PROPOSED GUIDELINES FOR REGISTRATION OF CHEMICAL PESTICIDES:
Nontarget plant testing and evaluation

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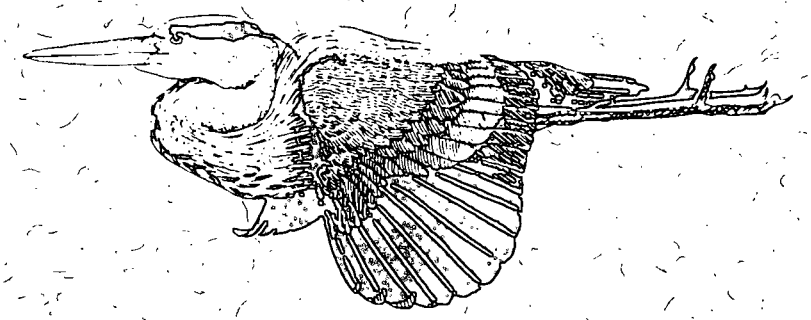

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Proposed guidelines for registration of chemical pesticides:

Nontarget plant testing and evaluation

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Foreword

The guidelines presented herein have been produced in consultation with the Commercial Chemicals Branch of Environment Canada, the Department of Fisheries and Oceans, Forestry Canada, Agriculture Canada and the chemical pesticide industry. The final draft was submitted to Agriculture Canada to become a Regulatory Proposal in October 1992. Before it can become a Regulatory Proposal, however, it has to undergo an economic impact assessment. This assessment will determine the economic, social and environmental costs and benefits to Canadian society of implementing these guidelines. The contract for the economic impact assessment was awarded in September 1993, and the assessment is due in February 1994. In the meantime the proposed Canadian guidelines are being extensively used as a core document by other countries to establish their own requirements for plant testing and risk assessment, e.g. the European Community through The Netherlands and Great Britain in particular. Also the OECD (Organization for Economic Co-operation and Development) is in the process of revising their own ecotoxicological guidelines for testing pesticides, and the USEPA (United States Environmental Protection Agency) will be presenting the proposed Canadian guidelines as a starting document. At the SETAC (Society for Environmental Toxicology and Chemistry) conference held in Houston, Texas (November 1993), a short course was organized on "Toxicity Assessment with Aquatic and Terrestrial Plants" where topics covered included "phytotoxicity testing with products (under FIFRA [Federal Insecticide, Fungicide, Rodenticide Act], TSCA [Toxic Substances Control Act], FDA [Food and Drug Administration], OECD, and Canadian guidelines)...". Also some pesticide companies have started following the guideline requirements for the development of new pesticides in anticipation of their impending acceptance. Pesticide companies generally support the development of these guidelines so that both industry and government have a clear understanding of the data required for registration.

Due to the extensive consultation process involved during the elaboration of these guidelines, several versions have been sent to reviewers and this may lead to confusion because of the current wide distribution of the document. At present the proposed guidelines have no official status and consequently are wrongly cited or referred to as the Canadian guidelines for testing effects of pesticides on nontarget plants.

It is for these numerous reasons that the Canadian Wildlife Service has decided to publish these proposed guidelines as a technical report pending the results of the economic assessment.

There is an increasing interest in strengthening the ecological impact assessment with emphasis on both direct and indirect effects of pesticides to wildlife. The proposed Canadian guidelines represent a significant advancement in the field of nontarget plant testing for pesticide impact.

Céline Boutin, PhD

Abstract

The chief objectives of these guidelines are to outline the data requirements for the nontarget plant testing of chemical pesticides, to suggest methods and approaches for generating and reporting the data required, and to facilitate and improve hazard assessment for nontarget plants (and habitats) as a result of chemical pesticide use.

These guidelines are divided into three main sections. The first section contains the nontarget plant guidelines proper, which include test requirements, refinements/changes to existing test protocols, and reporting details. The guidelines proper consist of a four-tier approach in which tests increase in complexity with tier progression. Tier I is a screening level with the objective of detecting phytotoxicity of a given chemical pesticide; therefore, minimum testing is required at one dose, the maximum recommended label rate. The aim of Tier II testing is to quantify the magnitude of toxicity of a chemical pesticide to different groups of plant species. At this tier, dose-response curves are established for different types of plants, both aquatic and terrestrial. Tier III includes additional single-species testing on aquatic plants as well as special tests on a case-by-case basis. Tier IV involves multispecies community testing conducted in microcosms, mesocosms, or the field. Scenarios for risk assessment are presented and criteria for tier progression are outlined following the descriptions of each test.

The second section, Appendix A, gives the scientific documentation and rationale for the different testing requirements. Also a comparison is presented of the disparities existing between these guidelines and those of the U.S. Environmental Protection Agency and the Organization for Economic Co-operation and Development.

Appendix B, the third and final section, demonstrates the statistics recommended to registrants and used by advisors to evaluate each test required in Tiers I and II of the guidelines proper. This section is authored by D.A. MacLeod.

The nontarget plant guidelines are very flexible, as registrants may be granted a waiver for any test required, provided the rationale is justified on appropriate scientific grounds.

Résumé

Ces lignes directrices ont pour objectifs principaux d'énumérer les types de données exigés pour évaluer l'effet des pesticides chimiques sur les plantes non ciblées par les pesticides, de suggérer les tests et les méthodes requises et finalement de faciliter et améliorer l'évaluation de l'impact des pesticides chimiques sur les plantes et les habitats.

Ces lignes directrices sont divisées en trois sections. La première section inclue les lignes directrices proprement dites dans lesquelles sont décrites les tests exigés, les modifications à apporter aux protocoles suggérés s'il y a lieu ainsi que les détails qui doivent être inscrits dans les rapports. Les lignes directrices proprement dites consistent en une approche à quatre niveaux avec une progression en complexité à mesure que le niveau augmente. Le niveau I s'avère un niveau de base ayant pour objectif de dépister l'occurrence de phytotoxicité d'un pesticide chimique donné sur un nombre restreint de plantes; ainsi à ce niveau, un seul taux est requis à la dose maximale recommandée sur l'étiquette. Le but du niveau II est de quantifier l'ampleur de la toxicité des pesticides chimiques. Pour ce faire, des courbes de dose-réponses sont établies pour différents groupes de plantes aquatiques et terrestres. Le niveau III inclue des tests supplémentaires sur d'autres types de plantes aquatiques de même que des tests spéciaux si nécessaire. Au niveau IV des tests impliquant des communautés d'espèces végétales sont demandés. Il s'agit de tests réalisés simultanément avec plusieurs types de plantes (microcosmes, mésocosmes) ou sur le terrain. Pour tous ces niveaux et tests, des scénarios d'estimation de risque sont présentés et des critères de progression d'un niveau à l'autre sont énumérés à la suite de la description de chacun des tests.

La deuxième section, Appendice A, présente la documentation scientifique utilisée lors de l'élaboration de ces lignes directrices et expose la justification reliée aux différents tests exigés. Cette section détaille et explique également les disparités entre ces lignes directrices et celles des Etats-Unis (U.S. Environmental Protection Agency) ainsi que celles de l'Organisation pour la Co-opération et le Développement (OCDE).

La section finale, Appendice B, donne un aperçu détaillé des analyses statistiques recommandées pour les différents tests et qui sont utilisées lors de l'évaluation des tests effectués aux niveaux I et II des lignes directrices présentées à la section I. Cette section a été écrite par D.A. MacLeod.

Les lignes directrices décrites ici sont très flexibles puisqu'une exemption peut être accordée pour tous tests requis, pourvu qu'une justification scientifique valable soit présentée.

Acknowledgements

We would like to thank Alain Baril, Pierre Mineau, and Bruce Pauli from the Canadian Wildlife Service of Environment Canada for their useful comments and discussion during the preparation of this document. Participants of the Interdepartmental Committee for Nontarget Plant Guidelines made an essential contribution; they are Ian Nicholson (Commercial Chemicals Branch of Environment Canada), Gary Rawn and Roger Garrod (Department of Fisheries and Oceans), Dean Thompson and Craig Howard (Forestry Canada), and Fa Yan Chang (Agriculture Canada). Brian Collins and Duncan Macleod (Canadian Wildlife Service), Glenn Atkinson (Applied Statistics Division of Environment Canada), and Brigit de March (Department of Fisheries and Oceans) gave valuable assistance on the statistics. Allan Brown from the Crop Protection Institute of Canada compiled comments from pesticide companies on earlier versions.

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1.0 Overview

1.1 Definition and importance of nontarget plants

For the purposes of these guidelines, the term *nontarget plant species* refers to plants occurring in nontarget areas and may include desirable species occurring in target areas of terrestrial and aquatic sites where total vegetation control is not intended.

Plants play critical roles in both aquatic and terrestrial ecosystems - in nutrient cycling, in primary production, and as food and habitat for other organisms. In these guidelines, several different aquatic and terrestrial species are included to represent different environmental compartments and trophic levels.

Algae are the primary carbon-fixing organisms in aquatic environments and are thus an indispensable link between solar radiation, the complex solution of chemicals in water, all aquatic animals, and humans, whose existence is dependent on the oxygen involved in photosynthesis. In freshwater ecosystems, particularly larger lakes, algae are more important than vascular plants in terms of primary production. In wetland communities, there is a delicate balance between the growth of algae and that of macrophytes (species with emergent, submerged, and floating leaves), which regulates the species composition of higher trophic levels. Terrestrial plants at the margin of crop fields are important constituents of habitats for several species of wildlife.

Both aquatic and terrestrial plants are essential for maintaining the quality of the aquatic habitat at a level suitable for fish. Algae, as primary producers, provide food for fish either directly or indirectly, by supporting populations of fauna that serve as fish food. Aquatic macrophytes provide cover from predators, serve as nursery areas, create a diversity of habitats, and help regulate the flow rate of water. Terrestrial plants stabilize soil, which reduces erosion and the deposition of soil in fish habitat. Shoreline vegetation provides an environment for terrestrial insects that contribute to the diet of fish. Vegetative cover of streams is also important for the regulation of water temperature. In addition, leaf fall from shoreline plants provides essential energy for the aquatic food chain.

Plant species may display different sensitivities to chemical pesticides because of their different biological characteristics, ecological roles, morphology, structure, and habitat (Swanson *et al.* 1991) and because they are exposed to pesticides by different

routes (i.e., via direct deposition, through water, or through sediment).

1.2 Objectives of guidelines

Pesticide registration in Canada is governed by the Pest Control Products Act. Under this act, there are no specific Canadian requirements for testing pesticide toxicity to nontarget plants other than agronomically important species (Agriculture Canada 1984). Data requirements are currently handled on a case-by-case basis only (Freemark *et al.* 1990).

The main objectives of these guidelines are to:

1) outline the data requirements for nontarget plant testing of chemical pesticides in Canada,

2) suggest methods and approaches for generating and reporting the data required, and

3) facilitate and improve hazard assessment for nontarget plants (and habitats) as a result of pesticide use.

1.3 Use of guidelines

1.3.1 Purpose

Data gathered to meet these guidelines may be used for:

1) evaluating pesticides proposed for Canadian registration and reevaluating previously registered pesticides (new active ingredients; major new uses, such as additional crops with large hectarage; new geographical areas; different application methods; new formulations, if very different; or any other situations that require assessment regarding hazards posed to wildlife, fish, and their habitats),

2) providing guidance in developing post-registration monitoring plans, and

3) evaluating the significance of pesticide contamination of nontarget areas and the potential for hazard mitigation.

1.3.2 Rationale

Pesticides other than herbicides can detrimentally affect nontarget plants (Thompson 1976; Swanson *et al.* 1991; K.E. Freemark and C. Boutin, unpubl. data; proprietary data); therefore, the assessment of hazard posed by nonherbicides on

beneficial plants is justified (as it is for other nontarget organisms, such as birds, mammals, fish, etc.). For the purpose of avoiding unnecessary testing, however, Tier I has been established as a screening level for detecting phytotoxicity through minimum plant toxicity testing.

For herbicides, testing performed according to these guidelines is needed to define their selectivity with respect to different types of plants (e.g., algae vs. floating plants vs. rooted vascular species) at critical life stages (e.g., seed germination vs. vegetative growth) or to test for specificity depending on the type of receiving environment (i.e., aquatic vs. terrestrial). Results of such testing will help to refine our knowledge of the general spectra of herbicide activities and to enhance our understanding of the potential detrimental effects of herbicides on nontarget plants, with emphasis, where possible, on species that are important to wildlife and fish. It is important to assess the potential effects of herbicides on nontarget plants in the vicinity of target areas in order to implement appropriate measures to protect wildlife and/or fish habitats. Protection of endangered species - plants and animals - is also considered essential.

1.3.3 Hazard and risk assessments

The approach developed for nontarget plant hazard assessment first considers the potential for contamination of the environment. Restricted uses, such as in closed-system greenhouses, indoors and swimming pools, do not trigger nontarget plant testing (Table 1). For other uses (Table 1), the toxicological endpoint of interest is compared with an expected environmental concentration (EEC) to determine the probable nature and magnitude of the hazard resulting from the release of the chemical into the environment. Uncertainty factors are applied in hazard assessment at Tiers II and III, based on current toxicological knowledge and the amount and quality of toxicological information provided. The information is then combined with current knowledge on environmental chemistry and fate as well as use patterns to evaluate the likelihood that a hazard to nontarget plants will be realized from the use patterns proposed for pesticide products submitted for registration.

Risk assessment extends hazard assessment by estimating the probability or likelihood that undesirable effects will occur. At present, assessments are semiquantitative and based in large part on expert judgement, particularly at Tier IV. In these guidelines, the terms hazard assessment and risk assessment are used interchangeably.

1.4 Comparison with other guidelines

Deviations that exist between the Canadian guidelines and those of the Organisation for Economic Co-operation and Development (OECD) and U.S. Environmental Protection Agency (EPA) in test requirements, protocol specifications, and risk assessments are outlined in Appendix A. Differences between the guidelines are due mainly to research conducted since the completion of the OECD guidelines (OECD 1981, 1984a) and the U.S. EPA guidelines (Holst and Ellwanger 1982; Holst 1986a, 1986b, 1986c, 1986d, 1986e).

1.5 Definitions and abbreviations

Active ingredient: the ingredient of a pesticide to which the effects of the pesticide are attributed

Adjuvant: any substance in a pesticide formulation added to the spray tank to modify the activity or application characteristics of a pesticide (see *formulant*)

Advisors or Advisory Departments: any regulators in one of four departments (Environment Canada, Department of Fisheries and Oceans, Forestry Canada, and Health and Welfare Canada) who will provide advice on products regulated under the Pest Control Products Act, which is administered by Agriculture Canada

Definitive test: a test conducted with a range of pesticide concentrations, in a geometric progression, covering the NOEC/NOEL and EC50 values for the test species selected

EC25 (effective concentration 25): the pesticide concentration that results in a 25% reduction in the test endpoint being measured relative to the control; it is used as a standard evaluation point in terrestrial toxicity testing under the Federal Insecticide, Fungicide and Rodenticide Act (see *FIFRA*) in the United States

EC50 (effective concentration 50): the pesticide concentration that results in a 50% reduction in the test endpoint being measured relative to the control; it is used as a standard international evaluation point in aquatic and terrestrial toxicity testing

EEC: expected environmental concentration (se sections 1.6.5.2 and 1.6.5.3)

Endpoint: a parameter measured during or at the end of a test, or calculated from test data, that is used for assessing the effects of a pesticide on the test organism (e.g., growth rate, biomass, cell concentration)

FIFRA (Federal Insecticide, Fungicide and Rodenticide Act): the act under which pesticides are registered in the United States and under which the U.S. EPA provides its testing requirements for registration (see Holst and Ellwanger 1982; Holst 1986a, 1986b, 1986c, 1986d, 1986e)

Final tank mix: Formulated pesticide (active ingredient + formulants) + adjuvants

Formulant: any substance added to the active ingredient to modify the activity or application characteristics of a pesticide

Formulated pesticide: a mixture containing the active ingredient and formulants; the pesticide formulation used for testing should be representative of the final formulation and should contain any adjuvants that would be added in the tank mix as specified on the label

Hazard or risk assessment: the identification and description of the potential for a pesticide to produce biological or ecological effects

Maximum challenge concentration (MCC): the pesticide concentration resulting from application at the maximum recommended label rate to the surface of a 15-cm-deep column of water or a 3-cm-deep column of soil with a bulk density of 1.5 g/cm^3

Maximum challenge rate (MCR): the maximum recommended label rate

Maximum challenge test: a test conducted with a pesticide applied at the maximum recommended label rate (MCC/MCR), simulating a worst-case scenario; the results determine whether definitive testing is required

Medium: refers to nutrient solution, water, or soil

NOEC/NOEL (no-observable-effect concentration/level): the highest pesticide level tested at which the observed endpoint is not statistically different from the control

Pesticide: a generic term that encompasses all chemical pest control products (fungicides, herbicides, insecticides, etc.)

Randomized complete block design: an experimental design where one replicate of each control and treatment is arranged randomly within a given space or block; the complete design will consist of several randomized blocks

Range-finding test: a test using more than one pesticide concentration, conducted to find the range of concentrations appropriate for the definitive test

TAI (technical active ingredient): the technical grade of the active ingredient representative of the active ingredient used in the final product before adjuvants are incorporated to enhance toxicity or delivery

TSCA (Toxic Substances Control Act): the act under which the U.S. EPA outlines its testing requirements for the registration of chemicals other than pesticides in the United States (see U.S EPA 1985a, 1985b, 1985c, 1985d, 1985e, 1987a, 1987b)

1.6 Description of guidelines

1.6.1 Background

These guidelines have been prepared based on a review and assessment of current protocols and guidelines for nontarget plant toxicity testing (Freemark *et al.* 1990), a review and assessment of aquatic plants for toxicity testing (Swanson *et al.* 1991), and independent guideline review and screening of the scientific literature.

1.6.2 Scope

The guidelines are concerned only with chemical pesticides and do not apply to microbial pesticides. The guidelines cover all chemical pesticides (herbicides, fungicides, insecticides, rodenticides, etc.) for use in terrestrial or aquatic environments. The aquatic environment includes open water as well as wetlands, and aquatic plants are considered to be emergents, submergents, and other plant species associated with aquatic environments. These guidelines are concerned with the examination of multigeneration effects of pesticides on aquatic plants (freshwater and marine algae, freshwater vascular floating plants) and single-generation effects on rooted terrestrial and aquatic vascular plants. Test requirements are tailored to reflect use patterns (Table 1).

1.6.3 Testing approach

Test types referred to above and repeatedly throughout the requirements section of this document (section 3.0) are defined in section 1.5.

A four-tiered approach to testing is outlined, with tests increasing in complexity with tier progression (Table 1).

At Tier I, which is a screening level, single species are used in maximum challenge tests (MCC/MCR). A rate higher (but not lower) than the maximum challenge rate may be acceptable for vascular

plants (test I-2) so that data that have already been generated in the routine pesticide development process (plant screening data, section 1.6.6) can be used. Tier I tests are conducted to assess the phytotoxic potential of a pesticide under a worst-case scenario on the growth and reproduction of aquatic algae (test I-1) and on vegetative growth and vigour of aquatic and terrestrial vascular plants (test I-2).

The aim of Tier II testing is to quantify the magnitude of toxicity to different groups of plant species for which test protocols are available. Definitive tests are carried out with a range of five concentrations in a geometric progression that span the NOEC/NOEL and EC50 levels for species tested in order to establish dose-response curves. Tier II tests are conducted to assess toxicity to the growth of aquatic algae (test II-I), the growth of a floating vascular plant (usually *Lemna gibba* strain G-3)(test II-2), seed germination and root elongation of terrestrial vascular plants (test II-3), and vegetative growth and vigour of rooted aquatic and terrestrial vascular plants (test II-4) (plant screening data, section 1.6.6). The appropriate concentration range for testing at Tier II should be determined on the basis of results from preliminary range-finding tests. The results of the range-finding tests need not be submitted for registration, with the *exception* of the plant screening data generated by registrants, which should all be submitted. Only the results of the maximum challenge tests (Tier I) and the definitive tests where appropriate (Tier II) are required for the following tests: algal growth, floating vascular plant growth, and seed germination/root elongation.

Tier III includes additional testing for aquatic plants. Tests are conducted with a rooted submerged species (test III-1) and an emergent species (test III-2) to indicate potential effects on a second type of aquatic plant - one with reliance on a vascular system that is exposed to the pesticide both in solution and in sediment. Emergent aquatic testing is conducted, following tests with a floating plant (test II-2), to indicate the potential effects of drift or overspray. At Tier III, registrants may also be requested to conduct, on a case-by-case basis, special tests with additional single species (test III-3), such as algal tests with formulated pesticides, whole-plant life cycle tests, tests to compare the toxicity of technical active ingredients with that of formulated pesticides, etc.

At Tier IV, multispecies community testing required on a case-by-case basis, is conducted in microcosms, mesocosms (test IV-1) or the field (test IV-2).

1.6.4 Waivers

Registrants may be granted a waiver for any test required in these guidelines if justified on appropriate scientific grounds. Some products may have physical, chemical, or biological properties or specific use patterns that would make it impossible to generate the required data, or the data generated would not be useful in the assessment of risk. For instance, if it can be demonstrated that the marine environment is unlikely to be exposed, the requirement for testing with three marine algal species can be waived.

Specific testing conditions are indicated for each test required in these guidelines. A waiver may be given for any modifications of these conditions if a sound scientific rationale is provided. For example, although its use is encouraged, a blank control may not be necessary if the test algal species demonstrated exponential growth during the experiment.

Registrants may choose to conduct Tier IV microcosm/mesocosm or field studies at any point in the testing process. In this case, additional single-species tests may still be required depending upon the use pattern of the pesticide and the effects observed in the Tier IV tests.

1.6.5 Tier progression

Following the descriptions of tests at each tier, criteria for progressing to the next level of testing are outlined. Specific tier progression triggers - i.e., fixed levels of specific criteria that dictate the need for progression to tests at a higher tier - have been set for the progression from Tier I to Tier II, from Tier II to Tier III, and, for some tests, from Tier III to Tier IV.

1.6.5.1 Progression from Tier I to Tier II

Progression from Tier I to Tier II is required when pesticide application results in statistically significant phytotoxicity (regardless of percent effect) or if inhibition is greater than 50% (algal growth) or 25% (vascular plant growth and vigour, section 1.6.6) relative to the control. The relationships between Tier I test results and Tier II test requirements are shown below (refer to Figure 1 for test requirements):

Tier I phytotoxicity		Tier II testing required*
Algae	Vascular plants	
No	No	No further testing
No	Yes	Herbicides: Tier II tests except algae Nonherbicides**: Tier I vascular plant growth expanded to include 30 species, 10 families, + Tier II tests except algae
Yes	No	Herbicides: Tier II tests except vascular plant growth Nonherbicides**: Tier I vascular plant growth expanded to include 30 species, 10 families, + Tier II tests
Yes	Yes	Herbicides: Tier II tests Nonherbicides**: Tier I vascular plant growth expanded to include 30 species, 10 families, + Tier II tests

* No further testing is required at Tier II for those species that showed no phytotoxic response at Tier I when tested in maximum challenge tests (MCC/MCR); species that showed a phytotoxic response at Tier I must be included at Tier II.

** When exposure to nonherbicides results in phytotoxicity to algae or vascular plants at Tier I, Tier II testing first involves expanding the Tier I maximum challenge test (test I-2, section 3.1.2.2) with vascular plants so that a total of 30 species and 10 families (preferably three species per family) are tested (including the 10 species from six families already tested at Tier I). Tier II tests described in section 3.2.1 are then required. (If none of the 30 species shows phytotoxicity in the maximum challenge test, the definitive vegetative growth test for vascular plants at Tier II [test II-4, section 3.2.1.4] would not be required.)

1.6.5.2 Progression from Tier II to Tier III

Progression from Tier II to Tier III, described briefly below, is discussed further after the appropriate tests. A pesticide is

considered to pose a hazard to nontarget plants, requiring progression to Tier III, if the EEC is greater than the concentration that resulted in reduction in the test endpoint being measured relative to the control divided by an uncertainty factor of 10. For vascular plant growth (plant screening data, section 1.6.6), progression to Tier III is required when the EEC is greater than the EC25 for 25% of the species or 50% of the families tested.

The EEC is determined for Tier II tests as follows:

Algae and species with submerged leaves

EEC = the concentration resulting from application at the maximum recommended label rate to a 15-cm-deep column of water

Seed germination/root elongation

EEC = the concentration resulting from application at the maximum recommended label rate to a 3-cm-deep column of soil with a bulk density of 1.5 g/cm^3

Lemna, *species with floating leaves, aquatic emergent and terrestrial vascular plants*

EEC = the concentration resulting from application at 100% of the maximum recommended label rate when sprayed over plants if the pesticide is likely to overspray nontarget habitats, *or* the concentration resulting from application at 10% of the maximum recommended label rate if the pesticide will reach nontarget plants through drift, runoff, and washoff *only*

1.6.5.3 Progression from Tier III to Tier IV

Progression from Tier III to Tier IV, described briefly below, is discussed further after the appropriate tests. A pesticide is considered to pose a hazard to nontarget plants, requiring progression to Tier IV, if the EEC is greater than the concentration that resulted in reduction in the test endpoint being measured relative to the control of any species tested divided by an uncertainty factor of 10.

The EEC is determined for Tier III tests as follows:

Species with submerged leaves

EEC = the concentration resulting from application at the maximum recommended label rate to a 15-cm-deep column of water

Species with floating leaves and emergent aquatic species

EEC = the concentration resulting from application at 100% of the maximum recommended label rate when sprayed over plants if the pesticide is likely to overspray nontarget habitats, *or* the concentration resulting from application at 10% of the maximum recommended label rate if the pesticide will reach nontarget plants through drift, runoff and washoff *only*

The need for additional special testing with single species and corresponding progression triggers will be determined on a case-by-case basis through discussions involving interested regulatory advisors and the registrant. Both toxicological effects (number and types of species affected, concentrations required to affect species) and exposure (environmental chemistry and fate characteristics, application rates and methods, and requested use patterns) will be considered in determining test requirements (see section 1.3.3).

1.6.6 Plant screening data

Vegetative growth and vigour of rooted vascular species will be assessed using the plant screening data routinely generated by registrants during the pesticide development process. Plant screening data are very valuable, as they include several families and species (Table 2) and, hence, the general spectrum of activity can be determined for each chemical. Table 2 represents a compilation of species used by many pesticide-developing companies.

During the pesticide development process, the effects of a chemical on plants are typically assessed by companies on a four-tiered basis. A primary screening for any herbicidal activity is first performed at one high rate on a newly discovered chemical. Once herbicidal activity has been demonstrated, several rates are used in a secondary screening to determine weed control and crop tolerance. A tertiary screening is used to define more precisely the rates of activity. Small-plot field trials are performed at the fourth level to determine the exact rates of application, most effective formulations, and the effect of adjuvants.

All plant screening data pertaining to the toxicity of the pesticide to terrestrial and/or aquatic vascular plants, from tests performed either in the greenhouse or in the field (i.e., pre-plant incorporated and pre- and post-emergence trials, from primary screening to field trials), must be submitted to meet the requirements of the vegetative growth and vigour tests (tests I-2 and II-4) in these guidelines. The requirements are as follows:

1) Tier I: all data from MCC and MCR tests using the TAI and, if

conducted, formulated pesticides, and

2) Tier II: all data from definitive tests using the TAI and, if conducted, formulated pesticides.

A minimum of 30 species from 10 families is required for herbicides. For nonherbicides, 10 species from six families are considered acceptable; this should be expanded to 30 species from 10 families if any phytotoxicity is demonstrated at the maximum recommended label rate. The main requirement, however, is that all plant screening data already generated should be submitted.

It is generally accepted that there are differences in the activities exhibited by a given pesticide in the greenhouse and in the field. Most frequently, effects of the herbicide in the field are reduced because of environmental factors (e.g., wind, temperature, rainfall conditions), plant anatomy (e.g., cuticle thickness), and physiological states of the plant (e.g., more active growth in the greenhouse) (Garrod 1989), although Fletcher *et al.* (1990) reached the opposite conclusion from their literature search. Nevertheless, plants used in the greenhouse are more uniform and represent a worst-case scenario. In the field, species and even individuals within a species often are at markedly different growth stages and, hence, differ in susceptibility; this introduces variability in the results, which makes interpretation difficult. In the assessment of products, both types of data will be considered.

1.6.7 Testing and the registration process

Based on the specific criteria outlined for progression from Tier I to Tier IV, registrants should be able to assess the need for pesticide testing. The registrant is encouraged to ask advice *before* starting special testing at Tier III or field testing at Tier IV.

The data/information submitted for a pesticide will be reviewed and evaluated, and potential hazards that may result from the use of the pesticide will be identified (see section 1.3.3). Based on this review, three scenarios may follow:

1) a final assessment of hazard to nontarget plants is made,

2) tests are repeated, and/or

3) additional testing is requested.

If the registrant makes major changes to a use pattern, formulation, or recommended label rate during or after the hazard assessment process, the changes will be reviewed and the need for additional testing assessed on a case-by-case basis. For

example, major changes in geographical region, crop, or application method (e.g., ground vs. aerial) will be reviewed.

1.6.8 Test requirements

The requirements listed for each tier in Table 1 are described by species, test type, and test substance (see individual requirement descriptions for more details). This table shows the use patterns for which testing is required. The omission of any "required" test must be justified on scientific grounds. Where they exist, internationally recognized protocols are recommended as standard approaches to specific tests, with modifications in some cases. Tests conducted according to other scientifically supportable protocols may also be acceptable but should be discussed with interested regulatory advisors prior to testing.

1.6.8.1 Test substance

Tests are conducted with either the TAI or a formulated pesticide. Initial testing (Tier I) is typically carried out with the TAI representative of the active ingredient used in the final product. This substance is also used in algal and vascular plant growth tests in Tier II. Vascular plant growth tests (plant screening data) that may have been conducted with the formulated pesticide should also be submitted. When the TAI has a solubility in water below 1000 mg/L (U.S. EPA 1985a) or vapour pressure above 5.20×10^{-3} Pa, a formulation of the pesticide could be considered as an alternative test substance.

