other causes as e.g. anaphylaxis[8]. Therefore, the feed must be free of antibiotics, test animals or plants should not have a history of disease, and the measures or manipulations in dealing with a disease — apparently not caused by the test compound — should be reported conscientiously (EPA, 1996).

The data requirements presented in the preceding chapters are generally aiming at the evaluation of microbial pesticides with totally unknown properties. However, dependent on the type and species, various data will be known, prior to the registration. Therefore waivers may be granted. In most countries it is common practice that preliminary testing plans of applicants for MPs, are thoroughly discussed with the CA. The OPPTS (1996) states that particularly definitive ecological exposure data indicating no persistence in the environment are the best support for granting waivers.

There appear to be differences between the data requirements as formulated by the CA in protocols on one hand, and the actual evaluation practice on the other hand: in many cases, only a few tests are required if the MP is non-indigenous. If the MP is indigenous, ecotoxicity and environmental fate tests are seldom required. Generally, the test batteries are composed on a case-by-case basis by the applicant and the CA by mutual arrangement. In the Netherlands a "core" program is often performed: infectivity, "classic" LD_{50} or LC_{50} test, and tests to check for the occurrence of toxins (pers. comm. CTB to RIVM, 1997).

Actual test protocols — e.g like those of the OECD, dealing with the scientific and technical aspects (incl. their quality) — are generally lacking. This correlates with the case-by-case evaluations in most European countries.

The complexity with respect to the environmental evaluations of microbial pesticides may be increased by a combinative effect of e.g. an MP with a chemical pesticide, or two different MPs. An example of the first is the use of *Chondrostereum purpureum*[9] — a fungus needing fresh wood wounds for infection — in combination with the herbicide glyphosate (Scheepens & Lotz, 1994). An example of the latter is de combination of the two fungi *Alternaria macrospora* and *Fusarium lateritium*, the first for making openings in the host plant, and the second for the

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6 A teliospore is a winter spore, formed in rust fungi, which remains dormant all winter, and germinates the following spring.

7 A virion is a fully-formed, mature virus. An infection in a cell is initiated by a virion.

8 Effect is due to e.g. sensibilisation; then, the immune system runs wild.

9 *Chondrostereum* has yet not been registered in the Netherlands.

actual destruction. It may be expected that such combinative effects are not noticed in case of evaluating the fungi separately.

The ecology of micro-organisms may be decisive in evaluating their environmental effects. This is illustrated by the following examples (Scheepens & Lotz, 1994). The efficacy of pathogenic mycoherbicides as e.g. *Alternaria cassiae* against *Cassia obtusifolius* in soya bean crops, is especially limited by the air humidity: infection of host plants at low humidities do not occur. Therefore, research is focused on the natural selection of fungi with a short period necessary for infection — thus removing the water requirement as a limiting factor for germination —, and on those components in the formulation, that enhance the fixation of water. Other Dutch current research aims at the package of spores into polymers, as to enhance the germination by fixing sufficient water. It may be expected that such "improved water fixing" properties can have environmental consequences as well.

Another example of using the ecology of micro-organisms in the development of soil mycoherbicides is the notion that it is important that the active ingredient remains active long enough to reach its target (Scheepens & Lotz, 1994). Therefore those parts of the life-cycle are of interest, that can survive the best under natural conditions. An example is the exploitation of chlamydospores[10] of the fungus *Phytophthora palmivora* in the MP DEVINE. It should be envisaged that such an advantage from an agricultural point of view, may influence the potential environmental impact of this MP as well.

Adjuvants in formulations — e.g. emulgators and spreading oils — can complicate the risk characterisation of an MP. Contrary to chemical pesticides, adjuvants may be added that are particularly effective for an MP. The efficacy of many perthotrofic[11] fungi e.g. may be increased by adding substances enhancing the germination of spores (Scheepens & Lotz, 1994).

The production of new microbial pesticides is not easy: the number of competitive, efficacious chemical pesticides on the markets is overwhelming, and the costs of development are high. The processing of living micro-organisms is limited by the potentially variable properties of these, much more than when chemical pesicides are processed. Some technical difficulties are not easy to overcome: e.g. baculoviruses — used in forestry against insects — are rapidly deactivated due to sunlight. Environmental concern in itself is probably

10 Thick-walled fungus spores capable of surviving conditions unfavourable to growth of the fungus as a whole.

11 Parasitic fungus that obtains nourishment from host tissues after having killed them by a poisonous secretion.

not sufficient enough to function as an incentive for the development of new microbial pesticides. It can be expected that it should join hands with agronomical — e.g. increasing resistance of TO against conventional pesticides — and economical motives.

