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PROPOSAL TO UPDATE NON-TARGET PLANT TOXICITY TESTING UNDER NAFTA

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ABBREVIATIONS

AAPCO - Association of American Pesticide Control Officials, Inc.
ACPA - American Crop Protection Association
ALS - acetolactate synthase
ASTM - American Society for Testing and Materials
EC_{25} - the concentration that results in a 25% reduction in the test endpoint being measured relative to the control
EC_{50} - the concentration that results in a 50% reduction in the test endpoint being measured relative to the control
ED_{25} - the dose that results in a 25% reduction in the test endpoint being measured relative to the control
EEC - expected environmental concentration
EER - estimated environmental rate
EPA -Environmental Protection Agency
FIFRA - Federal Insecticide, Fungicide, and Rodenticide Act
FWS - US Fish and Wildlife Service
GENEEC -
GLP - Good Laboratory Practices
LOC - Level of Concern
NAFTA - North American Free Trade Agreement
NEC - no-effect concentration
NOAEC - no observed adverse effects concentration
OECD - Organization for Economic Co-operation and Development
OPP - Office Of Pesticide Programs
OPPT - Office of Pollution Prevention and Toxics
OPPTS - Office of Prevention, Pesticides, and Toxic Substances
ORD - Office Of Research and Development
PAR - photosynthetically active radiation
PMRA - Pest Management Regulatory Agency
RQ - risk quotient
SAP - Scientific Advisory Panel
TEP - typical end use product
TGAI - technical grade active ingredient
TSCA - Toxic Substances and Chemicals Act
USGS - United States Geological Survey

1 OVERVIEW

1.1 Introduction

Under the North American Free Trade Agreement (NAFTA) the United States Environmental Protection Agency (EPA), Office of Pollution, Prevention, and Toxic Substances (OPPTS) and the Canadian Pest Management Regulatory Agency (PMRA) have agreed to develop and implement a compatible review program that will facilitate routine sharing of the work associated with the review of pesticide applications. An essential step in developing a compatible program between the two countries is harmonization of each country's data requirements and review procedures. The benefits of these harmonization efforts are many, including a savings of resources, improved communication within the EPA and among the two countries, improved science, and more consistent and better regulatory decisions.

To date, the United States and Canada have been successful in harmonizing the majority of their pesticide testing protocols. The Canadian PMRA agreed that, until the two countries finalize all their pesticide test guidelines, they would use EPA's testing requirements. One of the few testing protocols left for harmonization is non-target plant toxicity. In this document, the United States and Canada are proposing a four-tiered testing scheme for non-target toxicity testing for aquatic and terrestrial plants which is based on extensive collaboration between the two countries, international workshops, and literature searches.

For more than a decade, stakeholders, independent researchers, advisory groups, the states and EPA regional offices have urged the OPPTS/EPA and PMRA to modify their terrestrial and aquatic plant toxicity guidelines in ways that would allow the two agencies to conduct more refined estimates of risk to non-target plants. In this document, EPA and PMRA have proposed a four-level tiering structure for aquatic and terrestrial plants that will allow the two agencies to conduct more realistic and scientifically sound risk assessments and to focus on species, communities, and ecosystems at greatest risk. In the proposed schemes, testing at higher levels is determined by the extent of phytotoxic effects observed at lower levels. Progression to higher levels occurs only for those plant groups for which significant toxic effects have been determined.

The first level of testing, Level 1, is a deterministic assessment based on risk quotients. For this level, EPA and PMRA are proposing additional test species. Level II is a first-step refinement of the phytotoxic potential identified in Level 1 and utilizes dose-response data in conjunction with exposure distribution information. Level III is an expanded assessment that includes additional focal species testing and a more refined exposure assessment. Level IV is the final and most comprehensive testing step, consisting of microcosm, mesocosm, or field testing as well as post-registration monitoring. At this level, the objective is to address specific questions or issues raised with a chemical for a given terrestrial or aquatic environment.

Because of the wide variability in plant responses to chemicals and the large uncertainty in EPA's and PMRA's current plant risk assessments, the two agencies are proposing to expand the number of tested species. For the aquatic toxicity testing scheme, EPA and PMRA are proposing to expand the number of tested species from five to eleven and are recommending foliar spray applications for selected vascular plants to simulate spray drift. The proposed aquatic toxicity testing will include the addition of three microalgae, one submersed vascular plant, and two emergent vascular plants. For the terrestrial toxicity testing scheme, EPA and PMRA are proposing to expand the number of tested species from ten to twenty-six and to add reproductive tests. At the present time, EPA only requires testing on ten annual crop plants which represent two monocot families and four dicot families. In the new harmonized testing scheme, EPA and PMRA have proposed the addition of one monocot family, eight dicot families, four woody plants, one-life cycle test (reproductive test), and one partial life-cycle test. With better measurement endpoint data, the two agencies will be able to conduct more realistic and scientifically sound risk characterizations and will be able to substantially reduce the uncertainty in their risk assessments.

This document is divided into four chapters. The first chapter presents an overview and contains background information on the history of non-target plant toxicity tests, advances in environmental monitoring, a rationale for expansion of testing, and a summary of the proposed aquatic and terrestrial plant testing design. Chapter two describes the detailed proposed testing scheme for aquatic plants, while chapter three describes the testing scheme for terrestrial plants. The last part of the document contains appendices and references which support the proposed testing schemes.

EPA and the PMRA are requesting guidance from the SAP on the proposed four- tiered testing design for aquatic and terrestrial plants, the proposed endpoints and triggers for progression to higher levels, the proposed species for testing, post-registration monitoring programs, prioritized research needs to improve assessment of impacts on non-target plants, and any additional information that the two agencies need to consider to improve their assessments. A list of detailed questions for the SAP is found in the cover memorandum.

1.2 Regulatory History of Non-Target Plant Toxicity Tests

The Office of Pesticide Programs (OPP) in EPA presented its first proposal for aquatic and terrestrial non-target plant test guidelines to the FIFRA SAP in 1978. After an extensive public comment and revision period, the first non-target plant test guidelines were published in October 1982 and were titled: Pesticide Assessment Guidelines Subdivision J, Hazard Evaluation - Non-target Plants (Holst and Ellwanger 1982). These guidelines described three testing tiers for assessing the effects of pesticides on non-target plants. The first Tier assessed the effect of the maximum label dosage on plant appearance and growth. If Tier I testing showed growth reduction or visual phytotoxicity of >25% for terrestrial plants or >50% for aquatic plants, then Tier II tests were required. Tier II tests were dose- response tests that provided EC_{25}, EC_{50}, and NOAEC values. These endpoints were established after EPA received extensive public comments

from the public and the SAP. An EC_{50} value was established for aquatic plants because they have shorter recovery periods than terrestrial plants. On the other hand, an EC_{25} value was a more appropriate value for terrestrial plants and allowed the agency to account for low level damage of a cosmetic nature to high value ornamentals and fruits. The EPA recognized that some terrestrial plants may recover from a 25% defoliation or reduction in early growth (e.g. soybeans) with no adverse effect on yield, while other terrestrial plants such as corn may not recover from early growth injury resulting in a one bushel yield loss for every day delay in maturity. While the tested aquatic plants completed at least one full life cycle of growth, the terrestrial plants were not evaluated for effects beyond initial growth in the first 2-4 weeks (endpoints such as effects on flower formation, flower production, seed formation, delays in maturity, fruit or seed yield, pollen viability, or seed viability were not evaluated). In Tier III, field tests were designed to evaluate adverse effects on sensitive native plants in ecosystems. To date, only a few Tier III aquatic plant tests and one Tier III terrestrial plant test have been submitted to the Agency.

These non-target plant test guidelines (Subdivision J) established a regulatory precedent for non-target plant testing in the United States. At an international EPA Office of Research and Development (ORD) workshop in 1990, participants identified inadequacies and limitations in these guidelines, which included the inability to assess effects on plant reproduction, the need to expand terrestrial test species beyond only crop plants, and the capability to develop field test and monitoring protocols (Fletcher and Ratch 1991). According to Brown and Fletcher these Subdivision J guidelines only examined 20% of a plant's life cycle (i.e., seedling emergence and early seedling growth), overlooking the portion impacted by many herbicides (Brown 1996, Fletcher *et al.* 1996).

The original Subdivision J guidelines provided test protocols for the tiered testing of five aquatic plants: a floating aquatic macrophyte *Lemna gibba*, a green alga *Selenastrum capricornutum* recently renamed *Pseudokirchneria subcapitata*, a blue-green cyanobacteria *Anabaena flos-aquae*, a freshwater diatom *Navicula pelliculosa*, and a marine diatom *Skeletonema costatum*. For terrestrial plants, these guidelines provided test protocols for the tiered testing of 10 plants: corn (*Zea mays*), soybean (*Glycine max*), a root crop such as radish (*Raphanus sativus*), carrot (*Daucus carota*), or onion (*Allium cepa*) and seven other annual crop species for a balance of monocots and dicots. Three different types of plant tests were required by Subdivision J: seed germination, seedling emergence, and vegetative vigor. The seedling emergence and vegetative vigor test guidelines were modified in 1996 to allow the registrant the option of substituting weeds or native plants and/or adding species to the test battery of the 10 currently tested crop plants.

In 1990, Canada initiated the development of plant toxicity guidelines for testing pesticides (Freemark *et al.* 1990), and in 1993 Environment Canada published draft guidelines for non-target plant testing and evaluation (Boutin *et al.* 1993). Following publication of the Canadian plant toxicity guidelines, Freemark and Boutin conducted an analysis of existing test guidelines and literature (Freemark and Boutin 1994, Boutin *et al.* 1995). Later, EPA reassessed their non-target plant test guidelines and identified the following areas for improvement: 1) expand screens

to include all pesticides having outdoor uses; 2) expand initial screens to include untested groups of aquatic plants; 3) consider foliar toxicity resulting from pesticides drifting to above water aquatic plants; and 4) expand the number of terrestrial test species to include non-crop plants more representative of wildlife needs.

In a 1994 FIFRA SAP briefing on non-target plant data requirements, the Agency discussed expansion of data requirements to include a screen of all pesticides with outdoor uses, the requirement for the use of typical end use products (TEPs) in terrestrial plant tests, and the elimination of the seed germination test. The SAP recommended that the EPA rely on Tier I replicated tests using GLP procedures rather than on industry efficacy screening tests. The SAP noted the following deficiencies with the pesticide industry efficacy screening study methods: 1) plant injury rating systems differed from company to company; 2) test methods were inconsistent across the industry; 3) tests were not conducted with the TEP; 4) tests were often not replicated; and 5) tests only evaluated phytotoxicity to crops and weeds of interest to a particular company.

In 1994 when the SAP was asked to comment on the adequacy of the existing list of terrestrial test species, they replied: "The current list of 10 recommended test species probably serves as a good set of surrogates for cultivated crops, but it is extremely unlikely that the data collected on these plants is representative of how a new chemical impacts the growth and reproduction of non-target plants in natural plant communities. EPA recommends use of 10 annual crop species representing eight of 300 families in the plant kingdom. This list contains no perennial plants, no wetland plants, no non-aquatic macrophytes, no tree species, native plants, etc. Thus, the list is very narrow from both taxonomic and ecological standpoints. Further, there are almost no comparative toxicological data for these 10 plants in relation to the response in a broad spectrum of taxonomically and ecologically different plants. Haphazard augmentation of the current list of plants with a few native, perennial, woody plant species; or adoption of a new policy to use screening data collected on weeds (new Canadian policy)[1], is not considered an improvement over the current list of 10 plants. Such additions or changes serve no purpose until research is conducted to establish how selected plant surrogates represent specific segments of the overall taxonomic and ecological diversity present in agro-ecosystems. There is a critical need for research in this area . . . "

The 1994 SAP then suggested that the EPA identify research needs, assess the merits of field testing vs. monitoring programs, develop a plant reproduction test, and review national and international plant test requirements. They recommended the following areas of research: 1) comparative toxicology studies to permit accurate extrapolation from test species to the broad spectrum of different plant taxons present in agro-ecosystems; 2) development of reliable, inexpensive plant reproduction tests; and 3) development of field test protocols and/or monitoring techniques to ensure that the newly registered pesticides behave in the environment as predicted, and do not jeopardize crop yields or the welfare of native plant communities and dependent wildlife.

[1]A proposal not adopted by the PMRA

During the same year, OPPTS/EPA entered into a dialogue with the American Crop Protection Association (ACPA) to discuss the reasons for study rejections and published a document entitled "Pesticide Reregistration Rejection Rate Analysis, Ecological Effects" which analysed reasons for study rejection (US EPA 1994). Some of the non-target plant issues addressed in this document included: 1) acceptable plant germination rates; 2) use of pesticides other than the test chemical, including seed treatments; 3) test duration for *Lemna sp.* and algae; 4) use of TEP instead of technical grade active ingredient (TGAI); 5) proper watering methods for terrestrial plants; 6) plant pot densities; 7) geometric progression of test dosages; 8) numbers of aquatic plants tested; 9) use of OECD, Toxic Substances Control Act (TSCA) or the proposed Canadian guidelines in place of Subdivision J; 10) how to address failure to find a NOAEC value; and 11) degradation of the test substance during the study.

In 1996, the EPA completed a six-year ecological effects test guidelines harmonization project that produced agreement between the EPA Office of Pollution Prevention and Toxics (OPPT) and OPP. Under the Toxic Substances Control Act, the OPPT assesses the toxicity posed by the production/manufacture, use and disposal of industrial chemicals to aquatic and terrestrial organisms found in the environment. OPPT has a four tiered testing scheme which includes test guidelines that are unique, such as seed germination/root elongation toxicity test, early seedling growth toxicity test, *Rhizobium* legume toxicity, plant uptake and translocation test, and soil microbial community toxicity test. In their testing scheme, OPPT typically conducts a deterministic screening level aquatic or terrestrial risk assessment. Depending on the quality and quantity of the data, uncertainty factors ranging from 10 to 1,000 are used in their risk assessments (Smrchek and Morecock 1999). Toxicity data received for new (pre- manufacturing Notice, PMN) chemicals are often lacking, or are limited to microalgae studies, while existing chemicals (eg. metals and persistent, bioaccumulative organic chemicals such as PCB's and dioxins) often have more extensive plant toxicity data bases. In contrast to OPP and PMRA, OPPT does not routinely conduct acute risk assessments for aquatic and terrestrial vascular plants, but they do have the authority to request studies if necessary (Smrchek and Zeeman 1998).

After OPPT and OPP drafted their harmonized guidelines, they briefed the FIFRA SAP, published the draft guidelines in the Federal Register and solicited public comments in 1996 (US EPA 1996c). The non-target aquatic and terrestrial plant toxicity guidelines were included in Group D (850.4000-850.4800) and Group E (850.5100-850.5400).

Group D
850.4000 - Background - Non-target Plant Testing
850.4025 - Target Area Phytotoxicity
850.4100 - Terrestrial Plant Toxicity, Tier I (seedling emergence)
850.4150 - Terrestrial Plant Toxicity, Tier I (vegetative vigor)
850.4200 - Seed Germination/root elongation toxicity test
850.4225 - Seedling Emergence, Tier II
850.4230 - Early Seedling Growth Toxicity Test
850.4250 - Vegetative Vigor, Tier II

850.4300 - Terrestrial Plants Field Study, Tier III
850.4400 - Aquatic Plant Toxicity Test Using *Lemna sp.*, Tiers I and II
850.4450 - Aquatic Plants Field Study, Tier III
850.4600 - *Rhizobium*- legume Toxicity
850.4800 - Plant Uptake and Translocation Test

Group E
850.5100 - Soil Microbial Community Toxicity Test
850.5400 - Algal Toxicity, Tiers I and II

When the SAP was asked to comment on refined field tests to assess yield and reproductive effects, they stated: "The endpoints desired from Tier III field studies such as reproductive endpoints are critical to performing an accurate risk assessment. Two-week laboratory tests addressing vegetative growth do not necessarily reflect what happens to a plant in the long run. Life cycle and field testing will eliminate that vacuum of knowledge. Such studies are conducted under actual environmental conditions and can be useful in determining the actual ecological significance of a laboratory derived EC value. However, there are insufficient guidelines to conduct these assessments. The need for these procedures has been emphasized repeatedly at previous SAP-FIFRA meetings in addition to scientific meetings. The Agency needs to promote the development of these procedures through ORD or other avenues."

During the period of time that OPPT and OPP were finalizing their 850 series guidelines, PMRA/Canada was in the process of revising its proposed guidelines. Shortly after OPPTS' harmonized draft guidelines were published, PMRA agreed to harmonize its guidelines with OPPTS/EPA, and the two countries formed a non-target plant working group. In addition to these NAFTA harmonization efforts, both countries have participated in development of OECD (Organisation for Economic Cooperation and Development) guidance documents. At the present time, a draft OECD new test guideline for testing floating macrophytes *Lemna* spp. is near finalization, and OECD is working to update the microalgae toxicity test (OECD 201) and the terrestrial plant toxicity (OECD 208) guidelines. OPPTS/EPA has also participated in the development of several ASTM (American Society for Testing and Materials) guidance documents for non-target plant toxicity testing.

In 1999, the OPPTS sponsored an international workshop titled "Impacts Of Low-dose, High Toxicity Herbicides on Unintended Plant Species". This workshop, which was attended by 40 scientists from academia, government, industry, and ecological groups, provided a forum for discussion and analysis of public concerns regarding low dosage, high toxicity herbicides. During the workshop, five research papers were presented for discussion. A detailed outline of the workshop is provided in Appendix 1. In brief, issues which were identified by the workshop participants included the following:

- Alleged incidents of low-dose, high potency herbicide drift from application sites to distant non-target sites are of concern. Although analytical methods have been developed to

detect low-dose herbicides in food, they are not readily available for widespread application.

- The current plant species used for testing low-dose, high potency herbicides do not represent the key plant species found in eco-regions within Canada, the US, and Mexico.
- Assessing pesticide risk requires an improved understanding of the following data uncertainties: 1) adequacy of current test species to represent non-tested plant families and species; 2) predictive value of adverse effects on early plant growth (first 14-21 days) for extrapolation to adverse effects on reproduction and yield; 3) predictive value of adverse effects on plant growth observed in the greenhouse for extrapolation to adverse effects in the field; and 4) relationship of single exposures to multiple exposures.
- Factors responsible for low-dose, high potency herbicide movement at great distances from the target site of application are not well understood and must be identified and evaluated. The mechanisms responsible for movement of low-volatile pesticides many miles up wind or down wind from the treatment sites are, in some cases, not determined (Felsot 1996b).

Since the last SAP meeting, OPPTS/EPA and PMRA/Canada have made significant progress reviewing and incorporating the extensive comments received from the SAP, the pesticide industry, testing laboratories, the ILSI and ORD workshop participants, and other public sources. The proposed guidelines also include agreements reached in 1994 regarding the study rejection rate analysis. Some areas which were revised as a result of this analysis include: 1) reduction in the required algal test period from 5 to 4 days; 2) reduction in length of the *Lemna gibba* test from 14 days to 7 days; 3) addition of specific minimum germination percentages for terrestrial species; and 4) flexibility regarding terrestrial test plants to allow substitution of non-crop plants for some crop plants.