The formulation used for testing should be representative of the final formulation for which registration is being sought and should contain any adjuvants that would be added in the tank mix as specified on the label (see section 1.5). The same lot of pesticide, with purity reported, should be used in any one test. Foliar aquatic testing (Tier II) is conducted with a formulated pesticide. Seed testing is conducted with the TAI at Tier II or with the formulated pesticide at Tier III if necessary. Tier III testing and the microcosm/mesocosm test in Tier IV are generally conducted with a formulated pesticide, but the test substance used will depend on the results of previous testing and the tests to be conducted (e.g., testing with the submerged aquatic species may be conducted with the TAI). The tank mix with the final formulated pesticide is tested in the field at Tier IV.

Major changes in formulation after testing will be reviewed by interested regulatory advisors, and registrants may be asked to repeat tests using the new formulation.

Verification of test concentrations used in all toxicity tests in laboratory and greenhouse studies is a requirement at all four

tiers, because nominal concentrations may be highly inaccurate. It is preferred that concentrations be measured at the beginning and at the end of each test. At Tiers III and IV, the need to validate both concentrations in the tank and amounts actually applied must be determined on a case-by-case basis.

1.6.8.2 Endpoints

Clear, unambiguous endpoints (e.g., growth rate, biomass) have been chosen to assess pesticide toxicity and effects for each required test and to determine the need for progression to a subsequent tier. Visual evaluation (qualitative endpoint) is acceptable for plant screening data routinely generated by registrants (see section 1.6.6).

1.6.8.3 Experimental design

Appropriate controls are critical to the evaluation of the results. Three controls are required when testing a pesticide of limited water solubility:

1) *Test plants + medium + solvent* to determine effects of the solvent at rates used,

2) *Test plants + medium* to determine growth in the absence of solvent or pesticide, and

3) *Pesticide + medium + solvent* (blank) for chemical fate control measurement to account for losses of test substance through precipitation, transformation, volatilization, and sorption by container walls. Measurements should be performed at the start and end of the test to determine interactions between substances (e.g., turbidity) that could, for example, affect algal cell counts at the end of the test. A waiver can be given provided growth is typical of the species tested.

If the endpoints of controls 1 and 2 are significantly different statistically at the 0.10 level, only control 1 should be used in assessing the effects of the pesticide applied. Alternatively, a different solvent could be used. If there is no statistically significant difference between these controls, data should be pooled for the analysis of effects. The significance level recommended for this test is 0.10 instead of the 0.05 level used in statistical procedures. The reason is that the test of solvent is a preliminary test, carried out mainly to determine the proper control against which the pesticide treatment is to be tested. If a solvent effect is present but not detected, the test of pesticide effect could be adversely affected. Therefore, the significance level has been set to 0.10 in order to increase the chance of detecting a solvent effect (see also Appendix B,

section B2.3.6.1).

Two controls are required when testing a readily water-soluble pesticide:

1) *Test plants + medium* to determine growth in the absence of pesticide, and

2) *Pesticide + medium* (blank) for chemical fate control measurement at the start and end of the test (see control 3 above). A waiver can be given provided growth is typical of the species tested.

A randomized complete block design is recommended for laying out the replicates for tests outlined in Tiers I and II. Detailed experimental designs are described for specific tests in the section on registration requirements. In Tiers III and IV, appropriate experimental design will be determined on a case-by-case basis through discussions involving interested regulatory advisors and the registrant.

Refer to Appendix B for explanations of experimental design (examples provided) for the tests required in these guidelines.

1.6.8.4 Data analysis

Quantification of the test results and statistical assessment of their significance using standard scientific methods provide critical information upon which regulatory decisions should be based. This information is essential for conducting good hazard assessments and determining hazard mitigation options for nontarget plants.

In these guidelines, statistical analysis is used to evaluate the significance of the test results. A significance level of 0.05 is the scientific standard, and registrants should adopt it for toxicity testing (except for differences between solvent control and control, see section 1.6.8.3).

In the data analysis sections for tests in Tier I, procedures are described for determining the significance of inhibition due to pesticide application at the maximum recommended label rate relative to the control results. In Tiers II, III, and, in some cases, IV, the magnitude of the inhibition is determined over several concentrations. For tests with algae and *Lemna*, for which generation time is relatively short, 50% inhibition will trigger further testing. For tests with aquatic and terrestrial vascular plants that have longer generation times, 25% inhibition will trigger further testing (Holst and Ellwanger 1982). An excessively large variance in results may occur with insufficient or inaccurate data or may be due to high methodological

variability. If a 25% or 50% inhibition occurs that is not statistically significant, testing should be repeated with greater power (e.g., more replicates) to reduce variability. Alternatively, registrants must conduct testing at higher tiers.

Parametric statistical tests are preferred to nonparametric statistical tests because the parametric tests have higher overall power when all the assumptions are met (Sokal and Rohlf 1981). A one-tailed statistical test is required for determining the significance of inhibition. Two-tailed tests or nonparametric tests will be considered with justification (e.g., if stimulation occurs or if the assumptions of parametric tests are not met).

In cases in which several endpoints are reported in any one test (e.g., algal growth inhibition), the need for further testing will be determined according to the endpoint showing the greatest sensitivity. Alternatively, a waiver may be considered if appropriate scientific justifications are provided.

All details involved in the procedure for the statistical analysis should be provided - i.e., data transformations, statistical tests, and any modifications or decisions that might influence the assessment of the results.

Appendix B illustrates in detail how data submitted on nontarget plants will be assessed by government evaluators. Registrants are encouraged to follow a similar path for statistical analyses.

1.6.8.5 Reports

a) Experimental conditions

Detailed data/information on specimens (strains and source), pre-test conditions, test conditions, methods and precision for measuring all parameters reported, and experimental procedure (methods, experimental design) should be provided in all reports. Plant density (when appropriate) and pesticide concentration should be validated by measurement for every test at both the start and the end of the test.

b) Results

A copy of the raw, untransformed data is required for each replicate of a test to facilitate interpretation and evaluation of the results and to conduct additional analyses as necessary. Data should be provided on hard copy. Additionally, it is strongly encouraged that data be supplied in ASCII files on an IBM-compatible diskette; this will allow processing of data and evaluation of studies in a much shorter period. The report

should contain statistical details (methods, programs used, analysis results) and graphical presentations of the data. Further details on report requirements are given for each test.

1.6.9 Quality assurance, quality control, and good laboratory practice

The registrant is encouraged to develop a quality assurance (QA) program for toxicological nontarget plant testing. This program should be a comprehensive system of management and operational activities designed to ensure that the quality control (QC) system (the routine checks and procedures carried out within normal operations) is working effectively to ensure that quality data are produced (MacGregor and Doe 1987). The QA program should ensure that measures are taken to maintain and improve data quality and that the limits of uncertainty of the data are known.

In order to have effective QA and QC programs, the organizational process and the conditions under which laboratory studies are planned, performed, monitored, recorded, and reported ought to conform with standard good laboratory practice (GLP) (OECD 1982). At present, most laboratories in Canada are not certified; therefore, GLP is optional, although encouraged.

The use of QA and QC programs (for all testing) and GLP (for all tests except field efficacy testing) may reduce variance among replicate results, thus avoiding the need for repetition of required tests as a result of high methodological variance in replicate results. It will increase the utility of the data produced for fulfilling the requirements of the nontarget plant testing guidelines.

1.7 References

Full citations for the following references are provided in section 4.0.

Agriculture Canada 1984
Fletcher et al. 1990
Freemark et al. 1990
Garrod 1989
Holst 1986a, 1986b, 1986c, 1986d, 1986e
Holst and Ellwanger 1982
MacGregor and Doe 1987
OECD 1982
Sokal and Rohlf 1981
Swanson et al. 1991
Thompson 1976
U.S. EPA 1985a, 1985b, 1985c, 1985d, 1985e, 1987a, 1987b

2.0 Format of guidelines

The test requirements are outlined by tier, from I to IV. The general requirements for each tier are stated, followed by descriptions of supporting tests.

Tests are denoted by tier number and test number, test species, variable measured (e.g., growth, germination), test type (maximum challenge concentration, maximum challenge rate, definitive), and pesticide used (technical active ingredient, formulated pesticide). For example, the first test in Tier I is a maximum challenge concentration test to determine the effects of the technical active ingredient on aquatic algal growth and reproduction and is denoted by:

Test I-1 Algal growth and reproduction, maximum challenge concentration, technical active ingredient

Test requirements for each tier are summarized in Table 1. For each test, the following information is provided in the text:

A) Objective

B) When required

C) Methodology

1. Recommended protocol

Where available, one protocol is recommended for each test required to assist registrants in meeting the guideline requirements.

2. Protocol modifications

Under this heading, modifications of the recommended protocol (when specified) or additional information is outlined, or important elements of the recommended protocol are emphasized. Topics addressed include: a) test species, b) test substance, c) test conditions, d) experimental design, e) data collection, f) data analysis, and g) progression to next tier. Only those topics requiring modification or clarification are discussed for a test. The absence of any of these topics from a test methodology description indicates that coverage is adequate in the recommended protocol.

D) Test limitations and validation

E) Report

The report on experimental conditions for each test may include 1) tester identification, 2) test organisms, 3) test substance, 4) test conditions, and 5) experimental procedure. The report on results may include 1) calculation of tests variables, 2) test of pesticide effect on test variables and estimation of NOEC/NOEL and EC25 and EC50, and 3) other observed effects, and interpretation of results.

F) References

Following each test, the recommended protocol is fully cited. A list of references cited in the text and other relevant references is also provided for additional information. Full citations for these references are provided in section 4.0.

3.0 Testing requirements

3.1 Tier I requirements

Tier I tests are conducted to assess the general phytotoxic potential of a pesticide. At this tier, information on the mode of action of the pesticide and preliminary toxicological testing on aquatic and terrestrial plants, during critical stages of their development, is required. Maximum challenge tests at one concentration representing a worst-case scenario are used to rapidly assess the general phytotoxic potential of a pesticide with respect to nontarget plants.

3.1.1 Mode of action information

Information on the mode of action, if known, should be supplied for all pesticides (Table 1). A waiver may be given for this requirement when the mode of action is not known.

3.1.2 Tests

Testing requirements for Tier I are shown in Table 1. The relationship between Tier I and Tier II testing is illustrated in Figure 1 and section 1.6.5.1.

3.1.2.1 Test I-1: Algal growth and reproduction, maximum challenge concentration, technical active ingredient

A) Objective

This test is to determine the effects of a pesticide on growth and reproduction (over several generations) of algae representative of a wide variety of taxonomic groups in freshwater and marine environments.

B) When required

Testing with algae is required for all pesticides for nondomestic and domestic use in outdoor aquatic and terrestrial environments, except in greenhouses with closed systems, indoors and swimming pools (Table 1). Refer to section 1.6.7 for general testing requirements and the registration process. Testing with marine algae is required. This requirement may, however, be waived if the registrant can demonstrate that the product is not likely to occur in the marine environment.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

For freshwater and marine algae, the protocol prepared by the American Society for Testing and Materials (ASTM 1991a) is recommended as a basis for the Canadian protocol. Modifications to the recommended protocol are outlined in the following sections.

The ASTM protocol is recommended for general requirements such as apparatus, measurements, statistical analysis, etc. For some species that are suggested for testing, specific requirements are described in the references mentioned in Tables 3 and 4.

2. Protocol modifications

a) Test species

For *freshwater* testing, three algal species are to be tested - one from each of the Chlorophyceae (green), Cyanophyceae (nitrogen- or non-nitrogen-fixing blue-green), and Bacillariophyceae (diatoms). The list of species recommended in Table 3 is based on species listed by Swanson *et al.* (1991) as frequently tested in toxicological studies and species recommended in test protocols (Holst and Ellwanger 1982; OECD 1984b; U.S. EPA 1985a; Holst 1986c; ISO 1989; APHA 1989; ASTM 1991a). Beside each species are references to standard protocols or literature that may be useful in test design. As already stated, however, the ASTM (1991a) protocol is recommended for general requirements and methodology.

For *marine* testing, three algal species are to be tested - one from each of the Bacillariophyceae (diatoms), Dinophyceae (dinoflagellates), and Chlorophyceae (green) or Chrysophyceae (golden-brown). The list of species recommended in Table 4 is based on species listed by Swanson *et al.* (1991) as frequently tested in toxicological studies and species recommended in test protocols (Holst and Ellwanger 1982; U.S. EPA 1985a; Holst 1986c; APHA 1989; ASTM 1991a). Beside each species are references to standard protocols or literature that may be useful in test design. However, as already stated, the ASTM (1991a) protocol is recommended for general requirements and methodology.

Test species should be obtained from standard source collections.

b) Test substance

The type of test substance is not specified in the ASTM (1991a) protocol as it is for general chemical testing. A TAI representative of the active ingredient used in the final product (with purity reported) is to be tested at the concentration resulting from application at the maximum recommended label rate to a 15-cm-deep (Holst and Ellwanger 1982; Holst 1986c) column of water.

It is desirable to conduct tests with a reference toxicant or positive control to check algal sensitivity, which may differ greatly among species and between strains (Lewis 1990; Swanson *et al.* 1991; H. Peterson, pers. commun.). Potassium dichromate, copper sulphate, and several other metals in solution may be appropriate (OECD 1984b; Blanck *et al.* 1984; Blaise *et al.* 1986).

c) Test conditions

Optimum growing conditions vary among species. For species not listed in the recommended protocols, test conditions described in other protocols or in the references listed beside the species should be used as a guideline.

Cultures must be agitated continuously to keep the algae in suspension during the test and to maintain adequate carbon dioxide levels. Because continuous shaking of diatoms may cause clumping or retard growth, they should be hand-shaken twice daily (ASTM 1991a).

Either nitrate or ammonium can be used as a nitrogen source, provided pH deviation does not exceed one unit. Use of nitrate as the nitrogen source has the effect of increasing pH, which, with high biomass increase and carbon dioxide limitation, may lead to an unacceptable pH deviation.

Whatever the specific test procedure, axenic culture is preferred.

d) Experimental design

At least four replicates should be used to maintain confidence in the results.

e) Data collection

Observations on cell morphology, size, etc. should also be made for one treated replicate of each species and one control replicate upon termination of the test.

f) Data analysis

Methods for determining inhibition of biomass production by a pesticide, based on average growth rate or area under the growth curve, necessitate that the biomass (e.g., cell counts) be measured daily. For this test, however, only one pesticide concentration is being used, which alters and simplifies the analysis required.

For those experiments in which a solvent treatment was employed, a preliminary test is required for both growth and area under the growth curve to compare control and solvent (see section 1.6.8.3). The result affects the zero concentration (control) used.

It is preferred that the growth rate for each replicate be derived from the slope of the regression line of the natural log of cell count versus time rather than simply using the initial and final cell counts. Regression provides a better measure of the overall growth rate, as it takes into account the variation in growth rate during the entire test rather than emphasizing the start and end values. Biomass production is determined by calculating the area under the growth curve. For area under the growth curve, log transformation is recommended prior to analysis.

The growth rate and the log of the area under the growth curve should be calculated for each replicate and a mean and standard deviation determined for the pesticide treatment and control (plus solvent, if applicable). The significance of inhibition of biomass or growth rate due to pesticide application is assessed by conducting a one-tailed t-test to determine whether the mean for the pesticide treatment is significantly lower statistically than the mean for the control at the 0.05 level. The use of a two-tailed test or nonparametric statistics requires justification.

The test period selected for statistical analysis of the growth rate and area under the growth curve should correspond to the period of exponential cell growth (minimum 72 hours).

See Appendix B for further details on statistical analyses.

g) Progression to next tier

When treatment with a pesticide results in algal phytotoxicity that is statistically significant relative to the control, progression to Tier II testing is required.

For those algal species in test I-1 that show greater than 50%

inhibition relative to the control that is not statistically significant, the registrant must repeat the Tier I tests with increased power (e.g., more replicates) or proceed with Tier II testing (see section 1.6.8.4).

Fifty percent inhibition is used as the criterion for tests with algae, in contrast to 25% inhibition in tests with vascular plants (see section 3.1.2.2), because algae have shorter generation times, which allow assessment of the effects over several generations.

Under FIFRA (Holst and Ellwanger 1982; Holst 1986c), 50% inhibition is used to assess the toxicity of substances in all the aquatic testing protocols recommended and as a trigger for tier progression (there is no protocol for rooted aquatic plants). There are insufficient data to show that a different inhibition percentage is more appropriate in terms of potential for population recovery (Holst and Ellwanger 1982). As well, there is a large reference data base of EC50 values for many algae, which can be used for toxicity comparisons during hazard assessment.

D) Test limitations and validation

The test organisms should be cultured in the dilution water for at least two weeks prior to testing. Dilution water for tests with marine algae should be clearly described (ASTM 1991a).

The cell concentration in the control cultures should increase by a factor of at least 16 within three days for green algae (OECD 1984b). For other species, growth rate is to be determined when the algae are in a logarithmic phase of growth.

The test is most easily applied to pesticides that are soluble in water at concentrations above 1000 mg/L, but modifications (described in the OECD 1984b protocol) may be made for pesticides of lower solubility and for pesticides with a vapour pressure above 5.20×10^{-3} Pa. Henry's law constant can be used as an indicator of potential volatility from water. Alternatively, a formulated pesticide could be considered as the test substance.

The pH of the solutions should not deviate by more than one unit during the test (OECD 1984b).

A deviation in light intensity for any replicate of more than 15% from the selected intensity invalidates the test (ASTM 1991a).

A deviation in temperature of more than 4°C invalidates the test (ASTM 1991a).

E) Report

a) Experimental conditions

Reports should include, but not be limited to, the following:

1. identity of laboratory/individuals who performed the tests

2. test organisms: species, strain, origin

3. test substance: % TAI, concentration tested, analytical confirmation of test concentrations, solvents, reference toxicant

4. test conditions: pre-culture conditions; dates (start, end, observations), duration; medium composition; preparation of salt water; culture pH (start, end), light (source, quality [when possible], intensity [lux/m^2/s], photoperiod), temperature (°C), and agitation; culturing apparatus

5. experimental procedure: experimental design; description of controls; methods for measuring test conditions (#4 above); methods for measuring pesticide and solubilizing agent concentrations; methods for solubilizing test substance; cell counting methods; establishment of alternative endpoints

b) Results

1. calculation of test variables: a) cell counts for each flask at each measuring time (at the start of the test and every 24 hours), b) growth information for each replicate (plots of log cell counts vs. time, regression equations for log cell counts as a function of time, and plots of cell counts vs. time showing the area under the growth curve), c) growth rate and area under the growth curve for each replicate, d) test period over which growth rate and area under the growth curve were calculated, e) a statement as to whether any transformation was applied to either test variable (log transformation is recommended for area under the growth curve)

2. test of pesticide or solvent effect on each of the two test variables (i.e., growth rate and either the area or the log of the area under the growth curve): a) mean, standard deviation, and 95% confidence interval for the pesticide treatment, the control, and the solvent-only treatment, if employed, b) t-test results for those cases in which a solvent treatment was tested against the control, and a

statement as to what quantity was employed as the zero concentration (control) in tests of pesticide effect, c) t-test results comparing pesticide treatment with control for both the slope of and area under the frowth curve

3. other observed effects (cell size, shape, bacterial contamination, etc.), and interpretation of results

See Appendix B for further details on statistical analyses.

F) References

Recommended protocol:

ASTM (American Society for Testing and Materials). 1991a. Standard guide for conducting static 96-h toxicity tests with microalgae. Pages 845-856 *in* Annual book of ASTM standards. Vol. 11.04. Designation E 1218-90. Philadelphia, Pa.

References cited in text:

APHA 1989
Blaise *et al.* 1986
Blanck *et al.* 1984
Holst 1986c
Holst and Ellwanger 1982
ISO 1989
Lewis 1990
OECD 1984b
Swanson *et al.* 1991
U.S. EPA 1985a

Other relevant references:

Allen 1973
Fogg and Thake 1987
Freemark *et al.* 1990
Greene *et al.* 1989
Hayward 1968
Ibrahim 1983
Johnson and Sieburth 1979
Miles 1989
Nyholm 1985
Nyholm and Kallqvist 1989
Rippka *et al.* 1981
Sze 1986
Walsh 1988
Walsh *et al.* 1987a, 1987b

See Tables 3 and 4 for additional relevant references.

3.1.2.2 Test I-2: Vascular plant vegetative growth and vigour, maximum challenge concentration/rate, technical active ingredient/formulated

A) Objective

This test is to assess the effect of pesticide application on the vegetative growth of rooted aquatic and terrestrial vascular plant species.

B) When required

This test is required for all pesticides for nondomestic and outdoor domestic use in aquatic and terrestrial environments, except in greenhouses with closed systems, indoors and swimming pools (Table 1).

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

No protocol is recommended at this time. Effects on vegetative growth and vigour will be assessed using the plant screening data routinely generated by registrants for rooted aquatic and terrestrial vascular plants when determining the spectrum of phytotoxicity of a pesticide (refer to section 1.6.6).

2. Protocol modifications

Guidelines for screening experiments are described below.

a) Test species

Data should be provided on *all* vascular plants that have been tested during the screening process for pre-plant incorporated and pre- and post-emergence applications. Rooted aquatic species with submerged, emergent, or floating leaves and/or crop and noncrop terrestrial species that occur in Canada are preferred. For nonherbicides, at least 10 species from six families should be tested. For herbicides, 30 species from 10 families should be tested, preferably three species per family. Candidate test species are listed in Table 2. Species selected should be relevant to the use pattern of the pesticide. If any phytotoxicity is exhibited by nonherbicides at Tier I (algae or vascular species), testing of vascular species at Tier I should be expanded to a total of 30 species from 10 families.

b) Test substance

The results of all tests with the TAI (and formulated, if

conducted) should be reported. A TAI representative of the active ingredient used in the final product (with purity reported) should be tested. For aquatic species with submerged leaves, the concentration tested should equal the expected concentration in a column of water 15 cm deep (Holst and Ellwanger 1982; Holst 1986b) when the pesticide is sprayed at the maximum recommended label rate (MCC). Terrestrial and emergent aquatic species as well as species with floating leaves should be sprayed with pesticide at the maximum recommended label rate (MCR).

c) Test conditions

Detailed documentation should be provided on the testing procedure and conditions used by registrants for screening pesticide effects on plants.

d) Experimental design

The number of replicates per dose at any given time as well as the number of plants per dose (number of plants per pot) should be provided. A minimum of four replicates is recommended.

e) Data collection

A definition of the rating scale for endpoints used and its precision should be provided. Data are usually collected as herbicidal ratings - e.g., 1-9, 0-9, 0-10 or 0-100. Scales are based upon visual observations of plant stand, vigour, malformation, chlorosis, and overall plant appearance compared with a control.

f) Data analysis

Herbicidal qualitative ratings should be converted to percentages, setting each rating to the middle of its range:

Rating	Range	Midpoint	Rating	Range	Midpoint
9	100%	100%	4	30-44%	37%
8	91-99%	95%	3	16-29%	22.5%
7	80-90%	85%	2	6-15%	10.5%
6	65-79%	72%	1	1-5%	3%
5	45-64%	54.5%	0	0%	0%

A rating of 9 indicates full growth and vigour, 0, no growth.

A comparable method used by the Expert Committee on Weeds is

acceptable. Under this method, ratings from 0 to 9 are converted to percentages as follows:

Rating/9 = % control (e.g., 6/9 = 67%)

Endpoint values for each replicate should be determined and the mean value calculated for the pesticide treatment and control. Normally, each control unit will show full growth and vigour and thus will have a rating of 9 and a percent value of 100%. If the value is less than 100% for any of the control units, this should be clearly stated in the results, and the possible reasons for this should be specified.

No statistical tests of pesticide effect are carried out. Instead the units that received the high concentration of pesticide are compared directly with controls, and an effect equal to or greater than 25% is considered to be the threshold for further testing.

See Appendix B for further details on statistical analyses.

g) Progression to next tier

For those vascular plants screened for vegetative growth and vigour in test I-2 that show greater than 25% inhibition relative to the control, the registrant must proceed with Tier II testing (see section 1.6.8.4). Control units are expected to have a rating of 9 or 100%.

Twenty-five percent inhibition is used as a criterion for these tests, in contrast to 50% inhibition in tests with algae (see section 3.1.2.1), because these species have longer generation times, the test covers a much more limited portion of their life span (a short period in only one generation), and the test provides less information on toxicity.

The variation in plant response within a treatment can be expected to be at least 10% (Holst and Ellwanger 1982). Thus, in order to compare pesticide toxicities, an inhibition level greater than 10% must be used. Until there are sufficient data to show that a different inhibition level is more appropriate, 25% inhibition will be used as the progression criterion for vegetative growth and vigour testing, as is the case for terrestrial testing under FIFRA (Holst and Ellwanger 1982).

D) Test limitations and validation

Plants in the control should exhibit normal growth throughout the test.

E) Report

a) Experimental conditions

Reports should include, but not be limited to, the following:

1. identity of laboratory/individuals who performed the tests

2. test organisms: species (Latin and common names), source, stage of plant life cycle (seedling, leaf stages, etc.)

3. test substance: % TAI, formulation type, formulants, adjuvants, concentrations tested, analytical confirmation of test concentrations, solvents

4. test conditions: date and duration of test; geographic location; environment description (indoors, outdoors, experimental apparatus); application method (pre-plant incorporation, pre- and post-emergence); temperature (°C); light quality (when possible) and intensity (lux/m^2/s); relative humidity; watering method and frequency; soil (type, % organic carbon, pH)

5. experimental procedure: methods for measuring test conditions (#4 above); methods for determining pesticide concentrations; methods for solubilizing, incorporating, and applying pesticide; control description; number of replicates per pesticide treatment and control; number of plants per replicate and per pot

b) Results

1. calculation of the test variable: a) descriptions of visible effects related to treatment for each species, b) rating scale and percent growth and vigour for each replicate

2. test of pesticide effect: a) mean value of percent growth and vigour for pesticide treatment and for control, b) the difference between these mean values, c) examination of the difference to determine if it is greater than 25% of the mean value for control

3. other observed effects and interpretation of results

See Appendix B for further details on statistical analyses.

F) References

References cited in text:

Holst 1986b
Holst and Ellwanger 1982

Other relevant references:

Freemark *et al.* 1990
OECD 1984c
Sokal and Rohlf 1981
U.S. EPA 1985d

3.2 Tier II requirements

The purpose of Tier II testing is to quantify the magnitude of pesticide toxicity to different groups of plant species for which protocols are available when exposure is by way of the water column or by direct spraying and to demonstrate the phytotoxic potential of the formulated pesticide. Dose-response relationships for test substances and nontarget plants are examined for doses below the MCC/MCR. These definitive tests are carried out with doses covering the NOEC/NOEL and EC50 levels. In addition to algal growth and vegetative growth and vigour tests for vascular plants, Tier II also involves testing with a floating aquatic vascular plant (usually *Lemna gibba* strain G-3) and seed germination/root elongation tests for vascular plants.

Algal species used in test I-1 are tested at Tier II if pesticide application resulted in significant inhibition or in nonsignificant inhibition that was greater than 50% for any of the endpoints recorded.

For vascular species (aquatic and terrestrial) tested in the vegetative growth and vigour test (test I-2), inhibition that was greater than 25% for any endpoint is used as the criterion for species inclusion at Tier II.

When phytotoxicity to nonherbicides is exhibited by algae or vascular plants in Tier I testing, Tier II testing first involves expanding the Tier I MCC/MCR test (test I-2) with vascular plants so that a total of 30 species and 10 families (preferably three species per family) are tested (including the 10 species from six families already tested at Tier I). Tier II tests described in section 3.2.1 are then required, following the progression detailed in section 1.6.5 and in Table 1. (If none of the 30 species shows phytotoxicity, the definitive vegetative growth test for vascular plants at Tier II [test II-4] would not be required.)

3.2.1 Tests

Testing requirements for Tier II are shown in Table 1. The relationships between Tier II testing and testing at Tiers I and

III are illustrated in Figure 1 and section 1.6.5.

3.2.1.1 Test II-1: Algal growth and reproduction, definitive, technical active ingredient

This test is similar to the Tier I MCC test (test I-1), except that a range of concentrations, rather than one concentration, is used. Only the details specific to this test will be described below. All other details are as outlined in test I-1.

A) Objective

The objective of this test is to determine the dose-response relationship, NOEC, EC25, and EC50 for sensitive algal species that responded in the MCC test at Tier I (test I-1) and the TAI of a pesticide.

B) When required

This test is conditionally required (Table 1, see section 1.6.5). It is required for species exhibiting significant inhibition relative to the control at the 0.05 level in the Tier I MCC test (test I-1). This test may be conducted for species exhibiting greater than 50% inhibition at Tier I that is not statistically significant, instead of repeating the Tier I test.

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

The ASTM (1991a) protocol is recommended for freshwater and marine species.

2. Protocol modifications

Basic test methodology is described in section 3.1.2.1 (test I-1). Additional details are provided below.

a) Test species

See B) above.

b) Test substance

The TAI (with purity reported) tested should be representative of the active ingredient used in the final product. The test concentrations should span the NOEC and EC50 for each species.

A reference toxicant or positive control should be used (see section 3.1.2.1, test I-1).

c) Test conditions

See section 3.1.2.1.

d) Experimental design

See section 3.1.2.1.

e) Data collection

See section 3.1.2.1.

f) Data analysis

The growth rate and the log of the area under the growth curve should be calculated for each replicate and a mean and standard deviation determined for pesticide treatments and control (plus solvent, if applicable) prior to analysis. For area under the growth curve, log transformation is recommended prior to analysis.

For those experiments in which a solvent treatment was employed, a preliminary test is required for both growth rate and log of the area under the growth curve to compare control and solvent (see section 1.6.8.3). The result affects the zero concentration (control) used in the determination of the NOEC and the estimation of the EC50.