It is however generally recognised that the commercial agricultural market for microbial pesticides is expanding. This trend may be enhanced as more regular chemical pesticides and their applications will be banned. It is estimated that in 2020 about 15-20 mycoherbicides will be commercially available for Dutch farmers, market gardeners, and managers of the public green (Scheepens & Lotz, 1994). It can be predicted — based on current agricultural and economical trends — that around 2020 over the whole world c. 10 mycoherbicides may enter the commercial market per year. These figures support the necessity for adequate evaluation frameworks for microbial pesticides — at least for mycoherbicides. These increased interests in microbial pesticides, however, will be dependent on scientific research on the efficacy, the applicability, the commercial prospects, the (micro)biology, human and environmental impact of these pesticides.

5. CONCLUSIONS AND RECOMMENDATIONS

Data requirements

* It is considered useful to draw up a specifically Dutch data requirement framework as the starting point for evaluating the environmental impact of microbial pesticides. These data requirements must be the basis for environmental risk assessment. *As the EC and the OECD are currently discussing this issue, it is so much the better to draw up this framework, as the outcomes of a national discussion may directly feed the EC and OECD discussions. Of course, the communication with the EC and the OECD should be such that double work is avoided.*

* There is enough know-how and experience in and outside the Netherlands to base a data requirement framework upon. *It is preferable to construct a tiered system, in which the data requirements for the microbial pesticide (MP) are linked with the genetically modified organism (GMO). As the Canadian approach — though still under discussion — integrates the ecotoxicity and the environmental tests by a tiered system as being dependent on each other (see Fig. 1), the applicability of this approach in the Netherlands should be investigated.*

* It is recommended to reconsider the decision trees as proposed by Wäckers (1992) as a starting point for the further development of a Dutch data requirement framework. *This proposal lists for the applicant clearly which question, he/she has to answer, and when. Decisive questions are e.g. whether it can be demonstrated that the active ingredient during and after application comes into contact with the soil, and if this cannot be prevented, whether the active ingredient can naturally occur in the soil in comparable or even higher concentrations. Cut off values need to be implemented in these decision trees, and more detailed data requirements with respect to the quality of the scientific tests to be submitted should also be added.*

* It is useful to collect some finished environmental risk assessments from different countries as cases that can be compared with the data requirements "on paper". *Microbial pesticides in the European countries — with the exception of Denmark — are evaluated on a case-by-case basis. It would be of interest to pinpoint at the Danish regulatory practice to see whether the "American approach" is suitable for implementation in the Netherlands (additional to the EU guidance).*

Environmental risk assessment

* There is no expertise on a consistent environmental risk assessment for microbial pesticides, based on required test data. Therefore, such a framework should be developed simultaneously with a framework entailing which data to require for what purpose. *It is noted that the expertise on particularly GMOs may be helpful in this respect. It is also noted that a platform should be installed with various Dutch institutions to discuss further developments on the establishment of an evaluation framework for the environmental risk assessment of microbial pesticides. Relevant institutions are e.g. PD, CTB, VROM/DWL, LNV/Gewasbescherming, RIVM and RIZA. It is proposed to establish this platform as a part of the follow-up of this project. It should be noted that an internal note on the particular expertise within RIVM has already been made (Otermann & Linders, 1994). Examples of risk assessment schemes — primarily for chemical pesticides — can be found in EPPO (1993, 1994).*

* As research on the efficacy and applicability of microbial pesticides — apart from *Bacillus thuringiensis* — is in a relatively early phase, it is recommended to follow new developments, e.g. the recent research on mycoherbicides (see Scheepens & Lotz, 1994), and to structure and develop a framework for environmental risk assessment in dialogue with the researchers. *It may be speculated that probably within a short time, microbial pesticides — because of their selectivity — are only applicable in combination with chemical pesticides. Then an environmental evaluation should not only take the environmental impact of the individual MP into account, but also a decreased use of a compatible chemical pesticide.*

* It should be investigated whether a separate approach for agricultural versus non-agricultural microbial pesticides is useful or not. *No distinction is generally made in the current approaches for proper risk assessment of microbial pesticides. However, in view of differences between these two groups in e.g. application and emission type, this may be doubted.*

* It is recommended to subdivide the risk assessment framework into three subsequent parts (conforming to Van Leeuwen & Hermens, 1995): exposure assessment, effects assessment, and risk characterisation. It is also recommended to pay attention to the quality of data in relation to the intrinsic reliability of the tests, and to the usefulness for the risk assessment (for definitions see Mensink et al., 1995).