1.3 Environmental Monitoring Incidents

During the last decade, the Agency has received numerous incident reports concerning adverse effects to non-target plants from the use of pesticides. Studies in the scientific literature have shown that widespread use of pesticides are associated with damage to non-target terrestrial plants (Pimentel and Levitan 1986). Immediate adverse effects resulting from exposure to pesticides can range from plant death to reduction in biomass without recovery, to loss of biomass with recovery, to stimulation or excessive plant growth. Although effects can appear to be visibly minor, exposure at a sensitive growth stage may result in reduced ability to germinate, produce pollen, produce flowers, produce normal seed set, and ultimately result in reduced reproduction and survival capability. While some pesticides can cause severely acute visual damage to plant tissue (chlorophyll bleached), others may cause subtle adverse effects not readily visible to the naked eye. In many cases, a highly trained specialist is required to determine if the plant injury is caused by a phytotoxicant, lack of nutrients, poor soils or drainage, insect or fungal damage, or some other cause.

With improvements in analytical methodology and improved residue detection in various media, the Agency has received an increased number of reports documenting off-target movement of chemicals. Reports that document close range visual injury to adjacent plants of economic value are more numerous than those documenting injury to plants of value to fish and wildlife. Long-range residue transport has been well documented and residues have been found in remote areas thousands of miles from the treatment site. Residues can be transported atmospherically via rainfall, soil particles, fog, and mist. The chronic effects of long-range chemical transport (from the U.S. corn belt to the Arctic Circle via fog, for example) on plant growth and survival have not been studied to any great extent.

Many incidents are reported to the EPA from other Agencies such as the United States Geological Survey (USGS) and from states, industry, private citizens, and EPA regions under the agency's reporting provisions. Most of the FIFRA 6(a)(2) incidents reported to EPA provide few details on the incident, including final disposition of the complaints and can only be characterized as alleged. Nonetheless, alleged incidents do serve to indicate that incidents are occurring and provide some knowledge of potential trends. The number of alleged non-target plant incidents reported to EPA is considered to be grossly under-reported according to the literature, university agricultural extension websites, and data from the American Association of Pest Control Officials (AAPCO). For more information on environmental monitoring and incidents, refer to Appendix 16.

1.4 Update of Events Since the Last SAP Meetings

1.4.1 FIFRA 6(a)(2) Incident Reporting

In 1997, OPP strengthened its requirement to report pesticide adverse effects data under section 6(a)(2) of FIFRA. These incident reports include post registration field incidents, literature, or studies that demonstrate adverse acute effects to humans, domestic animals, wildlife, and plants. To date, OPP has received 1010 6(a)(2) reports of plant injury. This number, though, is probably a gross underestimation of the actual occurrence. Of the total reports, two-thirds did not identify specific non-target plants which were injured. Of the remaining one-third, trees were damaged the most (32%), followed by field crops (29%), vegetables (12%) and ornamentals (11%). Injury to gardens, berries, fruit vines, and turf made up the rest of the reports.

In general, OPP has found these incident reports to be extremely limited, and has requested that they include species and number of individuals per species affected, symptoms or adverse effects and severity of adverse effects, magnitude of effects (area), pesticide dosage per acre, laboratory analysis of samples, circumstances and description of habitat, distance from treatment, and name of the pesticide product with the EPA product registration number. While some of these reports can provide useful insight into the extent of adverse acute effects to certain non-target plants, they are only useful for chemicals that cause highly visible damage to leaf tissue, such as chlorophyll bleachers. Herbicides that are slow-acting, with less obvious visible injury may go unreported. If incidents with herbicides lack analytical methodology to measure the low levels that cause plant

damage or if the levels are below the food tolerance, no confirmatory analysis is performed and enforcement action is not reported or taken. Reproductive endpoints, such as effects on flowering or seed production are also not currently reported. Another weakness of 6(a)(2) data is the lack of information on adverse effects to non-target plants which have little or no economic value. Most 6(a)(2) reports in agricultural settings are initiated by growers seeking compensation for damages to nearby economic crops. More than 60% of incident reports contain no identification of the plant or plants injured, nor do they contain site maps or descriptions of the total area impacted. Most contain no assessment of damages beyond the economic crop of concern.

1.4.2 Recent Non-target Plant Field and Monitoring Studies under FIFRA

Under FIFRA, the Office of Pesticide Programs (OPP) can require field testing and monitoring for problematic pesticides. Some of the pesticides which OPP has received numerous complaints of non-target plant damage include the following herbicides: phenoxy herbicides, glyphosate, clomazone, ALS inhibiting herbicides, isoxaflutole, quinclorac and the following fungicides: benomyl, and azoxystroben. With the exception of benomyl and isoxaflutole, most of these complaints have involved long-range transport of residues many miles distant from the point of application. In the cases of ALS inhibitors and quinclorac, co-distillation and evapotranspiration are possible routes of dissipation post-application (Falsot, *et al.* 1996b and Bansal, *et al.* 1999).

Since 1996, OPP has required Level III aquatic and terrestrial field testing and monitoring for only one herbicide, isoxaflutole, following widespread complaints of adverse effects on plants. Edge of field studies and crop yield trials were conducted, using outdoor tubs to assess low level impacts on aquatic macrophytes . The edge of field studies assessed low level effects of isoxaflutole on native vegetation downwind, and downslope from the treated field. Although no adverse effects to aquatic macrophytes were observed at 10X the maximum expected environmental concentration (EEC), these field tests showed a significant reduction in cotton yield when only 1% of the registered label dosage was used. The edge of field studies are still in review (Davy 2001).

In 1998, the registrant voluntarily conducted field monitoring studies to assess long-range transport of quinclorac herbicide residues and their impact on tomato plants up to five miles from the application site. Monitoring included the use of tomato bioassay plants and high volume air samplers. Adverse impacts on tomato plant reproduction and survival were observed as far as five miles from the application site. Quinclorac residues were collected in high-volume air samplers at different locations up to five miles from the application site (Bansal *et al.* 1999).

Azoxystroben and benomyl fungicides have been studied extensively and voluntarily monitored for phytotoxic effects following numerous reports of plant injury post-registration. Azoxystroben was injurious to certain apple varieties if drift were to occur from treated vineyards. A registration requirements to utilize a separate sprayer for azoxystroben on vineyards was initiated due to the inability to properly rinse residues from the spray tank prior to use on apples. More

than 1800 reports of benomyl phytotoxicity to commercial ornamentals, hydroponic tomatoes, and field crops were received by the Agency following the introduction and use of the DF formulation (Davy 2001). The benomyl registrant conducted millions of dollars additional greenhouse and field research, extensive interviews, and grower settlements in their attempt to resolve the allegations.

1.4.3 Interactions with OECD and ASTM

Since the 1994 and 1996 SAP meetings, OECD has updated some of their plant toxicity protocols. The OECD is updating the OECD 201 algal toxicity test guidance to include blue-green cyanobacteria, a freshwater diatom, and a marine microalgae, and is developing a new guideline (OECD 202) for testing a floating aquatic macrophyte (*Lemna sp*.). The OECD is also updating the 208 terrestrial plant toxicity test guidance document. This effort has included an assessment of all currently tested species listed in terrestrial plant test guidance documents, but it has not been expanded to include non-crop species and woody plants or assessment of reproductive endpoints.

In 1998, the ASTM published a terrestrial plant test guidance document entitled, "Standard Guide for Conducting Terrestrial Plant Toxicity Tests", designation E 1963-98. This document provides a list of plant test species identified in regulatory documents, and another list of plants from the literature that have been tested for toxic effects. This ASTM document provides guidance for the following tests: seedling emergence, root elongation, *Brassica* life cycle, and woody plant species growth.

The OPPT has participated in the development of the following American Society for Testing and Materials (ASTM) guidance documents:

- Early seedling growth toxicity test, approved in 1994,
- Aquatic freshwater microcosm test, approved in 1996,
- Freshwater emergent macrophyte test, approved 1996,
- 14 day submersed macrophyte using *Myriophyllum sibiricum*, approved in 1997,
- 96 hour microalgae toxicity test, approved in 1997,
- Bioluminescent dinoflagellate test, approved in 1997,
- Algal growth test for *Selenastrum capricornutum*, re-approved in 1998,
- *Lemna gibba* static toxicity test, re-approved in 1998,
- Seaweeds sexual reproduction test, re-approved in 1998,
- Terrestrial plant toxicity test, approved in 1998

1.4.4 EPA/ORD Research in Support of Non-target Plants

While specific research goals were identified in the 1994 and 1996 SAP reports, the Office of Research and Development (ORD) has not incorporated these goals into the Agency's research budget. Since the last SAP meetings, though, OPPTS has increased its dialogue with ORD and has briefed them on its non-target plant toxicity research needs (Smrchek 1999, Petrie 1999). To date, ORD has developed test methods, such as the first *Lemna* and *Arabadopsis* life cycle tests and has participated in OECD round-robin testing of proposed guidelines. The ORD Corvallis Western Ecology Division (WED) laboratory has conducted comparative toxicity laboratory and field studies for herbicide effects on annual and woody plants. The EPA/ORD/WED laboratory and the EPA/Duluth laboratory have studied short- and long-range transport of toxicants, such as ozone and acid rain, and potential impacts of their deposition on sensitive plants, including endangered and forestry species. A long-range transport model was developed by EPA/Duluth and has been used to model atrazine herbicide transport. These studies and models will help OPPTS and PMRA improve their estimations of atmospheric transport of chemicals.

1.5 Rationale for Expansion of Testing

1.5.1 Valued Plant Resources

According to the most recent 1997 EPA estimates, approximately 4.6 billion pounds of pesticide active ingredients are used in the United States each year. Of this total amount, approximately 580 million pounds of herbicides are used, with 83% used in agriculture. Greater than 90% of all corn and soybean acreage in the U.S. are treated with one or more herbicides annually (Aspelin and Grube, 1999).

Plants are the immediate and ultimate source of all food and most shelter used by wildlife (Martin 1951). Groups of plants or plant communities provide habitats essential for productive fish and wildlife populations. In any healthy ecosystem, plants are the primary producers of energy for food chains in ecosystems and determine the amount of living mass (other organisms) that an ecosytem can support. Humans rely on plants for oxygen, food, fiber, shelter, erosion prevention, flood control, paper products, medicines, latex, waxes, essential oils, perfumes, spices, and for their aesthetic, recreational, and therapeutic value. Plants have high aesthetic value, remove pollutants from the air, and serve as buffers for stream pollutants (Hartman *et al.* 1981).

Pesticides, by design, alter the agro-ecosystems in which they are used, and thus have a high potential for impacting individual non-target plants, plant communities, and ecosystem function and structure. Depending on the mode of action and spectrum of pest control, toxicants can cause immediate visible damage to plants within hours, days, or weeks following exposure. Furthermore, the build up of persistent pesticides in sediments and/or organic matter will also eventually affect plants. The value of damaged plant resources to fish and wildlife often goes unnoticed and unaccounted. For example, a 5% (or even 1%) reduction in crop yield may be significant to the owner of a high value crop. However, a 5% reduction in biomass or

productivity of an adjacent hedgerow or estuary would probably go unnoticed. The impact of losing 5% productivity in non-crop areas may or may not be significant to animals that use the resource for food and shelter. The percentage of defoliation that plants may undergo in order to be considered significant for wildlife is uncertain. Valued resources can be individual species such as endangered/threatened plants or multiple species that provide function and structure. For more information on valued plant resources, refer to Appendix 17.

1.5.2 Reexamination of Non-target Plant Toxicity Tests

Several efforts which the Agency initiated to improve its ecological risk assessments led OPPTS/EPA to reexamine its non-target plant toxicity tests and risk assessments. One of the first efforts was the publication of "EPA's Framework For Ecological Risk Assessment" (1992) which required risk assessors and risk managers to identify valued resources and to assess impacts of stressors on multiple species. Other efforts which stimulated OPPTS to reexamine its testing programs included the emphasis on harmonization of data requirements with Canada and Mexico under NAFTA, the Agency's progression to higher tiered risk assessments (probabilistic risk assessments), the numerous adverse effects incidents for non-target plants, and improved analytical methodologies to detect low levels of chemical residues in air, water and soil.

With the publication of the "EPA Framework for Ecological Risk Assessment" (1992), OPPTS/EPA was directed to incorporate risk characterization into its assessment of ecological risk. The risk characterization phase described the likelihood of adverse effects on multiple species as a result of exposure to a stressor. Implementing this new paradigm required the Agency to examine the adequacy of its testing requirements to determine if the current testing scheme could support the risk characterization requirement. After reviewing its plant toxicity data requirements, OPPTS realized that additional plant toxicity studies were needed in order to adequately characterize the risk to all plants and to reduce the uncertainty in its risk assessments.

While the primary focus of non-target plant risk assessments in OPP/OPPTS has been on adverse effects to plants of economic value (e.g. corn, soybeans, and high value ornamental and nursery plants), the program is also responsible for protection of other valued plant resources. Under the adverse effects or 6(a)(2) provisions of FIFRA, OPP has received an increasing number of complaints associated with off-target plant damage. Responding to citizen complaints and Congressional inquiries regarding obvious, observable pesticide drift damage to crops and ornamental plants has demanded a large amount of scientific and risk management resources at regional offices, research laboratories, and EPA Headquarters. Many state and EPA regional offices do not have resources to conduct expensive residue analyses and/or collect yield data to document and analyse these numerous incidents. While minimal resources have been devoted to documentation of adverse effects of off-target pesticide movement on plants of little or no direct economic benefit, the large number of incident reports and grower complaints confirm that these plants are routinely exposed. A broader test species screen plus an understanding of the impacts of pesticides on the reproductive cycle of plants are needed to effectively respond to these

numerous complaints and incident reports and to better predict and mitigate risks to non-target plants. (Refer to Appendix 16 for more detailed information).

Harmonization efforts have also stimulated OPPTS to reexamine its plant toxicity tests. Under NAFTA, the U.S. and Canada agreed to harmonize their testing requirements and to share resources in reviewing test data. The Canada/U.S. Nontarget Plant Harmonization Workgroup reviewed Canada's "Proposed Guidelines for Nontarget Plant Testing and Evaluation" (Technical Report #145), "EPA's Framework for Ecological Risk Assessment", OPP's "Plant Toxicity Guidelines," the proceedings from the ILSI and ORD workshops on nontarget plants, previous plant and probabilistic risk assessment SAP comments, and the scientific literature. After extensive review and discussion, the U.S./Canada Workgroup concluded that the current testing schemes should be expanded and improved to allow the two agencies to better characterize risk to habitats, communities, and ecosystems and to reduce uncertainty in their risk assessments. Canada and the U.S. are also participating in harmonization efforts with OECD which will further strengthen the testing requirements, reduce resources needed for reviewing studies, and promote agreement on test methods among scientists worldwide.

Based on the recommendations of the SAP, OPP developed a four-tiered testing scheme for ecological risk assessment (probabilistic) which would allow the program to estimate the probability and uncertainties associated with exposure to a stressor. In this four-tiered system, Level I is a deterministic assessment, while Levels II, III, and IV are probabilistic assessments, representing higher levels of refinement. In Levels III and IV, multiple species testing and key species in an ecoregion are needed in order to fully understand impacts on nontarget species, including endangered/threatened plant species in use areas.

1.6 Limitations of the Current Testing Scheme

For twenty years, EPA has used a deterministic approach for assessing risk to non-target species. These assessments used gross estimates of hazard based on a pesticide's toxicity to the most sensitive tested species and predicted exposure concentrations. In a deterministic assessment, the risk assessor calculates a Risk Quotient (RQ) to determine whether a Level of Concern (LOC) has been exceeded. The LOC is a distinct level of concern that, if exceeded, triggers risk reduction measures and/or restrictions on use, and/or further testing in the field to refute the LOC estimate. Deterministic risk assessments for plants have many uncertainties, some of which are unique to plants, and some of which are also found in animal toxicity testing.

The following uncertainties have been identified in OPP's current plant toxicity testing:

- the use of crop plants as surrogates for non-crop or native plant species;
- the use of annual plants as surrogates for perennial or woody plants;
- the use of terrestrial vascular plants as surrogates for emergent rooted aquatic vascular plants;
- the use of one taxonomic group of plants to represent another group;

- the use of monocot macrophytes as surrogates for dicots;
- the relationship of early growth toxicity to reproduction and survival;
- the most sensitive plant stage(s) of growth;
- whether the choice of endpoints is dependent on the chemical mode of action;
- extrapolation of laboratory results to field conditions;
- the appropriate species for use in modelling and monitoring.

There are also various uncertainties related to exposure, for example:

- synergistic or antagonistic effects (active ingredients with formulation inerts, tank mix interactions, degradates, other pollutants such as ozone, pesticide interactions);
- mechanisms of long range transport (vapors, soil particles, co-distillation, transpiration, fog - dew - rainfall transport and deposition);
- persistence and degradation of phytotoxicants and metabolites on plant surfaces and cycling within ecosystems;

1.7 Proposed Testing Scheme

A tiered progression system, or an incremental refinement approach, is proposed for the harmonized PMRA/EPA non-target plant testing guidelines. As with the existing EPA guidelines and the draft Canadian guidelines for registration of chemical pesticides, each tier or level of progression requires a more refined assessment of hazard and exposure. The progression system is aimed at minimizing the cost of pesticide toxicity testing by avoiding generation of unnecessary data. With this system, a maximum of four testing levels is proposed for pesticides and other chemicals, allowing the regulatory agencies to focus on species, communities and ecosystems at greatest risk. Testing at higher levels is determined by estimated exposure and the extent of phytotoxic effects observed at lower levels. A set of representative plants is initially tested at the lowest level (Level I), and progression to higher levels occurs only when toxicity is a concern at a lower level. In this testing scheme, all pesticides and chemicals are tested for phytotoxicity when there is a potential for exposure to non-target plants. An overview of the levels of progression for phytotoxicity testing is illustrated in Appendix 3 of this document.

Level I is a "Deterministic Assessment" which includes acute and chronic tests and which determines if a pesticide or chemical is phytotoxic. If phytotoxicity is not observed at the Level of Concern (LOC), then no further testing is required. In OPP's assessments, a Risk Quotient (RQ) is calculated by dividing the Estimated Environmental Concentration (EEC) by the acute value (EC_x) and then comparing it to an established Level of Concern (LOC). Currently, the LOC for non-target plants is 1.0 or greater. If the risk quotient is less than 1.0, the LOC is not exceeded and the risk is considered acceptable. An LOC value of 1.0 or greater is expected to pose a potentially unacceptable risk which may require risk reduction measures. An EEC value may be generated by using a standard scenario, e.g., a 1-ha pond of 15-cm depth, or using a generic model, e.g., GENEEC. The EC_{25} value is currently used by OPP for non-target terrestrial plants, the EC_{50} value for non-target aquatic plants, and the NOAEC or EC_{05} value for

endangered/threatened plants. The PMRA also uses the EC_{25} value for terrestrial plants; however, for aquatic plants PMRA uses the NOAEC value.