The NOEC is determined as the highest pesticide concentration fo which the results (mean slope, mean area under the curve) are not statistically different from the control results at the 0.05 level. The NOEC must be determined for both growth rate and log of the area under the growth curve by testing the highest concentration first and proceeding to lower concentrations until

the effect is not significant. The NOEC should be determined using Williams' test (Williams 1971), which compares the means of ordered doses with the control mean. One-tailed tests should be used. Dunnett's test, sometimes recommended for determining the NOEC, assumes that there is no order to the doses being compared with the control and is therefore not appropriate.

For both growth rate and log of the area under the growth curve, the inhibition should be calculated for each concentration from:

$$\text{Inhibition} = 1 - \frac{\text{pesticide concentration mean}}{\text{control mean}}$$

The EC50 should be determined by fitting an equation for inhibition as a function of concentration using regression or other standard techniques. If appropriate, transformations such as the probit or logit may be applied to the inhibition, and the log transformation should be applied to the concentrations. The inhibition values and the dose-response relationship derived should be presented graphically.

See Appendix B for further details on statistical analyses.

g) Progression to next tier

Progression to Tier III and test III-1 is required under the following conditions:

$$\text{EEC} > \frac{\text{EC50}}{10} \text{ for any algal species}$$

where:

EEC = the concentration resulting from application at the maximum recommended label rate to a 15-cm-deep column of water

EC50 = the concentration resulting in a 50% reduction in the endpoint being measured relative to the control

10 = uncertainty factor

According to the calculations outlined by Blanck *et al.* (1984), an algal assay using only three species would underestimate the toxicity of a compound by a factor of 100 (at the 95% confidence level) when compared with the toxicity of the same compound to the most sensitive algal species. However, because the EEC, as calculated in these guidelines, is already a worst-case scenario, an uncertainty factor of 10 is used in assessing the hazard posed by a pesticide to nontarget plants.

D) Test limitations and validation

Refer to section 3.1.2.1 (test I-1).

E) Report

a) Experimental conditions

The basic topics on which to report are outlined in section 3.1.2.1 (test I-1).

b) Results

1. calculation of test variables: a) cell counts for each flask at each measuring time (at the start of the test and every 24 hours), b) growth information for each replicate (plots of log cell counts vs. time, regression equations for log cell counts as a function of time, and plots of cell counts vs. time showing the area under the growth curve), c) growth rate and area under the growth curve for each replicate, d) test period over which growth rate and the area under the growth curve were calculated, e) a statement as to whether any transformation was applied to either test variable (log transformation is recommended for area under the growth curve)

2. test of pesticide or solvent effect and estimation of NOEC and EC50 for each of the two test variables (i.e., growth rate and either the area or the log of the area under the growth curve): a) mean, standard deviation, and 95% confidence interval for each pesticide concentration, control, and solvent-only treatment, if employed, b) t-test results for those cases in which a solvent treatment was tested against the control, and a statement as to what quantity was employed as the zero concentration (control) for identifying the NOEC and estimating the EC50, c) results of Williams' test and identification of the NOEC for each species, d) inhibition values for each pesticide concentration (see the data analysis section above), e) discussion of any extreme inhibition values, such as values less than zero or greater than 100% (see Appendix B), f) the transformation applied to the inhibition or the concentration prior to fitting the equation, if any, g) the results of the least-squares fitting of the equation for the inhibition as a function of pesticide concentration, including the equation fitted, and the number of degrees of freedom of the residuals (which is equal to the number of data points minus the number of parameters in the equation), h) EC50 values for average growth rate and biomass for each

species, i) graph of the inhibition-concentration relationship, showing the inhibition values and the fitted equation

3. other observed effects (cell size, shape, bacterial contamination, etc.), and interpretation of results

See Appendix B for further details on statistical analyses.

F) References

Recommended protocol:

ASTM (American Society for Testing and Materials). 1991a. Standard guide for conducting static 96-h toxicity tests with microalgae. Pages 845-856 *in* Annual book of ASTM standards. Vol. 11.04. Designation E 1218-90. Philadelphia, Pa.

References cited in text:

Blanck *et al.* 1984
Williams 1971

Other relevant references:

Finney 1971
Fleiss 1973
OECD 1984b
Siegel 1956
Snedecor and Cochran 1967
Sokal and Rohlf 1981
U.S. EPA 1985a
Walsh *et al.* 1987a

Additional references pertaining to algae are cited under test I-1, section 3.1.2.1.

3.2.1.2 Test II-2: Lemna gibba *strain G-3, vegetative growth and reproduction, definitive, formulated*

A) Objective

The objective of this test is to determine the dose-response relationship, NOEL, EC25, and EC50 for growth (rate and biomass) and mortality of a floating aquatic vascular plant, *Lemna gibba* strain G-3, using a formulated pesticide when exposure is through a spray application and through the medium.

B) When required

This test is conditionally required (Table 1). It is required when algae or vascular plants exhibit a phytotoxic response in Tier I (see section 1.6.5).

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

The ASTM (1991b) protocol is recommended as the basis for the Canadian protocol because the methodology is described in detail.

2. Protocol modifications

a) Test species

Lemna gibba strain G-3 is preferred, but other floating aquatics such as *Lemna minor* can be used as test species.

b) Test substance

The ASTM (1991b) protocol does not specify the test substance, as it addresses general chemical testing, and it does not require testing with a spray application. In this guideline, a formulated pesticide (% TAI reported) representative of the final formulation is tested at concentrations covering the NOEL and EC50 levels. If adjuvants are specified on the label for normal use, they should also be added when testing (see definition of formulated pesticide in section 1.5). Use of the formulated pesticide is required to enhance contact and sorption of the product in a manner similar to the end-use scenario.

The pesticide should be sprayed over the test vessels at a given rate after *Lemna* is added, in a manner similar to application over test plants in the greenhouse (rates are calculated as application over a given surface). Plants should not be removed after spraying, so that they are exposed through spray *and* through the medium during the test period. A plate placed level with the vessels will serve to calibrate and monitor the application rate. Droplet spectrum and atomization technique

should be specified. Use of a fine spray corresponding to the usual lower range of droplet sizes (100-250 um) used in field operation for herbicides, insecticides, or fungicides (Sheehan *et al.* 1987) is preferred to ensure that fronds are thoroughly covered by the pesticide.

A reference toxicant or positive control should be used (see section 3.1.2.1, test I-1).

c) Test conditions

The basic pre-treatment and culture conditions outlined in the recommended protocol apply.

The test should be performed using the static technique. Replacement of the nutrient solution, as indicated in the TSCA (U.S. EPA 1985b) protocol, is not recommended. Over seven days, growth is not likely to become nutrient limited. Solution replacement would involve handling and disturbing the plants, thus introducing additional experimental error.

20X-AAP medium is preferred to Hoagland's medium (ASTM 1991b). pH should be maintained at 7.5.

Temperature should be recorded as one of the following:

1) daily growth medium temperature in a replicate containing growth medium only, or

2) daily maximum and minimum air temperature.

Light intensity should be recorded at the level of the growth medium surface at the position of each replicate before the test begins.

d) Experimental design

At least four replicates should be used to maintain confidence in the results. The number of plants is flexible, provided each test vessel receives an identical number of plants (4-5) and fully mature fronds (maximum of 16) at the start of any given experiment.

e) Data collection

The total number of living and dead (yellow or discoloured) should be counted at regular intervals (days 0, 3, 6, and 7) during the test for each replicate. Total biomass of whole plants (constant dry weight at 70°C) should be measured at the

end of the test (live fronds). Although biomass is usually correlated with other endpoints, it is the most objective and reproducible endpoint. As well, simply counting the number of fronds would not provide an accurate assessment of effects in a case in which frond size, but not frond number, was affected by pesticide application.

Changes observed in frond gloss, root length and number, and other parameters should also be recorded, as they may provide some information on the mode of action of the pesticide.

f) Data analysis

The value of each test variable should be calculated for each replicate. These variables are the frond growth rate, percent frond survival, and the final dry weight of the live fronds. Frond growth rate is defined as the slope of the regression of the log of the number of live fronds versus time in days. Percent frond survival is determined as the number of living fronds divided by the total number of fronds on day 7, multiplied by 100. Whole plant dry weight is defined as dry weight of live fronds on day seven.

The recommended transformations are the log transformation for final dry weight of live fronds and the angular transformation for percent frond survival. None is required for the frond growth rate.

The mean and standard deviation for the frond growth rate, whole-plant dry weight, and percent frond survival are determined for each concentration and for the control.

For those experiments in which a solvent treatment was employed, a preliminary test is required to compare control and solvent for each of the three variables (see section 1.6.8.3). The result affects the zero concentration (control) used in the determination of the NOEL and the estimation of the EC50.

The NOEL is determined as the highest pesticide concentration used for which the result is not statistically different from the control results at the 0.05 level. The NOEL must be determined for each variable by testing the highest concentration first and proceeding to lower concentrations until the effect is not significant. The NOEL should be determined using Williams' test (Williams 1971) as discussed under data analysis in test II-1 (see section 3.2.1.1).

Inhibition values (frond growth rate, whole-plant dry weight and percent frond survival) are then determined for each concentration (see section 3.2.1.1, test II-1, data analysis, calculation of inhibition). For each of the three endpoints, a

curve of inhibition as a function of concentration is plotted.

The EC50 should be determined by fitting an equation to the percent inhibition or survival as a function of concentration using regression or other standard techniques. If appropriate, transformations such as probit or logit may be applied to the inhibitions, and the log transformation should be applied to the concentrations. The inhibition values and the dose-response relationship derived should be presented graphically.

See Appendix B for further details on statistical analyses.

g) Progression to next tier

Progression to Tier III and test III-2 is required under the following conditions:

$$EEC > \frac{EC50}{10}$$

where:

EEC = the concentration resulting from application at 100% of the maximum recommended label rate when sprayed over plants if the pesticide is likely to overspray nontarget habitats, *or* the concentration resulting from application at 10% of the maximum recommended label rate if the pesticide will reach nontarget plants through drift, runoff, and washoff *only*

EC50 = the concentration resulting in a 50% reduction in the endpoint being measured relative to the control

10 = uncertainty factor

The same uncertainty factor is applied to this test as for the algal tests.

D) Test limitations and validation

The test organisms should be cultured in growth medium for at least eight weeks prior to the start of the test. A weekly transfer schedule into fresh growth medium is suggested (ASTM 1991b).

The results of the test are sensitive to minor variations in environmental variables such as light (Santelmann 1977). A

deviation in light intensity of more than 15% from the selected intensity for any replicate invalidates the test (ASTM 1991b).

The biomass in the control replicates should increase by a factor of five by the end of the test.

pH should be measured at the beginning and end of the test.

A deviation in temperature of more than 4°C invalidates the test (ASTM 1991b).

E) Report

a) Experimental conditions

The basic topics on which to report are outlined in the recommended protocol and in section 3.1.2.1. Additional details are provided below.

3. test substance: formulation type, % TAI, formulants, adjuvants, concentration tested, analytical confirmation of test concentrations, droplet spectrum

b) Results

1. calculation of test variables: a) number of plants and number of fronds for each plant that are alive or dead in each replicate at each sampling time, b) total dry weight of whole plants in each replicate at the end of the test, c) frond growth rate and percent frond survival for each replicate, d) a statement as to whether a transformation was applied to any test variables (such as log transformation for dry weight or an angular transformation for percent survival)

2. test of pesticide effect and estimation of NOEL and EC50 for each of the three test variables (i.e., growth rate of live fronds, percent frond survival, and final dry weight of live fronds): a) mean, standard deviation, and 95% confidence interval for each pesticide concentration, the control, and the solvent-only treatment, if employed, b) the t-test results for those cases in which a solvent treatment was tested against control, and a statement as to what quantity was employed as the zero concentration (control) for identifying the NOEL and estimating the EC50, c) results of Williams' test and identification of the NOEL, d) inhibition for each test variable for each pesticide concentration (see section 3.2.1.1, test II-1, data analysis, calculation of inhibition), e) discussion of any extreme inhibition values such as values less than zero or

greater than 100% (see Appendix B), f) the transformation applied to the inhibitions or the concentrations prior to fitting the equation, if any, g) the results of the least squares fitting of the equation for the inhibition as a function of pesticide concentration, including the equation fitted, and the number of degrees of freedom of the residuals (which is equal to the number of data points minus the number of parameters in the equation), h) EC50 values for average growth rate and biomass, i) a graph of the dose-response relationship showing inhibition values

3. other changes observed, such as frond gloss and root length and number, and interpretation of results

See Appendix B for further details on statistical analyses.

F) References

Recommended protocol:

ASTM (American Society for Testing and Materials). 1991b. Standard guide for conducting static toxicity tests with *Lemna gibba* G3. Pages 1137-1146 *in* Annual book of ASTM standards. Vol. 11.04. Designation E 1415-91. Philadelphia, Pa.

References cited in text:

Santelmann 1977
Sheehan *et al.* 1987
U.S. EPA 1985b
Williams 1971

Other relevant references:

Davis 1981
Freemark *et al.* 1990
Holst 1986c
Holst and Ellwanger 1982
Lockhart *et al.* 1989

3.2.1.3 Test II-3: Vascular plant, seed germination/root elongation, definitive, technical active ingredient

A) Objective

The objective of this test is to determine the dose-response relationship, NOEC and EC25 for the technical active ingredient in a pesticide and two critical stages of seedling establishment (seed germination, root elongation) for a variety of terrestrial plant species.

B) When required

This test is conditionally required (Table 1). It is required

when algae or vascular plants exhibit a phytotoxic response in Tier I (see section 1.6.5).

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

The protocol for seed germination and root elongation under TSCA (U.S. EPA 1985c) is recommended because the general procedure is outlined in detail; inert material is recommended rather than soil (variation in composition would affect the outcome and reduce the degree of test standardization), validity requirements are specified, and test duration is based on control results rather than a fixed length of time.

2. Protocol modifications

Basic test methodology is described in the recommended protocol. Additional details are provided below.

a) Test species

The species recommended for testing under the TSCA protocol (U.S. EPA 1985c) are all crops, although the protocol does suggest that other species of economic or ecological importance may be appropriate. In this guideline, at least half of the species must be noncrop in order to increase the diversity of species tested. At least 10 species should be chosen to reflect the environments likely to be contaminated by the pesticide. They must include members of the monocotyledons and dicotyledons and represent at least six plant families. Suggestions for species to be used are given in Table 2, which lists species from families that have already been used in testing.

b) Test substance

The recommended protocol does not indicate the type of substance to be used, as it is a general-purpose protocol. The TAI (with purity reported) tested should be representative of the active ingredient used in the final product. The test concentrations should span the NOEC and EC25 for each species.

A reference toxicant or positive control should be used. Suitable reference toxicants are given by Greene *et al.* (1989).

c) Test conditions

Pre-test germination requirements for temperature, light, scarification, etc. will vary among species and should be provided for each species.

The inert material used to fill the Petri dishes, as outlined in the recommended protocol, should be covered with filter paper (moistened with test solution) to keep the roots pressed against the upper surface of the Petri dish and facilitate measurement.

If severe fungal problems prevent the attainment of root growth to 20 mm for 15 seeds in control replicates, the test should be repeated. A chlorox rinse may be used on the seeds prior to testing.

d) Experimental design

Seeds of the same lot or source, year, and size (selected using dockage sieves) are used.

Four replicates, as outlined in the OECD (1984c) protocol, should be used, rather than the three required in the recommended protocol, to increase confidence in the results.

The number of seeds to be used per replicate is determined by the percent germination of the seed lot and subsequent loss, perhaps due to fungal problems. Enough seeds per replicate must be used so that at least 15 seeds in each control replicate germinate and produce roots at least 20 mm long. The test is complete when this has been achieved.

If chlorox is used to prevent fungal problems, three controls are required (see section 1.6.8.3).

e) Data collection

See recommended protocol

f) Data analysis

The value of each test variable should be calculated for each replicate. These variables are the mean root length and the percent germination. Mean root length is defined as the mean of root length of all germinating seeds. Percent germination is the

percentage of seeds that germinated. The recommended transformation for percent germination is the angular transformation. None is required for mean root length. The averages of the mean root length and mean percent germination are calculated for each pesticide concentration, control, and the solvent-only treatment.

For those experiments in which a solvent treatment was employed, a preliminary test is required to compare control and solvent for each of the two variables (see section 1.6.8.3). The result affects the zero concentration (control) used in the determination of the NOEC and the estimation of the EC25.

The NOEC is determined as the highest pesticide concentration used where the result is not statistically different from the control results at the 0.05 level. The NOEC must be determined for each test variable by testing the highest pesticide concentration first and proceeding to lower concentrations until the effect is not significant. The NOEC should be determined using Williams' test (Williams 1971), as discussed under data analysis in test II-1 (see section 3.2.1.1).

For both mean root length and percent germination, the percent inhibition should be calculated for each concentration (see section 3.2.1.1, test II-1, data analysis, calculation of inhibition).

The EC25 should be determined by fitting an equation using regression or other standard techniques. If appropriate, transformations such as probit or logit may be applied to the inhibitions and a log transformation should be applied to the concentrations prior to fitting the equation.

For each endpoint, a plot of percent inhibition as a function of concentration should be prepared.

See Appendix B for further details on statistical analyses.

g) Progression to next tier

Progression to Tier III and test III-3 is required under the following conditions:

$$EEC > \frac{EC25}{10}$$

where:

EEC = the concentration resulting from application at the maximum recommended label rate to a 3-cm-deep column of soil with a bulk density of 1.5 g/cm^3

EC25 = the concentration resulting in a 25% reduction in the endpoint being measured relative to the control

10 = uncertainty factor

D) Test limitations and validation

Because germination rates vary among species, a fixed percent germination is not used as a validation criterion as it is in the recommended protocol. Empirical performance criteria (i.e., mean % germination with 95% confidence intervals) are determined in advance of the test for seed lots of each species. Therefore, the performance of the control replicates should be tracked during the test period; if the percent germination of one of the control replicates falls below the confidence intervals at any time, the test must be repeated. If two consecutive tests fall below these intervals, the seed lot should be replaced.

A seed is considered to have germinated when the radicle is 5 mm long.

Sufficient numbers of seeds should be used to ensure that at least 15 seeds per control replicate produce healthy primary roots that are at least 20 mm long.

E) Report

a) Experimental conditions

The basic topics on which to report are outlined in the recommended protocol and in section 3.1.2.1.

b) Results

1. calculation of test variables: 1) empirical performance criteria and 95% confidence intervals for each seed lot tested, b) number and percentage of seeds germinating in each replicate, c) length of each primary root for germinated seeds for each replicate, d) a statement as to whether any transformation was applied to either test variable (angular transformation is recommended for percent germination)

2. test of pesticide or solvent effect and estimation of NOEC and EC25 for each of the two test variables (i.e., percent germination and root length): a) mean, standard deviation, and 95% confidence interval for each pesticide concentration, control, and solvent-only treatment, if

employed, b) t-test results for those cases in which a solvent treatment was tested against the control, and a statement as to what quantity was employed as the zero concentration (control) for identifying the NOEC and estimating EC25, c) results of Williams' test and the the identification of the NOEC for each species, d) inhibition of mean root length and percentage of seeds germinating for each pesticide concentration (see section 3.2.1.1, test II-1, results, calculation of % inhibition), e) discussion of any extreme inhibition values such as values less than zero or greater than 100% (see Appendix B), f) the transformation applied to the inhibitions or concentrations prior to fitting the equation, if any, g) the results of the least-squares fitting of the equation for the inhibition as a function of pesticide concentration, including the equation fitted, and the number of degrees of freedom of the residuals (which is equal to the number of data points minus the number of parameters in the equation), h) EC25 values for average root length and number of seeds germinating for each species, i) a graph of the dose-response relationship for each species, showing inhibition values

3. other changes observed, such as root thickness or colour, and interpretation of results

See Appendix B for further details on statistical analyses.

F) References

Recommended protocol:

U.S. EPA (United States Environmental Protection Agency). 1985c. Toxic Substances Control Act test guidelines: environmental effects testing guidelines. Seed germination/root elongation toxicity test. Fed. Regist. 50(188):39389-39391.

References cited in text:

Greene *et al.* 1989
OECD 1984c
Williams 1971

Other relevant references:

Edgington 1987
Finney 1971
Freemark *et al.* 1990
Hilman and Johndro 1986
Holst 1986a
Holst and Ellwanger. 1982
Linder 1989
Noreen 1989
Sokal and Rohlf 1981
Thomas and Cline 1985
Wang 1986
Wang and Elseth 1989

3.2.1.4 Test II-4: Vascular plant, vegetative growth and vigour, definitive, technical active ingredient/formulated

This test is similar to the Tier I test (test I-2), except that a range of concentrations, rather than one concentration, is used.

A) Objective

The objective of this test is to determine the dose-response relationship, NOEC/NOEL, and EC25 for rooted aquatic and terrestrial vascular plant species.

B) When required

This test is conditionally required (Table 1; see section 1.6.5). It is required for vascular species exhibiting significant inhibition relative to the control at the 0.05 level in the Tier I MCC/MCR test (test I-2). This test may be conducted for vascular species exhibiting greater than 25% inhibition that is not statistically significant at Tier I, rather than repeating the Tier I test.

When phytotoxicity to nonherbicides is exhibited by algae or vascular plants at Tier I, Tier II testing first involves expanding the Tier I MCC/MCR test (test I-2) with vascular plants so that a total of 30 species and 10 families are tested, preferably three species per family. (If none of the 30 species shows phytotoxicity, the definitive vegetative growth test for vascular plants [test II-4] would not be required.)

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

No protocol is recommended. Effects on vegetative growth and vigour will be assessed using the plant screening data routinely generated by registrants for rooted aquatic and terrestrial vascular plants when determining the spectrum of phytotoxicity of a pesticide (see section 1.6.6).

2. Protocol modifications

Basic test methodology is described in section 3.1.2.2 (test I-2). Additional details are provided below.

a) Test species

This test is required for species exhibiting greater than 25% inhibition relative to the control in the Tier I MCC/MCR test (test I-2).

b) Test substance

The TAI (with purity reported) tested should be representative of the active ingredient used in the final product. Test results for the formulated pesticide should be submitted if available. The test concentrations should span the NOEC/NOEL and EC25 for each species.

c) Test conditions

See section 3.1.2.2.

d) Experimental design

See section 3.1.2.2.

e) Data collection

See section 3.1.2.2.

f) Data analysis

Herbicidal qualitative ratings should be converted to percentages, with each rating set to the middle of its range. Endpoint values for each replicate should be determined and the mean value calculated for each pesticide concentration and control. Refer to section 3.1.2.2, data analysis, for further details.

The percent growth and vigour for each replicate should be determined and the mean value calculated for each pesticide concentration and control.

Each control will normally show full growth and vigour and thus will have a rating of 9 and a percent value of 100%. If the

value is less than 100% for any of the control units, this should be clearly stated in the results, and the possible reasons for this should be specified. The NOEC/NOEL is determined by direct observation, not by statictical analysis. It is the lowest concentration for which all of the replicates have a rating of 9 or 100%.

Inhibition is calculated for each pesticide concentration, (see section 3.2.1.1, test II-1, data analysis, calculation of inhibition).

The EC25 should be determined for each species by fitting an equation using regression or other standard techniques. If appropriate, transformations such as probit or logit may be applied to the inhibitions and a log transformation should be applied to the concentrations prior to fitting the equation. A plot of percent inhibition as a function of concentration should be prepared. The statistical procedure should be thoroughly described to assist evaluation.

See Appendix B for further details on statistical analyses.

g) Progression to next tier

Progression to Tier III and test III-3 is required under the following conditions:

EEC > EC25 for 25% of species or 50% of families

where:

Aquatic species with submerged leaves

EEC = the concentration resulting from application at the maximum recommended label rate to a 15-cm-deep column of water

Species with floating leaves, aquatic emergent and terrestrial vascular plants

EEC = the concentration resulting from application at 100% of the maximum recommended label rate when sprayed over plants if the pesticide is likely to overspray nontarget habitats, *or* the concentration resulting from application at 10% of the maximum recommended label rate if the pesticide will reach nontarget plants through drift, runoff, and washoff *only*

EC25 = the concentration resulting in a 25% reduction in the endpoint being measured relative to the

control

D) Test limitations and validation

Control plants should exhibit normal growth throughout the test.

E) Report

a) Experimental conditions

The basic topics on which to report are outlined in section 3.1.2.2 (test I-2).

b) Results

1. calculation of the test variable: a) descriptions of visible effects related to treatment for each species, b) rating scale and percent growth and vigour for each replicate

2. test of pesticide effect and estimation of NOEC/NOEL and EC25: a) mean values of percent growth and vigour for each treatment, b) NOEC/NOEL for each species, c) inhibition for each pesticide concentration relative to the control (see section 3.2.1.1, test II-1, data analysis, calculation of inhibition), d) the transformation applied to the inhibitions or the concentrations prior to fitting the equation, if any, e) the results of the least-squares fitting of the equation for the inhibition as a function of pesticide concentration, including the equation fitted, and the number of degrees of freedom of the residuals (which is equal to the number of data points minus the number of parameters in the equation), f) EC25 values, h) a graph of the dose-response relationship showing inhibition values

3. other observed effects, and interpretation of results

See Appendix B for further details on statistical analyses.

F) References

Relevant references are cited under test I-2, section 3.1.2.1.

3.3 Tier III requirements

Testing is conditionally required and involves testing a rooted submerged aquatic (test III-1), a rooted emergent aquatic (test

III-2), and special testing with single species (test III-3). Specific triggers indicating that testing at this tier is required are outlined after the appropriate tests and in Figure 1. Registrants may bypass Tier III and go directly to field testing at Tier IV.

3.3.1 Tests

Testing requirements for Tier III are shown in Table 1. The relationships between Tier III testing and testing at Tiers II and IV are illustrated in Figure 1 and section 1.6.5.

3.3.1.1 Test III-1: Rooted submerged aquatic vascular plant, vegetative growth and vigour, definitive, technical active ingredient/formulated

A) Objective

The objective of this test is to determine the dose-response relationship, NOEC and EC25 for a rooted aquatic vascular plant species with submerged leaves that is exposed to the TAI or the formulated pesticide.

B) When required

This test is conditionally required (Table 1). It is required if the EEC is greater than one-tenth the EC50 for any algal species tested at Tier II (test II-1).

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

No protocol is recommended at this time. Although regulatory advisors are *not* responsible for designing test protocols for registrants, they will advise registrants of particular requirements and comment on registrants' protocols if they are submitted before the tests are conducted.

2. Protocol modifications

Details on test species, test substance, and requirements for progression to Tier IV are provided below. All other test methodology must be designed by registrants.

a) Test species

Rooted, submerged aquatics that occur in Canada are preferred.

b) Test substance

A water application of the TAI of a pesticide (% purity reported) or the formulated pesticide representative of the final formulation (% TAI reported) is tested at concentrations covering the NOEC and EC25 levels for the test species.

g) Progression to next tier

Progression to Tier IV is required under the following conditions:

$$EEC > \frac{EC25}{10}$$

where:

submerged aquatics

EEC = the concentration resulting from application at the maximum recommended label rate to a 15-cm-deep column of water

EC25 = the concentration resulting in a 25% reduction in the endpoint being measured relative to the control

10 = uncertainty factor

3.3.1.2 Test III-2: Rooted emergent aquatic vascular plant, vegetative growth and vigour, definitive, formulated

A) Objective

The objective of this test is to determine the dose-response relationship, NOEL and EC25 for a rooted aquatic vascular plant species with emergent leaves that is exposed to a formulated pesticide.

B) When required

This test is conditionally required (Table 1). It is required if the EEC is greater than one tenth of the EC50 for *Lemna gibba* strain G-3 (test II-2).

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

No protocol is recommended at this time. Although regulatory advisors are *not* responsible for designing test protocols for registrants, they will notify registrants of particular requirements and comment on registrants' protocols if they are submitted for review before the tests are conducted.

2. Protocol modifications

Details on test species, test substance, and requirements for progression to Tier IV are provided below. All other test methodology must be designed by registrants.

a) Test species

Rooted, emergent aquatics that occur in Canada are preferred.

b) Test substance

A spray application of a formulated pesticide (% TAI reported) representative of the final formulation is tested at concentrations covering the NOEL and EC25 levels for each species. If adjuvants are specified on the label for normal use, they should be added when testing (see definition of formulated pesticide in section 1.5).

g) Progression to next tier

Progression to Tier IV is required under the following conditions:

$$EEC > \frac{EC25}{10}$$

where:

emergent aquatics or species with floating leaves

EEC = the concentration resulting from application at 100% of the maximum recommended label rate when sprayed over plants if the pesticide is likely to overspray nontarget habitats, *or* the concentration resulting from application at 10% of the maximum recommended label rate if the pesticide will reach nontarget plants through drift, runoff, and washoff *only*

EC25 = the concentration resulting in a 25% reduction in the endpoint being measured relative to the control

10 = uncertainty factor

3.3.1.3 Test III-3: Special testing with single species

A) Objective

The objective of further testing with single species is to address specific critical concerns.

B) When required

This testing is conditionally required (Table 1) on a case-by-case basis.

Refer to section 1.6.7 for general testing requirements and the registration process.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

Protocols are available for some special tests, but none is recommended at this time. Registrants will be notified of particular requirements and, in conjunction with registrants, interested regulatory advisors will decide on the most appropriate protocol for conducting the studies.

Tests might address such topics as:

1) acute lethality levels for algae and determination of effects as algistatic or algicidal,

2) seed germination/root elongation with a formulated pesticide,

3) reproduction (pollen viability, seed production and viability),

4) entire life cycles (phenology),

5) genotoxicity,

6) translocation and bioaccumulation, and

7) greenhouse tests tailored to specific questions.