Risk management

* There is no experience on *consistent* environmental risk management for microbial pesticides, as it is primarily on a case-by-case basis. Such *consistent* management should be based on *consistent* risk assessment. *It is important to establish cut-off criteria which allow policy makers to decide on the regulatory admission on the free market. However, it should be kept in mind, that such a framework for risk management can only be accomplished in an iterative way [e.g. if GMOs enter the market, the cut-off criteria can be adapted, as it concerns an unprecedented organism].*

GLOSSARY

Data requirements	the scientific data required by the CA in a more broader sense, including the dossier requirements, as the criteria for the dossiers should result into appropriate data that subsequently can be used for risk assessment. Therefore the term "data requirements" has been preferred above "dossier requirements"
Infective dose	the dose to use on a target species to cause an infection with the intended effect. Determined by a bioassay
Infectivity	the ability of a micro-organism to cross or evade natural barriers before entering and multiply within host cells
Micro-organism	any microbial entity, cellular or non-cellular, capable of replication or of transferring genetic material. Micro-organisms — defined as such — are e.g. bacteria, fungi, protozoa, viruses, and viroids
Pathogen	micro-organism able to cause pathogenicity
Pathogenicity	adverse effect — generally a disease — caused by the direct interaction of a micro-organism and a host, following an infection. Dependent e.g. on susceptibility of the host
Phenetic	pheno- and genotypic
Specificity	the number of target species, a micro-organism is able to injure
Toxicity	adverse effect via a metabolite — generally a poison or a toxin — not necessarily following an infection
Transmissibility	the possibility of spread of a micro-organism in the target population, but also from one target species to another (target) species
Virulence	the ability of a micro-organism to injure an individual organism

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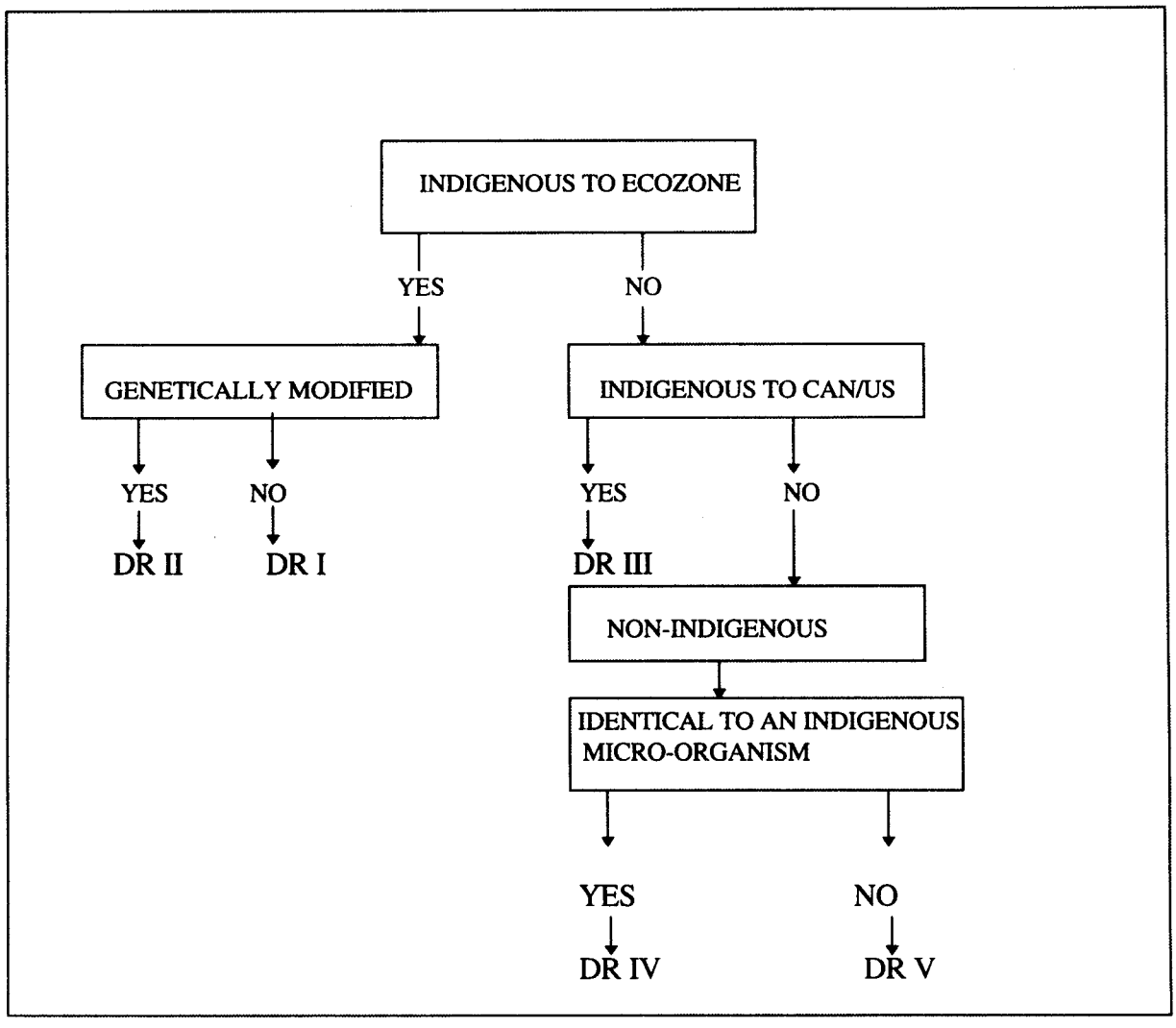
APPENDIX 1. Data Requirements as proposed by the NBCI in the US