If the LOC is exceeded at Level I, OPP initiates a Level II or a "Refined Assessment." The assessment at Level II compares the generic exposure distribution of dose-response data from plant groups shown to be sensitive at Level I and focuses on parameters that contribute most to variability and uncertainty. At this level, uncertainty factors may be required to estimate species sensitivity differences. The Level II assessment includes a refined exposure assessment using predictive models, such as PRZM/EXAMS, and may require additional definitive tests if necessary to perform a preliminary probabilistic risk assessment. At the present time, OPP is formulating the process for incorporating probabilistic risk assessment into its risk assessment for aquatic and terrestrial animals.

Level III testing or "Expanded Assessment" includes acute and chronic tests on keystone or ecologically significant species or groups and/or tests with region-specific (ecoregion) species or groups. The intent of Level III is to characterize the variability and decrease the uncertainty for scenarios of concern.

Level IV or "Comprehensive Assessment" focuses on specific taxa and use scenarios to confirm Level II and III estimates and includes multispecies testing (e.g., microcosm, mesocosm and field testing) and/or monitoring for toxicity. Level IV may be triggered not only by an identified risk following Level III testing, but also following Level I or Level II.

The amount of refinement needed at each level will depend on exposure and effects parameters, such as: 1) the extent of use; 2) the nature of the toxic effect; 3) the likelihood that a toxic effect will occur; 4) uncertainty surrounding variables that affect the risk assessment; 5) the numbers and types of organisms at risk; 6) the ability to detect the pesticide at plant effect levels; 7) persistence in soil or water; 8) the presence of toxic degradates; and 9) any incident or litigation reports.

A description of the specific levels, triggers and species recommended for testing aquatic and terrestrial plants at each level are provided in Sections 2 and 3, respectively.

1.8 Concerns with Existing Plant Protocols and Tests

1.8.1 Aquatic Plant Protocols and Tests

After the SAP endorsed the testing of all pesticides for phytotoxicity in 1994, the PMRA/EPA proposed that all outdoor use pesticides undergo a Tier I aquatic plant test screen for phytotoxicity. The PMRA/EPA NAFTA workgroup identified the following gaps in the existing aquatic plant tests and protocols:

- Algal species phyla currently used for testing do not represent the highly diverse plant types that exist in the environment. Only four phylogenetic classes of algae are tested. Each phylogenetic class contains hundreds of alga species.

- Aquatic macrophytes are seriously under represented. Of the many types of aquatic macrophytes (submerged, floating, emerged rooted), only one floating macrophyte, *Lemna* sp., is tested.
- According to studies in the scientific literature, foliar applications of phytotoxicants to floating and emerged aquatic macrophytes needs be tested. Vegetative vigor testing (foliar applications) are currently required for terrestrial vascular plants using the TEP.
- Differences in sensitivity to toxicants between algae and vascular plants (submersed and emersed species) are so large that algal toxicity testing should not be used as a surrogate for testing vascular plants or vice versa (Fletcher *et al.* 1990, Freemark *et al.*, 1990, Peterson *et al.* 1994).
- All potential routes of toxicant exposure in aquatic plants are not accounted for in the current testing guidelines. For example, exposure from the air-water interface vs the water column vs the sediment.
- No sexual reproduction tests are currently required for aquatic plants.
- The use of terrestrial vascular plants as surrogates for emersed rooted aquatic vascular plants is uncertain.
- The relevance of biochemical and/or physiological sub-lethal effects has not been examined.

Since 1982, a number of test methods have been developed by the ASTM, the EPA Office of Water, and independent researchers for marine dinoflagellates and aquatic macrophytes. If a broader initial species screen were used it would reduce the existing high level of scientific uncertainty associated with species under-representation and allow for a more meaningful risk assessment. In this document, the PMRA/EPA are proposing to expand the number of tested species from five to eleven and include foliar spray applications for selected vascular plants to simulate spray drift. The proposed aquatic toxicity testing will include the addition of three microalgae, one submersed vascular plant, and two emergent vascular plants. (Refer to Section 2 for a more detailed description of this proposed testing scheme).

1.8.2 Terrestrial Plant Tests and Protocols

In 1993, the Canadian Wildlife Service, Environment Canada, published "Proposed Guidelines For Registration Of Chemical Pesticides: Non-target Plant Testing and Evaluation", Technical Report Series No. 145. These proposed guidelines were different from the existing OPP terrestrial plant test guidelines because they expanded the number of test species to include non-crop plants. For known phytotoxicants such as herbicides, up to 30 individual plant species were required to be tested from a list of species ecologically significant.

The PMRA/EPA NAFTA project has identified significant gaps in the existing terrestrial plant tests and protocols, similar to those identified above, which result in a high level of uncertainty in terrestrial assessments.

- Currently 10 terrestrial crop plant species are tested and used to represent the highly diverse terrestrial plant species (>30,000) that exist in the environment.
- Crop plants serve as surrogates for non-crop or native plant species.
- Annual plants serve as surrogates for perennial or woody plants.
- Early growth toxicity serves to predict reproduction and survival.
- Laboratory results are extrapolated to field conditions.
- The most sensitive plant stage(s) of growth is tested.
- The measurement endpoints do not consider the mode of action.
- The relevance of biochemical and/or physiological sub-lethal effects is unknown.

In the proposed terrestrial toxicity testing scheme, PMRA and EPA are proposing to expand the number of tested species from ten to twenty-six and to add reproductive tests. The new harmonized testing scheme would include the addition of one monocot family, eight dicot families, four woody plants, one life-cycle test and one partial life-cycle test. (Refer to Section 3 for more detailed information on the terrestrial toxicity testing scheme).

1.8.3 Proposal For Additional Plant Tests

Currently, only 5 aquatic plants and 10 terrestrial crop plants are tested under OPP's non-target plant toxicity guidelines. These limited number of species serve as surrogates for all the plants in the United States and Canada. In a deterministic risk assessment, the most sensitive aquatic and terrestrial plant species are used to determine risk to all plants. With this approach, there are concerns that the most sensitive species may not be assessed or that the use of the most sensitive species may result in an overly conservative assessment.

For the Aquatic toxicity testing scheme, EPA and PMRA are proposing to expand the number of tested species from five to eleven and are recommending foliar spray applications for selected vascular plants to simulate spray drift. The proposed aquatic toxicity testing will include the addition of three microalgae, one submersed vascular plant, and two emergent vascular plants. For the terrestrial toxicity testing scheme, EPA and PMRA are proposing to expand the number of tested species from ten to twenty-six and to add reproductive tests. At the present time, EPA only requires testing on ten annual crop plants which represent two monocot families and four dicot families. In the new harmonized testing scheme, EPA and PMRA have proposed the addition of one monocot family, eight dicot families, four woody plants, one-life cycle test (reproductive test), and one partial life-cycle test. For more information, refer to Table 1 on p. 31 and Table 4 on p.62.

A review of the literature indicates that the current aquatic and terrestrial test species commonly used in plant toxicity tests cannot reliably serve as surrogates for untested plants. Swanson *et al.* (1991) assessed test data for microalgae, macroalgae, and macrophytes and concluded the following: 1) current microalgae testing does not include many untested phyla; 2) microalgae cannot serve as surrogates for aquatic vascular macrophytes; and 3) marine species are undertested. The authors concluded that an adequate test battery must include a test plant

representative from each phyla in order to reduce uncertainty associated with broad sensitivity ranges resulting from exposure to various toxicants.

Recently, Lytle and Lytle, 2001 reviewed the use of vascular plants for toxicity assessment of estuarine ecosystems by examining the EPA PHYTOTOX and AQUIRE databases (WWW.EPA.GOV/ECOTOX). The PHYTOTOX database contains published articles from the agrochemical industry and government agencies, and also contains the most frequently tested species in chemical application studies. The researchers found limited tests on aquatic plants, and none for marine submersed or emergent plants. Vascular plants have demonstrated greater sensitivity than marine algae in some studies, and rooted estuarine species are more sensitive than *Lemna sp.* in others. The authors concluded that an aquatic plant test battery should include rooted submersed and emersed estuarine and marine vascular plants. Although test protocols for estuarine and marine vascular plants have developed slowly due to difficulties in obtaining reliable seeds for culture and a readily available artificial sediment mixture, it is believed that these problems can be easily resolved. Researchers have already focused on rhizome collection, propagation, and storage techniques.

Regarding terrestrial plants, Fletcher (1991b) concluded from a review of the EPA PHYTOTOX data base that annual crop plants cannot reasonably serve as surrogates for species outside their family and cannot reasonably serve as surrogates for perennial or woody species. Current test species are not inclusive of some major native plants of widespread distribution and economic importance, such as pome and stone fruits, the Fagaceae (oak, beech, chestnut), and the Pinaceae (pine, spruce, fir) (Fletcher 1990). In a separate study, Cole, *et.al*, 1993 analyzed and prioritized crop and non-crop plants useful for regulatory testing and concluded that endangered species plant families should be considered in the testing scheme. They recommended expanding the initial Tier I maximum challenge test to include more untested plants.

In the publication, "Overview And Rationale For Developing Regulatory Guidelines For Nontarget Plant Testing With Chemical Pesticides," Environment Canada recommended the expansion of test species to include plants of importance to wildlife (Boutin *et al.* 1993). In addition, Kapustka *et al.* (1996) conducted a thorough analysis of existing vascular plant toxicity test methods and test species and concluded that the field of plant toxicity testing has lagged behind animal testing due to poor acceptance of methods to assess reproductive endpoints and woody plant species. Potential improvements could be obtained if testing included nonstandard species and a broader array of toxicity endpoints (Wang *et al.* 1997).

Fletcher *et al. (*1990) presented data from the PHYTOTOX data base showing variability among various species to different phytotoxicants. Questions posed by the authors included: "How well do laboratory results reflect the actual field toxicity of chemicals?". The responses of plants treated with the same toxicant in greenhouse vs field settings were examined for 13 plant species. Seventeen chemicals from eleven different classes were included. Analysis of the response ratios of twenty greenhouse vs field comparisons showed that greenhouse-treated plants were more

sensitive than field-treated plants in six cases; in three cases the responses were essentially equal; and in eleven cases the field-treated plants were more sensitive than the greenhouse plants.

Fletcher *et al.* (1990) also analyzed the PHYTOTOX data base for taxonomic differences and variability in plants when exposed to toxicants. Questions posed by the authors included: "Can the results collected from experiments on one species be extrapolated to another?". This effort compared responses of 151 plant species representing 43 plant families to one or more of 16 chemicals representing 11 different classes of chemicals from 230 published papers. This analysis was similar to one performed on fish toxicity data by Suter (1983). Fletcher concluded that the sensitivities of plant species to the same chemicals can be very broad for some chemicals, such as picloram (67X- 316X) or narrow for others, such as linuron (2-3X) with fourteen other chemicals in between. The pooled mean sensitivity ratio (lowest to highest within a chemical) for all chemicals and species studies was 10.5 with a confidence interval of 3.5. Further analysis of EC_{50} values for taxonomic differences among plants indicated that species' responses to herbicides are more similar within the same genera. The author concluded that care must be taken not to extrapolate test results from one species to another unless they belong to the same genus. This study also indicated that taxonomic differences among plants have a much greater influence on plant response to chemical treatment than the test condition (laboratory vs field testing). This analysis supported the need to expand a species screen to include additional plant families and to test native plant species. The author further stated that many species tested in the literature are sensitive native species that could be included in OECD and EPA testing schemes.

Boutin and Rogers (2000) assessed the EPA and PMRA terrestrial plant toxicity data bases for patterns of plant species sensitivity to various herbicides. The EPA Office of Pesticide Programs database contained early plant growth phytotoxicity data (first 14 to 21 days) on seedling emergence and vegetative vigor following dose-response exposure of 89 pesticides (80 of which were herbicides) to annual crop plants. The Canadian database contained efficacy data for 10 herbicides that included weed control and crop phytotoxicity information following exposure at specific label dosages. Boutin and Rogers' assessment was severely limited by the lack of dose response data for weeds tested and the general lack of data for plants other than annual crops and weeds considered of importance to pesticide producers for efficacy purposes. The crop and weed species contained in the data bases reviewed by Boutin and Rogers have adapted to open, frequently disturbed areas, and crops are genetically manipulated to further enhance their survival. The list of tested species did not include herbaceous plants in shaded areas, woody plants, and wetland plants which made it difficult to compare native plants with crops and weeds. Reproductive endpoints were also not measured in these tests. The author's conclusions were hampered by the severely limited databases; however, they did conclude that the more species tested, the broader the observed sensitivities for a given chemical. They further noted the need to include additional plant families that are representative of ecologically relevant native species or groups in the required test battery since it has not been demonstrated that crop species serve as surrogates for non-crop species found in nature.

In conclusion, the PMRA and EPA believe that the proposed plant toxicity testing scheme addresses the majority of the concerns identified in the 1994 and 1996 EPA SAP meetings, concerns identified in the scientific literature, and concerns raised by state and regional offices associated with the increasing number of non-target plant incidents. The proposed testing scheme will help the two agencies conduct more realistic and scientifically sound plant risk assessments, reduce the large uncertainty in the existing assessments, and more effectively mitigate risk to plants.

2 TESTING SCHEME FOR AQUATIC PLANTS

2.1 Introduction

This section focuses in detail on the proposed tiering structure and triggers, toxicity endpoints, recommended testing species, multispecies tests, monitoring, and reproductive tests for aquatic plants. In addition, various testing methods for proposed aquatic species are provided in Appendices 4-11. This section identifies the issues of concern with the current approach to testing aquatic plants and, thus, is aimed at stimulating discussion towards improving the testing system.

2.2 Tiering Structure

A four-level system is proposed for testing the effects of pesticides and chemicals on aquatic plants. Initially, a battery of representative aquatic plants is tested at the lowest level (Level I). Testing at higher levels is determined by the extent of phytotoxic effects observed at the preceding lower level. The extent of phytotoxicity is based on the comparison of a toxicity endpoint to the Estimated Expected Concentration (EEC). Progression to higher levels occurs only for those plant groups which show a significant level of phytotoxicity. An overview of the level progression scheme is illustrated in Appendix 3.

2.2.1 Level I: Deterministic Assessment

Level I or "Deterministic Assessment" is subdivided into Level I-A and Level I-B. Level I-A consists of "Maximum Challenge Tests" and/or "Range Finding Tests". Maximum challenge refers to testing at a single rate or concentration that is equivalent to the maximum expected exposure. For pesticides, the maximum labelled rate is tested In cases where more than one pesticide application per season is required, the frequency of applications and the environmental fate of the pesticide should be considered in determining the appropriate dose. For pesticides, the dose should be representative of the maximum exposure expected under operational uses. Range-finding tests involve more than one dose and are conducted to determine the range of concentrations or application rates to be used in definitive testing. Both tests serve as early screening tools to evaluate phytotoxic potential. The test concentration is calculated on the basis of a 1-ha, 15-cm deep pond that receives the maximum expected dose. This level is equivalent to the Tier I testing under the current EPA Guidelines.

If after Level I-A, the exhibited phytotoxicity is found to be a concern, then Level I-B or "Definitive Testing"is required. Level I-B is multiple concentration/rate testing that provides dose-response information from which toxicity endpoints (e.g., EC_{05}, EC_{25} or EC_{50}) can be estimated. There is the option, however, for initial testing at Level I-B rather than at Level I-A. As in the case for herbicide products where phytotoxicity is expected, testing of non-herbicides or

chemicals may also begin at Level I-B (equivalent to Tier II of current guidelines). The assessment at Level I-B involves the comparison of a point estimate of toxicity to a point estimate of exposure for each species.

2.2.2 Level II: Refined Assessment

The testing requirements and assessment approach at Level II are unclear until further developments are achieved in the EPA probabilistic risk assessment process for aquatic and terrestrial organisms. The assessment process for non-target aquatic plants will involve a refinement of exposure and better utilization of the dose-response data. In some cases, it may be necessary to require additional testing at the beginning of Level II.

Level II is a first-step refinement of the phytotoxic potential identified at Level I. Level II utilizes dose-response data for each species from Level I-B in conjunction with exposure distribution information. This assessment is the basis for determining whether Level III testing is necessary.

2.2.3 Level III: Expanded Assessment

As with Level II, until the EPA probabilistic risk assessment process has been further refined, there are uncertainties in the testing requirements and assessment approach for Level III.

Level III testing may include additional acute or chronic toxicity tests with species shown to be a concern (i.e., potentially at risk) at lower Levels and/or with species that are specific to the area(s) or region(s) where a pesticide would be used or where prolonged exposure to industrial chemicals may occur. For example, if the toxicity to a submersed vascular species was shown to be a concern at Level II, then testing at Level III could focus on region-specific submersed vascular species. The extent of testing at Level III is determined on a case-by-case basis, and species selection and duration of tests (i.e., acute or chronic) would be pesticide/chemical specific.

2.2.4 Level IV: Comprehensive Assessment

Level IV is the final and most comprehensive testing step. Level IV consists of microcosm, mesocosm or field testing as well as post-registration monitoring. As with Level III, the extent and complexity of testing is determined on a case-by-case basis. The aim is to address specific questions or issues raised with a pesticide or chemical for a given aquatic environment (e.g., wetlands, prairie potholes). If mitigative measures do not alleviate the concern or if there is major uncertainty with single species testing, then more ecologically relevant multispecies tests becomes necessary. Level IV may be triggered by the results or assessments from any previous level (see Section 2.3 Level Triggers) and can consist of multispecies testing, monitoring studies, or both.

2.3 Level Triggers

At Level I, a deterministic risk quotient (RQ), which is the ratio of the estimated environmental concentration or rate (EEC or EER) to a critical toxicity endpoint, ($EC_{25,}$ EC_{50} or NOAEC) will

be determined. The Level I 'trigger' or Level of Concern (LOC) is exceeded when the RQ exceeds one and a Level II analysis is warranted.

With progression through higher Levels, not only is there an increase in testing but also the opportunity to refine the exposure scenario by using scenarios which more accurately reflect the area of use and exposure models. With improved toxicity data, there is progression from a maximum EEC/EER at Level I to methods that consider the range of possible exposures at Levels II-IV. Levels II-IV assess the distributions of exposures and effects rather than point estimates. Canada's exposure scenarios are based on a direct over-spray of either a 1-ha, 30-cm deep water body or a 1-ha, 15- cm deep water body using the maximum label dosage.

As the exposure element is beyond the intentions of this proposal, the discussion of triggers essentially deals with the selection of toxicity endpoints. Although the PMRA and EPA agree that the endpoint of choice should reflect the significance of effects on a population or on individual organisms, there is disagreement between both agencies regarding this selection.