2. Protocol modifications

Details on test species and requirements for progression to Tier IV are provided below.

a) Test species

Additional species that might be tested at this tier include those characteristic of the receiving environment or those species that are known to be important to wildlife and/or fish habitat.

g) Progression to next tier

Progression to Tier IV is required under the following conditions:

$$EEC > \frac{EC25}{10} \text{ (vascular rooted plants)}$$

or

$$EEC > \frac{EC50}{10} \text{ (algae and } \textit{Lemna}\text{)}$$

where:

algae and species with submerged leaves

EEC = the concentration resulting from application at the maximum recommended label rate to a 15-cm-deep column of water

seed germination/ root elongation

EEC = the concentration resulting from application at the maximum recommended label rate to a 3-cm-deep column of soil with a bulk density of 1.5 g/cm^3

Lemna, *species with floating leaves, aquatic emergent and terrestrial vascular plants*

EEC = the concentration resulting from application at 100% of the maximum recommended label rate when sprayed over plants if the pesticide is likely to overspray nontarget habitats, *or* the concentration resulting from application at 10% of the maximum recommended label rate if the pesticide will reach nontarget plants through drift, runoff, and washoff *only*

EC25 or EC50
= the concentration resulting in a 25% or 50% reduction in the endpoint being measured relative to the control

10 = uncertainty factor

F) References

References concerning toxicity testing and aquatic vascular plants:

References for special tests discussed above:

Flemming and Momot 1988
Freemark *et al.* 1990
Macauley *et al.* 1989
Maury *et al.* 1989
Ribeyre and Boudou 1989
Sculthorpe 1971
Swanson *et al.* 1991
Bristow and Windom 1987
Church and Williams 1977
McFarlane *et al.* 1989
Ratsch 1989
Sandhu *et al.* 1989
Te-Hsiu 1989
U.S. EPA 1985e

3.4 Tier IV requirements

Testing is conditionally required on a case-by-case basis. Specific test requirements for Tier IV will depend on the results obtained from testing at Tiers I, II, and III. The experimental design will be determined through discussions involving interested regulatory advisors and the registrant. In general, testing at this level will focus on a community of species that make up a habitat rather than on specific plant species and will assist in answering specific questions that involve interactions with wildlife and/or fish habitat. This level of testing may involve microcosm/mesocosm studies or field studies. General guidelines for microcosm/mesocosm and field testing are outlined below.

Registrants may choose to go to Tier IV directly (bypassing Tiers I, II, or III), in which case additional single-species tests may still be required depending upon the use pattern of the pesticide and the effects observed in the Tier IV tests.

3.4.1 Tests

Testing requirements for Tier IV are shown in Table 1. The relationship between Tier IV and Tier III testing is illustrated in Figure 1 and section 1.6.5.

3.4.1.1 Test IV-1: Microcosm/mesocosm

Microcosms are multispecies subsets of the aquatic or terrestrial ecosystems that are tested in the laboratory. Mesocosms, also multispecies subsets, are larger in extent and commonly established outdoors. One of the most important aspects of mesocosms (outdoor testing) is that they incorporate natural dissipation mechanisms (photolysis, sorption, microbial degradation, etc.) that may mediate the inherent toxicity of pesticides. Microcosms and mesocosms are closer to natural ecosystem conditions than test conditions in previous tiers but are less complex than natural ecosystems, which facilitates interpretation of the results.

A) Objective

This test is conducted to address specific concerns that have been raised as a result of previous single-species testing with aquatic or terrestrial organisms. It may involve determining the effects of a pesticide on plants within an interactive group of species of other trophic levels.

B) When required

This test is conditionally required (Table 1). Based on the results of tests at Tiers I, II, and III, the need for this test will be determined on a case-by-case basis through discussions involving interested regulatory advisors and the registrant.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

Several standardized microcosm designs exist that are endorsed by regulatory agencies, such as the U.S. EPA (1987b). The major difference among these protocols is the origin of the species used. Some are artificially seeded (e.g., Taub 1989), whereas others are naturally derived (e.g., Leffler 1984). A particular design is not recommended in these guidelines, as the design should be based on the questions generated from the results of studies in previous tiers. Examples of microcosm/mesocosm studies are provided under references.

Although regulatory advisors are *not* responsible for designing test protocols for registrants, they will notify registrants of particular requirements and comment on registrants' protocols when submitted.

2. Protocol modifications

General guidelines for test development are given below.

a) Test species

Microcosms/mesocosms usually contain species representing several trophic levels. Ecological relevance, sensitivity, and previous use in testing should be included as species selection criteria.

b) Test substance

The test substance (usually the formulated pesticide with any adjuvants that would be added in the tank mix as specified on the label) and the appropriate concentrations required to span the NOECs/NOELs, EC25s, and EC50s for the microcosm/mesocosm endpoints will be determined by the results of tests in Tiers I, II, and III. It is likely that a maximum challenge test and range-finding tests will be required before a definitive test is begun.

c) Test conditions

The microcosms/mesocosms should be prepared well in advance and acclimated to test conditions before testing begins. Data on the baseline condition of the microcosms/mesocosms, including species present, population sizes, trophic structure, primary productivity, nutrient cycling, and nonbiological parameters such as water chemistry and sediment/soil characteristics, should be described before the test begins.

Detailed examples of test conditions are outlined in the references cited.

d) Experimental design

The experimental design will vary from case to case and should be appropriate for the questions being asked and the statistical methods chosen for data analysis. The details will be determined through discussions involving interested regulatory advisors and the registrant.

Test duration will depend upon the endpoints chosen and should be sufficient to examine the potential for species recovery and trends under control conditions in the microcosm/mesocosm.

e) Data collection

Ecologically relevant endpoints should be chosen once the precise objective of the test is defined.

f) Data analysis

The methods of data analysis should be chosen before the test is conducted. Parametric statistical tests are preferred to nonparametric tests for assessing the significance of the test results.

D) Test limitations and validation

Although microcosm/mesocosm tests provide a better indication of the effects of a pesticide at several trophic levels than did the single-species tests in Tiers I, II, or III, microcosms/mesocosms necessarily provide a more simple test environment than actual field conditions in terms of possible interactions and numbers and types of species involved. Appropriate microcosm/mesocosm design can reduce these limitations and increase the relevance of this test to field conditions.

E) Report

a) Experimental conditions

The report should include, but not be limited to, the following:

1. identity of laboratory/individuals who performed the tests

2. test species: identification, abundance of test plant species and other species

3. test substance: % TAI, formulation type, formulants, adjuvants, concentrations tested, analytical confirmation of test concentrations, verification of on-target deposit

4. test conditions: background monitoring data; dates (pre-test monitoring, test start, end, observations), duration; temperature, light, water chemistry (pH, dissolved oxygen, nutrient levels, etc.); physical environment (turbidity, water depth, exposure, tidal action, etc.); substrate characteristics (particle size, % organic carbon, soil moisture); experimental apparatus

5. experimental procedure: control description; methods for measuring test conditions (#4 above); methods for measuring pesticide, formulant, and adjuvant concentrations; methods for solubilizing, incorporating, and applying test substance; methods for measuring endpoint

b) Results

1. calculation of test variables, where appropriate

2. test of pesticide effect and estimation of NOEC/NOEL and EC25/EC50 where appropriate

3. description of supporting statistical analyses, and

interpretation of results

F) References

References cited in text:

Leffler 1984
Taub 1989
U.S. EPA 1987b

References relevant to aquatic and terrestrial microcosms/mesocosms:

Agriculture Canada 1987
Cairns 1985
Freemark *et al.* 1990
Holst and Ellwanger 1982
Moore and Keddy 1989

References relevant to aquatic microcosms /mesocosms:

Blaylock *et al.* 1986
Brazner *et al.* 1989
Cairns 1986
Giddings and Franco 1985
Hamala and Kollig 1985
Lamberti *et al.* 1989
Soloman *et al.* 1980
Stay *et al.* 1989

References relevant to terrestrial microcosms /mesocosms:

Gile *et al.* 1981
Gillett and Witt 1979
Hamill *et al.* 1977
Kromroy *et al.* 1989
Pfleeger 1989
Tolle *et al.* 1989

3.4.1.2 Test IV-2: Field testing

Field testing could be coupled with testing under an efficacy research permit.

As with mesocosms (outdoor testing), field testing incorporates natural dissipation mechanisms (photolysis, sorption, microbial degradation, etc.) that may mediate the inherent toxicity of pesticides.

A) Objective

The objective of field testing is to address specific concerns that have not been resolved in previous testing with aquatic or terrestrial organisms. It will necessarily involve several trophic levels - that is, interactions among wildlife, fish, and plant species that are part of wildlife and/or fish habitat.

B) When required

This test is conditionally required (Table 1). Based on the results of tests at Tiers I, II, and III, the need for this test will be determined on a case-by-case basis through discussions involving interested regulatory advisors and the registrant.

C) Methodology

Refer to sections 1.6.8 and 1.6.9 for a general discussion of testing methodology.

1. Recommended protocol

No protocol is recommended. Field studies are conducted on a case-by-case basis and tailored to answer questions being asked as a result of the tests performed for a particular pesticide with a particular use pattern. Some guidance concerning field testing is provided below.

Although regulatory advisors are *not* responsible for designing test protocols for registrants, they will notify registrants of particular requirements and comment on registrants' protocols when submitted.

2. Protocol modifications

a) Test species

The species requirements will be determined on a case-by-case basis. Species that dominate plant communities or are significant for wildlife and/or fish habitat (e.g., cover, food) and species that have been used in tests at previous tiers may be included.

The results of previous single-species tests cannot be used to partially satisfy the species requirements for field testing as in the FIFRA guidelines, which accept the results of previous tests.

b) Test substance

The tank mix with the final formulated pesticide is tested. The concentrations to be tested will be determined by the objectives of the test but should normally follow maximum recommended label rates. The results of tests in previous tiers may also serve as concentration range indicators.

c) Test conditions

Field tests should be carried out in geographic locations where the pesticide is expected to be used, based on use pattern. The variation in test conditions that must be represented will be determined by the vegetation heterogeneity and wildlife and/or fish of concern in the intended region of application in Canada.

The nontarget phytotoxicity testing will be established based on the registered method of application considered to have greatest potential effects on nontarget species. Detailed examples of test condition descriptions are outlined in the references cited.

d) Experimental design

Once the test objectives and endpoints are established, the appropriate experimental design should be determined in conjunction with the data analysis methods.

Test duration will depend on the endpoints chosen and should be sufficient to examine the potential for recovery of species and recovery to control conditions. As well, test duration must allow for multiple applications, if these are recommended on the label. Discussions involving interested regulatory advisors and the registrant will serve to determine appropriate questions to be answered and the corresponding experimental design.

e) Data collection

Ecologically relevant endpoints can be chosen once the precise objective of the test is defined.

f) Data analysis

In keeping with good experimental design, the methods of data analysis should be chosen before the test is conducted. Standard statistical techniques are to be used to determine the significance of the effects observed.

D) Test limitations and validation

Appropriate experimental design should be used to minimize the limitations of the test performed.

E) Report

a) Experimental conditions

The report should include, but not be limited to, the following:

1. identity of laboratory/individuals who performed the tests

2. test species: identification, abundance of test plant species and other species; development stages of test species

3. test substance: % TAI; formulation type; formulants, adjuvants, and pesticide concentrations, analytical confirmation of test concentrations; verification of on-target deposit

4. test conditions: location and description of test site; background monitoring data; dates (pre-test monitoring, test start, end, observations), duration; temperature, light, rainfall, water chemistry (pH, dissolved oxygen, nutrient levels, etc.), or soil chemistry (pH, nutrient levels, % organic carbon, etc.); physical environment (turbidity, exposure, tidal action, etc., or soil particle size, depth, drainage, etc.); substrate characteristics; description of experimental apparatus

5. experimental procedure: control description; methods for measuring test conditions (#4 above); methods for measuring pesticide, formulant, and adjuvant concentrations; methods for solubilizing, incorporating, and applying test substance; number of applications; methods for measuring endpoint

b) Results

1. calculation of test variables, where appropriate

2. test of pesticide effect and estimation of NOEC/NOEL and EC25/EC50 where appropriate

3. description of supporting statistical analyses, and interpretation of results

F) References

References relevant to aquatic and terrestrial field testing:

Agriculture Canada 1987
Freemark *et al.* 1990
Holst and Ellwanger 1982

References relevant to aquatic field testing:

Brazner *et al.* 1989
Cairns 1985, 1986
Holst 1986e

References relevant to terrestrial field testing:

Borders and Shiver 1989
Frans and Talbert 1977
Holst 1986d

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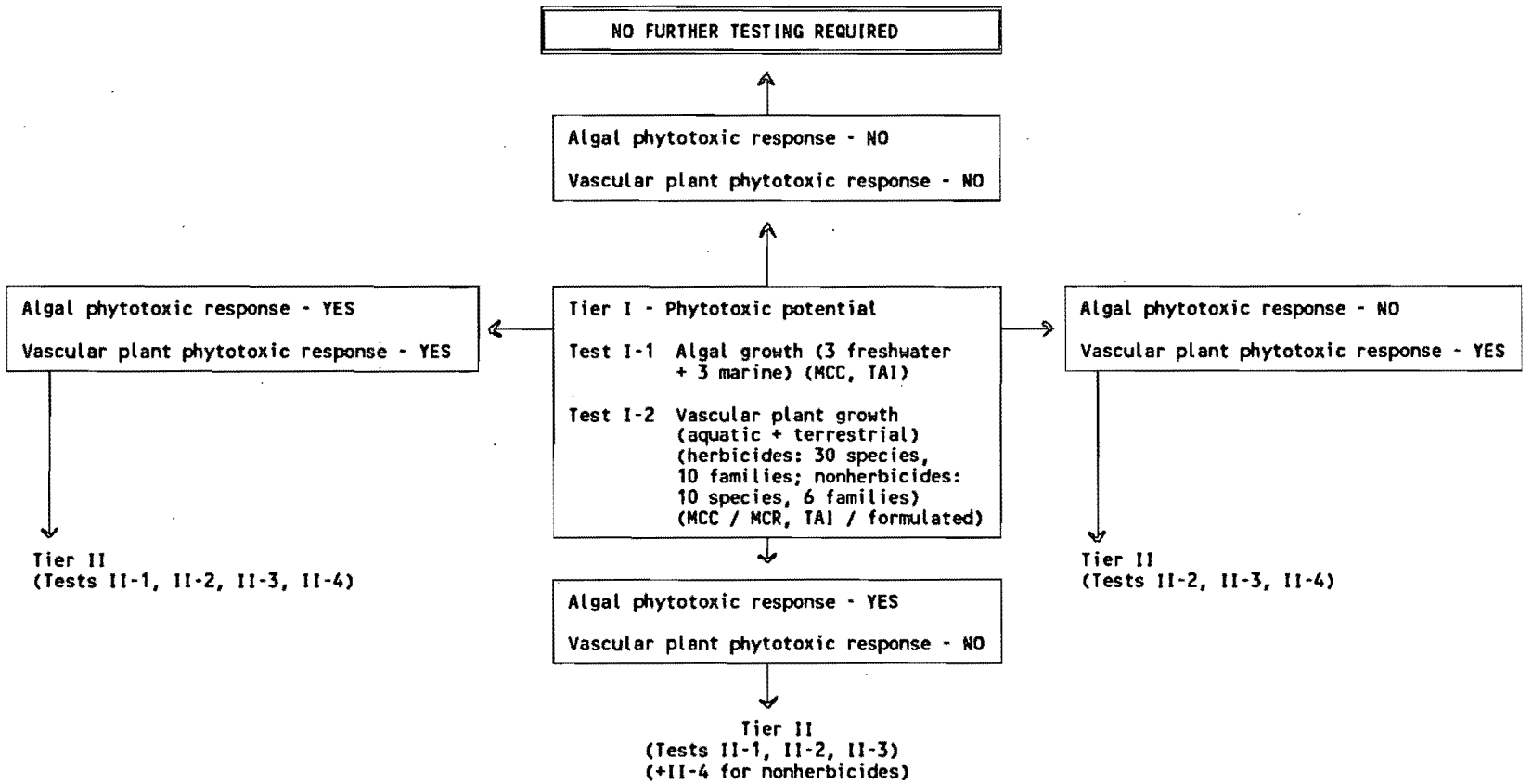
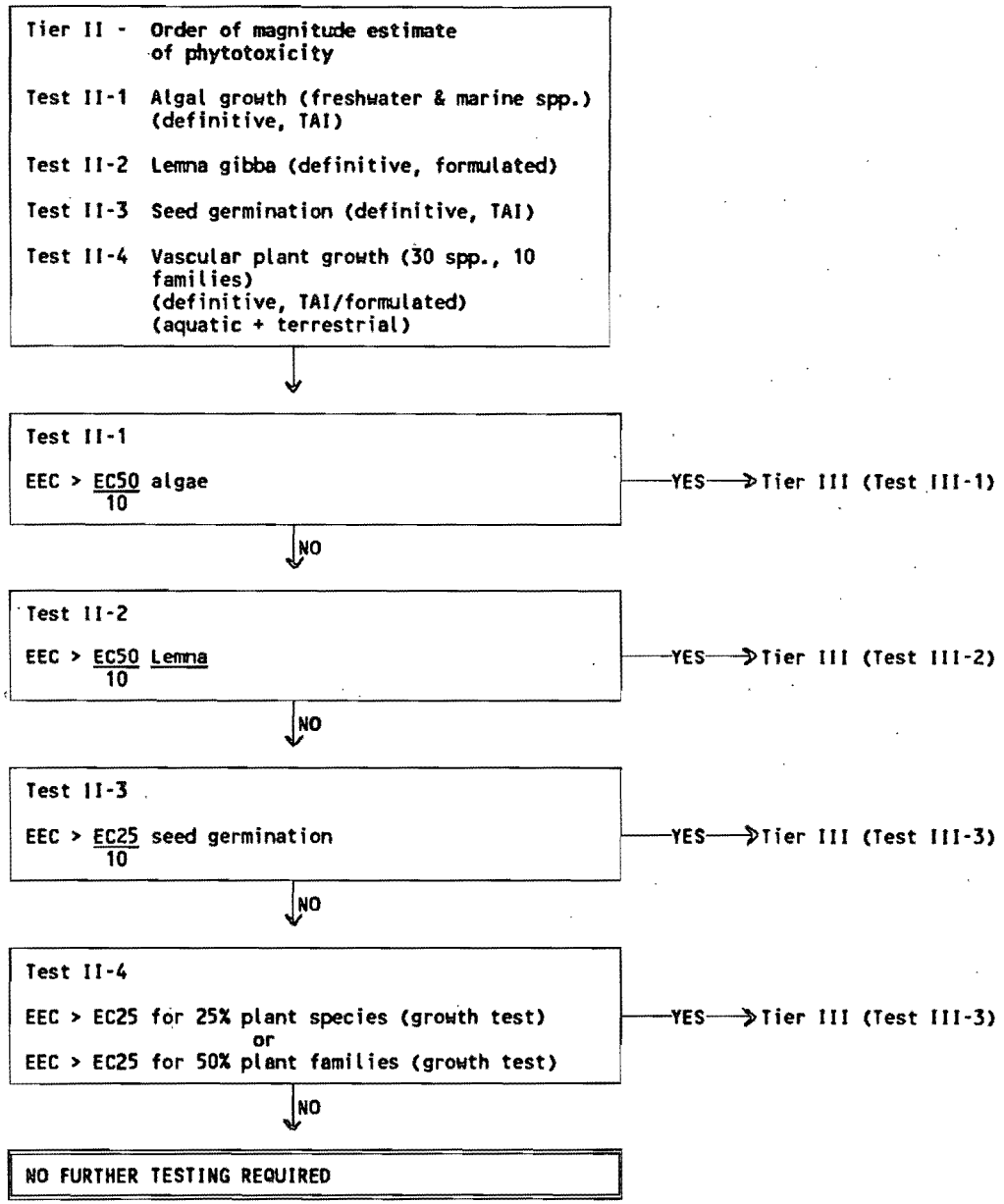


Figure 1. The relationships among tests for nontarget plants and tier progression (see also section 1.6.5)

(Figure 1. continued)



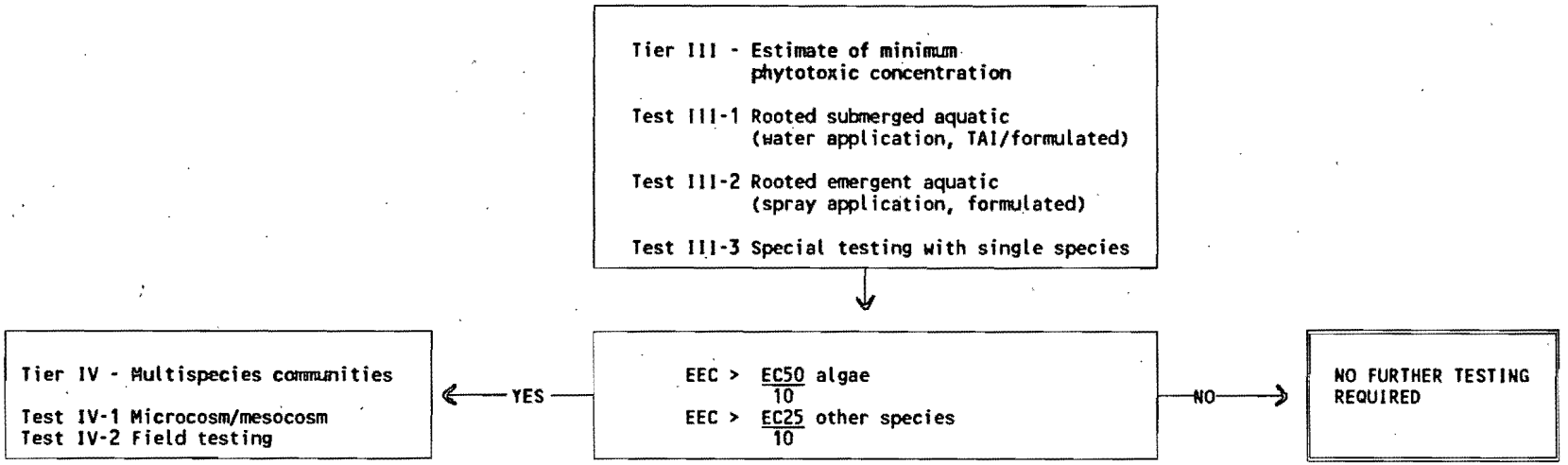

Tier III - Estimate of minimum phytotoxic concentration
Test III-1 Rooted submerged aquatic (water application, TAI/formulated)
Test III-2 Rooted emergent aquatic (spray application, formulated)
Test III-3 Special testing with single species
EEC > EC50 algae / 10
EEC > EC25 other species / 10
YES
NO
Tier IV - Multispecies communities
Test IV-1 Microcosm/mesocosm
Test IV-2 Field testing
NO FURTHER TESTING REQUIRED

Table 1. Test requirements for nontarget plant testing in Canada, by use pattern. Tests and conditional requirements are described in detail in the text.

Tier/ Tests	Test type	Test substance	Nondomestic use			Domestic use	
			Terrestrial	Aquatic			
			Food crop/ Nonfood crop/ Forestry	Food/Nonfood D/S	Green-house*	Indoor/Pool	Outdoor
Tier I							
Mode of action information	-	-	R	R	R	R	R
Tests							
I-1 Algae	MCC	TAI	R	R	CR	NR	R
I-2 Vascular vegetative growth	MCC/MCR	TAI/FORM	R	R	CR	NR	R
Tier II							
Tests							
II-1 Algae	DEF	TAI	CR	CR	CR	NR	CR
II-2 *Lemna*	DEF	FORM	CR	CR	CR	NR	CR
II-3 Seed germination	DEF	TAI	CR	CR	CR	NR	CR
II-4 Vascular vegetative growth	DEF	TAI/FORM	CR	CR	CR	NR	CR

Tier/ Tests	Test type	Test substance	Nondomestic use: Terrestrial — Food crop/ Nonfood crop/ Forestry	Nondomestic use: Aquatic — Food/Nonfood D/S	Nondomestic use: Aquatic — Greenhouse*	Domestic use — Indoor/Pool	Domestic use — Outdoor
Tier III							
Tests							
III-1 Submerged aquatics	DEF	TAI/FORM	CR	CR	CR	NR	CR
III-2 Emergent aquatics	DEF	FORM	CR	CR	CR	NR	CR
III-3 Special testing	TBD	TBD	CR	CR	CR	NR	CR
Tier IV							
Tests							
IV-1 Microcosm/ mesocosm	TBD	TBD	CR	CR	CR	NR	CR
IV-2 Field	TBD	FORM	CR	CR	CR	NR	CR

Test level
I = Tier I
II = Tier II
III = Tier III
IV = Tier IV

Test substance
FORM = Formulated pesticide
TAI = Technical active ingredient

Application method
D = Dissolved in medium
S = Sprayed over surface

Test type
MCC = Maximum challenge concentration (exposure through the medium)
MCR = Maximum challenge rate (aerial exposure)
DEF = Definitive
TBD = To be determined

When required
R = Required
CR = Conditionally required
NR = Not required
* = Required if system open

Table 2. List of vascular plant species and families routinely tested during pesticide development. The list is compiled from herbicide registration submissions and information provided by the Crop Protection Institute of Canada. The list of plant species relevant to forestry use comes primarily from Swanson *et al.* (1991). At this point, it is incomplete because of a lack of documentation.

TERRESTRIAL PLANTS

Amaranthaceae

Amaranthus retroflexus

Apiaceae

Apium graveolens
Daucus carota

Asteraceae

Ambrosia artemisiifolia
Bidens aurea
Helianthus annuus
Lactuca sativa
Matricaria inodora
Xanthium canadense
Xanthium orientale
Xanthium pensylvanicum

Brassicaceae

Brassica kaber
(=Sinapsis arvensis)
Brassica napus

Chenopodiaceae

Beta vulgaris
Chenopodium album
Spinachia oleracea

Convolvulaceae

Convolvulus arvensis
Ipomoea hederacea
Ipomoea purpurea

Cucurbitaceae

Cucumis sativa

(Table 2. continued)

Cyperaceae

Cyperus rotundus

Fabaceae

Arachis hypogaea
Cassia obtusifolia
Cassia tora
Glycine max
Glycine soja
Medicago sativa
Phaseolus vulgaris
Pisum sativum
Sesbania spp.
Trifolium pratense
Vicia alba
Vicia sativa

Linaceae

Linum usitatissimum

Malvaceae

Abutilon theophrasti
Gossypium hirsutum
Sida spinisa

Poaceae

Agropyron repens
Alopecurus myosuroides
Alopecurus pratensis
Avena fatua
Avena sterilis
Cynodon dactylon
Digitaria sanguinalis
Echinochloa crus-galli
Hordeum vulgare
Oryza sativa
Panicum dichotomiflorum
Panicum miliaceum
Phalaris canariensis
Phalaris minor
Secala viridis
Sorghum halepense
Sorghum vulgare
Triticum aestivum
Zea mays

Polygonaceae

Polygonum persicaria

Portulacaceae

Portulaca oleracea

(Table 2. continued)

Rubiaceae

Galium aparine

Solanaceae

Datura stramonium
Lycopersicon esculentum
Nicotiana tabacum
Solanum tuberosum

AQUATIC PLANTS

Alismaceae

Alisma trivale
Sagittaria latifolia
Sagittaria pygmae

Cyperaceae

Cyperus difformis
Cyperus seretinus
Eleocharis acicularis
Scirpus juncoides
Scirpus maritimus
Scirpus mucronatus

Pontederiaceae

Monochoria vaginalis

FORESTRY SPECIES

Betulaceae

Alnus spp.
Betula spp.

Cornaceae

Cornus stolonifera

(Table 2. continued)

Cupressaceae

Thuya occidentalis
Thuya plicata

Pinaceae

Abies balsamea
Picea mariana
Picea sitchensis
Pinus banksiana
Pinus contorta
Pinus strobus
Pseudotsuga menziesii
Tsuga canadensis
Tsuga heterophylla

Rosaceae

Prunus virginiana
Rubus spp.

Salicaceae

Populus tremuloides
Salix spp.

Table 3. Candidate freshwater algal species for tests I-1 and II-1 that have a history of chemical testing.

Species	References
Green algae (Chlorophyceae)	
Ankistrodesmus falcatus	Paromenskaya and Lyalin 1968 Ibrahim 1984 Burrell *et al.* 1985 Larsen *et al.* 1986
Chlamydomonas reinhardii	Loeppky and Tweedy 1969 Hollister and Walsh 1973 Moore 1973 Stevenson *et al.* 1983 Yee *et al.* 1985 Hersh and Crumpton 1987
Chlorella pyrenoidosa	Wells and Chappell 1965 Kratky and Warren 1971 Virmani *et al.* 1975 Birmingham and Coleman 1983 Stevenson *et al.* 1983 Maule and Wright 1984 Stratton 1984
Chlorella vulgaris	OECD 1984a U.S. EPA 1985a Holst 1986c OECD 1984a
Oedogonium cardiacum	Moore 1973 U.S. EPA 1979
Scenedesmus obliquus	Larsen *et al.* 1986
Scenedesmus quadricauda	U.S. EPA 1985a
Scenedesmus subspicatus	OECD 1984a ISO 1989 ASTM 1991a

(Table 3. continued)

Species	References
Selenastrum capricornutum	Holst and Ellwanger 1982 OECD 1984a U.S. EPA 1985a Holst 1986c APHA 1989 ISO 1989 ASTM 1991a
Blue-green algae (Cyanobacteria)	
Anabaena cylindrica	Moore 1973 Wright *et al.* 1977 U.S. EPA 1979 Yee *et al.* 1985
Anabaena flos-aquae	Holst and Ellwanger 1982 Holst 1985c APHA 1989 ASTM 1991a
Microcystis aeruginosa (=*Anacystis cyanae*)	APHA 1989 ASTM 1991a
Diatoms (Bacillariophyceae)	
Cyclotella sp.	APHA 1989
Navicula sp.	Hollister and Walsh 1973 Birmingham and Coleman 1983 Mayer 1987 Hughes *et al.* 1988 ASTM 1991a
Nitzschia sp.	APHA 1989
Synedra sp.	APHA 1989

Table 4. Candidate marine algal species for tests I-1 and II-1 that have a history of chemical testing.