Data Requirements for the release of an unprecedented organism into the environment as proposed by the NBCI in the US (Delfosse, 1996)

	DATA REQUIREMENTS
TARGET	**1. Scientific name (order, family, genus, author) and common name(s) if any, of target species, or host and site of action for antagonists, and competitors** **2. Native range, and if determinable, probable center of origin** **3. Current and estimated potential distribution in North America** **4. Related species in North America (at least to family, if there are many species)** **5. Pest status, including nature of damage, extent of losses, and availability of other control methods available for the target**
TIER 1 **[PLANT-FEEDING OR - ATTACKING AGENT]**	**1. Scientific name (order, family, genus, species, and author) and common name(s) if any of the biological pesticide, and name and qualifications of identifier** **2. Native range, and if determinable, probable center of origin** **3. Brief biology, including mode of action and potential for control of target** **4. Proposed source (country, laboratory, etc.)** **5. Related species in North America, a summary of their host range, and discussion of potential risk of population-level effects to non-target species** **6. Possible interactions with existing biological control programs and how these would be resolved** **7. Host-specificity testing program to be proposed, or which has been accepted by APHIS and FWS (includes list of test organisms, methods of testing, and results)** **8. Date(s) and location(s) of proposed release(s), collaborators, and methods to be used for evaluating establishment, dispersal, and effect on target, and for what period of time**
TIER 2 **[NON-PLANT FEEDING OR - ATTACKING AGENT]**	**The same requirements as for Tier 1 (see above), except no. 7**

APPENDIX 2. Data Requirements for field studies in Canada

Tiered Data Requirements (DR) for performing a small-, medium-, or large-scale field study on environmental fate and behaviour or ecotoxicology: a Canadian proposal (see also Fig.1) (more explanation in Table 3)(Anon., 1993b)



DR. I: for micro-organisms indigenous to the ecozone of intended use. DR include a small set of notification criteria.

DR II for genetically modified micro-organisms indigenous to the ecozone of intended use. DR II include DR I, and quality control information.