Currently 50% or greater inhibitory effect is used by EPA to progress from Level I-A to Level I-B (the current Tier I and Tier II, respectively) for aquatic plants. In the proposed Canadian guidelines for registration of chemical pesticides (Boutin *et al.* 1993), the authors indicated that any phytotoxic effect that is statistically significant relative to the control warrants definitive testing for both aquatic and terrestrial plants. This, therefore, suggests that endpoints could be less than 50% inhibition. In addition, the authors indicate that, if greater than 50% inhibition is not shown to be statistically significant from the control, then Level I-A tests should be repeated with higher replication or proceed directly to Level I-B. The PMRA proposes that any statistically significant effect serves as a trigger for progression to Level 1-B for aquatic plants.

Following Level I-B, the PMRA uses the NOAEC value to determine the risk. Use of the NOAEC value to determine toxicity has been subject to criticisms for being poorly defined (Bruce and Versteeg 1992, Kooijman *et al.* 1996). Instead, the use of regression models to determine parametric no-effect concentration (NEC) is advocated. The variability in the NEC, however, is typically quite large and as the inhibition level increases, the variability decreases. The likelihood of obtaining good data is, therefore, greater at the higher inhibition levels. Boutin *et al*. (1995) has suggested the use of the EC_{50} for algae and duckweed and the EC_{25} for other species followed by an uncertainty factor of 10 to address interspecific variation. Blanck (1984) suggests that the use of a small battery of species requires an uncertainty factor of 100. Boutin *et al*. (1995) suggests that the uncertainty factor be lowered to 10 in Canada because of the use of a rather conservative exposure scenario which is a direct overspray of a 15-cm or 30-cm deep pond at the maximum application rate. Neither the PMRA nor EPA /OPP are currently using uncertainty factors but EPA/OPPT does. The PMRA's use of NOAEC for algae and *Lemna* is considered to be similar to an EC_{50} with a uncertainty factor of 10, making the PMRA's assessment similar to that suggested by Boutin *et al*. (1995).

The question, therefore, remains whether to use a statistically-significant inhibition at Level 1-A or set the endpoint to some higher value (e.g., 25% inhibition). One approach is to keep the

endpoint consistent throughout the testing progression. For example, use a 10% inhibition for all species and tests. Another approach is to use 10% inhibition for tests that examine population growth (e.g., cell number, biomass) and 25% inhibition for tests that examine individual growth parameters (e.g., shoot length). A third approach is to use ≥50% inhibition for population growth parameters and 25% inhibition for individual growth parameters. In addition, uncertainty factors could be introduced. For example, an uncertainty of 10 could be used (e.g., (EC_{50}/10 and EC_{25}/10) for progression to Level II.

Appropriate toxicity endpoints for progression between Levels is unresolved and, thus, requires guidance or further research in this area. The following summarizes some possible endpoints used in triggering progression to higher Levels.

For progression from Level I-A to Level I-B:

Option 1 any statistically significant effect relative to the control

Option 2 ≥10% effect

Option 3 ≥10% effect on population growth parameters
≥25% effect on individual growth parameters

Option 4 ≥50% effect on population growth parameters
≥25% effect on individual growth parameters

For progression from Level I-B to Level II:

Option 1 EC_{10} on population growth parameters
EC_{25} on individual growth parameters

Option 2 EC_{50} on population growth parameters
EC_{25} on individual growth parameters

Option 3 EC_{50}/10 on population growth parameters
EC_{25}/10 on individual growth parameters

2.4 Alternative Testing Endpoints

Currently, the testing endpoints of plant toxicity are largely gross acute endpoints. These include: number of individuals; growth rate; biomass measurements such as dry or wet weight of roots, shoots, stems, leaves and nodes; and visual symptoms, e.g., chlorosis, necrosis. Toxicity in plants is first manifested at the biochemical level before effects are evident at the whole-organism level, consequently, biochemical effect parameters can be early warning indicators of environmental stressors. However, they are not considered in regulatory data submissions. These sublethal effects can include inhibition in oxygen evolution and carbon fixation, changes in plant pigments,

carbohydrate content, cytochrome f, ethylene/ethane, oxidative enzyme activity and protein concentration, enzyme levels, changes in antioxidant levels, formation of stress proteins, chlorophyll fluorescence and lipid peroxidation (Sprecher and Netherland 1995, Ramanathan *et al.* 1996, Lewis and Wang 1999, Lytle and Lytle, 2001). These effects are often more sensitive, but their environmental relevance and relationship with gross parameters such as biomass are not known (Lytle and Lytle 2001). Nevertheless, effects on biochemical parameters have been examined in algae. For example, the effects of atrazine and cadmium on carbon, protein, lipid and chlorophyll content in *Selenastrum capricornutum* have been reported (Abou-Waly *et al.* 1991, Thompson and Couture 1991).

Biomass is often used in the estimation of productivity. Another way of measuring productivity is photosynthetic activity. The photosynthetic response is generally measured by carbon uptake or oxygen evolution. Oxygen evolution and consumption in aquatic plants can be measured using an oxygen/ meter or a colorimetric method. The use of ^{14}C is commonly used on plant segments or on whole plants to determine carbon uptake (Lewis and Wang 1999, Lewis 1995). Despite the short test duration, in one case the EC_{50} values for changes in photosynthesis were greater than those based on population growth in standard 3-4 day tests (Turbak *et al.* 1986, Versteeg 1990).

Pigment content has been used to determine physiological status of aquatic plants. One advantage in using pigment is that samples can be frozen and held for months before analysis. The most commonly measured pigments are the chlorophylls. In particular, chlorophyll *a* content has been used to measure biomass and can be used as an indicator of water quality. Reduction in chlorophyll can result in inhibition of carbohydrate synthesis (Lewis and Wang 1999).

Plant enzymes that detoxify the toxicant and whose activity is increased with increased toxicant exposure have possible value as early indicators of physiological status. For example, peroxidase and its isozymes have been used as nonspecific indicators of metabolic shifts that results from toxicant exposure. One of the primary mechanisms of cell injury by xenobiotics is the free radical oxidation of polyunsaturated fatty acids that are abundant in cell membranes. This process is sometimes referred to as lipid peroxidation. Antioxidants such as ascorbic acid and gluatathione act by scavenging free radicals thus reducing cell damage. Increased peroxidase activity and levels of ascorbic acid and gluatathione are believed to protect plant cells from free radical oxidation (Byl and Klaine 1991, Byl *et al.* 1994). Peroxidase activity has been used to evaluate contaminant exposure to aquatic plants. For example, *Spartina alterniflora* showed a significant increase in peroxidase activity when exposed to 9.9 µg/L atrazine (De Souza and Yodh 1997). Similarly, there was a significant increase in peroxidase activity in *Scirpus olneyi* with exposure to metolachlor at a concentration of 9.9 µg/kg in estuarine sediment (Lytle and Lytle 1996).

A novel approach has been developed to detect phytotoxicants to plants by monitoring the CO_2 content of the air stream that has passed through a culture at a constant rate. Hannan (1995) studied the effects of seven sulfonylurea and four triazine herbicides on the freshwater green alga *Chlorella pyrenoidosa*. He found that 10 ppt of bensulfuron methyl, the amount that was leached from an apple leaf where 0.01 µg was applied, could be detected by way of CO_2 uptake by the alga in 15 minutes. This study demonstrated that the gas-exchange method of measuring

microbiological growth rates can be used to detect submicrogram amounts of sulfonylurea and triazine herbicides within minutes to several hours.

These tests are of short-duration and, hence, could facilitate more convenient and cost-effective methods for assessing phytotoxicity. Although these biochemical endpoints have been used as early warning signals, there are few reports of the relevance to growth, survival and reproduction of the whole plant and effects on the plant community (Lewis and Wang 1999). Hence, this is an area that requires guidance and/or further research efforts before these endpoints can be utilized in aquatic assessment methods.

2.5 Species Selection For Aquatic Toxicity Testing - Level I

2.5.1 Rationale

Aquatic plants are important components of aquatic ecosystems for a number of reasons. They contribute to primary productivity, generate oxygen, affect flow patterns (Dennis 1984), provide habitat and food for other organisms (Dewey 1986), stabilize sediment (Lembi and Netherland 1990), are utilized by detritivors (Wallace 1989), are involved in nutrient cycling (Pimentel and Edwards 1982) and improve water quality (Catallo 1993, Hook 1993). The biomass of aquatic vascular plants and algae amount to less than 1% of the total plant biomass on earth, yet it is estimated that the net primary production from aquatic plants amount to 35-50 giga-tons of carbon (giga-ton = 10^{15} g) with terrestrial plant production being only slightly greater at 50-70 giga-tons (Falkowski and Raven 1997). During photosynthesis, carbon dioxide is sequestered and oxygen is released with aquatic and terrestrial plants being responsible for similar rates of initial carbon sequestration and oxygen production. Algae and vascular aquatic plants can also degrade pesticides (O'Kelley and Deason 1976, Boyle 1984) and have been used to remove contaminants from wastewater including nutrients, metals and coliforms (Lavoie and de la Noue 1985, Tripathi and Shukla 1991).

Phytotoxic chemicals entering aquatic environments can trigger a series of effects with far-reaching implications on carbon sequestration, atmospheric oxygen levels, the structure and composition of aquatic habitats, the quality and quantity of available food sources, and the quality of the physico-chemical environment (e.g., reduction in the quantity of oxygen dissolved in the water). The effects generated by pesticides can be stimulatory or inhibitory and both effects can have a detrimental impact (Lewis 1995).

Aquatic plants can exhibit several orders of magnitude more sensitivity to pesticides than aquatic animals (Peterson *et al.* 1997, Roshon *et al.* 1999). Habitat alteration in aquatic systems through adverse effects on plants can ultimately affect non-target animals to a greater extent than that caused by direct toxic effects (Freemark *et al.* 1990, Freemark and Boutin 1994). Huxley (1984), reported that for every plant species that becomes extinct, 10-30 other non-plant organisms may also become extinct. Thus, the primary purpose of generating more data with these groups of organisms (i.e., test more species) is to refine the assessment of toxicity. Currently, the assessment for plants is based on a deterministic approach on the most sensitive species. Obviously, this approach does not account for the range of species sensitivities that are inherent in any aquatic environment.

The relative sensitivities in plants and animals are species and chemical specific and, are thus, unpredictable. The concept, therefore, of utilizing a universal group of sensitive organisms is not realistic (Lewis 1995). For example, Blanck *et al.* (1984), showed that there was a 200-fold difference in the EC_{100} for 13 algal species exposed to disodium hydrogen arsenate. Lewis (1995) indicates that interspecific variation in toxicity of one to two orders of magnitudes are not uncommon in microalgae. Fletcher (1990) found that algae were less sensitive than vascular

aquatic plants with 16 herbicides, more sensitive with10 herbicides, and equally sensitive with seven herbicides. This author suggested that herbicides that affected basic metabolic processes common throughout the plant kingdom are more likely to affect algae, and those that disrupt processes unique to the physiologically more complex vascular aquatic plants may not be as toxic to algae. For example, metals, which typically exert nonspecific toxicity, are frequently more toxic to algae than to vascular aquatic plants (Miller *et al.* 1985). By contrast, submersed vascular plants were shown to be more sensitive to certain pesticides than freshwater or marine algae or *Lemna*, another vascular plant(Swanson *et al.* 1991). For example, the estuarine submersed macrophyte, *Vallisneria americana* was shown to be more sensitive than *Lemna* to certain pesticides and other submersed species such as *Potamogeton perfoliatus, Ruppia maritima, Myriophyllum spicatum*. The estuarine species, *Zannichellia palustris*, was more sensitive to atrazine than *Lemna* (Jones and Winchell 1984, Hughes *et al.* 1988).

In algae, the large variation in toxicity to pesticides can be highlighted by a 77% inhibition in a diatom (*Nitzschia* sp.) by glyphosate at the maximum challenge dose (EEC), while a blue-green algal species (*Microcystis aeruginosa*) was stimulated by 41%. Similar variation in sensitivity to atrazine was demonstrated in *Chlorella pyreniodosa* (green alga) and *Gloecapsa alpicola* (blue-green alga), where the 4-day EC_{50}s were 60 and 5360 µg/L, respectively (Maule and Wright 1984). Large variation may also occur within the same genus. For example, the 4-day EC_{50}s of atrazine in the blue-green algal species, *Anabaena inaequalis* and *Anabaena variabilis* were 30 and 4000 µg/L, respectively (Stratton, 1981).

Sensitivity variation also occurs between distinct taxonomic groups. For example, triasulfuron stimulated carbon uptake in the diatom *Nitzschia* sp. (39%), while the floating aquatic vascular plant *Lemna minor*, was inhibited by 91% (Peterson *et al.*, 1994).

Fairchild *et al.* (1997) conducted a study that specifically compared the relative sensitivity of the green alga, *Selenastrum capricornutum* and *Lemna minor* to16 herbicides (atrazine, metribuzin, simazine, cyanazine, alachlor, metoachlor, chlorsulfuron, metsulfuron, triallate, EPTC, trifluralin, diquat, paraquat, dicamba, bromoxynil and 2,4-D) representing a total of 9 herbicide classes (Appendix 4 and 5). The triazine, sulfonylurea, dinitroaniline and pyridine classes were highly toxic to both species with EC_{50} values ranging from 0.4-198 µg/L. *Lemna minor* was highly sensitive to the sulfonylurea herbicides, chlorosulfuron (EC_{50} = 0.7 µg/L) and metsulfuron (EC_{50} = 0.4 µg/L), whereas *Selenastrum capricornutum* was less sensitive by two orders of magnitude as the EC_{50}s were 135 µg/L (chlorosulfuron) and 190 µg/L (metsulfuron). Similarly, *Lemna* was more sensitive to the pyridines with EC_{50} values of 18 µg/L (diquat) and 51 µg/L (paraquat) compared to *Selenastrum* where the EC_{50}s were 80 µg/L (diquat) and 559 µg/L (paraquat). Dicamba (benzoic acid class) and 2,4-D (phenoxy class) were relatively non-toxic to both species as the EC_{50}s were greater than 35,000 µg/L. This result of low toxicity to both species was expected as these herbicides are auxin mimics intended to control broadleaf weeds. The thiocarbamate class (EPTC and triallate) which is known to inhibit multiple biochemical pathways including synthesis of fatty acids, lipids, proteins, isoprenoids and flavonoids was expected to be toxic to both species. Neither species, however, was shown to be sensitive to EPTC. For triallate, *Selenastrum* was sensitive (EC_{50} = 47 µg/L), whereas *Lemna* was relatively insensitive

(EC_{50} >10,000 µg/L). The authors indicated that their study supports the contention that a suite of plant species must be used to perform an accurate risk assessment as neither *Selenastrum* nor *Lemna* are universally sensitive across or within classes of herbicides. Furthermore, the authors indicated that both species are inadequate for conducting evaluations on herbicide classes that are selective for broadleaf weeds (i.e., dicots) and have recommended the addition of a dicot species such as *Myriophyllum* sp.

Variation in species sensitivity is also documented for rooted aquatic plants. In four submersed species, *Ceratophyllum demersum*, *Myriophyllum heterophyllum*, *Elodea canadensis* and *Najas* sp., the 14-day EC_{50}s (wet weight) were similar (EC_{50} = 14-24 µg/L) for exposure to the triazine herbicides, atrazine and metribuzin; the exception was *Myriophyllum heterophyllum* exposed to atrazine where the EC_{50} was 132 µg/L. By contrast, there were substantial differences in sensitivity for exposure to the acetanilide herbicides, alachlor and metolachlor. For example, in *Ceratophyllum demersum*, *Najas* sp., *Elodea canadensis* and *Myriophyllum heterophyllum*, the 14-day EC_{50}s (wet weight) of metolachlor were 70, 242, 2355 and >3000 µg/L, respectively (Fairchild *et al.* 1998).

The variation in sensitivity, therefore, warrants the testing of sufficient species to facilitate a more realistic assessment of impacts resulting from phytotoxic compounds. As such, both Agencies have recognized the need to expand from the traditional deterministic evaluation by conducting an assessment of the distribution in species sensitivity.

Thus, the number of test species is a critical factor in the assessment. The predictive value of a test battery improves with size. Blanck (1984) stated that if only three species are used, toxicity of a chemical could be underestimated by a factor of 100 (95% confidence level) compared with the most sensitive species. To reduce this factor to 10, a nine species battery is required. Boutin and Rogers (2000), examined the pattern of sensitivity of plant species to various herbicides using industry-sponsored data and data submitted to the EPA. These authors found that the range of species sensitivity increases with an augmentation of numbers of species tested, which suggests that the number of species tested in the current guidelines is insufficient. Furthermore, the authors suggested that an improved database on phytotoxicity is a prerequisite to refine the risk assessment of pesticide effects on non-target plants.

Blanck's (1984) suggestion of an uncertainty factor of 100 was based on conventional pesticides. In general, most of these conventional pesticides have less specific modes of action, while the majority of new pesticides have more specific modes of action. While it may be relatively simple to generate reasonable toxicity data for compounds with less specific modes of action, the increasing development and use of pesticides with higher specificity is making non-target assessments more difficult. These pesticides are designed to inhibit a specific organism while leaving even closely related species unaffected. It is, therefore, not reasonable to assume that for regulatory testing purposes a very small number of testing organisms will be able to precisely identify the environmental hazard posed by a pesticide (Peterson 2001, personal communication with the PMRA).

Swanson *et al.* (1991) assessed the literature to determine variability in species responses to toxicants. The range of responses among different marine algae can be as great as for freshwater species. A seven-fold difference in response to atrazine occurred among 17 different algal species. Four marine species exposed to dieldrin insecticide showed responses ranging over five orders of magnitude. Based on high variability in response among classes of algae, the authors stated that a test battery including species of diatoms, green algae, and dinoflagellates as well as golden-brown algae would be prudent. Swanson *et al.* (1991) identified seven marine algal species from three classes that are suitable for inclusion in a species battery. Several other researchers have indicated that it would be beneficial to implement a test battery utilizing a wide taxonomic range of plants (Swanson *et al.* 1991, Nyholm and Peterson 1997, Peterson *et al.* 1997; Roshon *et al.* 1999).

2.5.2 Overview of Species Testing

Level I is comprised of 14 tests in total that are divided among seven groups of organisms (Groups 1-7). The first four groups (Groups1-4) are aquatic exposure tests in which the organisms are exposed to the substance dissolved in the growth media. The remaining three groups (Groups 5-7) are aerial exposure tests in which the floating or emergent plant species are exposed through overspray of their foliage. Of the aerial exposure tests, Group 7 encompasses reproduction testing.

A total of 11 species are tested and consist of 3 freshwater algae, 4 marine algae, 1 floating macrophyte, 1 submersed macrophyte and 2 emergent macrophytes (Table 1). Protocols are available for all of the proposed tests except sexual reproduction tests for rice and nodding smartweed (Table 2), however standard greenhouse methods can be employed. The rationale for the species selected may be found in the subsequent relevant sections following the tables.

Table 1: Recommended species for Level I testing.