Species	References
Green algae (Chlorophyceae)	
Chlorococcum sp.	Walsh and Grow 1971 Hollister and Walsh 1973 Maly and Ruber 1983 Mayer 1987
Diatoms (Bacillariophyceae)	
Nitzschia sp.	Moore 1973 Davis *et al.* 1979 Walsh and Alexander 1980 Borthwick and Walsh 1981 Maly and Ruber 1983
Skeletonema costatum	Holst and Ellwanger 1982 Holst 1985c U.S. EPA 1985a APHA 1989 ASTM 1991a
Thalassiosira fluviatilis	Hollister and Walsh 1973 Moore 1973 Sikka and Rice 1974 Davis *et al.* 1979 Mayer 1987
Thalassiosira pseudonana (=*Cyclotella nana*)	U.S. EPA 1985a APHA 1989 ASTM 1991a

(Table 4. continued)

Species	References
Golden-brown algae (Chrysophyceae)	
Emiliania huxleyi (=*Coccolithus*)	Maestrini *et al.* 1984
Isochrysis galbana	U.S. EPA 1985a
Pavlova (Monochrysis) lutheri	Walsh and Grow 1971 Hollister and Walsh 1973 Moore 1973 Bonin *et al.* 1986 Mayer 1987
Phaeodactylum tricornutum	Hayward 1968 Hollister and Walsh 1973 Ibrahim 1983, 1984 Bonin *et al.* 1986 Mayer 1987
Dinoflagellates (Dinophyceae)	
Dunaliella tertiolecta	Walsh and Grow 1971 Hollister and Walsh 1973 Moore 1973 Sikka and Rice 1974 Walsh *et al.* 1977 Walsh 1983 Bonin *et al.* 1986 Mayer 1987 APHA 1989 ASTM 1991a

Appendix A

Comparison between the proposed Canadian guidelines and other existing guidelines

Contents

Disparities that exist between the Canadian guidelines, the OECD guidelines, and the U.S. EPA guidelines in test requirements, protocol specifications, and hazard and risk assessments are mainly due to research conducted since the completion of the OECD guidelines in 1981 and 1984a, the U.S. EPA guidelines in 1982, the establishment of the U.S. Standard Evaluation Procedure in 1986, as well as experience acquired to date.

As also stipulated in the U.S. EPA guidelines, Canadian registrants may be granted a waiver for any tests required or modifications to recommended protocols or specific conditions if justified on appropriate scientific grounds.

A1.0 Potential for exposure and toxicity testing for all pesticides

In the Canadian guidelines, minimum testing (Tier I) is required on all chemical pesticides, as it has been demonstrated that pesticides other than herbicides detrimentally affect nontarget plants (see section 1.3.2 in the guidelines) (Thompson 1976; Swanson *et al.* 1991; K.E. Freemark and C. Boutin, unpublished manuscript; proprietary data). In addition, when a pesticide is released into the environment, except in a closed-system greenhouse, indoors and swimming pools (Table 1), exposure to nontarget plants will occur (Norby and Skuterud 1975; Bode *et al.* 1976; Maybank *et al.* 1978; Grover *et al.* 1979).

Current policy as stated in the U.S. EPA guidelines is as follows (quoted from Lewis and Petrie 1991):

"1- Determine if the chemical is toxic to plants. If phytotoxicity, proceed to tier I. If an herbicide, proceed to tier II.

2- No herbicide phytotoxicity data are required if applied solely to food/feed crops; and, if applied with ground equipment only; and, if the herbicide volatility is less than 1.0×10^{-5} mm Hg and if the herbicide is less than 10 ppm water solubility. Exceptions to these rules include: known cases of documented adverse effects in the field, potential for adverse effects to endangered species, or if the pesticide is in Special Review at EPA."

The OECD guidelines were developed for testing chemicals in general, including pesticides.

A2.0 Testing approach

In the Canadian guidelines, a four-tiered approach (I to IV) to testing is outlined, as opposed to a three-tiered system in the OECD guidelines (basic, confirmatory and definitive) and in the U.S. EPA guidelines (1 to 3). The tier system is very effective, as it reduces repetitive consultation between registrants and advisors, thereby decreasing time for development of data for registration.

A2.1 Tier I

At the basic level, OECD recommends that simple tests be used. The only phytotoxity test recommended at this level is an algal growth inhibition test (1984b). OECD is currently in the process of collating information from member countries in order to revise existing guidelines and develop new guidelines to address pesticides, particularly in the area of environmental fate and ecotoxicology.

In the United States, the Tier 1 requirement for aquatic species consists of testing with one or four species of algae, depending on the use pattern, and *Lemna gibba*. For terrestrial species, seed germination, seedling emergence, and vegetative vigour tests are required for dicotyledons on six crop species of at least four families, one species of which is soybean (*Glycine max*) and a second a root crop, and for monocotyledons on four species of at least two families, one species of which is corn (*Zea mays*). The quantity of test substance to be tested for aquatic species should be equivalent to the maximum recommended label rate as though it were directly applied to the surface of a 15-cm deep water column. For terrestrial species, one concentration level equal to no less than the maximum recommended label rate should be tested. In either case, if it can be determined that the maximum quantity that will be present in the nontarget area is significantly less than the maximum recommended label rate, a concentration or rate equal to no less than three times that quantity may be tested.

In the Canadian guidelines, Tier I is a screening level with the purpose of detecting the phytotoxicity potential to some aquatic species (three freshwater and three marine algal species) and terrestrial species (vegetative growth and vigour using the plant screening data routinely generated by registrants during their product development). Only one dose, the maximum recommended label rate, is required.

It cannot be assumed that toxicity tests on algae can be used to predict possible effects on aquatic vascular plants (Swanson *et*

al. 1991) or on terrestrial vascular species. The intent of this tier is, however, to detect any potential phytotoxicity with a minimum of testing.

The Canadian guidelines do not require testing with *Lemna*, as do the U.S. EPA guidelines, nor is seedling emergence testing required in the Canadian guidelines.

A2.2 Tier II

The confirmatory level of the OECD guidelines suggests tests on additional algal species, as well as testing on *Lemna* and other additional aquatic vascular species (OECD 1980). Recommended testing on terrestrial species includes a germination test, growth tests and partial life-cycle tests on both monocots and dicots. A protocol is available for terrestrial plant growth test (OECD 1984c). The confirmatory level tests should yield more complete information if "suspicions as to the acceptability of a chemical has been previously raised at the basic level" (OECD 1984a).

In the United States, the Tier 2 requirement consists of testing those plant species that exhibited a 25% phytotoxic effect at Tier 1 on terrestrial species and a 50% phytotoxic effect on aquatic species. A dose-response curve is required with five doses, including a nontoxic and a subtoxic concentration.

The purpose of Tier II in the Canadian guidelines is to quantify the order of magnitude of the phytotoxicity (dose-response curves) of an array of plant types that could be exposed to pesticides: algae, a floating vascular species (usually *Lemna gibba*), rooted aquatic and terrestrial vascular plants (seed germination/root elongation, vegetative growth and vigour using the plant screening data). Species tested in Tier I that showed no toxicity at the maximum recommended label rate are not required to be tested at Tier II. The Canadian guidelines do not require seedling emergence studies at Tier II, as do the U.S. EPA guidelines.

A2.3 Tiers III and IV

The OECD does not recommend any particular definitive tests. General categories include tests with confined natural communities, aquarium tests with artificial communities, and compartment tests with separate trophic levels. This level is needed if "appreciable environmental concentrations of the chemical are likely to be involved and /or some indication of possible environmental hazard exists".

In the United States, the Tier 3 requirement consists of aquatic

or terrestrial field testing. In aquatic testing, dicots, monocots, ferns and allies, algae, cyanobacteria, mosses, and liverworts should be represented. In terrestrial testing, dicots, monocots, ferns and allies, mosses, liverworts, and conifers should be included in the testing.

Tier III testing in the Canadian guidelines is aimed at aquatic emergent or submerged species. Only a minimum testing with aquatic species is requested at Tiers I and II, and, given the ecological importance of rooted aquatic vascular macrophytes in aquatic ecosystems, testing is requested if toxicity is detected at lower tiers. Special single-species tests may also be requested.

Tier IV of the Canadian guidelines entails a microcosm/mesocosm or field study. It involves focusing on a community of species in order to address specific questions raised by a particular pesticide; it may also involve interactions with wildlife and/or fish habitats. In contrast, Tier 3 of the U.S. EPA guidelines consists of testing a diversity of plant groups regardless of the specific concern. No microcosm or mesocosm studies are mentioned.

A3.0 Tier progression

In the Canadian guidelines, progression from Tier I to Tier II occurs if any phytotoxicity is detected. An uncertainty factor of 10 is used for progression between Tiers II and III and between Tiers III and IV for algal testing, *Lemna*, seed germination/root elongation, and rooted aquatic vascular plants. The results of Blanck *et al.* (1984) with algae suggest that ratios greater than 0.01 (uncertainty factor of 100) should be of concern when only three species are tested. Because any given pesticide is tested on several types of species, however, and because EECs are estimated from worst-case scenarios, the uncertainty factor is reduced to 10 for assessing the hazard posed by a pesticide to nontarget plants. No uncertainty factor is used by the U.S. EPA.

In the Canadian guidelines, the ratio method is not used to calculate hazard scores for plant screening data because of uncertainties in the experimental design (10-30 species qualitatively assessed). Progression to Tier III is required if the EEC is greater than EC25 for 25% of species or 50% of families based on our experience to date. In contrast, the vegetative plant growth and vigour test of the U.S. EPA (10 species quantitatively assessed) triggers further testing if the EEC is greater than the EC25 for *one* or more of the test species.

No quantitative tier progression criteria are specified by the OECD.

A4.0 Algal testing

The recommended protocol in the Canadian guidelines is ASTM (1991a) rather than the OECD protocol (1984b) or the FIFRA protocol (Holst and Ellwanger 1982; Holst 1986c), as it is more detailed and more up-to-date with respect to current research and methodologies. The protocol recently developed by ASTM (1991a) contains most of the necessary information for testing several classes of freshwater and marine algal species.

In the Canadian guidelines, testing is required with three freshwater species and three marine species from separate classes. It has been demonstrated that variability among species and classes of algae was so great and unpredictable that a battery of species is necessary in order to detect toxicity and offer a universal protection of algae in aquatic environment (Swanson *et al.* 1991).

A5.0 *Lemna* testing

The recommended protocol in the Canadian guidelines is ASTM (1991b) rather than the FIFRA protocol (Holst and Ellwanger 1982; Holst 1986c), as it is more detailed and more up-to-date with respect to current research and methodologies. No protocol on *Lemna* species has been developed by OECD.

In the Canadian guidelines, testing is required with the formulated pesticide sprayed over the plants, exposing them through the leaves and through the medium at the onset of testing. In contrast, the U.S. EPA guidelines require exposure through the medium with the active ingredient. *Lemna* as an aquatic floating species is likely to be exposed to pesticides through overspray, drift, and, to a lesser extent, runoff. For instance, it has been established that *Lemna minor* is much more susceptible to glyphosate when the herbicide is sprayed over the plants than when the plants are exposed through the medium only (Lockhart *et al.* 1989). In contrast, other pesticides are more sensitive when exposed through the medium (proprietary data) — hence the need for testing the two modes of exposure.

Testing with a representative of the formulated pesticide is required to enhance contact and sorption in a manner similar to the end-use scenario. It is believed that testing with the formulated pesticide is possible at this level, especially

because the *Lemna* test is a short-term one, lasting only seven days. Moreover, testing with the formulated pesticide is currently performed at an early stage with mammals.

A6.0 Seed germination/root elongation

The recommended protocol in the Canadian guidelines is TSCA (U.S. EPA 1985) rather than the FIFRA protocol (Holst and Ellwanger 1982; Holst 1986a), as it is more detailed and more up-to-date with respect to current research and methodologies. No protocol on seed germination/root elongation has been developed by OECD.

Germination and the first days of seedling growth are often the most sensitive stages of plant development. Adverse effects due to chemical substance exposure are most likely to take place during these phases. Because of the rapid growth phase, damage to the plant roots will be most readily discernible. A test using seed germination alone as an endpoint (as in the U.S. EPA) is considered less sensitive than if root elongation is also measured (Ratsch 1983; Wang 1985). The seed germination/root elongation test requires minimum time, space, equipment, and cost.

A7.0 Vegetative growth and vigour

For terrestrial plants, OECD (1984c) recommends testing with three species from two or three families. Emergence and average weight are the endpoints required.

Ten crop species from six families are recommended in the U.S. EPA guidelines, with quantitative endpoints measured. Plant screening data routinely generated by companies can also be used. The following statement can be found in Subdivision J of the U.S. EPA guidelines: "The Agency realizes that registrants who desire to market herbicides and other pesticides have tested their products extensively for phytotoxic effects. The information to be reported for Tiers 1, 2 and 3 have [*sic*] generally been generated during these tests. Therefore, to satisfy the requirements for phytotoxicity data ... the registrant would simply have to make the data from these investigative tests presentable and provide them to the Agency."

As explained in section 1.6.6 of the guidelines proper, the vegetative growth and vigour of rooted vascular species are assessed, in the Canadian guidelines, using the plant screening data routinely generated by registrants during the pesticide development process. This set of data, readily available to

registrants, is very valuable, as it includes several families and species (weeds and crops); hence, the general spectrum of activity can be determined for each pesticide. The Canadian guidelines require at least 10 species from six families for nonherbicides and a minimum of 30 species from 10 families for herbicides at Tier I. If a nonherbicide shows phytotoxicity at Tier I, testing should be expanded to include 30 species from 10 families.

Several common weeds tested by registrants for their product development represent important species used by wildlife for food (as well as crop species) and cover (K.E. Freemark and C. Boutin, unpubl. data). Because of uncertainties in the experimental design and because the endpoint for measuring effects is qualitative, it is believed, however, that several species are needed for assessing pesticide effects.

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Appendix B

Recommended statistical methods for nontarget plant testing

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Contents

B1.0 Introduction

This appendix contains additional information on the statistical procedures required by the Tier I and Tier II tests set out in these guidelines.

The Tier I and Tier II tests are discussed separately because of the differences in statistical procedures involved. The Tier I tests, which are run first, involve the comparison of a single dose level against a control in order to examine the effect of the pesticide at a high concentration. The Tier II tests, which are run if an effect is found in one of the Tier I tests, involve the comparison of several dose levels against the control.

In addition, either the Tier I or Tier II tests may involve the inclusion of a solvent-only treatment, in order to evaluate the effect of the solvent independently of the pesticide.

These tests are:

Tier I tests

Test I-1 - Algal growth
Test I-2 - Vascular plant growth

Tier II tests

Test II-1 - Algal growth
Test II-2 - *Lemna* growth
Test II-3 - Vascular plant seed germination/root elongation
Test II-4 - Vascular plant growth

B2.0 Tier I tests

The test procedures for test I-1 and test I-2 variables are summarized in Figures B1 and B2.

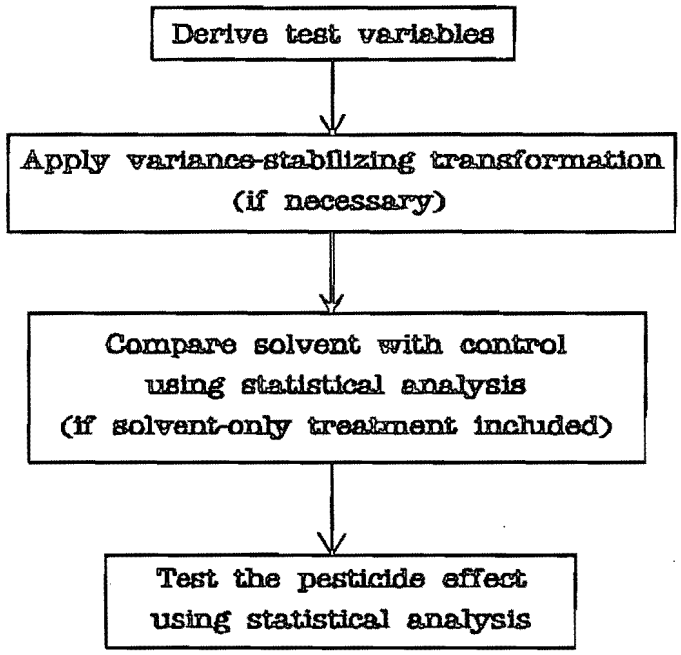

Figure B1. Summary of the test procedure for test I-1 variables

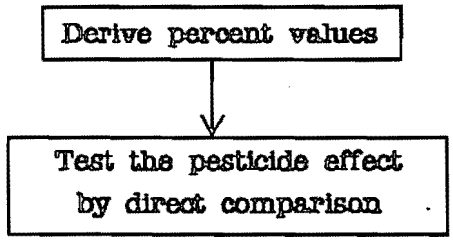

Figure B2. Summary of the test procedure for the test I-2 variable

B2.1 Outline of the Tier I test procedures

B2.1.1 Treatments applied

The treatments in the experiment depend on whether or not the pesticide is dissolved in a solvent that could have an effect on the test plants that is in addition to the effect of the pesticide. If no solvent is used, there are two treatments in the experiment:

1) a control, and

2) a single high concentration of pesticide.

On the other hand, if a solvent is used, the experiment includes three treatments:

1) a control,

2) a solvent-only treatment, and

3) a single high concentration level of pesticide (including solvent).

The solvent-only treatment is included for the purpose of testing the effect of the solvent (see section B 2.3.2).

B2.1.2 Experimental design

Each treatment is applied to a certain number of units (minimum of four), each unit consisting of a cluster of cells or a number of whole plants. At the start of the experiment, the units are assigned to the treatments according to either a blocked design or a one-way design:

Blocked design: Units are grouped into blocks, with each block containing one unit from each treatment. Within each block, one unit is assigned at random to each treatment.

One-way design: An equal number of units are assigned at random

to each treatment.

It is recommended that a blocked design be employed for these experiments, as it compensates for the fact that growth conditions (e.g., temperature, humidity, lighting) may not be completely uniform throughout the growth chamber. If a one-way design is employed, the lack of uniformity in growth conditions can make it more difficult to compare treatments, as the units for one treatment will be exposed to conditions somewhat different from those of another treatment. However, if a blocked design is employed in which the conditions within each block are relatively uniform, the conditions to which the treatments are exposed will be equalized to a large extent, and the problem of nonuniformity will be minimized.

Note that the advantages of blocked designs apply only if there are at least four units per treatment. If there are fewer than four, the advantage of blocking is offset by the reduction in the number of degrees of freedom in the statistical error. See Annex B1 for more information on the application of blocked and one-way designs to these experiments.

B2.1.3 Derivation of the test variables

Most of the test variables that are analyzed statistically are not measured directly but must be derived from raw measurements made on the units. For example, in the algal growth test, the raw measurements are cell counts, and the test variables (the cell growth rate and the area under the growth curve) are derived from these counts. The process of deriving the test variables is described in detail in section B2.2.

B2.1.4 Analysis of the test I-1 variables

For these variables, the effect of the high concentration of pesticide (and of the solvent if a solvent treatment is employed) is analyzed using statistical procedures. This involves the calculation of a mean value for each treatment and the comparison of these means using t-tests. The analysis procedures are

described in detail in section B2.3.

B2.1.5 Analysis of the test I-2 variable

For this variable, the effect of the high concentration of pesticide is analyzed by direct comparison of the pesticide data values with the control values, not by statistical procedures.

B2.2 Derivation of the test variables

B2.2.1 Test I-1 (algal growth)

B2.2.1.1 Basic information

Composition of unit: Each unit consists of a cluster of algal cells, the clusters being as close as possible to a specified size (e.g., 10 000 cells).

Minimum number of units per treatment: 4.

Raw measurements: Cell counts are made after 0, 24, 48, 72, and 96 hours.

Test variables derived:
- Cell growth rate, and
- Area under the growth curve.

B2.2.1.2 Derivation of the cell growth rate

a) Selection of the time period

The first step in deriving the cell growth rate of the log cell counts is to select the time period over which this rate will be measured. This period should be that portion of the 96-hour measurement period during which the growth in the number of cells is exponential for the control units and should be a minimum of 72 hours.

The most suitable time period can be selected by examining the growth in the log cell counts for the control units and identifying the period over which they show a linear increase with time. Thus, the first step in the selection of the time period is to calculate the daily averages of the log cell counts for the control units and plot these averages against time. A typical plot is shown in Figure B3.

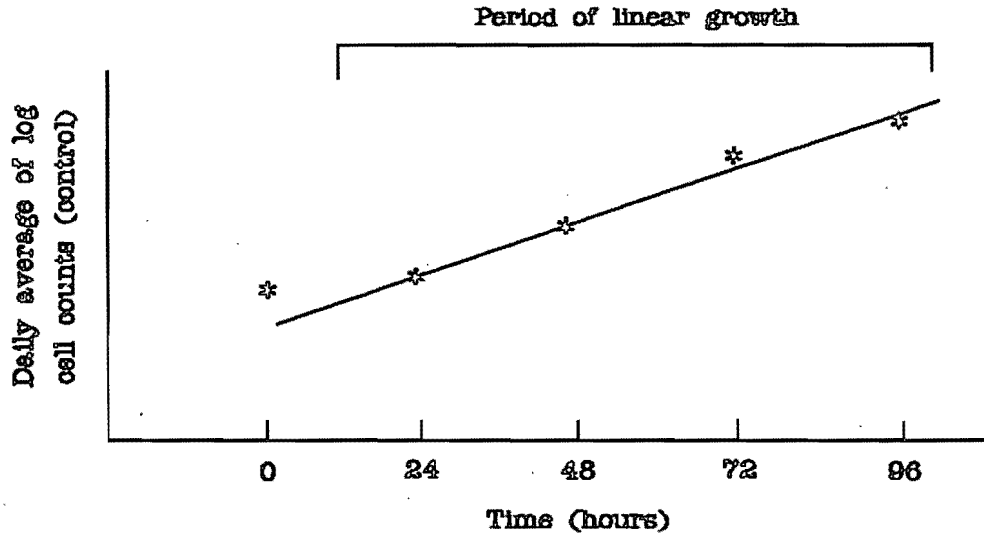

Figure B3. Selection of the test period

The period during which the growth in log cell counts is linear is then selected from this plot. As the test period should be at least 72 hours in length, the options are to select the total measurement period (0-96 hours), the first 72 hours (0-72 hours), or the last 72 hours (24-96 hours). For the plot in Figure B3, the best choice would be 24-96 hours.

b) Calculation of the cell growth rate

The cell growth rate G, which is the rate of growth of the log cell counts, is then obtained for each unit over the selected time period. The procedure recommended is to carry out a linear regression of log cell counts versus time in days. The growth

rate G is equal to the slope parameter, and its formula is:

$$G = \Sigma (Y_i - \bar{Y})(X_i - \bar{X}) / \Sigma (X_i - \bar{X})^2$$

The Y_i values are the logs of the cell counts throughout the selected test period (either natural logs or logs to base 10 may be used), the X_i values are the times in days at which the counts were made, and $\bar{Y}$ and $\bar{X}$ are the means of the Y_i and X_i, respectively.

Note: All summations (Σ) in this appendix are carried out over i through its full range of values, unless indicated otherwise.

For example, if the test period is 24-96 hours, the Y_i values are the logs of the counts at 24, 48, 72, and 96 hours and the X_i values are 1, 2, 3, and 4, as illustrated in Figure B4.

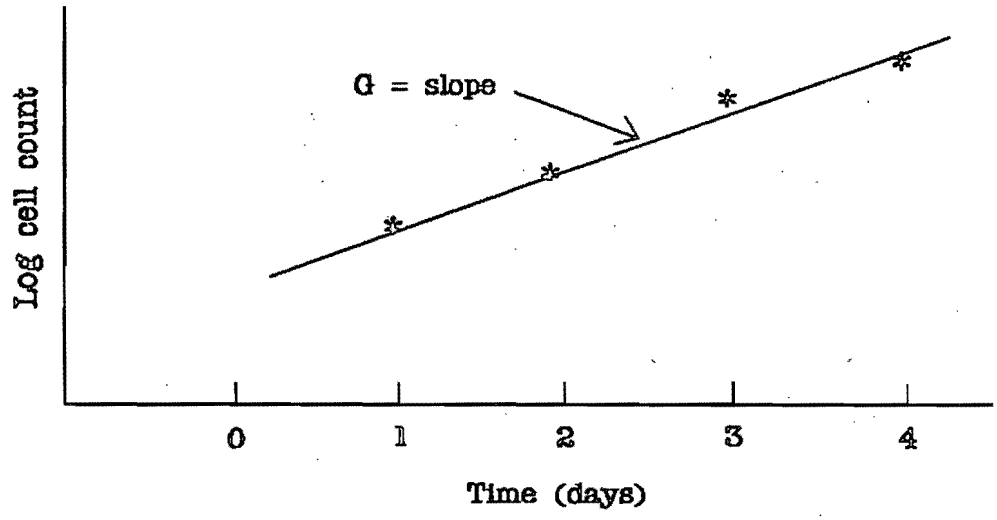

Figure B4. Calculation of the cell growth rate

B2.2.1.3 Derivation of the area under the growth curve

The other test variable derived for each unit is the area under the plot of cell counts versus time, as illustrated below. This is measured over the same time period that was selected for calculating growth rates, as described in section B2.2.1.2.

The cell counts used in the calculation are the counts in excess of the count at the start of the test. As illustrated in Figure B5, the area under the growth curve is the total area under the line segments AB, BC, and CD but above the horizontal line EF.

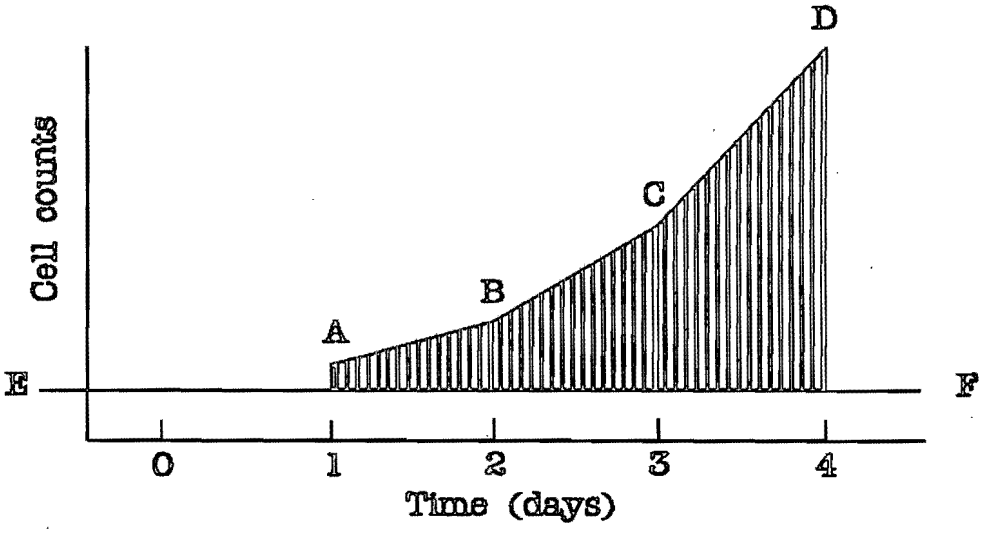

Figure B5. Area under the growth curve

To calculate the area under the growth curve, let the cell count at time i be C_i. Then the area J for the example in Figure B5 is calculated using the formula:

$$J = \text{Area under AB} + \text{Area under BC} + \text{Area under CD}$$

$$= \frac{(C_1-C_0)}{2} + \frac{(C_2-C_0)}{2} + \frac{(C_2-C_0)}{2} + \frac{(C_3-C_0)}{2} + \frac{(C_3-C_0)}{2} + \frac{(C_4-C_0)}{2}$$

B2.2.2 Test I-2 (vascular plant growth)

B2.2.2.1 Basic information

Composition of unit: Each unit consists of a number of whole plants.

Minimum number of units per treatment: 4.

Raw measurements: A visual rating is made after a specified period that depends on the species (usually 14 or 21 days).

Test variables derived: Percent growth and vigour.

B2.2.2.2 Derivation of percent growth and vigour

The subjective rating made on each unit indicates its growth and vigour relative to what is expected for an untreated unit. This rating is an integer on a scale of 0 (indicating no growth at all) to 9 (indicating full growth and vigour).

The rating is converted to a percent value ranging from 0% (no growth) to 100% (full growth and vigour). It is up to the experimenter to select an appropriate conversion method. The method recommended is to employ a conversion table based on the percent ranges set out in the guidelines (see section 3.1.2.2, test I-2). This table was obtained by converting a rating value to the midpoint of its corresponding range (e.g., a rating of 6 is converted to 72%).

Rating	Range	Midpoint	Rating	Range	Midpoint
9	100%	100%	4	30-44%	37%
8	91-99%	95%	3	16-29%	22.5%
7	80-90%	85%	2	6-15%	10.5%
6	65-79%	72%	1	1-5%	3%
5	45-64%	54.5%	0	0%	0%

Normally, each control unit will show full growth and vigour and thus will have a rating of 9 and a percent value of 100%. If the

value is less than 100% for any of the control units, this should be clearly stated in the results, and the possible reasons for this should be specified. It may be necessary to repeat the experiment in this case.

B2.3 Statistical analysis procedures for the test I-1 variables

The statistical procedures presented here are general in nature and apply to both of the test I-1 variables (except for the variance-stabilizing transformations applied prior to the analysis, which are specific to particular variables).

B2.3.1 Variance-stabilizing transformations

For some test variables, a transformation of the data is recommended prior to the statistical analysis in order to equalize the error variance throughout the range of the data as much as possible. The choice of transformation will depend on the nature of the variable. The transformations recommended are:

Test variable	Transformation
Cell growth rate	None required
Area under growth curve	Log transformation (see below)

For the log transformation, either natural logs or logs to base 10 may be used.