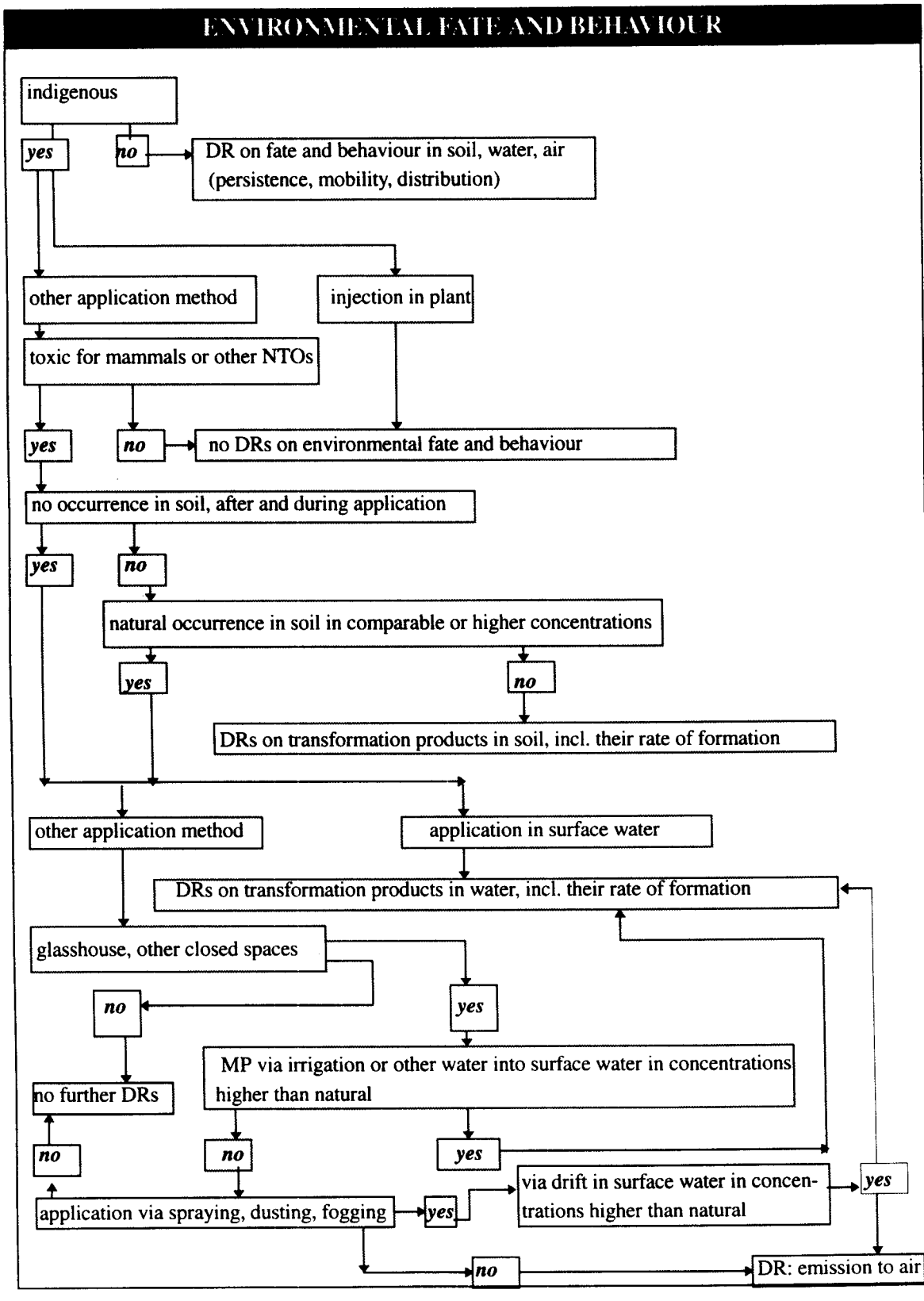
DR III for micro-organisms not indigenous to the ecozone of intended use, but indigenous to continental Canada or US. DR III include DR II, the geographical range of the MP, the target, and affected non-target organisms, and a comprehensive literature review on the effects and environmental fate and behaviour of taxonomically related species.

DR IV for micro-organisms not indigenous to the ecozone of intended use, but claimed to be identical to an indigenous micro-organism in continental Canada or US. DR IV include DR III, and the verification of the taxonomic similiarity of the MP and the indigenous micro-organism.

DR V for micro-organisms not indigenous to continental Canada or US, and not identical to an indigenous micro-organism in continental Canada or US. DR V include DR IV and all other relevant required data as in Anon. (1993a) (see Table 3 and Chapter 3).