Group		Exposure	Species
1	Freshwater algae	Aquatic	Green algae: *Selenastrum capricornutum* Blue-green algae: *Anabaena flos-aquae* Diatom: N*avicula pelliculosa*
2	Marine algae	Aquatic	Diatom: *Skeletonema costatum* Dinoflagellate: *Gonyaulax polyedra* or *Pyrocystis lunula* Red algae: *Champia parvula* Golden-brown algae: Phaeodactylum tricornutum
3	Floating vascular	Aquatic	*Lemna* sp. (*L. minor* or *L. gibba*)
4	Submersed vascular	Aquatic	*Myriophyllum sibiricum*
5	Floating vascular	Aerial	*Lemna* sp. (*L. minor* or *L. gibba*)
6	Emergent vascular	Aerial	Monocot: *Oryza sativa* Dicot: *Polygonum muhlenbergh*
7	Emergent vascular (reproduction)	Aerial	Monocot:*Oryza sativa* Dicot: *Polygonum muhlenbergh*

Table 2: Protocols for Proposed Test Species.

Species/Test Type	Protocol Available	Protocol Title
Red macro algae (*Champia parvula*) ***Growth***	Yes	"Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms."; EPA/600/4-91/003.
Marine dinoflagellates *(Gonyaulax polyedra* or *Pyrocytis lumula*) ***Growth***	Yes	"Standard Guide for Conducting Toxicity Tests with Bioluminescent Dinoflagellates."; ASTM Annual Book of Standards; Vol. 11.05; In Press.
Marine golden-brown algae (*Phaeodactylum tricornutum*) ***Growth***	Yes	"Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae"; ASTM Annual Book of Standards; Vol. 11.05; E 1218-97a
Lemna sp. - foliar exposure ***Growth***	Yes	"Bioassays with a floating aquatic plant (*Lemna minor*) for effects of sprayed and dissolved glyphosate."; Lockhart *et al*. (1989); Hydrobiologia 188/189: 353-359.
Northern watermilfoil *(Myriophyllum sibiricum*) ***Growth***	Yes	"Standard Guide for Conducting Static, Axenic, 14-Day Phytotoxic Tests in Test Tubes with the Submersed Aquatic Macrophyte, Myriophyllum sibiricum Komarov"; ASTM Annual Book of Standards; E 1913 97; Vol. 11.05.
Rice (*Oryza sativa*) ***Growth***	Yes	"Development of a Plant Bioassay to Assess Toxicity of Chemical Stressors to Emergent Macrophytes."; Powell *et al*. (1996); Environmental Toxicology and Chemistry, Vol. 15, No. 9, pp. 1570-1576. "Standard Guide for Conducting Renewal Phytotoxicity Tests with Freshwater Emergent Macrophytes". ASTM Annual Book of Standards; D1841; Vol. 11.05.
Nodding smartweed (*Polygonum muhlenbergh*) ***Growth***	Yes	"Development of a Plant Bioassay to Assess Toxicity of Chemical Stressors to Emergent Macrophytes."; Powell *et al*. (1996); Environmental Toxicology and Chemistry, Vol. 15, No. 9, pp. 1570-1576. "Standard Guide for Conducting Renewal Phytotoxicity Tests with Freshwater Emergent Macrophytes". ASTM Annual Book of Standards; D1841; Vol. 11.05.
Rice (*Oryza sativa*) ***Sexual reproduction***	No	-
Nodding smartweed (*Polygonum muhlenbergh*) ***Sexual reproduction***	No	-

2.5.3 Freshwater Algae

Algae is an inclusive term used for the large number of photosynthetic organisms of varying form and complexity; the majority of which are truly aquatic (Bold *et al.* 1980, Palmer 1977). Algae are the primary carbon-fixing organisms in aquatic environments (Reynolds 1984, DeLorenzo *et al.* 2001), and play critical roles in nutrient cycling and are sources of food for other organisms (Boutin *et al.* 1995). In large freshwater lakes, algae are greater contributors to primary productivity than aquatic vascular plants. In wetlands, the balance between the growth of algae and aquatic macrophytes has a direct influence on the species composition at higher trophic levels (Boutin *et al.* 1995). Detrimental effects on microalgae may have subsequent impacts on organisms at higher trophic levels (DeLorenzo *et al.* 2001). For example, changes in the phytoplankton species composition can affect the growth of zooplankton grazers (Ahlgren *et al.* 1990).

There are approximately 11,000 species of freshwater algae (Nalewajko and Olaveson 1998). In the US, there are approximately 500 genera (Lewis 1990). The classification of freshwater algae is based upon biochemical characteristics (e.g., type of pigments), cell wall composition and morphological characteristics (e.g., presence and type of flagella) (Nalewajko and Olaveson 1998). The majority of freshwater algae belong to eight classes, including the cyanobacteria (blue-green algae) bacillariophyceae (diatoms) and chlorophyceae (green algae) (Nalewajko and Olaveson 1998). Of these eight, the majority of freshwater phytoplankton is made of blue-green algae, diatoms and green algae (Sze 1986). The presence of algal species in freshwater systems fluctuates throughout the year. Even though more than 200 species of algae may be present on a yearly basis, only 20 to 40 species may be present at any one time with 6-8 of the most abundant species composing approximately 90% of the biomass (Nalewajko and Olaveson 1998).

Algae are useful organisms for toxicity testing because they are small, require little laboratory space, and are easily cultured (Addison and Bardsley 1968). Standardized pesticide bioassays for algae are quick, simple, inexpensive and reproducible (Swanson and Peterson, 1988). Canada and the US currently require the testing of three freshwater species representing a green alga, a blue-green alga and diatom.

2.5.3.1 Green algae (Division Chlorophycophyta)

Ecologically, the green algae (chlorophytes) are important in planktonic, benthic and epiphytic communities (Bold *et al.* 1980, Sze 1986). Toxicity testing with freshwater green algae has been conducted for decades and the methodology is fairly well established. Recommended test methodologies for toxicological testing include a flask method (APHA 1998a, ASTM 1998c, 1997a, Miller *et al.* 1978, OECD 1984, US EPA 1971, US EPA 1996a) and a microplate test (Environment Canada 1992). The preferred test species, *Selenastrum capricornutum*, is the most commonly tested algal species, partially because it is the easiest species to culture and use (Mason 1988). Other acceptable test species include *Scenedesmus subspicatus* and *Chlorella vulgaris* (ASTM 1997a, OECD, 1984). *Chlorella pyrenoidosa* (Kratky and Warren 1971, Thomas *et al.* 1990, Sikka and Pramer 1968), *Chlamydomonas eugametos* (Hess 1980, Loeppky and Tweedy

1969) and *Chlamydomonas reinhardi* (Loeppky and Tweedy 1969) have also been used successfully for toxicity testing and would be acceptable species for regulatory purposes.

2.5.3.2 Blue-green algae or cyanobacteria (Division Cyanochloronta)

The blue-green algae (cyanobacteria) differ morphologically and physiologically from other algae. The blue-greens are prokaryotic organisms that are capable of fixing nitrogen. *Anabaena flos-aquae* is the blue-green alga that is recommended for testing. *Microcystis aeruginosa* is another species that has been used in toxicity testing. These species may be tested following the flask method. (ASTM 1997a, Peterson *et al.* 1998). The microplate test has also been used with both of these species (Nalewajko and Olaveson 1998, Peterson *et al.* 1998) and has been successfully used with *Anabaena cylindrica* (Day and Hodge 1996).

2.5.3.3 Diatoms (Division Chrysophycophyta; Class Bacillariophyceae)

The diatoms (class Bacillariophyceae) are morphologically different from other algae. Diatoms have specialized cell walls containing silica. The diatoms are important components of benthic and planktonic communities (Sze 1986). *Navicula pelliculosa* is the diatom currently being recommended by the PMRA and the EPA for use in pesticide regulation. This species of diatom can be tested using the flask method (ASTM 1997a). If the microplate test method is followed, *Navicula pelliculosa* (Nalewajko and Olaveson 1998) and *Nitzschia* sp. (Nalewajko and Olaveson 1998, Peterson *et al.* 1998) have been used successfully. The benthic diatom *Craticula cuspidata* has also been used successfully to determine chronic toxicity of atrazine in which the test was extended for 67 days (Nelson *et al.* 1999).

2.5.4 Marine Algae

Estuarine environments serve as critical feeding and nursery grounds for many marine organisms, including commercial fish and shellfish species. These productive and diverse ecosystems are particularly vulnerable because they act as repositories for pollutants from sources upland. In the US, it is estimated that millions of pounds of pesticides end up in coastal watersheds each year. For example, in a southern Florida district which is adjacent to the Everglades National Park and Florida Bay, pesticide usage comprises an estimated 1415 tons of atrazine, 36 tons of endosulfan and 622 tons of chlorpyrifos (DeLorenzo *et al.* 2001).

Marine systems are dominated by algae, with few vascular aquatic species present. Marine algae are either benthic or pelagic. The phytoplankton account for 90 – 95% of the total plant biomass produced in marine systems (estuaries, salt marshes, continental shelf and open ocean). Seventy-two percent of the total marine plant biomass occurs in the open ocean; a total of 20.9×10^9 tons of carbon. The total carbon production in marine systems equals 27.8×10^9 tons (Sumich 1988). Marine phytoplankton contribute 1.0-4.5 m. t dry organic matter/ha to the annual net primary productivity. Marine macroalgae contribute between 25-40 m. t dry organic matter/ha to the annual net primary productivity (Westlake 1969).

When pesticides are likely to enter coastal areas (e.g., through surface runoff, transport in streams or rivers or by atmospheric transport), estuarine/marine organisms should be used in toxicity testing. There are two types of estuarine/marine algae, phytoplanktonic microalgae (similar to the algae discussed for freshwater systems) and macrobenthic algae. Common phytoplanktonic microalgae representatives are diatoms, chrysophytes and green algae. Phytoplanktonic microalgae used in toxicity testing include *Skeletonema costatum, Nitzschia* spp., *Isochrysis galbana, Phaeodactylum, Chlorococcum* sp. and *Dunaliella tertiolecta* (Swanson *et al.,* 1991).

In coastal areas, estuarine/marine macrobenthic algae (seaweeds) play a dominant role in providing habitat and food for many organisms, including fish. Two types of algae are particularly important, the brown algae (belonging to Phaeophyta) and the red algae (belonging to Rhodophyta). The most critical stage in the life of macrobenthic marine algae is sexual reproduction. Any toxic effect on sexual reproduction could have far-reaching and long-term consequences for the seaweed population. For the brown algae, a sexual reproduction test using the cosmopolitan, temperate-zone *Laminaria saccharina,* is in development (Steele and Thursby 1983). For the red algae two sexual reproduction tests have been developed. One is for the warm water species *Champia parvula* (ASTM 1993) and the second is for a temperate species, *Ceramium strictum* (Eklund 1995).

Given the ecological importance of these different classes of marine algae, it would be appropriate to include representative species in a test battery where there is potential for entry of chemicals or pesticides into marine habitats (e.g., estuaries, salt marshes). Thus, the PMRA and EPA are recommending the testing of three additional marine algal species in addition to *Skeletonema costatum*. These species would be representative of the divisions Chrysophycophyta (includes diatoms and golden brown algae), Pyrrhophycophyta (dinoflagellates) and Rhodophyta (red algae).

2.5.4.1 Diatoms (Division Chrysophycophyta; Class Bacillariophyceae)

In the diatoms (Division Chrysophycophyta; Class Bacillariophyceae), there are approximately 200 genera, and estimates of species range from 5000 (Bold *et al.* 1980) to 10,000, of which 30–50% are marine species. (Sumich 1988). The diatoms are important components of the phytoplankton in temperate and polar oceans (Sze 1986). Diatoms are also components of the benthic community in shallow marine environments where these organisms make a significant contribution to the primary productivity of estuaries, bays and other shallow areas (Sumich 1988).

The marine diatom *Skeletonema costatum* is currently recommended and is widely tested with the standard flask method. *S. costatum* has also been tested following the microplate procedure (Gilbert *et al.* 1992, Blaise *et al.* 1998). *Thalassiosira pseudonana* is another marine diatom that can be used following the flask method (APHA 1998a, ASTM 1997a).

2.5.4.2 Dinoflagellates (Division Pyrrhophycophyta)

There are over 1,100 species of dinoflagellates, 93% of which are marine. Dinoflagellates are

either unicellular or colonial and typically have two flagella. Rather than cell walls, the cells are surrounded by a theca composed of plate-like units. Some species are bioluminescent and produce light when luciferin is oxidized in the presence of luciferase (Sumich 1988). During the day, dinoflagellates inhabit the euphotic zone near the surface to maximize their photosynthesis and during the night, they migrate to the deeper waters where nutrients are more abundant. Dense growth of dinoflagellates, called red tides, often occur after a period of upwelling that enriches the surface waters with nutrients (Sze 1986).

The inhibition in bioluminescence is used to measure toxicity in marine dinoflagellates. In the proposed methodology two species can be used, *Gonyaulax polyedra* or *Pyrocystis lunula* (ASTM 1997c). In this method, the light generated by the bioluminescence of the dinoflagellate culture is measured with a photomultiplier tube. Appendix 6 outlines the methodology for toxicity testing with marine dinoflagellates.

2.5.4.3 Red Algae (Division Rhodophyta)

The red algae consist of approximately 4,000 species, of which 98% are marine. The red algae are mostly multicellular and can reach a maximum length of one metre. All species are benthic and are found most often in the tropics and subtropics with some species in cold-water regions (Sumich 1988). They flourish in both littoral and sublittoral zones. Some red algae precipitate calcium carbonate on their cell surfaces, become calcareous and are important in reef formation (Bold *et al.* 1980). A few species of red algae are commercially harvested as food crops. Agar and carrageenan are produced by red algae and are economically important in stabilizers and gels (Sze, 1986).

The red algae recommended for pesticide and chemical testing is *Champia parvula*. It has been used to determine the toxicity of organic compounds (Thursby *et al.* 1985, Thursby and Steele 1986) and metals (Steele and Thursby 1983, Thursby and Steele 1984). It also has broad geographic distribution, extending from Cape Cod to the Caribbean and into the Gulf of Mexico (eastern North America) and from southern California into Mexico (western North America) (ASTM 1998a).

The proposed methodology is a sexual reproduction test where female and male gametophytes are exposed to the toxicant in test chambers for two days under static or renewal conditions. At the end of the exposure period, female gametophytes are removed and incubated (if necessary) for an additional period of time in toxicant-free medium to allow development and germination of the zygote. At the end of the development period, the number of sexually produced structures is determined (ASTM 1998a, Steele and Thursby 1983, Thursby and Steele 1984, Thursby and Steele 1986, Thursby *et al.* 1985). Appendix 7 outlines the methodology for toxicity testing with red algae.

2.5.4.4 Golden-Brown Algae (Division Chrysophycophyta; Class Chrysophyceae)

There are 650 species of golden-brown algae with about 20% of these found in marine

environments (Sumich 1988). Some genera are heterotrophic and differ in their ability to utilize sugars, organic acids, amino acids, and alcohols (Nalewajko and Olavseon 1998). The golden-brown algae are abundant in planktonic communities and most are flagellated single cells or colonies (Sumich 1988).

The species, *Phaeodactylum tricornutum*, is recommended for toxicity testing. This species can be tested with the standard flask method (ASTM 1997a). Even though *P. tricornutum* is recommended for toxicity testing, other test methods exist for this division of marine algae (Chrysophycophyta). For example, kelp gametophytes (order Laminariales) are being used by the State of California for effluent monitoring purposes. The species most commonly used is *Macrocystis pyrifera* (Anderson *et al.* 1998). Appendix 8 outlines the methodology for toxicity testing with golden-brown algae.

2.5.5 Floating Vascular Species

Of the floating aquatic plant species, duckweed (i.e., *Lemna* sp.) is perhaps the most commonly used in toxicity testing. Actually, "duckweed" can refer to both *Lemna* sp. and *Spirodela* sp. (greater duckweed), although, it is usually associated with *Lemna* sp. (Newmaster *et al.* 1997). Duckweeds are floating non-rooted aquatic plants with a reduced root system and lack stems and true leaves. It has been speculated that the roots serve as anchors to keep the fronds right side up and to form the tangled masses which are of some importance in dispersal and protection from water movement. Mats of duckweed are habitat for small invertebrates, for example, the ephydrid fly (*Lemnaphila scotlandae*) and the rhyncophorous beetle *(Tansyphyrus lemnae*). Other invertebrates such as hydras, flatworms and snails are common just beneath the duckweed mat (Hillman 1961). Waterfowl and marsh birds such as coots, black ducks, mallards, teals, wood ducks, buffleheads and rails eat duckweed in large quantities. Duckweed also provides food and shelter for fish (Newmaster *et al.* 1997).

Most toxicological studies with aquatic vascular plants expose the test organism to the toxicant through the exposure medium. Testing procedures can follow the ASTM (1998b), Environment Canada (1999), APHA (1998b) or the EPA (1996b) test method guidelines. The methodology recommended for use in pesticide registration is a static test with *Lemna* sp., similar to the flask method for algae. Briefly, the test is initiated by the introduction of *Lemna* fronds into test vessels. Colonies are inspected for changes in frond number and appearance at initial stages of exposure and at the end of exposure, the total number of living and/or dead fronds are enumerated.

Testing with other floating vascular plants is not well documented. Some of the obvious choices for other floating species are pond lily (*Nuphar* sp.), water lily (*Nymphaea* sp.) and greater duckweed (*Spirodela* sp.). There are however, no known test protocols for these species.

There are currently no required data for the exposure of aquatic vascular plants to pesticide spray drift. *Lemna* species can easily be used for the examination of pesticide exposure through water,

for the study of pesticide drift and research into the effects of surface films at the air-water interface (Swanson 1989, Taraldesen and Norberg-King 1990). Lockhart *et al.* (1989) have shown that the sensitivity of *Lemna* to glyphosate increased several-fold with a foliar exposure compared to the conventional exposure through the growth medium. In this method, glyphosate was first sprayed onto Petri dishes containing *Lemna* fronds in growth medium. The sprayer consisted of a spray nozzle which moved along a rigid track. The sprayer was calibrated using dyes to allow for selection of the desired application rate. After spraying, fronds were allowed to stand for 6-24 hours before being removed to flasks containing clean culture medium. Subsequently, fronds were counted several times over a 2-week period.

The difficulty with recommending this type of test is that there is only one published report where exposure was examined through aerial deposit (Lockhart *et al.* 1989). In the case of herbicides, it may be useful to consider the mechanism of action before requiring this study. For example, if a herbicide is intended as a contact toxicant, then it would be useful to explore a foliar test. By contrast, it may not be necessary to examine systemic herbicides by this type of exposure provided that the mechanism of action is well characterized.

2.5.6 Submersed Vascular Species

It is recognized that submersed vascular plants play an important ecological role in the littoral zones of lakes, estuaries and oceans (Hutchinson 197,; Thayer *et al.* 1975, Den Hartog 1977). Submersed plants provide food and shelter for waterfowl, fish, and invertebrates (Hurley 1990) and are major contributors to primary productivity, nutrient absorption and oxygen production (Boynton and Heck 1982, Stevenson and Confer 1978). As food, submersed plants are a major portion of the diet of several species of duck, geese and swans. Some of these species include wild celery (*Vallisneria americana*), sago pondweed (*Potamogeton pectinatus*), redhead grass (*Potamogeton perfoliatus*), widgeon grass (*Ruppia maritima*) and eelgrass (*Zostera marina*) (Hurley 1990).