B2.3.2 Effect of solvent treatment on the data analysis

The presence or absence of a solvent treatment affects the number and the makeup of the treatments in the experiment, as described in section B2.1:

Treatments if no solvent	Treatments if solvent included
- control	- control
- high pesticide concentration	- solvent
	- high pesticide concentration (+ solvent)

The presence or absence of solvent also affects the analysis of the data:

Analyses run if no solvent	Analyses run if solvent included
- test of pesticide effect	- test of solvent effect
	- test of pesticide effect

If solvent is included, the procedure for the test of the pesticide depends on the results of the test of the solvent. If the solvent is found to have a significant effect, the pesticide (plus solvent) is tested by comparison with the solvent treatment only. If the solvent effect is not significant, the solvent is considered to be a second control. The results for the control and the solvent are averaged, and the pesticide is compared with this solvent-control average.

B2.3.3 Requirement for different versions of the analysis procedure

A number of different versions of the basic statistical procedure are required, because of the different sets of treatments and experimental designs that could be employed. As there are two possible sets of treatment (with and without solvent) and two possible experimental designs (blocked and one-way), there are four cases to be covered:

Case	Treatments	Experimental design
1	Control, high pesticide concentration	Blocked
2	Control, high pesticide concentration	One-way
3	Control, solvent, solvent + high	Blocked

	pesticide concentration	
4	Control, solvent, solvent + high pesticide concentration	One-way

The procedures for these four cases are discussed in sections B2.3.4 to B2.3.7, respectively.

B2.3.4 Case 1: Control, high pesticide concentration blocked design

Let N be the number of blocks, with each block containing one control unit and one high pesticide concentration unit,
C_i and T_i be the values for the control and high pesticide concentration units, respectively, in block i (i = 1 to N), and
$\overline{C}$ and $\overline{T}$ be the means of the C_i and T_i, respectively.

The quantity to be tested is the difference $\overline{D}$ between treatment means:

$$\overline{D} = \overline{C} - \overline{T}$$

To test $\overline{D}$, its variance must first be calculated. Let D_i be the difference between the control and high concentration values in the i'th block:

$$D_i = C_i - T_i$$

Let S_D^2 be the variance of the D_i:

$$S_D^2 = \sum (D_i - \overline{D})^2/(N - 1)$$

Then the variance $S_{\overline{D}}^2$ of $\overline{D}$ is given by:

$$S_{\overline{D}}^2 = S_D^2/N$$

To test $\overline{D}$, calculate the t-value:

$$t = \overline{D}/S_{\overline{D}}$$

The test is carried out by comparing t with the critical value for a one-tailed t-test at the 5% significance level with N-1 degrees of freedom. If t is greater than this value, $\overline{D}$ is significantly greater than zero, and the pesticide is considered to have a significant effect at the high concentration.

It is recommended that a one-tailed test be employed instead of the two-tailed test, to increase the power of the test to detect significant effects. Further discussion of the rationale for the use of a one-tailed test is given in Annex B2.

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added: if $\overline{D}$ is greater than 0.5 $\overline{C}$ but is not statistically significant, the data are not adequate, and the test must be repeated. See Annex B3 for further information on this condition.

B2.3.5 Case 2: Control, high pesticide concentration — one-way design

Let N be the number of units per treatment,
(C_i, i = 1 to N) be the data values for the control units,
(T_i, i = 1 to N) be the data values for the high pesticide concentration units, and
$\overline{C}$ and $\overline{T}$ be the means of the C_i and T_i, respectively.

The quantity to be tested is the difference $\overline{D}$ between treatment means:

$$\overline{D} = \overline{C} - \overline{T}$$

To test $\overline{D}$, its variance must first be calculated. Let S_C^2 and S_T^2 be the variances of the C_i and T_i, respectively:

$$S_C^2 = \sum (C_i - \overline{C})^2/(N - 1)$$

and

$$S_T^2 = \sum (T_i - \overline{T})^2/(N - 1)$$

Then the variance $S_{\bar{D}}^2$ of $\bar{D}$ is given by:

$$S_{\bar{D}}^2 = S_C^2/N + S_T^2/N$$

To test whether $\bar{D}$ is significantly greater than zero, calculate the t-value:

$$t = \bar{D}/S_{\bar{D}}$$

and test it using a one-tailed t-test at the 5% level with 2(N-1) degrees of freedom. A discussion of the rationale for the use of a one-tailed test is given in Annex B2.

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added: if $\bar{D}$ is greater than 0.5 $\bar{C}$ but is not statistically significant, the data are not adequate, and the test must be repeated. See Annex B3 for further information on this condition.

B2.3.6 Case 3: Control, solvent, solvent + high pesticide concentration — blocked design

Let N be the number of blocks, each containing one unit from the control, solvent, and (solvent + high pesticide concentration) treatments,

C_i, L_i, and T_i be the data values for these respective treatments in the i'th block (i = 1 to N), and

$\bar{C}$, $\bar{L}$, and $\bar{T}$ be the means of the C_i, L_i, and T_i, respectively.

There are two comparisons to be made:

1) comparison of the solvent against control, and

2) comparison of the solvent + high pesticide concentration against an appropriate zero concentration.

B2.3.6.1 Comparison of solvent against control

The quantity to be tested is the mean difference $\bar{D}$:

$$\overline{D} = \overline{C} - \overline{L}$$

To test $\overline{D}$, its variance must first be calculated. Let D_i be the difference between the control and solvent in the i'th block:

$$D_i = C_i - L_i$$

Let S_D^2 be the variance of the D_i:

$$S_D^2 = \sum_i (D_i - \overline{D})^2/(N - 1)$$

Then the variance $S_{\overline{D}}^2$ of $\overline{D}$ is given by:

$$S_{\overline{D}}^2 = S_D^2/N$$

To test $\overline{D}$, calculate the t-value:

$$t = \overline{D}/S_{\overline{D}}$$

This value is then used to test whether or not $\overline{D}$ is significantly greater than zero using a one-tailed t-test at the 10% level with N-1 degrees of freedom. The rationale for a one-tailed test is the same for this test as for the other tests in this experiment (see Annex B2 for further discussion).

In addition, the significance level recommended for this test is 10% instead of the 5% level normally used in statistical procedures. The reason is that the test of solvent is a preliminary test, carried out mainly to determine the proper zero concentration against which the pesticide treatment is to be tested. If a solvent effect is present but not detected, the test of the pesticide effect could be adversely affected. Therefore, the significance level has been set to 10% in order to increase the chance of detecting a solvent effect. This practice of employing a 10% significance level for preliminary tests is common in statistical analysis.

B2.3.6.2 Comparison of high concentration of pesticide against zero concentration

The quantity employed as the zero concentration for this test depends on whether or not the effect of the solvent was found to be significant. If it was not significant, the solvent is treated as a second control, and the pesticide is tested against the average of the control and solvent treatments. If it was significant, the pesticide is tested against the solvent only. Thus, there are two cases to be covered:

Case 3-1: Pesticide compared with average of solvent and control:

In this case, the mean $\overline{T}$ of the high concentration is tested against the average of $\overline{C}$ and $\overline{L}$.

Set $\overline{D} = (\overline{C} + \overline{L})/2 - \overline{T}$

Case 3-2: Pesticide compared with solvent alone:

In this case, the mean $\overline{T}$ of the high concentration is tested against $\overline{L}$ alone.

Set $\overline{D} = \overline{L} - \overline{T}$

Then the variance $S_{\overline{D}}^2$ of $\overline{D}$ is calculated. This involves carrying out a two-way analysis of variance (ANOVA) on the data (as described in standard texts, e.g., Snedecor and Cochran 1967:302). Using this ANOVA, the variation of the data can be partitioned into three sources:

1) variation between treatments,

2) variation between blocks, and

3) the interaction of treatments × blocks.

The quantity obtained from this ANOVA is S_{TB}^2, the mean square of treatments × blocks. The formula for this mean square is given in Annex B4.

Once it is obtained, the variance $S_{\overline{D}}^2$ of $\overline{D}$ can be calculated:

Case 3-1: $S_{\overline{D}}^2 = (1.5/N)S_{TB}^2$ *Case 3-2*: $S_{\overline{D}}^2 = (2/N)S_{TB}^2$

The t-value:

$$t = \overline{D}/S_{\overline{D}}$$

is then calculated and used to test whether or not $\overline{D}$ is significantly greater than zero, using a one-tailed t-test at the 5% level with 2(N-1) degrees of freedom. A discussion of the rationale for the use of a one-tailed test is given in Annex B2.

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added: if $\overline{D}$ is greater than 0.5 $\overline{C}$ but is not statistically significant, the data are not adequate, and the test must be repeated. See Annex B3 for further information on this condition.

B2.3.7 Case 4: Control, solvent, solvent + high pesticide concentration — one-way design

Let N be the number of units per treatment,
(C_i, i = 1 to N) be the data values for the control units,
(L_i, i = 1 to N) be the data values for the solvent units,
(T_i, i = 1 to N) be the data values for the units with the (solvent + high pesticide concentration) treatment, and
$\overline{C}$, $\overline{L}$, and $\overline{T}$ be the means of the C_i, L_i, and T_i, respectively.

There are two comparisons to be made:

1) comparison of the solvent against control, and

2) comparison of the solvent + high concentration of pesticide against an appropriate zero concentration.

B2.3.7.1 Comparison of solvent against control

The quantity to be tested is the mean difference $\overline{D}$:

$$\overline{D} = \overline{C} - \overline{L}$$

Before $\overline{D}$ can be tested, its variance must be calculated. Let S_C^2 and S_L^2 be the variances of the C_i and L_i, respectively:

$$S_C^2 = \sum (C_i - \overline{C})^2/(N - 1)$$

and

$$S_L^2 = \sum (L_i - \overline{L})^2/(N - 1)$$

Then the variance $S_{\overline{D}}^2$ of $\overline{D}$ is given by:

$$S_{\overline{D}}^2 = S_C^2/N + S_L^2/N$$

To test whether $\overline{D}$ is significantly greater than zero, calculate the t-value:

$$t = \overline{D}/S_{\overline{D}}$$

and test whether it is significantly greater than zero using a one-tailed t-test at the 10% level with 2(N-1) degrees of freedom. The rationale for a one-tailed test is discussed in Annex B2. The 10% significance level is employed to increase the chance of detecting a solvent effect, as this test is preliminary in nature and its outcome affects the procedure for the test of the pesticide. This is explained further in the test of solvent against control in Case 3 (see section B2.3.6.1).

B2.3.7.2 Comparison of high concentration of pesticide against zero concentration

The quantity employed as the zero concentration for this test depends on whether or not the effect of the solvent was found to be significant. If it was not significant, the solvent is treated as a second control and the pesticide is tested against the average of the control and solvent. If it was significant, the pesticide is tested against the solvent only. Thus, there are two cases to cover:

Case 4-1: Pesticide compared with average of solvent and control:

In this case, the mean $\overline{T}$ of the high concentration

Case 4-2: Pesticide compared with solvent alone:

In this case, the mean $\overline{T}$ of the high concentration

is tested against the average of $\overline{C}$ and $\overline{L}$.

Set $\overline{D} = (\overline{C} + \overline{L})/2 - \overline{T}$

is tested against $\overline{L}$ alone.

Set $\overline{D} = \overline{L} - \overline{T}$

To obtain the variance of $\overline{D}$, first calculate the average within-treatment variance S_{WT}^2. For both cases, this is:

$$S_{WT}^2 = (S_C^2 + S_L^2 + S_T^2)/3$$

The variance $S_{\overline{D}}^2$ of $\overline{D}$ can then be calculated from S_{WT}^2. The formula is different for the two cases:

Case 4-1: $S_{\overline{D}}^2 = (1.5/N)S_{WT}^2$ *Case 4-2:* $S_{\overline{D}}^2 = (2/N)S_{WT}^2$

The t-value:

$$t = \overline{D}/S_{\overline{D}}$$

is then calculated and used to test whether or not $\overline{D}$ is significantly greater than zero, using a one-tailed t-test at the 5% level with 3(N-1) degrees of freedom. A discussion of the use of a one-tailed test is given in Annex B2.

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added: if $\overline{D}$ is greater than 0.5 $\overline{C}$ but is not statistically significant, the data are not adequate, and the test must be repeated. See Annex B3 for further information on this condition.

B2.4 Analysis procedures for the test I-2 variable

The analysis of the data for the test I-2 variable (percent growth and vigour) is much simpler than the analysis for the test I-1 variables, for the following reasons:

1) The only treatments in the experiment are the control and the high pesticide concentration. No solvent treatment is present.

2) No statistical analysis is carried out. The pesticide effect

is tested by a simple comparison of the percent values for the control and pesticide units.

3) The procedure for this comparison is the same whether a blocked
or a one-way design was employed for the experiment.

The first step is to examine the percent values for the control units and confirm that they are all equal to 100%. (As stated earlier, if any of them are less than 100%, the reasons for this should be explained. It may be necessary to repeat the experiment.)

The next step is to examine the percent values for the units that received the high concentration of pesticide. If the effect is equal to or greater than 25%, it is concluded that a pesticide effect has been detected.

B3.0 Tier II tests

The test procedures for test II-1, II-2, II-3 and II-4 variables are summarized in Figures B6 and B7.

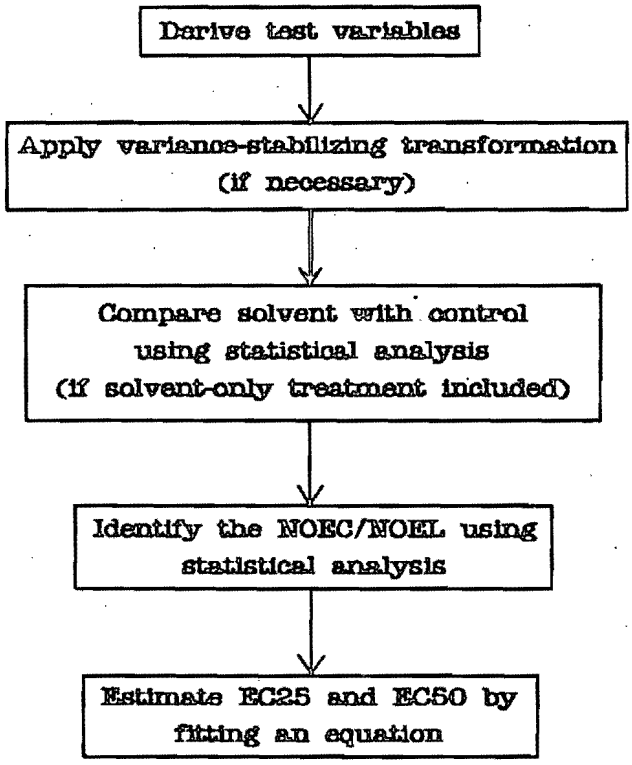

Figure B6. Summary of the test procedure for test II-1, II-2, and II-3 variables

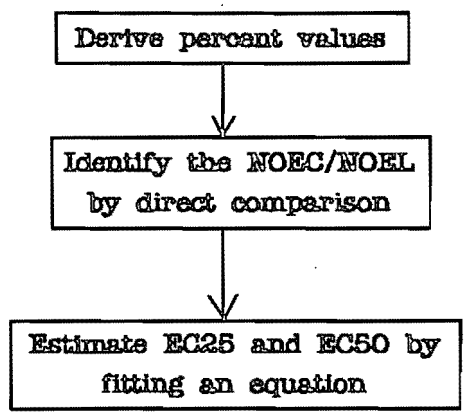

Figure B7. Summary of the test procedure for the test II-4 variable

B3.1 Outline of the Tier II test procedures

B3.1.1 Treatments applied

The treatments in the experiment depend on whether or not the pesticide is dissolved in a solvent that could have an effect on the test plants that is in addition to the effect of the pesticide. If no solvent is used, the treatments in the experiment are:

1) a control, and

2) a series of pesticide concentrations.

On the other hand, if a solvent is used, the treatments consist of:

1) a control,

2) a solvent-only treatment, and

3) a series of pesticide concentrations, each of which also includes solvent.

The solvent-only treatment is included for the purpose of testing the effect of the solvent (see section B3.3).

B3.1.2 Experimental design

Each treatment is applied to a certain number of units (minimum of four), each unit consisting of a cluster of plant cells, a number of fronds, a group of seeds, or a number of whole plants. At the start of the experiment, the units are assigned to the treatments according to either a blocked design or a one-way design:

Blocked design: Units are grouped into blocks, with each block containing one unit from each treatment. Within each block, a unit is assigned at random to each treatment.

One-way design: An equal number of units are assigned at random to each treatment.

It is recommended that a blocked design be employed for these experiments, in order to compensate for the fact that growth conditions (e.g., temperature, humidity, lighting) may not be completely uniform throughout the growth chamber. If a one-way design is employed, the lack of uniformity in growth conditions can make it more difficult to compare treatments, as the units for one treatment will be exposed to conditions somewhat different from those of another treatment. However, if a blocked design is employed in which the conditions within each block are relatively uniform, the conditions to which the treatments are exposed will be equalized to a large extent, and the problem of nonuniformity will be minimized.

Note that the advantages of blocked designs apply only if there are at least four units per treatment. If there are fewer than four, the advantage of blocking is offset by the reduction in the number of degrees of freedom in the statistical error. See Annex B1 for more information on the application of blocked and one-way designs to these experiments.

B3.1.3 Derivation of the test variables

Most of the test variables that are analyzed statistically are not measured directly but must be derived from raw measurements made on the units. For example, in the algal growth test, the raw measurements are cell counts, and the test variables (the cell growth rate and the area under the growth curve) are derived from these counts. The process of deriving the test variables is described in detail in section B3.2.

B3.1.4 Analysis of the test II-1, II-2, and II-3 variables

For each of these variables, two procedures are carried out:

1) a test to identify the NOEC/NOEL, and

2) a procedure to estimate the EC25 and EC50 parameters.

If a solvent treatment is employed, a third procedure is also run:

3) a test of the effect of the solvent.

The procedure to examine the effect of the solvent is described in section B3.4. The procedure to identify the NOEC/NOEL is described in section B3.5, and the procedure to estimate EC25 and EC50 is described in section B3.6. Further information on the role of the solvent treatment in the analysis is given in section B3.3.

B3.1.5 Analysis of the test II-4 variable

For this variable, the analysis procedures are:

1) the identification of the NOEC/NOEL, which is carried out by direct comparison of the pesticide and control data values rather than by a statistical procedure (see section B3.7.1), and

2) the estimation of the EC25 parameter, which is carried out by

the same procedure that is used for the test II-1, II-2, and II-3 variables (see section B3.6).

B3.2 Derivation of the test variables

B3.2.1 Test II-1 (algal growth)

B3.2.1.1 Basic information

Composition of unit: Each unit consists of a cluster of algal cells, the clusters being as close as possible to a specified size (e.g., 10 000 cells).

Minimum number of units per treatment: 4.

Raw measurements: Cell counts are made after 0, 24, 48, 72, and 96 hours.

Test variables derived:
- Cell growth rate, and
- Area under the growth curve.

B3.2.1.2 Derivation of the cell growth rate

a) Selection of the time period

The first step in deriving the cell growth rate is to select the time period over which this rate will be measured. This period should be that portion of the 96-hour measurement period during which the growth in the number of cells is exponential for the control units and should be a minimum of 72 hours.

The most suitable time period can be selected by calculating the daily averages of the log cell counts for the control units and plotting them against time. The best test period is the maximum period over which these averages show a linear increase with time (see Figure B3 in section B2.2.1.2). As the test period should be at least 72 hours in length, the options are to select the full period (0-96 hours), the first 72 hours (0-72 hours), or the

last 72 hours (24-96 hours).

b) Calculation of the cell growth rate

The cell growth rate G for each unit is then calculated from the rate of growth of the log cell counts over the selected time period. The formula recommended is the formula for the slope parameter in the linear regression of log cell count versus time in days:

$$G = \sum (Y_i - \overline{Y})(X_i - \overline{X}) / \sum (X_i - \overline{X})^2$$

where the Y_i values are the logs of the cell counts throughout the selected time period (either natural logs or logs to base 10 may be used), the X_i values are the times in days at which the counts were made and $\overline{Y}$ and $\overline{X}$ are the means of the Y_i and X_i, respectively. An example is given in section B2.2.1.2.

B3.2.1.3 Derivation of the area under the growth curve

The area under the growth curve for a given unit is the total number of cells added to that unit over the test period, as approximated by the area under the plot of cell counts versus time. The test period is the same period that was employed for calculating the growth rates of the log cell counts as described in section B3.2.1.2. A description of the calculation procedure is given in section B2.2.1.3.

B3.2.2 Test II-2 (*Lemna* growth)

B3.2.2.1 Basic information

Composition of unit: Each unit consists of four or five *Lemna* plants, having a total of 16 fronds.

Minimum number of units per treatment: 4.

Raw measurements:

- Counts of the total number of fronds (both live and dead) and of the total number of live fronds are made on days 3, 6, and 7, and
- Dry weight of live fronds is measured on day 7.

Test variables derived:

- Frond growth rate,
- Percent frond survival, and
- Final dry weight of live fronds.

B3.2.2.2 Derivation of the test variables

a) Frond growth rate

The frond growth rate F for each unit is calculated from the rate of growth of the log of the counts of live fronds over the seven days. The formula recommended is the formula for the slope parameter in the linear regression of log frond count versus time in days:

$$F = \sum (Y_i - \overline{Y})(X_i - \overline{X}) / \sum (X_i - \overline{X})^2$$

where the Y_i values are the logs of the frond counts on days 0, 3, 6, and 7 (either natural logs or logs to base 10 may be used), the X_i values are equal to 0, 3, 6, and 7, and $\overline{Y}$ and $\overline{X}$ are the means of the Y_i and X_i, respectively (see Figure B8).

Note: If the number of live fronds is zero at one or more of the four times (on days 0, 3, 6, and 7), the log of the frond count would be undefined. In these cases, all of the frond counts should be increased by 1 prior to taking the logarithms.

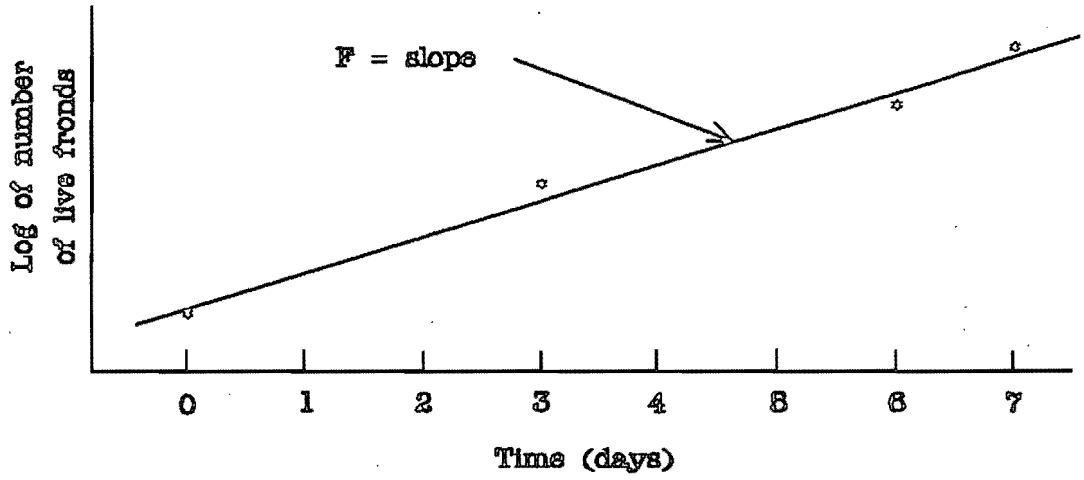

Figure B8. Calculation of the frond growth rate

b) Percent frond survival

$$\text{Percent frond survival} = \frac{\text{No. of living fronds on day 7}}{\text{Total no. of fronds on day 7}} \times 100$$

c) Final dry weight

This is the dry weight of live fronds measured on day 7.

B3.2.3 Test II-3 (vascular plant seed germination and root elongation)

B3.2.3.1 Basic information

Composition of unit: Each unit consists of a certain number of seeds. This number should be large enough to ensure that, for the units assigned to the control, at least 15 seeds will germinate and develop roots that reach a specified length (typically 20 mm) within the test period.

Minimum number of units per treatment: 4.

Raw measurements:
- A count of the number of seeds that have germinated at the end of the test period, and
- Measurements of the root lengths of the germinated seeds.

Test variables derived:
- Percent seed germination, and
- Mean root length.

B3.2.3.2 Derivation of the test variables

a) Percent germination

$$\text{Percent germination} = \frac{\text{No. of germinated seeds}}{\text{Total no. of seeds}} \times 100$$

b) Mean root length

This is the average of the root lengths for the germinated seeds.

B3.2.4 Test II-4 (vascular plant growth)

B3.2.4.1 Basic information

Composition of unit: Each unit consists of a number of whole plants.

Minimum number of units per treatment: 4.

Raw measurements: A visual rating is made after a specified period that depends on the species (usually 14 or 21 days).

Test variables derived: Percent growth and vigour.

B3.2.4.2 Derivation of percent growth and vigour

The subjective rating made on each unit indicates its growth and vigour relative to what is expected for an untreated unit. This rating is usually an integer on a scale of 0 (indicating no growth at all) to 9 (indicating full growth and vigour).

The rating is converted to a percent value ranging from 0% (no growth) to 100% (full growth and vigour). It is up to the experimenter to select an appropriate conversion method. The method recommended is to employ a conversion table based on the percent ranges set out in the guidelines (see section 3.1.2.2, test I-2). This table was obtained by converting a rating value to the midpoint of the corresponding range (e.g., a rating of 6 is converted to 72%).

Rating	Range	Midpoint	Rating	Range	Midpoint
9	100%	100%	4	30-44%	37%
8	91-99%	95%	3	16-29%	22.5%
7	80-90%	85%	2	6-15%	10.5%
6	65-79%	72%	1	1-5%	3%
5	45-64%	54.5%	0	0%	0%

Normally, each control unit will show full growth and vigour and thus will be rated at 100%. If the rating is less than 100% for any of the control units, this should be clearly stated in the results, and the possible reasons for this should be specified. It may be necessary to repeat the experiment in this case.

B3.3 Effect of solvent treatment on the data analysis

The presence or absence of a solvent treatment affects the number and the makeup of the treatments in the experiment, as described in section B2.1:

Treatments if no solvent	Treatments if solvent included
- control	- control
- several pesticide concentrations	- solvent
	- several pesticide concentrations (+ solvent)

The presence or absence of solvent also affects the analysis of the data. If solvent is not included, the analyses carried out are:

1) identification of the NOEC/NOEL by testing the mean of the pesticide concentrations against the control mean, and

2) estimation of EC25 and EC50, using the percent inhibition relative to control.

If solvent is included, the situation is more complicated. The analyses carried out are:

1) a test of the effect of the solvent,

2) identification of the NOEC/NOEL by testing the mean of the pesticide concentrations against an appropriate zero concentration mean, and

3) estimation of EC25 and EC50, using the percent inhibition relative to an appropriate zero concentration.

The zero concentration mean employed in these last two analyses depends on the results of the test of the solvent. If the solvent is found to have a significant effect, the zero concentration mean is the mean of the solvent treatment. If the solvent effect is not significant, the zero concentration mean is the average of the control and the solvent means.

B3.4 Procedure #1 for the II-1, II-2, and II-3 test variables: Testing the effect of solvent

The basic procedure involves calculating mean values for the control and solvent treatments and comparing these means using a t-test.

The procedure uses only the data from the control and solvent treatments. The data from the pesticide concentrations are not employed, even for purposes of calculating variances. Thus, the procedure is identical to the procedure for testing for the

effect of the solvent in the Tier I tests.

The statistical procedures presented here are general in nature and can be applied to any of the test variables (except for the variance-stabilizing transformations applied prior to the analysis, which are specific to particular variables).

B3.4.1 Variance-stabilizing transformations

See section B3.5.1.

B3.4.2 Requirement for different versions of the procedure

Two different versions of the procedure are required, because of variation in the choice of experimental design:

Case	Experimental design
1	Blocked
2	One-way

The procedures for these cases are discussed in sections B3.4.3 and B3.4.4.

B3.4.3 Case 1: Blocked design

Let N be the number of blocks, each containing one unit from the control and solvent treatments,
C_i and L_i be the data values for these respective treatments in block i (i = 1 to N), and
$\overline{C}$ and $\overline{L}$ be the means of the C_i and L_i, respectively.

The quantity to be tested is the mean difference $\overline{D}$:

$$\overline{D} = \overline{C} - \overline{L}$$

To test $\overline{D}$, its variance must first be calculated. Let D_i be the difference between the control and solvent values in the i'th block:

$$D_i = C_i - L_i$$

Let S_D^2 be the variance of the D_i:

$$S_D^2 = \sum (D_i - \overline{D})^2/(N - 1)$$

Then the variance $S_{\overline{D}}^2$ of $\overline{D}$ is given by:

$$S_{\overline{D}}^2 = S_D^2/N$$

To test $\overline{D}$, calculate the t-value:

$$t = \overline{D}/S_{\overline{D}}$$

and test whether it is significantly greater than zero using a one-tailed t-test at the 10% level with N-1 degrees of freedom. The rationale for a one-tailed test is the same for this test as for the other tests in this experiment (see Annex B2 for further discussion).

The 10% significance level is employed to increase the chance of detecting a solvent effect, as this test is preliminary in nature and its outcome affects the procedure for the test of the pesticide. This is explained further in the description of the Tier I test of solvent against control (see section B2.3.6.1).

B3.4.4 Case 2: One-way design

Let N be the number of units per treatment,
(C_i, i = 1 to N) be the data values for the control units,
(L_i, i = 1 to N) be the data values for the solvent units,
and
$\overline{C}$ and $\overline{L}$ be the means of the C_i and L_i, respectively.