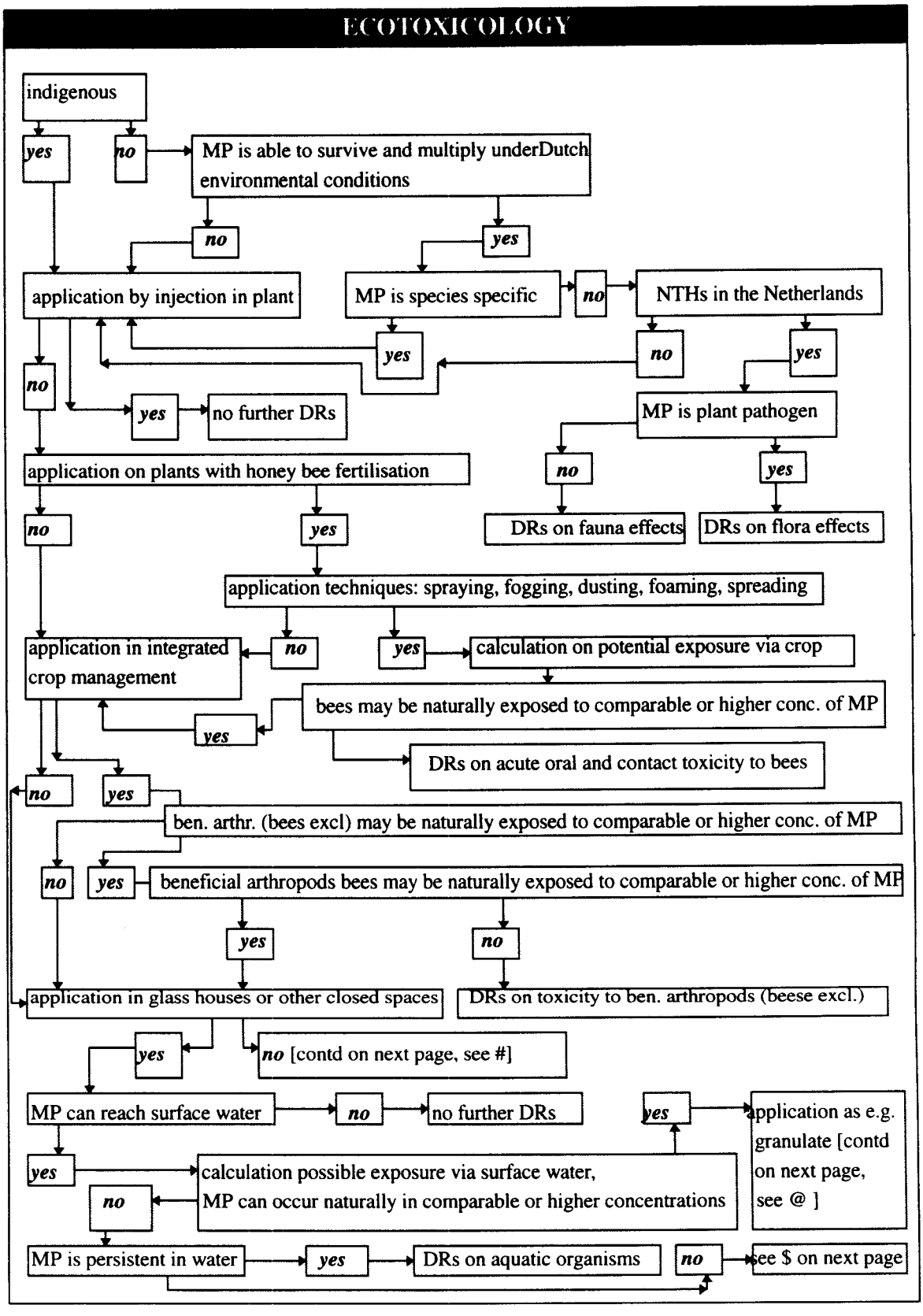
APPENDIX 3. Data Requirements on environmental fate and behaviour (a Dutch proposal)

Data requirements (DRs) for performing laboratory tests: a Dutch proposal by Wäckers (1992)



APPENDIX 4. Data Requirements on ecotoxicology (a Dutch proposal)

Data requirements (DRs) for performing laboratory tests: a Dutch proposal by Wäckers (1992)



Appendix 4 *(contd)*