Submersed aquatic vascular plants are morphologically different from terrestrial vascular plants. Submersed plants often have a reduced root system, decreased proportion of woody tissue, have sparse cuticles and thin leaves that are often dissected. The characteristic that distinguishes submersed leaves from aerial leaves is the thinner cuticle. Submersed vasculars have the ability to absorb nutrients through their leaves as well as through their roots. The thin, dissected leaves also increase the surface area for gas diffusion and light absorption (Moss 1988). Inclusion of submersed vasculars would account for the potential effects on aquatic plants which rely on a vascular system that is exposed to a pesticide or chemical in the water column and in sediment (Boutin *et al.* 1995). A chemical or pesticide entering an aquatic system (through spray drift or surface runoff), could either remain largely in the water column or partition into sediment. Thus, the inclusion of rooted submersed plants (e.g., *Myriophyllum* sp.) is advantageous as it would account for both routes of exposure.

In addition, the routes of uptake in submersed vasculars are different when compared to algae

which are also submersed. As algae are non-vascular, the only route of uptake is across the cell-membrane. Due to these differences in uptake, toxicity may be quite different for a chemical or pesticide.

Most scientists who have evaluated the limitations of present testing protocols have suggested that a battery of species representing different functional groups and indeed morphologies need to be used to achieve a reasonable level of confidence in the test data (Nyholm and Kallqvist 1989, Swanson *et al.* 1991, Peterson *et al.* 1994, Nyholm and Peterson 1997, Peterson *et al.* 1997, Roshon *et al.* 1999). Lewis (1995) concluded that the sensitivities of rooted vascular plants relative to algae, duckweed, and animal test species are largely unknown. Building a broader database of effects of pesticides on rooted aquatic vascular plants is therefore essential.

Duckweed is the only aquatic vascular plant that is currently required for pesticide and chemical testing. Development of submersed vascular plant tests is recommended as no single species can be representative of the majority of vascular plant species even if *Lemna* has a global distribution. *Lemna* is somewhat unique in its floating, unrooted growth habit and exposure can be both aerial and aquatic. Furthermore, *Lemna* is a monocotyledon species. As there may be differences in herbicide sensitivity between monocot and dicot species, it may be beneficial to include dicot species in the testing scheme

There are nine species of the dicotyledonous macrophyte, *Myriophyllum*, native to North America (*M. pinnatum, M. farwellii, M. heterophyllum, M. humile. M. laxum, M. tenellum,, M. alternifolium, M. verticillatum, and M. exalbescens*) and one species that was introduced (*M. spicatum*; or Eurasian water milfoil) (Cook 1985). *M. exalbescens* is also known as *M. sibiricum* (Aiken and Cronquist 1988, Ceska and Ceska 1986, Ricketson, 1989) or has the common name, northern water milfoil.

M. sibiricum is ecologically important as it provides food and shelter for other organisms (Fink 1994). The seeds and foliage provide 0.5 to 5% of waterfowl diet and the seeds are 0.5 - 2% of the diet of marsh and shore birds (Martin *et al.* 1951). *Myriophyllum* is an important food for moose. The many aquatic invertebrates that live in these plants serve as food for fish and waterfowl (Newmaster *et al.* 1997). *Myriophyllum* species are also important in nutrient cycling and reducing the erosional impact of wind and wave action (Sutton 1985).

M. sibiricum has a broad ecological range as it is found in eutrophic waters, marl lakes, slightly alkaline lakes and brackish water (Ceska and Ceska 1985). Geographically, this species occurs in northern latitudes where there is a 0 °C January isotherm, as it requires cold temperatures for successful turion formation (Couch and Nelson 1985). Thus, it is a circumpolar species that has been found in northern Europe and northern Asia in addition to North America (Ceska and Ceska 1985). *M. sibiricum* is found in the Eastern United States (Connecticut, Delaware, Indiana, Kentucky, Maine, Maryland, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Ohio, Pennsylvania, Rhode Island, Vermont, Virginia, West Virginia) and north-central United States (Steward 1993).

A standardized toxicity test for *M. sibiricum* has been published in the ASTM Annual Book of Standards (ASTM 1997b). The axenic toxicity test with *M. sibiricum* is a replicable and repeatable system. Features of this method that enhance standardization include a chemically defined medium (Roshon *et al.* 1996), an artificial rooting substrate and inoculation of each tube with an axenic macrophyte segment. The artificial rooting substrate is extremely easy to prepare and provides consistent test results. It is a static, partial life-cycle laboratory test that determines the toxicant effect over fourteen days. This species is easily cultured in test tubes under laboratory conditions. Every second day, plant shoot height can be measured to allow for the generation of growth curves. Endpoints that can be measured include total plant height, root number and length, fresh weight, dry weight, plant area, oxygen production, change in membrane integrity, chlorophyll *a*, chlorophyll *b* and carotenoid content (Roshon 1997) and chlorophyll fluorescence (McCann 1997). Root length was shown consistently to be the most sensitive endpoint for detecting the effects of $ZnCl_2$ and phenol, in *M. sibiricum* (Roshon 1997, Roshon and Stephenson 1997). Appendix 9 outlines the methodology for toxicity testing with submersed vascular plants.

Other species of *Myriophyllum* have been used to examine the effects of toxicants. *M. heterophyllum* was used in toxicity tests by researchers at the Saskatchewan Research Council and the University of Saskatchewan. Researchers at the Canadian Forestry Centre used *M. spicatum* to determine the effects of forestry pesticides (McCann 1997).

Another research group developed and evaluated a pesticide toxicity test for submersed macrophytes using an aquarium culturing and beaker testing system. The researchers tested numerous macrophyte genera, including *Ceratophyllum, Myriophyllum, Egeria* and *Najas* with four pesticides (atrazine, alachlor, metolachlor and metribuzin) (Fairchild *et al.* 1994). Another tested genus was *Vallisneria* with metribuzin (Nelson and Fairchild 1994). *Ceratophyllum* was shown to be the most sensitive genus to these compounds (Fairchild *et al.* 1994).

A laboratory toxicity test with *Vallisneria americana* Michx. (wild celery) has been developed for testing contaminated sediment. This toxicity test involved planting *V. americana* shoots into glass jars containing sediment from different sites. The jars were placed into aquaria and exposed to a natural photoperiod. After one week of exposure, the plants were measured for the number of leaves, length and width of each leaf, number of roots, diameter and length of each root and the biomass of leaves and roots. This toxicity test successfully detected the effects of contaminated sediments on *V. americana* (Biernacki *et al.* 1997).

Other submersed species used in pesticide testing (atrazine, metribuzin and glyphosate) include *Cabomba caroliniana* Gray (fanwort), *Elodea canadensis* Michx. (American elodea), *Egeria densa* Planch. (Brazilian elodea), *Myriophyllum spicatum* L. (Eurasian watermilfoil), *Vallisneria americana* and *Potamogeton perfoliatus* (Forney and Davis 1981).

2.5.7 Emergent Vascular Species

Emergent plants are critical for wetland environments as they provide habitat in the form of nursery areas and cover for wildlife (Powell *et al.* 1996). These plants also regulate the temperature and flow of water (Boutin *et al.* 1995). Despite evidence indicating that wetland plants may be the most sensitive to toxicants (Swanson *et al.* 1991, Thomas *et al.* 1986), testing with these plants have been largely ignored. Due to the close proximity of wetlands to agricultural areas, data on phytotoxicity to emergent plants may be required for pesticide registration (Powell *et al.* 1996).

There are no available data to support the idea that terrestrial species are surrogates for emergent aquatic plants. Even within terrestrial plants, for example, there is no definitive evidence that crop species can serve as surrogates for non-crop species (e.g., weeds or native plants) or vice versa. Traditionally, crop species have been used to extrapolate to native or wild species that are ecologically relevant and, thus, used to characterize the risk to habitats of concern. Similarly, there is no scientific basis for extrapolating from terrestrial species to aquatic emergent species simply because of the limited database for making such a comparison.

Both emergent aquatic and terrestrial plants are similar in that their foliage may be exposed to pesticide spray drift. It is not known if there are substantial physiological or morphological differences (e.g., cuticle thickness of leaves) between emergent and terrestrial species to warrant the testing of both types of plants. Emergent species, however, differ in that their submersed stem may potentially be exposed to contaminants in the water column.

Some plant species inhabit both aquatic and terrestrial systems. For example, wetland plants prefer to live either in moist soil or in sediment with less than 18" (45.7 cm) of overlaying water (Powell *et al.* 1996). In these cases, it can be argued that some wetland plants could also serve as terrestrial species, however, these species do not represent the wide range of plant families found in strictly terrestrial habitats.

Obviously, to resolve this issue of aquatic emergents vs terrestrials, testing should be based on species (or plant families) that are relevant to the area or habitat of concern rather than continuing with the general assumption that rooted vascular plants have similar sensitivities. The inclusion of data on aquatic emergent species would, therefore, reduce the uncertainty of extrapolating from one plant group to another and provide a more comprehensive and scientifically defensible position for establishing the risk to aquatic ecosystems. Furthermore, it would facilitate more appropriate mitigative measures for the off-target movement of pesticides and chemicals into aquatic ecosystems (i.e., estimating buffer zones).

There have been recent advances in research on aquatic macrophytes other than duckweed. In 1996, an emergent macrophyte testing protocol was developed (Powell *et al.* 1996) and published by the American Society for Testing and Materials (ASTM 1996). Numerous emergent wetland species were screened and rated based on their suitability for toxicity testing. Based on this rating, the following is listed from the best to worst: *Oryza sativa* (domestic rice); *Spartina*

pectinata (prairie cordgrass); *Phalaris arundinacea* (reed canary grass); *Polygonum muhlenbergh* (nodding smartweed); *Scirpus acutus* (hardstem bulrush); *Typha latifolia* (narrow-leaf cattail); *Iris versicolor* (blue water iris); *Trifolium repens* (white clover); *Zinania aquatica* (giant wild rice); *Alisma plantago aquatica* (water plantain); *Onobrychis viciaefolia*; *Carex rostrata* (beaked sedge); and *Juncus effusus* (soft rush) (Powell *et al.* 1996). The authors recommended the monocot, *Oryza sativa* (domestic rice), and alternative monocots, *Spartina pectinata*, *Scirpus acutus* and *Phalaris arundinacea*. The alternative dicot was *Polygonum muhlenbergh.*

Domestic rice is recommended for toxicity testing because the seeds are easily obtained, it is easily cultured in the greenhouse, and it is economically important and produces consistent results. Rice has also been used to examine the toxicity of effluents to seed germination (Wang 1991b, Wang 1991c). Chlorophyll *a* is being emphasised as the most sensitive endpoint, except that more time and equipment are needed to conduct this analysis. No toxicological information from this research is presented in this synopsis as boron was the test compound (Powell *et al.* 1996). Details of this growth test with emergent macrophytes (ASTM 1996) are summarized in Appendix 11.

An alternative method was designed to evaluate the effects of contaminates in water on the germination and seedling growth (root elongation, root and shoot dry weight) of emergent plants. Inhibition of germination or seedling growth will affect the ability of the plants to compete and survive. The recommended species in this method are *Echinochloa crusgalli* (Japanese millet), *Leersia oryzoides* (rice cutgrass), *Nelumbo lutea* (American lotus), *Oryza sativa* (domestic rice), *Rorippa nasturtium-aquaticum* (watercress), and *Zinania aquatica* (wild rice). These tests may be either static, renewal or flow-through systems (APHA 1998c).

Furthermore, emergent macrophytes are exposed to contaminants not only through their foliage, but also through their submersed stem and roots. Some pesticides and chemicals may favor partitioning into sediments rather than remaining in the water column. The use of aquatic plants, however, to determine toxicity from contaminated sediments is uncommon. Aquatic plants can be affected by contaminated sediments. Root growth can be inhibited and assimilation of these substances can lead to leaf injury, growth inhibition and reduced seed production (Lewis 1995). For the proposed tests with the emergent macrophytes (rice and nodding smartweed), it may be possible to modify the test methodology to account for exposure through the water column or contaminated sediment which would account for any potential exposure through root uptake.

2.6 Reproduction

Reproduction endpoints in vascular plants (rooted aquatic and terrestrial) has not been required by regulatory agencies in an effort to minimize testing. With the use of some of the newly developed low-dose high-potency herbicides, there have been reports of severe effects on reproduction in terrestrial plants at very low levels of these herbicides (Bhatti *et al.* 1995, Fletcher *et al.* 1995). Bhatti *et al.* (1995) and Al-Khatib *et al.* (1992a) reported that non-target terrestrial plants (e.g., sweet cherry) in areas near application of these herbicides showed reductions in flowering and fruiting under experimental field conditions. These reproductive effects occurred at

rates far less than the recommended application rates and did not elicit visible foliar damage. Fletcher *et al.* (1993, 1996) demonstrated a similar pattern under laboratory and greenhouse conditions with a range of plant species. The researchers demonstrated that the reproductive yield of some herbaceous species was only 1% of control plants when growth was unaffected at rates that were 0.004-0.008 of the recommended field rate.

Similar effects are hypothesized for aquatic plants. In conventional testing with algae and duckweed, the effects measured are essentially on asexual reproduction as this is the dominant mode of proliferation in these organisms. In some emergent aquatic species, however, the primary mode of propagation is sexual reproduction (Arber 1963, Peterson 2001). If sexual reproduction is more severely affected than growth, then more appropriate endpoints may need to be examined (e.g., seed set, pollination and flower development).

Although, a shift from sexual to asexual (vegetative) reproduction is often associated with the evolution of aquatic plants, a complete absence of flowering and seed set characteristics occurs only in a few species. Most aquatic angiosperms retain the ability to flower and set seed and this is obviously important for many aquatic plant groups. Some of these species that have retained aerial flowers include bladderwort (*Utricularia* sp.), *Megalodonta* (Asteraceae), *Limnophila* (Scrophulariaceae) and *Ranunculus* (Ranunculaceae) (Philbrick and Les 1996).

When sexual reproduction is the major mode of reproduction in some emergent plants, (Arber 1963, Peterson 2001) it is feasible to include the examination of relevant sexual reproduction endpoints in plant testing. Some of the obvious measurement endpoints are flower formation, pollen formation and seed production. Protocols to determine reproductive impairment in emergent aquatic species, though, are lacking. It is unclear how to select the appropriate assessment endpoints (e.g., EC_{05}, EC_{25} or EC_{50}) relative to reproduction parameters; however, the same assessment endpoints used in assessing growth inhibition could be used (i.e., 25% effect or EC_{25}).

Asexual reproduction includes both seed production without fertilization (agamospermy) and vegetative reproduction (Philbrick and Les 1996). Asexual reproduction is important in the establishment, growth and maintenance of aquatic plant populations (Cook 1985, Spencer and Bowes 1993). The principal means of population increase in the three growth forms of aquatic plants (i.e., floating, submersed and emergent) is by vegetative reproduction (Spencer and Bowes 1993). For example, the water hyacinth (*Eichhornia crassipes*; Pontederiaceae) and Eurasian watermilfoil (*Myriophyllum spicatum*; Haloragaceae) have spread over vast areas by means of vegetative reproduction (Philbrick and Les 1996). Most aquatic plants, however, are monocots which explains why there is a much higher association of rhizomatous growth in aquatic habitats than terrestrial habitats (Tiffney and Niklas 1985). By contrast, there are far fewer dicots that are rhizomatous (Grace 1993). Perennial aquatic plants possess several forms of vegetative reproduction, including corms, rhizomes, stolons, tubers and turions (Grace 1993, Hutchinson 1975, Sculthorpe 1967, Vierssen 1993). In particular, aquatic plants have developed a variety of highly specialized structures that function as propagules (i.e., functions in propagation and dispersal, e.g., spores or seeds). The most prominent vegetative propagule in aquatic plants is the

turion. Turions, sometimes called, “winter buds” are dormant vegetative buds enclosed by specialized leaves that are substantially different from foliage leaves.

In considering some of these vegetative structures of asexual reproduction, it may be appropriate to examine the important measurement endpoints (e.g., number of turions, extent of tuber formation, length and weight of rhizomes and stolons) to assess the impact on asexual reproduction. The selection of the measurement endpoint(s), however, would likely be species dependent. Protocols that examine the effects of pesticides or chemicals on asexual reproduction in rooted aquatic macrophytes are not available. As with sexual reproduction, it is unclear how to select the appropriate assessment endpoint (e.g., EC_{05}, EC_{25} or EC_{50}) as it relates to asexual reproduction parameters. We propose that the same assessment endpoints used in assessing growth inhibition are applicable (i.e., 25% effect or EC_{25}).

2.7 Species and Protocols - Higher Levels

As previously discussed in Section 2.5.1, the predictive value of a test battery improves with size. The PMRA and EPA, therefore, believe that testing should also be expanded on the Group(s) shown to be sensitive at Level I. Table 3 lists plant species which have acceptable methodologies for testing and can be used in higher level testing.

For the additional species of freshwater green algae, several protocols can be followed, including ASTM, APHA, EPA and OECD methodologies (see section 2.5.3.1). For blue-green algae and freshwater diatoms, ASTM protocols and methodologies used by several researchers can be followed (see sections 2.5.3.2 and 2.5.3.3). Similarly, for the marine diatom and golden-brown alga, published methodologies could be followed (sections 2.5.4.1 and 2.5.4.4).

Additional species of dinoflagellates or red algae are not proposed at this time as there is no available information on recommended species and protocols. There are methodologies, however, for the submersed and emergent vascular species (sections 2.5.6 and 2.5.7).

Table 3: Other Species for Testing.

Group		Additional Species
1	Freshwater algae	Green algae: *Scenedesmus subspicatus*; *Chorella vulgaris*; *Chlamydomonas reinhardi*; *Chlamydomonas eugametos*. Blue-green algae: *Anabaena cylindrica*; *Microcystis aeruginosa* Diatom: *Nitzchia* sp.; *Craticula cuspidata*
2	Marine algae	Diatom: *Thalassiosira pseudonana* Dinoflagellate: ND Red algae: ND Golden-brown algae: *Macrocystis pyrifera*
3, 5	Floating vascular	*Nuphar* sp.; *Nymphaea* sp.; *Spirodela* sp.
4	Submersed vascular	*Ceratophyllum* sp.; *Vallisneria americana*; *Elodea canadensis*; *Egeria densa*; *Potamogeton perfoliatus*; *Najas* sp.
6, 7	Emergent vascular	Monocot: *Spartina pectinata*; *Scirpus acutus*; *Phalaris arundinacea* Dicot: *Nelumbo lutea; Rorippa nasturium-aquaticum*

ND - not determined

2.8 Specialized Testing - Higher Levels

The lipophilic nature of many pesticides and industrial chemicals may pose a threat to higher organisms. For example, although endosulfan was shown to be non-toxic to algae at concentrations likely found in the environment (<1 µg/L), it may bioaccumulate in algae and be consumed at higher concentrations by grazing organisms (DeLorenzo *et al.* 2001). It was demonstrated by Rao and Lal (1987), that the blue-green algae, *Aulosira* and *Anabaena*, accumulated endosulfan to levels that were 700 times the exposure level within 48 hours. The amount of pesticide or chemical that is accumulated depends not only on the adsorptive properties and rate of uptake, but also on the amount of substance that is loaded into the aquatic environment and the rate of transformation (or persistence) of that substance. Thus, there is the potential for pesticide and chemical accumulation in grazers which subsequently may be toxic to other organisms that consume them or continue to bioconcentrate through the food web. This is especially true in estuaries where contaminated sediments are continually resuspended due to tidal action and dredging (DeLorenzo *et al.* 2001).