The quantity to be tested is the mean difference $\overline{D}$:

$$\overline{D} = \overline{C} - \overline{L}$$

To test $\overline{D}$, its variance must first be calculated. Let S_C^2 and S_L^2 be the variances of the C_i and L_i:

$$S_C^2 = \sum (C_i - \overline{C})^2/(N - 1)$$

and

$$S_L^2 = \sum (L_i - \overline{L})^2/(N - 1)$$

Then the variance $S_{\overline{D}}^2$ of $\overline{D}$ is given by:

$$S_{\overline{D}}^2 = S_C^2/N + S_L^2/N$$

To test $\overline{D}$, calculate the t-value:

$$t = \overline{D}/S_{\overline{D}}$$

and test whether it is significantly greater than zero using a one-tailed t-test at the 10% level with 2(N-1) degrees of freedom. The rationale for using a one-tailed test is the same for this test as for the other tests in this experiment (see Annex B2 for further discussion).

The 10% significance level is employed to increase the chance of detecting a solvent effect, as this test is preliminary in nature and its outcome affects the procedure for the test of the pesticide. This is explained further in the description of the Tier I test of solvent against control (see section B.2.3.6.1).

B3.5 Procedure #2 for the II-1, II-2, and II-3 test variables: Identifying the NOEC/NOEL

The basic procedure for identifying the NOEC/NOEL is to start with the highest pesticide concentration in the experiment and compare its mean with the mean for zero concentration (which will be either the solvent mean or an average of the control and solvent means). If the difference between the means is significant, the next lowest pesticide concentration is tested. This proceeds until a concentration is found for which the difference is not significant. This concentration is then identified as the NOEC/NOEL.

The statistical procedures presented here are general in nature and can be applied to any of the test variables (except for the

variance-stabilizing transformations, which are specific to particular variables).

B3.5.1 Variance-stabilizing transformations

For some test variables, a transformation of the data is recommended prior to the statistical analysis, in order to equalize the error variance throughout the range of the data as much as possible. The choice of transformation will depend on the nature of the variable. The transformations recommended are:

Test	Test variable	Transformation
II-1	Cell growth rate	None required
II-1	Area under growth curve	Log transformation (see below)
II-2	Frond growth rate	None required
II-2	Percent frond survival	Angular transformation (see below)
II-2	Final dry weight of live fronds	Log transformation (see below)
II-3	Percent seed germination	Angular transformation (see below)
II-3	Mean root length	None required

B3.5.1.1 Log transformation

Either natural logs (to base e) or logs to base 10 may be used.

B3.5.1.2 Angular transformation

The formula is:

$$Y = \arcsin(P^{0.5})$$

where P is the percentage expressed as a proportion between 0 and 1. If this formula is employed, it is recommended that the

following end value adjustments be applied:

1) If P = 0, set P to 1/4M, where M is the total number of fronds or seeds (e.g., if P = 0 and M = 20, set P to 1/80 or 0.0125).

2) If P = 1, set P to 1 - 1/4M (e.g., if P = 1 and M = 20, set P to 1 - 1/80 or 0.9875).

These adjustments are standard for the angular transformation and are suggested in most statistical texts (e.g., Snedecor and Cochran 1967:328).

An alternative formula for the angular transformation that is used in toxicological studies (e.g., Haseman and Kupper 1979) is the Freeman-Tukey binomial formula. This has the advantage that it does not require an end value adjustment. Let X be the number of live fronds or germinated seed, and M be the total number of fronds or seeds (thus, P = X/M). The Freeman-Tukey binomial transformation is:

$$Y = 0.5\ [\arcsin[(X/(M+1))^{0.5}] + \arcsin[((X+1)/(M+1))^{0.5}]]$$

B3.5.2 Requirement for different versions of the procedure

A number of different versions of the basic statistical procedures are required, because of the different sets of treatments and experimental designs that could be employed. As there are two possible sets of treatments (with and without solvent) and two possible experimental designs (blocked and one-way), there are four cases to be covered:

Case	Treatments	Experimental design
1	Control, several pesticide concentrations	Blocked
2	Control, several pesticide concentrations	One-way
3	Control, solvent, solvent + several pesticide concentrations	Blocked
4	Control, solvent, solvent + several pesticide concentrations	One-way

The procedures for these four cases are discussed in sections B3.5.3 to B3.5.6, respectively.

B3.5.3 Case 1: Control and several pesticide concentrations — blocked design

Let N be the number of blocks,
C_i be the value for the control unit within block i (i = 1 to N),
T_{ki} be the value for the unit of the k'th pesticide concentration within block i, and
$\overline{C}$ and $\overline{T}_k$ be the means of the C_i and T_{ki}, respectively, averaged over all blocks i.

B3.5.3.1 Calculation and adjustment of mean differences

The first step is to calculate the set of mean differences $\overline{D}_k$ between the control mean and the mean for the k'th pesticide concentration:

$$\overline{D}_k = \overline{C} - \overline{T}_k$$

The $\overline{D}_k$ will normally form an increasing series for any test variable as the concentration increases. However, by chance there may arise an irregularity, in that the value of $\overline{D}_k$ for a particular concentration may be less than that for the next lowest concentration. This could cause error in the identification of the NOEC/NOEL.

In these cases, it is recommended that a procedure proposed by Williams (1971) be employed to modify these particular means so as to remove the irregularity. These modified means (denoted by $\overline{D}_k$(mod)) are then employed in the identification of the NOEC/NOEL in place of the original means. Additional information on this modification procedure is given in Annex B5.

B3.5.3.2 Variance of the mean differences

In order to test whether the $\overline{D}_k$ are significant, their variance must be calculated. This involves carrying out a two-way ANOVA on the data (as described in standard texts, e.g., Snedecor and Cochran 1967:302). Using this ANOVA, the variation of the data can be partitioned into three sources:

1) variation between treatments,

2) variation between blocks, and

3) the interaction of treatments × blocks.

All treatments are included in the ANOVA, including the control. The quantity obtained from this ANOVA is S_{TB}^2, the mean square of treatments × blocks. The formula for this mean square is given in Annex B4. Once it is obtained, the variance $S_{\overline{D}}^2$ of any mean difference $\overline{D}_k$ can be calculated:

$$S_{\overline{D}}^2 = (2/N) S_{TB}^2$$

B3.5.3.3 Testing the mean differences

The first test is the test of the highest pesticide concentration. If the mean difference $\overline{D}_H$ for the highest concentration was not modified by Williams' procedure, the t-value for this test is:

$$t_H = \overline{D}_H / S_{\overline{D}}$$

If $\overline{D}_H$ was modified, it is replaced in the t-value by $\overline{D}_H(\text{mod})$:

$$t_H = \overline{D}_H(\text{mod}) / S_{\overline{D}}$$

The test is carried out by comparing t_H to the critical value for a one-tailed Williams' test at the 5% level with $(N_T-1)(N-1)$ degrees of freedom, where N_T is the total number of treatments in the experiment (including control).

Williams' test involves the same t-value as a t-test; however, the critical values are different. A special table of critical values must be employed because of the use of Williams' procedure to remove irregularities in the series of mean differences. (This special table is necessary even if no irregularities occurred.) This table is given in Annex B5, taken from Williams (1971).

A one-tailed test is recommended to increase the power of the test to detect significant effects. The rationale for the use of a one-tailed test instead of the more common two-tailed test is given in Annex B2.

If t_H is not significant, the NOEC/NOEL is set at a value greater than or equal to the highest concentration. However if t_H is found to be significant, the t-value for the next highest concentration is calculated and tested in the same fashion. The process proceeds until a nonsignificant t-value is found. The concentration corresponding to this t-value is taken as the NOEC/NOEL. If no such nonsignificant t-value is found, the NOEC/NOEL is set at a value that is less than the lowest concentration tested.

Note: In looking up the critical value in Williams' table, the "number of treatments" parameter is N_T-1 for the test of the highest concentration and reduces by 1 for each move to a lower concentration. The number of degrees of freedom for all tests is $(N_T-1)(N-1)$.

B3.5.3.4 Adequacy requirement

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added. Let $\overline{D}_{NO}$ be the mean difference for the pesticide concentration identified as the NOEC/NOEL. If this mean difference was modified by Williams' procedure, then $\overline{D}_{NO}$ is the modified value. The added condition is: if $\overline{D}_{NO}$ is greater than 0.5 $\overline{C}$ (for the test II-1 and II-2 variables) or 0.25 $\overline{C}$ (for the test II-3 variables), the data are not adequate and the test must be repeated. Alternatively, registrants must conduct testing at

higher tiers. See Annex B3 and section 1.6.8.4 for further information on this condition.

B3.5.4 Case 2: Control and several pesticide concentrations — one-way design

Let N be the number of units per treatment,
(C_i, i = 1 to N) be the data values for the control units,
(T_{ki}, i = 1 to N) be the values for the k'th concentration units, and
$\overline{C}$ and $\overline{T}_k$ be the means of the C_i and T_{ki}, respectively.

B3.5.4.1 Calculation and adjustment of mean differences

The first step is to calculate the set of mean differences $\overline{D}_k$ between the control mean and the mean for the k'th pesticide concentration:

$$\overline{D}_k = \overline{C} - \overline{T}_k$$

The $\overline{D}_k$ will normally form an increasing series for any test variable as the concentration increases. However, by chance there may arise an irregularity, with the value of $\overline{D}_k$ for a particular concentration being less than that for the next lowest concentration. This could cause error in the identification of the NOEC/NOEL.

In these cases, it is recommended that a procedure proposed by Williams (1971) be employed to modify these particular means so as to remove the irregularity. These modified means (denoted by $\overline{D}_k$(mod)) are then employed in the identification of the NOEC/NOEL in place of the original means. Additional information on this modification procedure is given in Annex B5.

B3.5.4.2 Variance of the mean differences

In order to test whether the $\overline{D}_k$ are significant, their variance must be calculated. This involves carrying out a one-way ANOVA

on the data (as described in standard texts, e.g., Snedecor and Cochran 1967:258) and obtaining the within-treatments mean square S_{WT}^2. See Annex B4 for the formula for this mean square. All treatments are included in the ANOVA, including the control.

Once S_{WT}^2 is obtained, the variance $S_{\overline{D}}^2$ of any mean difference $\overline{D}_k$ can be calculated:

$$S_{\overline{D}}^2 = (2/N) S_{WT}^2$$

B3.5.4.3 Testing the mean differences

The first test is the test of the highest pesticide concentration. If the mean difference $\overline{D}_H$ for the highest concentration was not modified by Williams' procedure, the t-value for this test is:

$$t_H = \overline{D}_H / S_{\overline{D}}$$

If $\overline{D}_H$ was modified, it is replaced in the t-value by $\overline{D}_H(\text{mod})$:

$$t_H = \overline{D}_H(\text{mod}) / S_{\overline{D}}$$

The test is carried out by comparing t_H with the critical value for a one-tailed Williams' test at the 5% level with $N_T(N-1)$ degrees of freedom, where N_T is the total number of treatments in the experiment (including control). A table of critical values for Williams' test is given in Annex B5.

If t_H is not significant, the NOEC/NOEL is set at a value greater than or equal to the highest concentration. However, if t_H is found to be significant, the t-value for the next highest concentration is calculated and tested in the same fashion. The process proceeds until a nonsignificant t-value is found. The concentration corresponding to this t-value is taken as the NOEC/NOEL. If no such significant t-value is found, the NOEC/NOEL is set at a value that is less than the lowest concentration tested.

The test procedure is the same as that employed in case 1

(section B3.5.3), except that there are $N_T(N-1)$ degrees of freedom. Further information on the use of Williams' test for this analysis is given in section B3.5.3. The rationale for using a one-tailed test instead of the more common two-tailed test is given in Annex B2.

B3.5.4.4 Adequacy requirement

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added. Let $\overline{D}_{NO}$ be the mean difference for the pesticide concentration identified as the NOEC/NOEL. If this mean difference was modified by Williams' procedure, then $\overline{D}_{NO}$ is the modified value. The added condition is: if $\overline{D}_{NO}$ is greater than 0.5 $\overline{C}$ (for the test II-1 and II-2 variables) or 0.25 $\overline{C}$ (for the test II-3 variables), the data are not adequate and the test must be repeated. Alternatively, registrants must conduct testing at higher tiers. See Annex B3 and section 1.6.8.4 for further information on this condition.

B3.5.5 Case 3: Control, solvent, several pesticide concentrations — blocked design

Let N be the number of blocks,
C_i be the value for the control unit within block i ($i = 1$ to N),
L_i be the value for the solvent unit within block i,
T_{ki} be the value for the unit of the k'th pesticide concentration within block i, and
$\overline{C}$, $\overline{L}$, and $\overline{T}_k$ be the means of the C_i, L_i, and T_{ki}, respectively, averaged over all blocks i.

B3.5.5.1 Calculation and adjustment of the mean differences

The first step is to calculate the set of mean differences $\overline{D}_k$ between the mean for the k'th pesticide and the appropriate mean value for the zero concentration. There are two cases to

consider, depending on whether or not the pesticide means are compared with the average of the solvent and control means or with the solvent mean alone (as discussed in section B3.3).

Case 3-1: Pesticide compared with average of solvent and control:

In this case, the zero concentration mean is the average of $\overline{C}$ and $\overline{L}$.

Set $\overline{D}_k = (\overline{C} + \overline{L})/2 - \overline{T}_k$

Case 3-2: Pesticide compared with solvent alone:

In this case, the zero concentration mean is $\overline{L}$.

Set $\overline{D}_k = \overline{L} - \overline{T}_k$

The $\overline{D}_k$ will normally form an increasing series as the concentration increases. However, by chance there may arise an irregularity, in that the $\overline{D}_k$ value for a particular concentration may be less than that for the next lowest concentration. In these cases, it is recommended that a procedure proposed by Williams (1971) be employed to modify these particular means so as to remove the irregularity. These modified means (denoted by $\overline{D}_k$(mod)) are then employed in the identification of the NOEC/NOEL in place of the original means. Additional information on this modification procedure is given in Annex B5.

B3.5.5.2 Variance of the mean differences

In order to test whether the $\overline{D}_k$ are significant, their variance must be calculated. This involves carrying out a two-way ANOVA on the data (as described in standard texts, e.g., Snedecor and Cochran 1967:302). Using this ANOVA, the variation in the data can be partitioned into these three sources:

1) variation between treatments,

2) variation between blocks, and

3) the interaction of treatments × blocks.

All treatments are included in the ANOVA, including the control and the solvent. The quantity obtained from this ANOVA is S_{TB}^2,

the mean square of treatments × blocks. The formula for this mean square is given in Annex B4.

Once S_{TB}^2 is obtained, the variance $S_{\bar{D}}^2$ of any mean difference $\bar{D}_k$ can be calculated. There are again two cases to be covered:

Case 3-1: Pesticide compared with average of solvent and control:

$$S_{\bar{D}}^2 = (1.5/N) S_{TB}^2$$

Case 3-2: Pesticide compared with solvent alone:

$$S_{\bar{D}}^2 = (2/N) S_{TB}^2$$

B3.5.5.3 Testing the mean differences

The first test is the test of the highest pesticide concentration. If the mean difference $\bar{D}_H$ for the highest concentration was not modified by Williams' procedure, the t-value for this test is:

$$t_H = \bar{D}_H / S_{\bar{D}}$$

If $\bar{D}_H$ was modified, it is replaced in the t-value by $\bar{D}_H(\text{mod})$:

$$t_H = \bar{D}_H(\text{mod}) / S_{\bar{D}}$$

The test is carried out by comparing t_H with the critical value for a one-tailed Williams' test at the 5% significance level with $(N_T-1)(N-1)$ degrees of freedom, where N_T is the total number of treatments in the experiment. A table of critical values for Williams' test is given in Annex B5.

If t_H is not significant, the NOEC/NOEL is set at a value greater than or equal to the highest concentration. However, if t_H is found to be significant, the t-value for the next highest concentration is calculated and tested in the same fashion. The process proceeds until a nonsignificant t-value is found. The concentration corresponding to this t-value is taken as the NOEC/NOEL. If no such nonsignificant t-value is found, the NOEC/NOEL is set at a value that is less than the lowest concentration tested.

The test procedure is the same as that employed in case 1 (section B3.5.3). Further information on the use of Williams' test for this analysis is given in section B3.5.3. The rationale for using a one-tailed test instead of the more common two-tailed test is discussed in Annex B2.

B3.5.5.4 Adequacy requirement

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added. Let $\overline{D}_{NO}$ be the mean difference for the pesticide concentration identified as the NOEC/NOEL. If this mean difference was modified by Williams' procedure, then $\overline{D}_{NO}$ is the modified value. The added condition is: if $\overline{D}_{NO}$ is greater than 0.5 $\overline{C}$ (for the test II-1 and II-2 variables) or 0.25 $\overline{C}$ (for the test II-3 variables), the data are not adequate and the test must be repeated. Alternatively, registrants must conduct testing at higher tiers. See Annex B3 and section 1.6.8.4 for further information on this condition.

B3.5.6 Case 4: Control, solvent, several pesticide concentrations — one-way design

Let N be the number of units per treatment,
(C_i, i = 1 to N) be the data values for the control units,
(L_i, i = 1 to N) be the data values for the solvent units,
(T_{ki}, i = 1 to N) be the values for the k'th concentration units, and
$\overline{C}$, $\overline{L}$, and $\overline{T}_k$ be the means of the C_i, L_i, and T_{ki}, respectively.

B3.5.6.1 Calculation and adjustment of mean differences

The first step is to calculate the set of mean differences $\overline{D}_k$ between the mean for the k'th pesticide and the appropriate mean value for the zero concentration. There are two cases to consider, depending on whether or not the solvent mean is treated as a second control for the purposes of identifying the NOEC/NOEL (as discussed in section B3.3).

Case 4-1: Pesticide compared with average of solvent and control:

In this case, the zero concentration mean is the average of $\overline{C}$ and $\overline{L}$.

Set $\overline{D}_k = (\overline{C} + \overline{L})/2 - \overline{T}_k$

Case 4-2: Pesticide compared with solvent alone:

In this case, the zero concentration mean is $\overline{L}$ alone.

Set $\overline{D}_k = \overline{L} - \overline{T}_k$

The $\overline{D}_k$ will normally form an increasing series as the concentration increases. However, by chance there may arise an irregularity, in that the value of $\overline{D}_k$ for a particular concentration may be less than that for the next lowest concentration.

In these cases, it is recommended that a procedure proposed by Williams (1971) be employed to modify these particular means so as to remove the irregularity. These modified means (denoted by $\overline{D}_k$(mod)) are then employed in the identification of the NOEC/NOEL in place of the original means. Additional information on this modification procedure is given in Annex B5.

B3.5.6.2 Variance of the mean differences

In order to test whether the $\overline{D}_k$ are significant, their variance must be calculated. This involves carrying out a one-way ANOVA on the data (as described in standard texts, e.g., Snedecor and Cochran 1967:258) and obtaining the within-treatments mean square S_{WT}^2. The formula for this mean square is given in Annex B4. All treatments are included in the ANOVA, including the control and the solvent.

Once S_{WT}^2 is obtained, the variance $S_{\overline{D}}^2$ of any mean difference $\overline{D}_k$ can be calculated. There are again two cases to be covered:

Case 4-1: Pesticide compared with average of solvent and control:

$S_{\overline{D}}^2 = (1.5/N) S_{WT}^2$

Case 4-2: Pesticide compared with solvent alone:

$S_{\overline{D}}^2 = (2/N) S_{WT}^2$

B3.5.6.3 Testing the mean differences

The first test is the test of the highest pesticide concentration. If the mean difference $\overline{D}_H$ for the highest concentration was not modified by Williams' procedure, the t-value for this test is:

$$t_H = \overline{D}_H / S_{\overline{D}}$$

If $\overline{D}_H$ was modified, it is replaced in the t-value by $\overline{D}_H(\text{mod})$:

$$t_H = \overline{D}_H(\text{mod}) / S_{\overline{D}}$$

The test is carried out by comparing t_H with the critical value for a one-tailed Williams' test at the 5% level with $N_T(N-1)$ degrees of freedom, where N_T is the total number of treatments in the experiment. A table of critical values for Williams' test is given in Annex B5.

If t_H is not significant, the NOEC/NOEL is set at a value greater than or equal to the highest concentration. However, if t_H is found to be significant, the t-value for the next highest concentration is calculated and tested in the same fashion. The process proceeds until a nonsignificant t-value is found. The concentration corresponding to this t-value is taken as the NOEC/NOEL. If no such nonsignificant t-value is found, the NOEC/NOEL is set at a value that is less than the lowest concentration tested.

The test procedure is the same as that employed in case 1 (section B3.5.3), except that there are $N_T(N-1)$ degrees of freedom. Further information on the reasons for employing Williams' test are given in Annex B5. The rationale for using a one-tailed test instead of a two-tailed test is discussed in Annex B2.

B3.5.6.4 Adequacy requirement

In order to ensure that the data are adequate to detect biologically important effects, a further condition was added.

Let $\overline{D}_{NO}$ be the mean difference for the pesticide concentration identified as the NOEC/NOEL. If this mean difference was modified by Williams' procedure, then $\overline{D}_{NO}$ is the modified value. The added condition is: if $\overline{D}_{NO}$ is greater than 0.5 $\overline{C}$ (for the test II-1 and II-2 variables) or 0.25 $\overline{C}$ (for the test II-3 variables), the data are not adequate and the test must be repeated. Alternatively, registrants must conduct testing at higher tiers. See Annex B3 and section 1.6.8.4 for further information on this condition.

B3.6 Procedure #3 for the II-1, II-2, and II-3 test variables: Estimating EC25 and EC50

The basic procedure for estimating these parameters is the same for all test variables and involves the following steps:

1) Calculate an inhibition value I_k for each concentration.

2) Screen the I_k to ensure that they are suitable for estimation purposes.

3) If appropriate, apply a transformation to the concentrations, to the I_k, or to both.

4) Fit an equation (to either the original or the transformed data) to model inhibition as a function of concentration.

5) Set EC25 and EC50 equal to the concentrations corresponding to inhibitions of 25% and 50%, respectively.

Once they are estimated, EC25 and EC50 are compared directly with the value specified in the guidelines for a given species, parameter, and test. This value is either the EEC (the expected environmental concentration, which is set at the maximum recommended label rate) or a specified percentage of the EEC (see section 1.6.5 of the guidelines). This is a direct comparison, not a statistical test. Standard errors are not required for these estimates of EC25 and EC50.

B3.6.1 Calculation of the inhibition values

The first step is to obtain the inhibition values I_k for the different concentrations. The calculation depends on whether or not a solvent treatment was included in the experiment in addition to the control. Thus, there are two cases to be covered:

Case	Solvent included	Treatments
1	No	Control, several pesticide concentrations
2	Yes	Control, solvent, solvent + several pesticide concentrations

The reasons why the presence or absence of a solvent treatment affects the calculation of the inhibition values are discussed in section B3.3.

B3.6.1.1 Case 1: Solvent not included

Let $\overline{C}$ be the mean of the control data values, and
$\overline{T}_k$ be the mean of the values for the k'th pesticide concentration.

The inhibition I_k for the k'th pesticide concentration is calculated from:

$$I_k = 1 - \overline{T}_k/\overline{C}$$

B3.6.1.2 Case 2: Solvent included

Let $\overline{C}$ be the mean of the control data values,
$\overline{L}$ be the mean of the solvent data values, and
$\overline{T}_k$ be the mean of the values for the solvent + the k'th pesticide concentration.

The formula for the inhibition I_k for the solvent + k'th pesticide concentration depends on whether the denominator of the ratios is the average of the control and solvent means, or the

solvent mean alone (as discussed in section B3.3).

Case 2-1: The denominator is the average of the control and solvent means:

$I_k = 1 - \overline{T}_k/\overline{Z}$

where $\overline{Z} = (\overline{C} + \overline{L})/2$

Case 2-2: The denominator is the solvent mean alone:

$I_k = 1 - \overline{T}_k/\overline{L}$

B3.6.2 Screening the inhibition values

Once the I_k have been calculated, their suitability for purposes of estimating EC25 and EC50 should be assessed by examining them to determine:

1) whether or not any I_k are outside the range of 0-100%,

2) whether there are enough I_k values in the analysis, and

3) whether the I_k cover a sufficiently wide range of percentages.

B3.6.2.1 Presence of extreme values of I_k

a) Values of I_k less than zero

An I_k is less than zero if the treatment mean $\overline{T}_k$ is greater than the control mean $\overline{C}$. This can occur at low concentrations of a pesticide. In general, there are two explanations for this:

1) a stimulation effect, by which plants that are exposed to a low concentration of pesticide actually grow at a faster rate than plants exposed to zero concentration, and

2) random variation in the data.

The experimenter should indicate any such values of I_k and should state whether in his/her opinion these values are the result of

stimulation or of random variation. The treatment of the data depends on this decision.

If the less-than-zero I_k are due to stimulation, it is recommended that the minimum I_k be identified and that the I_k for all lower concentrations be removed from the data set. Thus, the set of I_k to be employed in the estimation of EC25 or EC50 consists only of those I_k that are on the rising portion of the plot of I_k versus concentration, as illustrated in the examples in Figure B9.

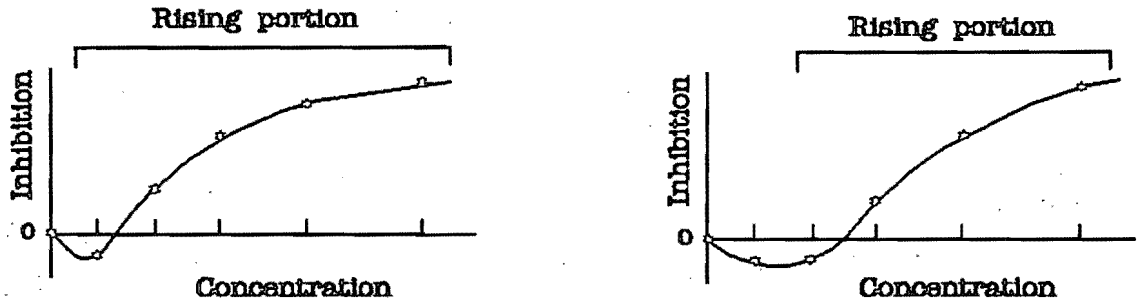

Figure B9. The rising portion of the I_k - concentration curve

The reason for removing the I_k on the descending portion of the plot is that they do not in general provide useful information on the values of EC25 or EC50, and their presence in the analysis may complicate the fitting of the equation. For example, it is often desirable to fit an equation in which the I_k increase monotonically as concentration increases. The presence of I_k values on the descending portion of the plot would make it difficult to fit such an equation.

If the less-than-zero I_k are due to random variation in the data, none of the I_k should be removed from the analysis.

b) Values of I_k greater than 100%

An I_k is greater than 100% if the treatment mean $\overline{T}_k$ is less than

zero. This can occur at high concentrations of a pesticide, but only with certain test variables. For example, it can occur with data on the growth rate of the log of the live fronds for *Lemna* growth, as this growth rate will be negative if the pesticide effect is so strong that the frond numbers decline over the test period; on the other hand, a negative $\overline{T}_k$ could not occur with data on the percent germination of seedlings, for example, as the germination rate cannot be less than zero.

The experimenter should indicate any values of I_k that are greater than 100% and should explain their occurrence. However, the values should not be removed from the analysis.

B3.6.2.2 Number of the I_k

All of the I_k values should be included in the estimation of EC25 and EC50, except for those low-concentration I_k that are removed because they are on the descending portion of the curve (as described in section B3.6.2.1). There should be a minimum of four I_k values in the estimation.

In addition, the number of I_k should exceed the number of parameters in the equation by at least two. This ensures that the residuals will have at least two degrees of freedom.

B3.6.2.3 Range covered by the I_k

The set of I_k values used in the estimation of EC25 and EC50 should cover a sufficiently wide range of percentages and should be spaced sufficiently closely within this range. The I_k are considered to provide good coverage of the range of percentages if the following conditions are met:

1) They provide continuous coverage over a range of at least 50%, where "continuous coverage" is defined to mean that the gaps between consecutive I_k are not larger than 35%. For example, the following set of I_k (5%, 10%, 30%, 60%, and 97%) provides continuous coverage over a range of 55%, extending from 5% to 60%.

2) The range of continuous coverage includes at least a 10% margin on either side of each parameter to be estimated. For example, if EC25 is to be estimated the range includes the 15-35% interval, and if EC50 is to be estimated, it includes the 40-60% interval.

In practice, it may not be possible to select the series of pesticide concentrations so that these conditions will be met. However, if the coverage of the range of percentages is very narrow or uneven, the reasons for this should be discussed.

B3.6.3 Rules and guidelines for fitting the equation

The sole objective of the fitting of the equation of I_k as a function of concentration is to obtain estimates of EC25 or EC50. The equation needs only to be accurate enough for this purpose and to be applicable over the 25-50% range of inhibitions. It does not have to be accurate over the entire range of inhibitions or concentrations.

The following rules and guidelines have been established for these equations:

1) The I_k values should not be adjusted prior to fitting the equation. Thus, for example, it is not appropriate to apply Williams' procedure (as described in Annex B5) to remove irregularities in the series of I_k values.

2) The equation must be obtained by least-squares regression methods. It is not valid to employ linear interpolation between data points (as in Figure B10a below), for example, or to fit a very flexible curve that passes through every data point (as in Figure B10b).

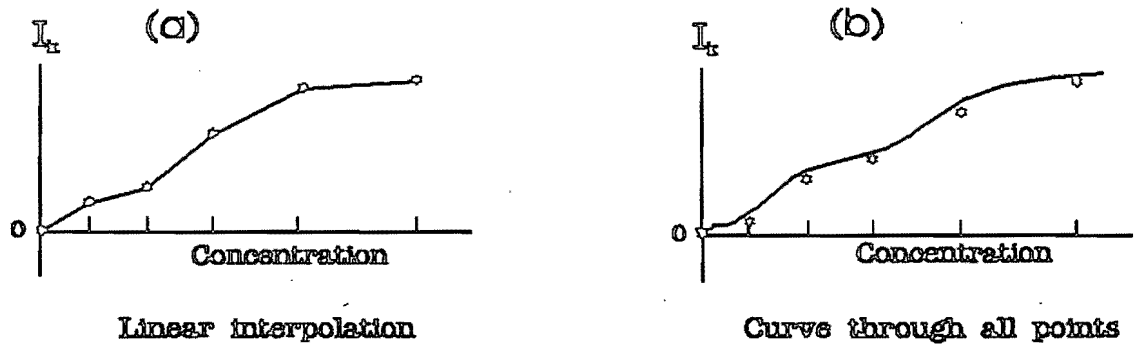

Figure B10. Examples of invalid estimation methods

3) The number of fitted parameters in the equation must be no greater than $N_I - 2$, where N_I is the number of I_k used in the derivation of the equation. This ensures that the residuals will have at least two degrees of freedom.