Hinman and Klaine (1992), have developed a methodology for measuring the uptake of pesticides from sediments by the aquatic macrophyte, *Hydrilla* sp. It may also be possible to conduct uptake/accumulation tests with the proposed submersed and emergent species (i.e., *Myriophyllum*, *Oryza* and *Polygonum*).

Furthermore, it may be necessary to conduct partial- or full-life cycle tests to fully characterize the toxicity of certain chemicals and pesticides. This becomes even more apparent with persistent compounds where chronic toxicity may be more critical than acute effects. Longer-term tests could also be used to determine the toxicities of intermediate biotransformation products (Payne and Hall 1979, Holst and Ellwanger 1982). For terrestrial plants, Wang and Freemark (1995), report that life-cycle tests can provide more in-depth information than the traditional germination and root elongation tests. The authors recommend the use of mouse-ear cress (*Arabidopsis thaliana*) because of its small size, short life-cycle, large seed production and ease of culturing. There are currently, however, no available information on aquatic species with similar characteristics that would make it suitable for partial- or full-life cycle tests.

2.9 Multispecies Testing

Often, it has been argued that results from single-species laboratory studies are not reflective of situations in the field. For example, the ecological relevance of laboratory-derived toxicity endpoints for single-species cultured algae is not known for most pesticides and chemicals. The large interspecific response and unrealistic experimental conditions in standard toxicity tests are factors that limit our ability to extrapolate laboratory-based results to impacts on natural plant communities. This has led to the use of more realistic conditions in laboratory studies, such as the use of river water, mixtures of test substances and exposure of two or more species simultaneously. Several multispecies tests have been used to evaluate toxicity (Lewis 1995). Wang and Freemark (1995) report that microcosm tests with aquatic plants have been standardized and are reproducible, sensitive and comparable with field data. Mesocosm and field testing have been developed but require further development (Wang and Freemark 1995).

Microcosms are multispecies systems that are tested under laboratory conditions whereas, mesocosms are usually larger multispecies systems that are tested outdoors. Mesocosm studies would also account for the natural dissipation processes of the pesticide or chemical (e.g., photolysis, biotransformation), thus, making the test conditions closer to that of ecosystem conditions (Boutin *et al.* 1993). The selection criteria for species should be ecological relevance for the area(s) of concern and ease of culturing. Both microcosm and mesocosm studies are less complex that field studies.

Field testing (and monitoring studies) could consist of in-situ limnocorrals or enclosures or whole-systems (e.g., entire pond). As with mesocosm studies, these studies incorporate natural dissipation processes. Studies should be conducted in the location(s) where the pesticide or chemical is used or area(s) of concern where there is potential exposure. The study should also account for multiple applications (pesticides) or continuous exposure (chemicals). The test

duration should be sufficient to examine the potential for recovery of plant communities. The selection of species for consideration would be based on their dominance in plant communities and/or their importance to organisms at higher trophic levels (e.g., food and shelter for invertebrates; cover for fish). Thus, the species requirement and experimental design would be determined on a case-by-case basis. It may be necessary to include several trophic levels (e.g., invertebrates, fish) into these studies to reflect the natural interactions between plants and wildlife (i.e., herbivore and predation).

Ecosystem pesticide exposure studies have been conducted to assess the prevalence and impacts of pesticide residues in prairie pot holes and wetlands on plants and aquatic invertebrates used by prairie nesting ducks for food and shelter (Sheehan *et al.* 1987). The prairie pot hole study focused on some commonly used herbicides and insecticides. The prairie pot hole region of North America covers approximately 300,000 miles, largely in agricultural areas of south-central Canada (Alberta, Saskatchewan, Manitoba) and the north-central United States (Minnesota, North Dakota, South Dakota, Iowa, Montana). Indirect (sublethal) toxicity of herbicide drift and surface runoff residues to algae and macrophytes in prairie potholes was the primary concern. The authors concluded that herbicides may reduce storage of energy reserves in tubers or seeds eaten by ducks and their nesting cover, reduce plant diversity, and cause replacement of sensitive species with resistant ones. Due to the close association between macrophytes and invertebrates, a major concern is the reduction or loss of plants of importance to gastropods, crustaceans, and aquatic insects; and ultimately the reduction in food for fish and birds (Sheehan *et al.* 1987).

In whole-system studies (e.g., entire ponds), the selection of trophic levels is not an issue. With in-situ limnocorral/enclosure studies, however, there is debate over which organisms (other than plants) to exclude; the only practical choice may be to exclude fish. Testing non-herbicide products with trophic level systems might present some difficulties. For example, an insecticide (having herbicidal properties), may reduce herbivore by reducing the aquatic invertebrate population, thereby, affecting results. If the sole purpose is to determine phytotoxicity, then it may be not suitable to conduct in-situ studies with non-herbicidal products. On the other hand, however, it would be meaningful to determine the overall effects on an aquatic ecosystem (i.e., other than phytotoxic effects).

In several cases, the results of multispecies tests have been compared to those of single-species laboratory tests with the same toxicant (Plumley and Davis 1980, Boyle *et al.* 1985, Larsen *et al.* 1986, Lewis *et al.* 1986, Stay *et al.* 1989). These comparisons which were species- and compound-specific, indicated that extrapolation from single algal species to a plant community level should be approached with caution (Lewis 1995). Although, there are limited data on aquatic vascular plants to make similar conclusions, it is intuitive that the same rationale also applies for conducting multispecies testing with higher plants.

With this type of testing, parameters should be selected that reflect the key precesses in the maintenance and productivity of the aquatic ecosystem in question. For example, in microcosm and in-situ studies with multiple algal species, the measured parameters have often been biomass, O_2 production, carbon uptake and species abundance (Brockway *et al.* 1984, Larsen *et al.* 1986,

deNoyelles *et al.* 1982). It would also be appropriate to examine these parameters in vascular aquatic plants in multispecies/community level tests.

2.10 Monitoring

Algae and macrophytes are essential components of aquatic ecosystems. Adverse effects on nontarget aquatic plants are of particular concern because of the widespread and increasing worldwide use of pesticides (especially herbicides) (Pimental *et al.*, 1991). From 1975-1995, the annual application of herbicides in the US and Canada has increased by 3 to 5-fold (Nielsen and Lee 1987, Freemark *et al.* 1990, Pimental *et al.* 1991). This increased use of herbicides has been implicated as the cause of reduced aquatic vegetation (Bellrose *et al.* 1983, Kemp *et al.* 1984). In addition, industrial effluents shown to be practically non-toxic to fish and aquatic invertebrates were very toxic to aquatic vegetation when discharged into receiving waters (Ashton and Crafts 1981, Berry 1984, Gersich and Mayes 1986, Presing and Ponyi 1986, Mayes *et al.* 1987). In the field, submersed plants appeared to be more sensitive to effluents and were less able to compete with more tolerant emergent plant species (Dickman *et al.* 1983). Several reports have suggested that lethal effects of toxicants on plants have profound ecological and economic impacts (Altieri and Letourneau 1982, Freemark and Boutin 1994). Even with sublethal effects on plants there are dramatic impacts on natural vegetation and food production (Benenati 1990, Hunsaker *et al.* 1990, Weinstein *et al.* 1990).

Arguments have been presented to the PMRA and EPA, claiming that a 50% inhibition in plant growth or biomass in laboratory studies is not necessarily meaningful from an ecological perspective as plants may recover once the toxicant is removed. Toxic effects in the laboratory can be temporary or permanent and depend largely on the toxicant, its concentration, its persistence, the route of exposure, the plant species, the life-stage and the plant's health (Hughes *et al.* 1988). Also, plants that are stressed or injured by toxicants are more vulnerable to disease and are out-competed by more tolerant species (Wang and Freemark 1995). For example, Dickman *et al.* (1983), demonstrated that in the field, submersed plant species were more sensitive to effluent toxicity and/or less able to compete with the more-tolerant emergent species.

There is always uncertainty when assessing the risk to aquatic plant communities as many of the phytotoxic tests are conducted under controlled laboratory conditions that are often unrealistic for extrapolation to field situations. One method of detecting ecosystem response to phytotoxic chemicals is by monitoring. Aquatic plant species, populations and communities should be used as indicators of the aquatic ecosystem response to different stressors. Some of these indicators include decline in sensitive species, decline in species richness (i.e., species diversity) and trophic level changes The loss or predominance of certain species may indicate the presence of toxicants. Toxicity and bioaccumulation studies using aquatic vascular plants can provide information about the effects of toxicants, bioaccumulation and biomagnification (Stewart *et al.*, 1999).

The PMRA and EPA recommend a monitoring system for toxicants that are found to pose a high risk to non-target plants during Level IV testing.

Duckweed is most often recommended in biomonitoring studies for effluents and pesticides (American Public Health Association *et al.* 1992, American Society for Testing and Materials, 1994). Macrophytes, however, are becoming more critical as test species for monitoring pesticides, effluents and industrial chemicals (Wang and Freemark 1995). Some of these species include *Hydrilla*, lettuce, millet and rice (Behera and Misra 1982, Cassidy and Rodgers 1989, Wang 1990, Wang 1991, Wang 1992, Lewis 1995). Freemark *et al.* (1990) and Swanson *et al.* (1991), indicated that many ecologically relevant emergent and submersed macrophytes are potential biomonitors of the aquatic environment. These include sago pondweed (*Potamogeton pectinatus*), arrowhead (*Sagittaria*), cattail (*Typha*) and common waterweed (*Elodea canadensis*). Wang (1991, 1992), indicates that millet has been used in toxicity testing of organic substances, industrial and municipal effluents, surface and ground waters and sediments. Millet is found along rivers, lake shore and in wetlands environments. It is an important food source for native and migratory waterfowl and other wildlife. It is often planted in flood plains and wetlands for wildlife management (Wang 1991, Wang 1992). Hence, millet could be one of several important indicator species for adverse impacts resulting from off-target movement of phytotoxic compounds.

3 TESTING SCHEME FOR TERRESTRIAL PLANTS

3.1 Introduction

This section focuses in detail on the proposed tiering structure, toxicity endpoints, various testing methods, recommended testing species, multispecies tests, monitoring, and reproductive tests for terrestrial plants. This section identifies the issues of concern with the current approach to testing terrestrial plants and, thus, is aimed at stimulating discussion towards improving the testing system.

3.2 Tiering Structure

The proposed four level system, introduced in Section 1.5, is recommended for pesticides and chemical tests on terrestrial plants. In this proposed system, testing at higher levels is determined by the extent of phytotoxic effects observed at lower levels. The EPA and PMRA recommend that a set of 25 representative terrestrial plant species be initially tested at the lowest level, Level I. Progression to higher levels occurs only for those plant groups for which toxicity has been assessed as significant. There would be no further testing required for the group(s) showing non-significant toxicity. An overview of the level progression design is illustrated in Appendix 3.

The EPA and PMRA have been urged for more than a decade to modify terrestrial plant toxicity test guidelines in ways that would support harmonization between the US and Canada (Fletcher *et al.* 1988, Fletcher 1990, Freemark *et al.* 1990, Fletcher 1991a, Aldridge *et al.* 1993, Smrchek *et al.* 1993, Boutin *et al.* 1995, Freemark and Boutin 1995, Boutin and Rogers 2000). A goal of toxicity testing is to gather empirical evidence about toxic effects on representative non-target species, and to generalize these effects to all potentially-exposed non-target species for the purpose of assessing risk. The EPA and PMRA have recognized the need to improve the non-target plant toxicity test requirements by expanding test species and family representation to include plants other than annual crop plants and to screen pesticides and chemicals for their effects on plant reproduction and survival.

3.2.1 Level I: Deterministic Assessment

Level I or “Deterministic Assessment” is subdivided into Level I-A and Level I-B. Level I-A consists of “Maximum Challenge Tests”. Maximum challenge refers to testing at a single rate or concentration that is equivalent to the maximum expected exposure. For pesticides, the maximum labelled rate is tested. In cases where more than one pesticide application per season is required, the frequency of applications and the environmental fate of the pesticide should be considered in determining the appropriate dose. For pesticides, the dose should be representative of the maximum exposure expected under operational uses. Range-finding tests involve more than one dose and are conducted to determine the range of concentrations or application rates to be used in definitive testing. Both tests serve as early screening tools to evaluate phytotoxic potential. The

test concentration is calculated on the basis of a 1-ha, 15-cm deep pond that receives the maximum expected dose. This level is equivalent to the Tier I testing under the current EPA Guidelines.

If after Level I-A, the exhibited phytotoxicity is a concern, then Level I-B or "Definitive Testing"is warranted. Level I-B is multiple concentration/rate testing that provides dose-response information from which toxicity endpoints (e.g., EC_{05}, EC_{25} or EC_{50}) can be estimated. There is the option, however, for initial testing at Level I-B rather than at Level I-A. As in the case for herbicide products where phytotoxicity is expected, testing of non-herbicides or chemicals may also begin at Level I-B (equivalent to Tier II of current guidelines). The assessment at Level I-B involves the comparison of a point estimate of toxicity to a point estimate of exposure for each species.

Two species, cherry and *Arabidopsis thaliana* (thale cress or mouse-ear cress) or *Brassica rapa* (canola) are recommended for testing to generate reproductive endpoints. These species are tested at 15% of the maximum dose concentration to simulate the maximum amount of aerial drift that can be expected. The NOAEC is determined in reproductive testing.

3.2.2 Level II: Refined Assessment

The testing requirements and assessment approach at Level II are unclear until further developments are achieved in the EPA probabilistic risk assessment process for aquatic and terrestrial organisms. The assessment process for non-target terrestrial plants will involve a refinement of exposure and better utilization of the dose-response data for each species. In some cases, it may be necessary to require additional testing at the beginning of Level II.

Level II is, therefore, a first-step refinement of the phytotoxic potential identified in Level I. This assessment is the basis for determining whether Level III testing is necessary.

3.2.3 Level III: Expanded Assessment

As with Level II, until the EPA probabilistic risk assessment process has been further refined, there are uncertainties in the testing requirements and assessment approach for Level III.

Level III testing may include additional acute or chronic toxicity tests with species shown to be a concern (i.e., potentially at risk) at lower Levels and/or with species that are specific to the area(s) or region(s) where a pesticide would be used or where prolonged exposure to industrial chemicals may occur. For example, if the toxicity to a terrestrial species was shown to be a concern at Level II, then testing at Level III could focus on region-specific or keystone species. Dependancies between plants and animals must be understood if keystone plants are to be identified (Mills *et al.* 1993). Some plants are dependent on animals for seed dispersal and pollination whereas some animals are dependent on specific plants for food and shelter.

The extent of testing at Level III is determined on a case-by-case basis with species selection, and duration of tests (i.e., acute or chronic) would be pesticide/chemical specific.

3.2.4 Level IV: Comprehensive Assessment

Level IV is the final and most comprehensive testing step. Level IV consists of microcosm, mesocosm or field testing as well as post-registration monitoring. The aim is to address specific questions or issues raised with a pesticide or chemical for a given terrestrial environment (e.g., wetlands, forests, woods, rangeland, etc.). If mitigative measures do not alleviate the concern or if there is major uncertainty with single species testing, then more ecologically relevant multispecies tests becomes necessary. Level IV may be triggered by the results or assessments from any previous level and can consist of multispecies testing, monitoring studies, or both.

3.3 Alternative Testing Endpoints

Powell (1997) states that "Responses may be measured at several different levels of biological organization, starting at the subcellular level and moving toward whole ecosystems. At the subcellular level, changes may be biochemical or physiological in nature. Organismal-level alternations may involve anatomical or morphological changes, as well as reduction in reproductive success and shortened life span. On a larger scale, whole populations or communities may be altered, while ecosystem structure and function may be impacted... Historically, many environmental concerns have been handled in a reactive manner... The challenge today is to be more proactive and to prevent major environmental alterations or, at least minimize their impact. An 'early warning' system should provide rapid, reliable detection of effects at the lowest practical level. It is recommended that a suite of responses or endpoints from different organizational levels be included when making ecological decisions".

3.3.1 Foliar Measurements (acute effects):

Progression through the level system is influenced by the sensitivity of the plant to the test chemical, and detection of this sensitivity depends on the endpoints selected as well as when they are measured during the plant life cycle. Selected endpoints should be easily testable, reproducible and, of course, highly sensitive to any potential effects. It is also preferable to have cheaper and faster tests that result in rapid endpoint determination. Endpoints currently assessed by guideline seedling emergence and vegetative vigor phytotoxicity tests typically include percent emergence, seedling height, seedling weight, and phytotoxicity rating (an arbitrary ranking defined by the severity of observable adverse effects). However, common herbicide modes of action are varied, including perturbation of photosystems, membrane proton gradients, respiration, metabolic pathways, and inhibition of fatty acid biosynthesis and enzymes (Merlin 1997). Endpoints assessed in seedling emergence and vegetative vigor tests may not fully detect these physiological changes.

Numerous cases have been documented showing that herbicides can increase plant susceptibility

to attack by pests and pathogens (Freemark and Boutin 1994). Physiological processes such as photosynthesis may be altered, affecting whole plant carbon balance and, thus, plant growth. Foliar exposure to herbicides through spray drift could impact stomatal conductance, having consequences on CO_2 uptake and transpirational water loss. In light of these facts, some studies have broadened their focus beyond pesticide impacts on plant growth and toward determining the effects of pesticides on plant physiology (Haile *et al.* 1999; Krugh and Miles 1996).

Peroxidase enzyme: Researchers have noted that increases in peroxidase activity are dose dependent. For several chemicals, the increase in peroxidase enzyme occurs prior to vegetative growth reduction. The use of peroxidase as an indicator of plant stress may be a more sensitive endpoint than vegetative growth (Powell, 1997).

Oxygen evolution and consumption: Biomass is often used in the estimation of productivity. Another way of measuring productivity is photosynthetic activity. The photosynthetic response is generally measured by carbon uptake or oxygen evolution. Oxygen evolution and consumption in plants can be measured using an oxygen/ meter or a colorimetric method. The use of ^{14}C is commonly used on plant segments or on whole plants to determine carbon uptake (Lewis and Wang 1999, Lewis 1995).