4) The slope of the inhibition-concentration curve should be steep enough that the estimates of EC25 and EC50 are not excessively sensitive to small changes in the parameters (as illustrated in Figure B11).

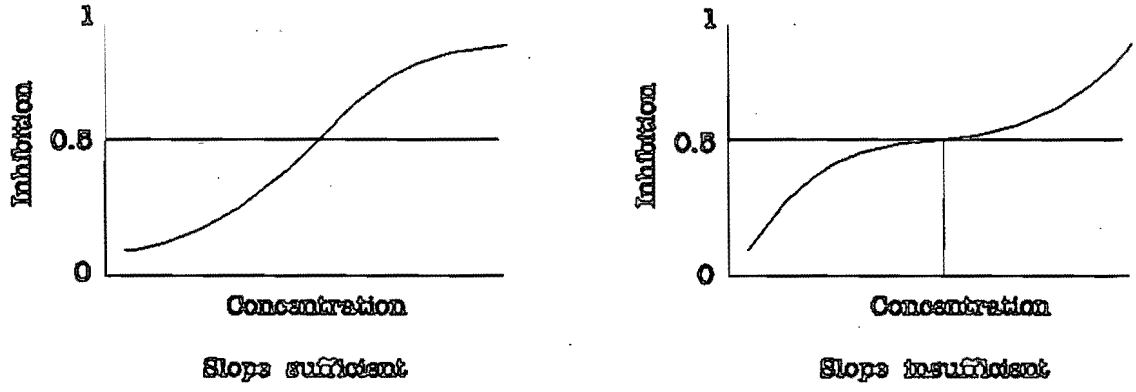

Figure B11. Examples of sufficient and insufficient slopes at $I_k = 0.50$

Although there is no specific guideline as to the minimum slope required, the experimenter should provide a plot of the curve and should discuss the situation if the curve flattens out at inhibition levels of 0.25 or 0.50. In these cases, the experimenter should indicate whether in his/her opinion the flattening of the curve is a real feature of the relationship between inhibition and concentration or is the result of random variation in the data.

B3.6.4 Data transformation prior to fitting the equation

It is usually advantageous to transform either the I_k or the concentrations (or both) prior to fitting an equation, rather than to fit the equation directly to the untransformed values. The use of appropriate transformations can facilitate the fitting of the equation by modifying the data so that they can be fitted by a relatively simple equation such as a straight line. In addition, certain transformations of the I_k produce statistical benefits, in that they tend to equalize the error variance over all concentrations. However, it is up to the experimenter to decide whether or not to apply transformations.

In general, the most useful transformations are logarithmic ones. For concentration, the transformation suggested is:

$$X_k = \log(\text{concentration})$$

Transformations suggested for the I_k are:

$$Y_k = \log(I_k)$$
$$Y_k = \log(1-I_k)$$
$$Y_k = \log(I_k/[1-I_k]) \text{ [the logit transformation]}$$
$$Y_k = \text{probit}(I_k) \text{ [the probit transformation]}$$

Note: The I_k must be expressed as a proportion, not as a percentage. For the log transformations, either logs to base e or logs to base 10 can be employed.

The transformations $Y_k = \log(I_k)$, $\log(I_k/[1-I_k])$, and $\text{probit}(I_k)$ cannot be applied in their basic form if any of the I_k are less

than zero; similarly, the transformations $Y_k = \log(1-I_k)$, $\log(I_k/[1-I_k])$, and $\text{probit}(I_k)$ cannot be employed in basic form if any of the I_k are greater than one. However, it is still possible to employ them in modified form by fitting an extra parameter, as discussed in Annex B6.

B3.6.5 Suggested procedure for fitting the equation

The fitting procedure must be adapted to the nature of the relationship between inhibition and concentration. Although this relationship can vary considerably from one data set to another, in many cases it has approximately the form illustrated in Figure B12a. For these cases, assuming that the I_k are within the range of 0 to 1, the following transformations are recommended:

$$X_k = \log(\text{concentration})$$
$$Y_k = \log(I_k/[1-I_k]) \text{ [the logit transformation]}$$

Taking the logs of the concentrations will transform the relationship to one of a symmetric S-shaped curve with evenly spaced points, as in Figure B12b. (Note that the data value [I_k = 0, concentration = 0] has been dropped.)

Applying the logit transformation to the I_k will then transform the relationship to an approximately linear one, as in Figure B12c, and will also tend to equalize the variance of the inhibition over all concentrations.

Note: The probit transformation would also tend to linearize the relationship and equalize the variance of the inhibition; however, the logit is preferred, as the probit involves specific assumptions about the distribution of the I_k that may not hold in some cases.

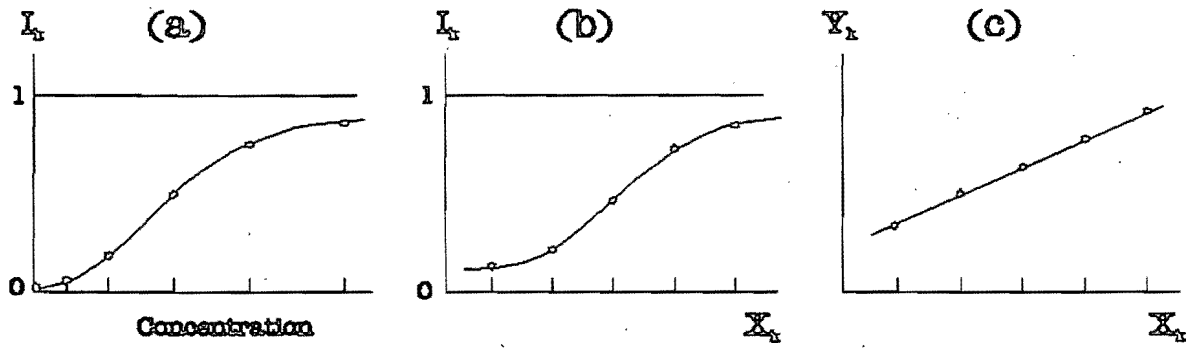

Figure B12. Effects of the log and logit transformations

The transformed data should then be plotted and examined. If the relationship is sufficiently linear, it is recommended that the standard equation for a straight line be fitted:

$$Y_k = a + b X_k$$

In some situations, the above procedure requires modification. Two such situations are:

1) cases in which the I_k are not all within the range of 0 to 1, and

2) cases in which the inhibition-concentration relationship is not linear, even after transformation.

The following suggestions may be useful in these cases; however, it is up to the experimenter to decide what action should be taken.

If the I_k are not all within the range of 0 to 1, some possible options are as follows:

1) The logit transformation could be modified by adding a constant, as described in Annex B6.

2) A different transformation could be applied to the I_k, such as $Y_k = \log(I_k)$ or $Y_k = \log(1-I_k)$.

3) The equation could be fitted to the untransformed I_k.

If the relationship is not linear, even after transformation, the three options presented above are still possible. A fourth possibility as follows:

4) A quadratic equation $Y_k = a + b X_k + c X_k^2$ could be fitted.

Note: Fitting a quadratic equation or modifying the logit transformation will result in a three-parameter equation. For these options to be applicable, there should be at least five I_k values in the analysis in order to satisfy the condition that the residuals have at least two degrees of freedom.

B3.6.6 Estimating EC25 and EC50

Estimating EC25 and EC50 is straightforward once the equation has been fitted. For example, suppose that the transformations recommended in section B3.6.5 were applied and a linear equation was fitted of the form:

$$Y_k = a + b X_k$$

where the Y_k are the transformed inhibition values and the X_k are the logs of the concentrations.

The EC25 value is then estimated by:

1) setting the inhibition to 0.25

2) calculating the corresponding transformed value $Y_{0.25}$:

$$Y_{0.25} = \log(0.25/(1-0.25)) = \log(3) = 1.099$$

3) calculating the corresponding log concentration value $X_{0.25}$ using the parameter values a and b obtained in the regression:

$$X_{0.25} = (Y_{0.25} - a)/b$$

4) calculating EC25:

$$EC25 = \exp(X_{0.25})$$

(*Note*: The above calculations have employed logarithms to base e.)

EC50 can be estimated in the same manner, starting with a value of 0.50 for the inhibition.

B3.7 Procedures for the II-4 test variable

The analysis of the data for the test II-4 variable (percent growth and vigour) is much simpler than the analysis for the test II-1, II-2, or II-3 variables, for the following reasons:

1) The only treatments in the experiment are the control and the pesticide concentrations. No solvent treatment is present.

2) No statistical tests of significance are carried out. The NOEC/NOEL for the pesticide effect is identified by direct comparisons between the control data values and the data values for the pesticide concentrations.

3) The procedure for this comparison is the same whether a blocked or a one-way design was employed for the experiment.

B3.7.1 Identifying the NOEC/NOEL

The first step is to examine the data values for the control units and confirm that they are all equal to 100%. (As stated earlier, if any of them are less than 100%, the reasons for this should be explained. It may be necessary to repeat the experiment.)

The data values for the units that received the pesticide concentrations should then be examined, and the lowest concentration level for which the percent value is less than 100% for at least one of the units should be identified. The NOEC/NOEL is taken as the concentration level immediately below this identified level. If this identified level is already the

lowest level in the experiment, the NOEC/NOEL is set to a value that is less than the lowest concentration tested.

If none of the data values is less than 100% for any of the pesticide concentrations, the NOEC/NOEL is set to a value that is greater than the highest concentration tested.

The procedure is the same regardless of whether a blocked or a one-way design was employed in the experiment.

B3.7.2 Estimation of EC25

The procedure for estimating the EC25 parameter for the test II-4 variable is the same as that described for the test II-1, II-2, and II-3 variables (see section B3.6).

B4.0 References

Haseman, J.K., and L.L. Kupper. 1979. Analysis of dichotomous response data from certain toxicological experiments. Biometrics 35:281-293.

Snedecor, G.W., and W.G. Cochran. 1967. Statistical methods. 6th ed. Iowa State University Press, Ames, Iowa.

Williams, D.A. 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27:103-117.

Annex B1. One-way and blocked designs

Growing conditions are not completely uniform in many growth chambers but vary from one location to another as a result of, for example, variations in temperature, humidity, or light conditions. In these cases, a random assignment of units may happen to place the units for one treatment in conditions that are generally more favourable than the conditions for the second treatment. The comparison of treatments would then be adversely affected by a location bias.

Blocked designs are commonly employed to minimize such biases, with blocks being set up so that conditions within a block are as uniform as possible.

To illustrate the use of one-way and blocked designs, consider a simplified example. Suppose that there are two treatments in an experiment and eight units in total, with four units to be assigned to each treatment. Suppose that the experiment is to be conducted in a growth chamber that contains two levels, with space for four units on each level.

The simplest design for this experiment is the one-way design, in which the units are located randomly within the chamber. An example of a random assignment is illustrated in Figure B-A1.

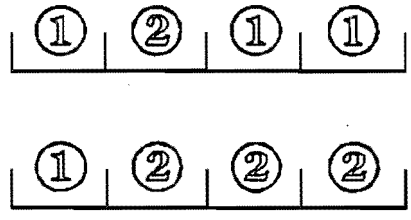

Figure B-A1. Example of assignment of units for a one-way design

Suppose that, for the chamber used in this experiment, there is vertical variation between the upper and lower levels and also horizontal variation between the centre of the chamber and the outside.

A possible blocked design for this experiment would be to group the units into four blocks of two units each, with one block occupying the centre of the top row, one occupying the outside locations in the top row, one occupying the centre of the bottom row, and one occupying the outside of the bottom row, as illustrated in Figure B-A2.

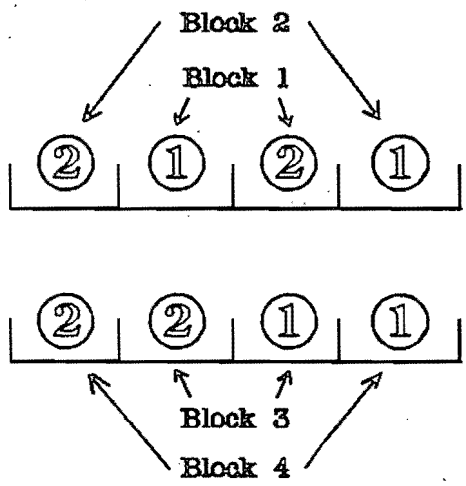

Figure B-A2. Example of assignment of units for a blocked design

The desirability of employing a blocked design depends upon the amount of variation in the conditions to which the units are exposed. However, it is not the only method for removing location bias. Another solution to this problem is to rotate units or trays within the chamber throughout the course of the experiment so that all units are exposed to the full range of conditions.

Annex B2. One-tailed tests versus two-tailed tests

For any statistical test of significance, the first step in the procedure is to formulate the assumption that the effect being tested for is completely absent. For example, in testing the effect of the high concentration of pesticide, the assumption is first made that it has no effect whatsoever. Under this assumption, the difference $\overline{C} - \overline{T}$ between the means for the control and the high concentration has an expected value of zero and will be negative as often as it is positive.

In the statistical test, the decision is made as to whether this no-effect assumption is consistent with the data. The key to this decision is the distribution of the test statistic that would occur if the no-effect assumption is correct. In this report, the test statistic is always a t-value, which has a known distribution (Student's t-distribution with the appropriate number of degrees of freedom) under the no-effect assumption. An example of the t-distribution is shown in Figure B-A3.

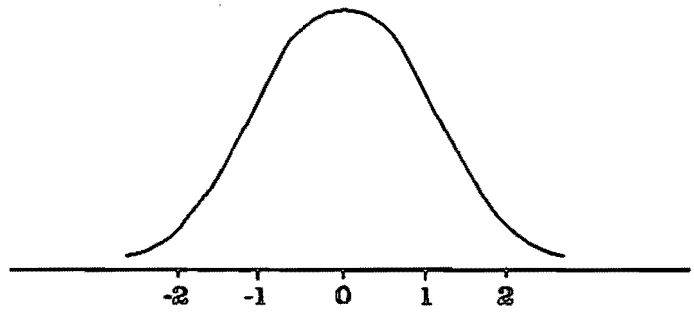

Figure B-A3. Example of the t-distribution

The data from the experiment are considered to be inconsistent with the no-effect assumption if the t-value obtained from the data is within the most extreme 5% of the distribution, referred to statistically as the "critical region". If the t-value falls within this region, the effect being tested for is considered to be significant at the 5% confidence level.

The difference between the one-tailed and two-tailed tests is in the definition of the critical region. For the one-tailed test, it is the upper 5% of the distribution (Figure B-A4), whereas for the two-tailed test it is the upper 2.5% and the lower 2.5 % (Figure B-A5. Thus, values of t do not have to be as large to be significant using a one-tailed test as they do if a two-tailed test is employed.

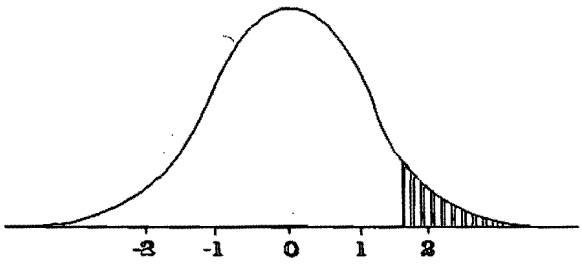

Figure B-A4. Critical region for a one-tailed test

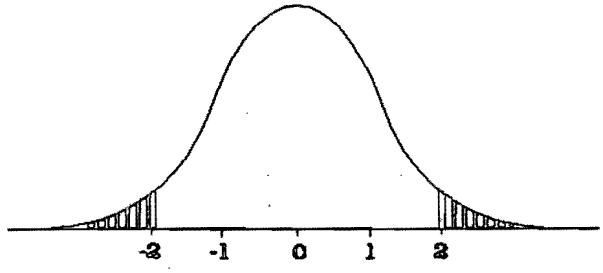

Figure B-A5. Critical region for a two-tailed test

The critical region must be matched to the effect that is being tested for, in that it must be the best region for discriminating between situations where an effect occurs and situations where it does not. In most statistical situations, the effect being tested for could occur in either a positive or negative direction, and the best critical region is the one for the two-tailed test. (The exact definition of the best critical region involves complex mathematical concepts such as maximum likelihood and is discussed in some statistics texts.)

The critical region for the one-tailed test is justified only if the effect being tested for can produce a positive t-value only and never a negative one; thus, the decision to be made in the test is that of whether there is a positive effect or no effect. This appears to be appropriate for the statistical tests described in this report, as in each case the effect being tested for is expected to produce a positive mean difference.

The disadvantage of using a one-tailed test is that if a large negative t-value should unexpectedly occur, no conclusion could be drawn as to whether or not the treatment being tested was causing a negative effect. The only conclusions that are consistent with the use of a one-tailed test are that there is a positive effect or that there is no effect.

Annex B3. Use of an added condition to ensure data adequacy

The significance of a particular mean difference $\overline{D}$, as tested by calculating its t-value $\overline{D}/S_{\overline{D}}$, depends on its standard deviation $S_{\overline{D}}$ as much as it does on $\overline{D}$ itself. If $S_{\overline{D}}$ is large, the test loses power, in that even a large $\overline{D}$ may not be significant. Thus, large treatment effects may go undetected. An excessively large $S_{\overline{D}}$ is caused by either insufficient data or inaccurate data.

In order to ensure that the t-tests carried out have sufficient power to detect effects that are large enough to be biologically important, an extra condition was imposed to identify those situations where the power is clearly not sufficient. This condition varies from one test variable to another.

B3-1.0 Test of I-1, II-1, and II-2 variables

A treatment effect is considered to be biologically important if the treatment mean $\overline{T}$ is less than 50% of the control mean $\overline{C}$. Therefore, the condition was imposed that if a mean difference $\overline{D}$ is greater than 0.5 $\overline{C}$ but is not significantly greater than zero, the test is inadequate.

B3-2.0 Test of II-3 variables

A treatment effect is considered to be biologically important if the treatment mean $\overline{T}$ is less than 25% of the control mean $\overline{C}$. Therefore, the condition was imposed that if a mean difference $\overline{D}$ is greater than 0.25 $\overline{C}$ but is not significantly greater than zero, the test is inadequate.

In botth cases, the experiment must be repeated. Alternatively, registrants must conduct testing at higher tiers. See section 1.6.8.4 for further information on this condition.

These conditions apply to both the standard t-tests and the Williams' tests.

Annex B4. ANOVA mean-square formulas

B4-1.0 The one-way ANOVA

Let N_T be the number of treatments,
N be the number of units per treatment,
X_{ki} be the data value for the i'th unit in the k'th treatment, and
$\overline{X}_{k.}$ be the mean value for the k'th treatment.

Then the formula for the within-treatments mean square S_{WT}^2 is:

$$S_{WT}^2 = (\sum_k \sum_i [X_{ki} - \overline{X}_{k.}]^2) / (N_T[N-1])$$

B4-2.0 The two-way ANOVA

Let N_T be the number of treatments,
N be the number of blocks,
X_{ki} be the data value for the k'th treatment within block i,
$\overline{X}_{k.}$ be the mean value for the k'th treatment over all blocks,
$\overline{X}_{.i}$ be the mean value for the i'th block over all treatments, and
$\overline{X}_{..}$ be the mean over all data values.

Then the formula for the mean square S_{TB}^2 for the interaction of treatments × blocks is:

$$S_{TB}^2 = (\sum_k \sum_i [X_{ki} - \overline{X}_{k.} - \overline{X}_{.i} + \overline{X}_{..}]^2) / ([N_T-1][N-1])$$

Note: In the above formulas, K is summed from 1 to N_T and i is summed from 1 to N.

Annex B5. Williams' procedure for removing irregularities in a series of mean values

There is a chance that the series of means $\overline{D}_k$ employed in the identification of the NOEC/NOEL will contain one or more irregularities, in that the mean value for a particular concentration may be less than the mean for the next lowest concentration, as illustrated in Figure B-A6.

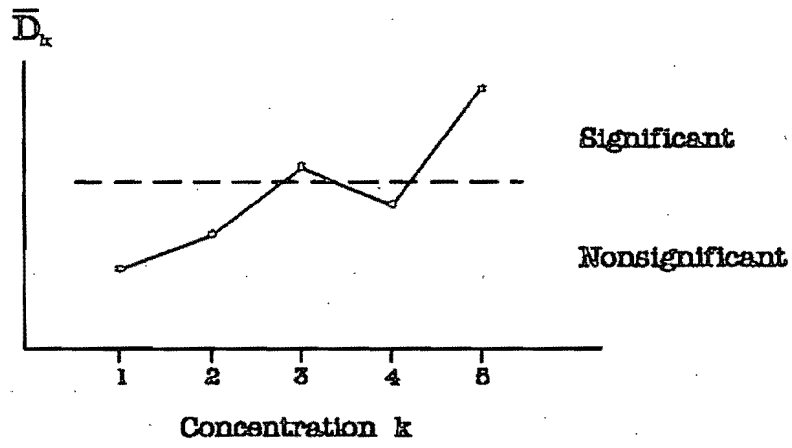

Figure B-A6. Example of an irregularity in a series of means

This is a condition that could cause ambiguity or error in the identification of the NOEC/NOEL. It is possible that the effect of the pesticide could be nonsignificant at a certain concentration but significant at a lower concentration. This would be the case if the dividing line between significant and nonsignificant $\overline{D}_k$ values occurred at the irregularity, as in the figure above.

In these cases, it is recommended that a modification procedure proposed by Williams (1971) be employed to remove the irregularity. Suppose that a certain mean difference $\overline{D}_L$ is lower than the difference $\overline{D}_{L-1}$ for the next lowest concentration, instead of being higher as expected. In Williams' procedure, $\overline{D}_L$

and $\overline{D}_{L-1}$ are averaged, and this average then replaces both $\overline{D}_L$ and $\overline{D}_{L-1}$ in the series of mean differences to produce a modified series of means that no longer has an irregularity, as illustrated in Figure B-A7. This modified series is then employed in the identification of the NOEC/NOEL in place of the original series.

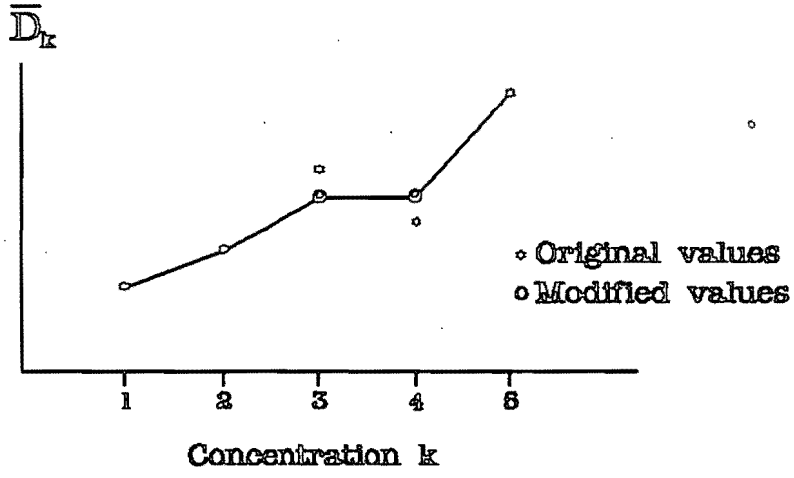

Figure B-A7. The modified means

Because of this removal of irregularities in the series of mean differences, a special table of critical values must be employed in determining whether or not the t-values are significant when these differences are tested. A set of such tables has been produced by Williams (1971). The one employed for the tests set out in this appendix is the one for one-tailed tests at the 5% significance level, which is given in Table B-A1. When t-values are tested using these special tables, the test is referred to as a Williams' test.

Note: In Williams' paper, the removal of the irregularities is carried out on the series of treatment means rather than on the series of differences between treatment and control; however, the result is the same.

Table B-A1. Critical values for a one-tailed Williams' test at the 5% significance level.

Degrees of freedom	Number of dose levels							
	1	2	3	4	5	6	7	8
5	2.02	2.14	2.19	2.21	2.22	2.23	2.24	2.24
6	1.94	2.06	2.10	2.12	2.13	2.14	2.14	2.15
7	1.89	2.00	2.04	2.06	2.07	2.08	2.08	2.09
8	1.86	1.96	2.00	2.01	2.02	2.03	2.04	2.04
9	1.83	1.93	1.96	1.98	1.99	2.00	2.00	2.01
10	1.81	1.91	1.94	1.96	1.97	1.97	1.98	1.98
11	1.80	1.89	1.92	1.94	1.94	1.95	1.95	1.96
12	1.78	1.87	1.90	1.92	1.93	1.93	1.94	1.94
13	1.77	1.86	1.89	1.90	1.91	1.92	1.92	1.93
14	1.76	1.85	1.88	1.89	1.90	1.91	1.91	1.91
15	1.75	1.84	1.87	1.88	1.89	1.90	1.90	1.90
16	1.75	1.83	1.86	1.87	1.88	1.89	1.89	1.89
17	1.74	1.82	1.85	1.87	1.87	1.88	1.88	1.89
18	1.73	1.82	1.85	1.86	1.87	1.87	1.88	1.88
19	1.72	1.81	1.84	1.85	1.86	1.87	1.87	1.87
20	1.72	1.81	1.83	1.85	1.86	1.86	1.86	1.87
22	1.72	1.80	1.83	1.84	1.85	1.85	1.85	1.86
24	1.71	1.79	1.82	1.83	1.84	1.84	1.85	1.85
26	1.71	1.79	1.81	1.82	1.83	1.84	1.84	1.84
28	1.70	1.78	1.81	1.82	1.83	1.83	1.83	1.84
30	1.70	1.78	1.80	1.81	1.82	1.83	1.83	1.83
35	1.69	1.77	1.79	1.80	1.81	1.82	1.82	1.82
40	1.68	1.76	1.79	1.80	1.80	1.81	1.81	1.81
60	1.67	1.75	1.77	1.78	1.79	1.79	1.80	1.80
120	1.66	1.73	1.75	1.77	1.77	1.78	1.78	1.78
∞	1.645	1.716	1.739	1.750	1.756	1.760	1.763	1.765

Note: The values for one dose level (in the first column on the left) are the same as the values in the standard t-table for a one-tailed test at the 5% significance level.

Source: Williams, D.A. 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27:103-117.

Annex B6. Modifications of the transformations

B6-1.0 Accommodating inhibition values that are less than zero

If any of the I_k are less than zero, the transformations

$$Y_k = \log(I_k)$$
$$Y_k = \log(I_k/[1-I_k])$$
$$Y_k = \text{probit}(I_k)$$

cannot be used in their basic form, as the transformed value would be undefined.

However, they can be applied in modified form by selecting a small positive constant U such that (I_k+U) is greater than zero for all concentrations. For example, if the minimum value of the I_k is -0.05, assigning a value of 0.10 to U ensures that the quantity (I_k+U) is positive for all concentrations.

The modified forms of these transformations are:

$$Y_k = \log(I_k+U)$$
$$Y_k = \log([I_k+U]/[1-I_k])$$
$$Y_k = \text{probit}([I_k+U])/[1+U])$$

B6-2.0 Accommodating inhibition values that are greater than one

Similarly, if any of the I_k are greater than one, the transformations

$$Y_k = \log(1-I_k)$$
$$Y_k = \log(I_k/[1-I_k])$$
$$Y_k = \text{probit}(I_k)$$

cannot be used in their basic form, as $(1-I_k)$ is negative. However, they can be applied in modified form by selecting a small positive constant V such that $(1-I_k+V)$ is greater than zero for all concentrations.

The modified forms of these transformations are:

$$Y_k = \log(1-I_k+V)$$
$$Y_k = \log(I_k/[1-I_k+V])$$
$$Y_k = \text{probit}(I_k/[1+V])$$

The addition of the U parameter enables a set of I_k to be modelled over the range of -U to 1 instead of the normal range of 0 to 1, as illustrated in Figure B-A8. The addition of the V parameter enables a set of I_k to be modelled over the range of 0 to (1+V), as illustrated in Figure B-A9.

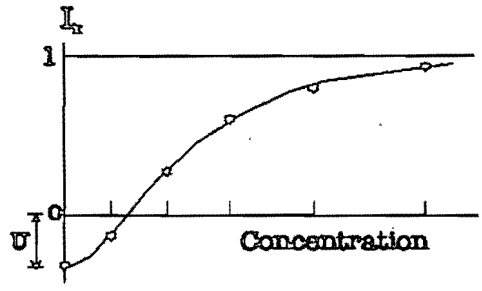

Figure B-A8. Effect of U parameter

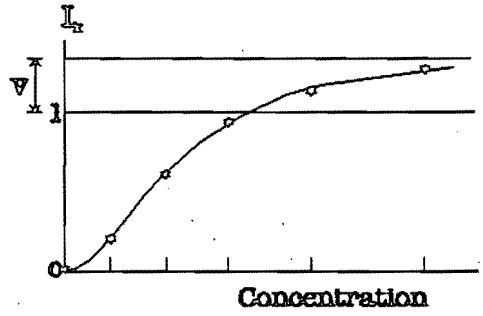

Figure B-A9. Effect of V parameter

Note: Care should be taken to ensure that the proper estimate is obtained for EC25 and EC50. For example, if a parameter U is added, care should be taken to ensure that EC25 and EC50 are the concentrations at which the value of I_k is 0.25 or 0.50, not the concentrations at which (I_k+U) is 0.25 or 0.50.