3.3.2 Physiological Measurements

3.3.2.1 Sub-Lethal Effects

Measurements that may be useful for detecting phytotoxicity include growth rate and gas-exchange parameters such as photosynthesis, transpiration, respiration, and stomatal conductance (Breeze 1993). Nondestructive measures such as these can be monitored continuously throughout the experiment and can provide valuable information about temporal changes in phytotoxicity. This section describes various sub-lethal effects that can be measured in terrestrial plant tests. The endpoints measured include chlorophyll induction analysis, gas-exchange method, chlorophyll production and chlorophyll *a* measurements, transpiration, peroxidase, leaf area, enzyme kinetics, mineral nutrient status, plant water content, and ethylene production.

Chlorophyll induction analysis: Krugh and Miles (1996) measured the quantum efficiency (electron transport) and the fluorescence-quenching capacity of mung bean plants hours after herbicide exposure. They showed that chlorophyll induction analysis is a useful, nondestructive method for determining the adverse effects of photosynthesis-inhibiting pesticides on plants.

Chlorophyll production: Gealy *et al.* (1995) sprayed pea and lentil plants with 2, 4-D or a mixture of thifensulfuron and tribenuron in the field to simulate drift of sulfonylurea and phenoxy herbicides from spring cereal fields. In this study, bleaching of newly emerged leaves and stipules occurred in low dose treatments (3.3%) one week after application, and chlorophyll content was reduced as much as 40% in the 10% treatment group. As a result, early canopy closure was reduced which increased the fraction of incident PAR (photosynthetically active radiation) reaching the soil surface and the potential for weedy growth and competition. Blossom formation

of pea and lentil plants was also reduced, flowering was delayed, and seed yield was lower in the 10% thifensulfuron:tribenuron treatment.

Pigment content has been used to determine physiological status of plants. One advantage in using pigment is that samples can be frozen and held for months before analysis. The most commonly measured pigments are the chlorophylls. In particular, chlorophyll *a* content has been used to measure biomass. Reduction in chlorophyll can result in inhibition of carbohydrate synthesis (Lewis and Wang 1999).

Transpiration measurements: Transpiration has also been shown to be an easily obtainable, nondestructive measurement that can be continuously measured as an indicator of chemical toxicity. Thompson *et al.* 1998 exposed hybrid, pre-rooted poplar cuttings to TNT (2,4,6-trinitrotoluene) solutions in a growth chamber experiment and found that transpiration, measured gravimetrically, was negatively related to TNT concentration. Reductions in transpiration were also associated with foliar damage, such as chlorosis and leaf abscission.

Gas exchange measurements: In another study, wine grapes were repeatedly sprayed with low doses (1/100 of the field application rate) of an SU herbicide (Bhatti *et al.* 1998). Chlorosis and other visible injuries were observed and plant growth was significantly reduced. Frequently exposed plants also experienced a 33% reduction in photosynthesis and a 60% increase in stomatal resistance, compared to untreated control plants. There were significant negative relationships between chlorosulfuron exposure and photosynthetic rate and between photosynthetic rate and percent chlorosis. These results support the inclusion of physiological endpoints (e.g., gas exchange measurements) as useful, nondestructive indicators of plant status during low dose herbicide exposure.

Hannan (1995) studied the effects of seven sulfonylurea and four triazine herbicides on the freshwater green alga *Chlorella pyrenoidosa*. He found that 10 ppt of bensulfuron methyl, the amount that was leached from an apple leaf where 0.01 µg was applied, could be detected by way of CO_2 uptake by the alga in 15 minutes. This study demonstrated that the gas-exchange method of measuring microbiological growth rates can be used to detect submicrogram amounts of sulfonylurea and triazine herbicides within minutes to several hours. Such studies can also be done on terrestrial plants.

Peroxidase tests: Increased peroxidase activity and levels of ascorbic acid and gluatathione are believed to protect plant cells from free radical oxidation (Byl and Klaine 1991, Byl *et al.* 1994). Peroxidase activity has been used to evaluate contaminant exposure to plants. For example, *Spartina alterniflora* (an aquatic plant) showed a significant increase in peroxidase activity when exposed to 9.9 µg/L atrazine (De Souza and Yodh 1997). Similarly, there was a significant increase in peroxidase activity in *Scirpus olneyi* (an aquatic plant) with exposure to metolachlor at a concentration of 9.9 µg/kg in estuarine sediment (Lytle and Lytle 1996).

Other endpoints measured: Other proposed endpoints include chlorophyll production, leaf area, enzyme kinetics, mineral nutrient status, plant water content, and ethylene production (Rodecap

and Tingey 1981, Breeze 1988, Fletcher 1991a).

In some cases, the interpretation of endpoint response may be misleading and complicate risk assessment. Certain growth indices may exhibit positive responses at low herbicide doses. For example, Breeze and Timms (1986) reported that oilseed rape plants exposed to the phenoxyalkanoic herbicide mecoprop exhibited higher leaf expansion rates than control plants. This stimulation was not a direct response to the pesticide, but a compensatory response due to pesticide-induced losses in leaf area. The potential for misinterpretation of pesticide effects, such as these, illustrates the need for time course measurements and non-destructive repeated measurements (Breeze 1988). Furthermore, compensatory responses typically involve resource allocation from storage organs to sites of new growth. Such a redistribution of internal resources during vegetative growth may ultimately impact the availability of internal resources for reproduction later in the plant life cycle. This study supports incorporating the evaluation of reproductive parameters in phytotoxicity tests.

In another study, tomato plants exhibiting moderate and severe symptoms from exposure to quinclorac drift appeared to recover a few days after exposure; however, symptoms reoccurred days later and, in cases where foliar symptoms ceased, bloom abortion continued (Bansal *et al.* 1999). This example illustrates the danger of misinterpreting recovery symptoms and emphasizes the importance of post-registration monitoring.

All of the above tests were of short-duration and could facilitate more convenient and cost-effective methods for assessing phytotoxicity. Although these biochemical endpoints have been used as early warning signals, there are few reports associated with their relevance to growth, survival and reproduction of the whole plant and effects on the plant community (Lewis and Wang 1999). Further research is needed before these endpoints can be utilized in aquatic assessment methods.

3.3.2.2 Acute Effects

Chlorophyll a fluorescence: Cucumber plants were adversely affected by formulation products of the fungicide benomyl (Gaffney et al. 1998). Dibutylurea (DBU) reduced shoot and root biomass at the highest concentration (94 mg/L), and DBU-exposed plants also had lower peak-to-terminal chlorophyll *a* fluorescence ratios compared to control plants. Since chlorophyll *a* fluorescence is a measure of electron transport chain activity, it provides information on the photosynthesis impact of DBU and can be used as a nondestructive means of monitoring the physiological status of herbicide-exposed plants.

3.3.2.3 Root Measurements

Westra *et al*. (1990) described the measurement of several nontraditional endpoints for potato plants from a field experiment conducted to determine the injury to plant growth, foliage, and crop yield to various herbicides. Some of the endpoints examined in this experiment which are not considered in current US phytotoxicity test guidelines include flower number, tuber number, tuber weight, and tuber quality (e.g., normal, cracked, folded). Many of these endpoints are easy to quantify and could serve as useful, nondestructive indicators of phytotoxicity throughout the experiment.

Several studies have shown that root bioassays are highly sensitive indicators of pesticide toxicity, and extensive information has been collected for a wide variety of crop species (Strek *et al.* 1989, Stephenson *et al.* 1997, Jourdan *et al.* 1998a, Jourdan *et al.* 1998b, Szmigielska *et al.* 1998). Tomato *(Lycopersicon esculentum*) root bioassays are more sensitive to sulfonylureas than bioassays using corn, lentils and peas, although sensitivity has been shown to vary depending on the type of sulfonylurea (De Barreda and Lorenzo 1991, De Barreda *et al.* 1993). In these tests, pre-germinated seeds are exposed to the test substance via soil or water media, and the average length of the main root is measured. These tests are statistically powerful because they use large replicate numbers, thus minimizing the variability not attributed to treatment (De Barreda *et al.* 1993). However, the EPA phytotoxicity tests do not require root bioassays to examine root length or biomass because of the difficulty in separating roots from organic media during harvest. Separation usually results in destruction of root architecture and the inevitable attachment of rooting media to harvested roots, which can falsely inflate or deflate root biomass values.

If phytotoxicity tests fail to examine roots, the toxicity of some compounds may not be adequately assessed. For example, slow rates of reduction in shoot density of Canada thistle were shown over a four-year period of 2,4-D and chlorsulfuron application, while root production was limited to depths of 50 cm (Donald 1992). In this case, the adverse effects of the herbicides on root production were more apparent than the effects on shoot production. Shoot data (e.g., leaf area, leaf number, and stem height) can be obtained nondestructively and at various intervals throughout the experiment. In some cases, causes for changes in shoot density may be revealed through information provided by changes in root production, as tradeoffs between these plant organs illustrate the dynamics of internal carbon budgeting. The effects of residual soybean herbicides were determined for sugar beet planted one, two, and three years following application (Renner and Powell 1991). Visual injuries were observed in sugar beet plants in almost all treatment conditions. Root yield reductions ranged from 30 to 100% for imazaquin, imazethapyr and chlorimuron treatments, despite little to no detection of soil residues.

Strek *et al.* (1989) showed that lentil root bioassays conducted in growth chambers overestimated herbicide injury to crops in the field and could, therefore, serve as a useful and conservative tool in risk assessment. Although low topographical areas had no observable injury reported, the bioassay showed injury up to 50%. In contrast, ridge, hilltop, and mid-ridge areas showed up to two times more injury than reported in the bioassay.

3.3.2.4 Other Influences on Phytotoxicity Tests

Several studies have shown that young plants are more susceptible to herbicides than older plants (Marrs *et al.* 1991). Current phytotoxicity tests are conducted on very young plants, but more developed plants provide the opportunity to observe phytotoxic responses not otherwise seen with current phytotoxicity methods. In a test conducted with five wetland and terrestrial plant species, plants were most sensitive to sulfonylurea herbicides during the seedling stage, but reductions in reproductive endpoints were noted in older plants (Boutin *et al.* 2000). Again, these results emphasize the need to evaluate reproductive endpoints, which may require conducting tests with older plants.

The timing and mode of herbicide exposure can also influence phytotoxic response. For example, ED_{25} values from plants studied in phytotoxicity tests were compared across Canadian and US databases (Boutin and Rogers 2000). This comparison of databases revealed that plants were more sensitive to pre-emergent herbicide applications than post-emergent applications; however, another study has shown greater sensitivity with postemergent application (Snipes *et al.* 1992). In most phytotoxicity studies, plants are exposed to liquid or vapor application of herbicides, either directly or through spray drift. A concern has been raised, however, over the exposure of non-target plants to herbicides through contaminated, wind-blown soil. These effects were examined in a study where the foliar absorption and translocation of thifensulfuron, chlorsulfuron, glyphosate, and 2, 4-D was studied by applying them to foliage of alfalfa, grape, and pea plants in aqueous form or as herbicide-treated soil (Al-Khatib *et al.* 1992a). After seven days of treatment, foliar absorption of herbicide-treated soil was minimal, ranging from 0.8 to 4.5% of applied concentrations. 2,4-D was absorbed the most by alfalfa and pea, while bromoxynil and glyphosate were absorbed the most by grape plants. Humidity and simulated dew increased foliar absorption from herbicide-treated soil by as much as 20% and three times, respectively. Some phytotoxic symptoms were observed in grape plants as a result of herbicide-treated soil exposure (mild chlorosis and leaf cupping), but they did not adversely impact plant growth at the applied concentrations. The investigators of this study concluded that exposure to extremely high concentrations of herbicide-contaminated soil would be required to cause injury in field-grown plants.

3.3.2.5 Laboratory and Field Study Conditions

Aside from soil conditions, plant age, and method of exposure, test conditions may also limit the range of phytotoxic responses exhibited by terrestrial plants. Greenhouse studies control more factors, so they provide conservative estimates of toxic effects, while field studies test phytotoxic effects under a more realistic set of environmental conditions (Boutin *et al.* 1995). To accurately assess phytotoxic response, data should be compared across multiple experimental settings.

Obrigawitch *et al.* (1998) observed "where limited field study data are available for certain sulfonlyureas on non-target crops, it is important to recognize the need for data from multiple locations before definitive conclusions can be reached. Such studies need to include economic or ecological impact measurements (e.g. yield, quality) in addition to short-term response data (i.e.

visual measurements)".

In examining a limited database, Fletcher *et al.* (1990) observed an average of two fold variability of EC_{50} values calculated from field and laboratory testing. These results, although limited to 13 species tested, show some variability between field and laboratory. Additional research may be needed to settle the question of differences between field and laboratory results. Moyer (1995) showed similar results in field and growth chamber experiments for alfalfa plants exposed to sulfonylurea herbicide soil residues. When the SAP was questioned in 1994 about the application of laboratory results to field scenarios, it maintained that controlled environment tests should bear close approximation to responses in realistic field settings. The SAP also noted that the stability and mobility of the test chemical should be the factor showing the greatest discrepancy between lab and field settings, because it demonstrates the greatest dependence on environmental conditions. Similarities between lab and field studies should provide assurance that laboratory-based Level I and Level II tests adequately assess toxicity as it would be expressed under field conditions, and provide support that field testing at Level III and (potentially) Level IV will be informative and not superfluous in cases where extreme phytotoxicity of a test compound has been shown at lower levels.

The EPA and PMRA recommends that gross acute endpoints (plant height, plant weight and symptomology) continue to be used at this time. At this time, we are uncertain as whether physiological and biochemical testing should be requested.

3.4 Species Selection for Terrestrial Phytotoxicity Testing

The PMRA and EPA recommend expansion of test species to include species with ecological or conservation interest.

Key species should be identified and tested when the productivity of a given plant species might impact community or ecosystem stability. The scientific literature may identify test species that are useful indicators of phytotoxicity. (Appendices 13,14). Species selected for dependent responses are typically easy to cultivate and measure. Additional tests, such as those that gauge reproductive yield or carbon assimilation, could be added at any testing level.

When selecting test species from these families, Cole *et al.* (1993) recommended that care should be taken to avoid variegated species, species with natural epidermal blemishes, or fine-structured species, the characters of which may mask the detection of low-level damage. Tissue culture testing has been recommended as a useful method to determine phytotoxic effects in slow-growing and difficult to measure woody perennials, including forest (canopy and understory) and wetland species (Fletcher and Ratsch 1991).

In 1994, the SAP concluded that the group of ten test species recommended by the US phytotoxicity test guidelines (6 dicots and 4 monocots) was an adequate number of surrogate species for crops. They further concluded that this list of species inadequately represented the broad range of responses exhibited by natural plant communities, or by any species other than

annual crops. The SAP indicated that rigorous testing would have to be conducted to support the addition of new non-target species whose responses would closely resemble the diversity of species from pesticide-impacted ecosystems.

Additional phytotoxicity data has been entered into EPA's toxicity database since 1994. Many of the herbaceous crop and non-crop species that have not been tested previously do have similar growth habits and parameters as the often tested crop species. The EPA believes that it will not be necessary to have rigorous testing for such new herbaceous species to support the addition of these new species. The general framework for the vegetative vigor and seedling emergence testings should be able to accept these new species without extensive modifications.

In response to the question "Can the results collected from experiments on one species be extrapolated to another?", Fletcher (*et al.*1990) presented data from the PHYTOTOX database showing the phytotoxicity variability among various species. These data showed that the sensitivities of plant species to each other from the same chemicals can be very broad for picloram (67X- 316X) or much narrower for linuron (2-3X) with 14 other chemicals in between. Another analysis was performed with EC_{50} values which showed that taxonomic differences among plants have a much greater influence than a laboratory to field response. The analysis indicated that species responses to herbicides are more similar within the same genera or family. The data supports testing from different families and genera. Fletcher (*et al.*1990) further stated that many of the species tested in PHYTOTOX are sensitive native species which are overlooked in the present OECD and EPA testing scheme.

ASTM guidelines for terrestrial plant toxicity tests (Allen *et al.*1999) provide a more extensive list of plant test species than those identified by FIFRA (>25). The list from these guidelines encompasses species that have been identified in regulatory documents, standard test procedures, and in studies of toxicity effects. This set of guidelines also describes protocols for a root elongation assay, a brassica life cycle test, and contains a separate section for tests concerning the growth and development of woody plant species; examples of acceptable ASTM endpoints (root length) and tests not addressed by EPA FIFRA guidelines. The ASTM E 1963-98, however, currently does not contain the vegetative vigor test.

The species selection process should be based on functional differences expressed by plants (Boutin and Keddy 1993). Boutin and Rogers (2000) found that species with similar growth habits, physiology and reproductive cycles generally exhibit similar toxicity responses. Also, the authors found that species responded variably across studies, and that they could not be generally classified as sensitive or insensitive to chemical herbicides. They also found that there was a greater variability among broadleaf species than grass species, indicating that fewer grass species and a greater number of broadleaf species should be selected in order to fully represent the variation within these families (Boutin and Rogers 2000). Other studies have also recognized greater consistency of responses within plant families compared to across them (Chapman et al. 1998), as well as the unlikelihood of identifying an ideal sensitive species.

Stephenson *et al.* (1997) exposed a battery of 30 plant species to boric acid and found that

phytotoxic responses of most species tested resembled those of corn (C4 species or mostly monocots) or canola (C3 species or mostly dicots). However, this database has a very limited number of species tested and contains data on the use of boric acid and artificial and contaminated soils rather than pesticides. The authors evaluated perennial species red clover, alfalfa, wheatgrass, bluejoint, and Canada bluegrass as well as some annual crop species. Criteria used to select tests included germination time, crop vs. non-crop species, monocot vs. dicot species, C3 vs. C4 photosynthesis system, source and availability of seed, critical variable requirements (pH, nutrients, etc.), root formation and relative sensitivities to known contaminants. Environmental Canada (1998) used 33 testing endpoint criteria (including 2 different soils for LC20 and LOAEC) each with a ranking factor to select species for testing contaminated soils. The criteria for species selection in this study included percent emergence in controls, time of emergence, ease of root separation from soil, duration of test, sufficient biomass at end of test, seed size, and soil effect on growth. The study was limited to contaminated soils (no pesticides) and only acute endpoints were measured.

Freemark *et al.* (1990) noted that there was great variation in phytotoxic response among and within species as well as cultivars, and attributed much of this variation to differences in plant physiology and age. There is evidence to suggest that dicots (mustard) show greater sensitivity to phosphoamidates than monocots (wheat), illustrating that patterns of herbicide response may develop between morphologically different plant types for some herbicides (Das and Roy 1998).

Hageman and Behrens (1984) compared the amount of chlorsulfuron needed to reduce the growth of two weed species by 50% and found a difference of 21,000 fold. Sweetser et al. (1981) stated that "tolerant plants, such as wheat, oats, and barley, rapidly metabolize chlorsulfuron to a polar, inactive product" but "sensitive broadleaf plants show little or no metabolism of chlorsulfuron."

Species with similar structural features have also been shown to respond in similar ways. For example, plants with a higher potential for foliar absorption of pesticides because of their reduced cuticular wax or abundant leaf hair showed greater phytotoxic response (Dayan *et al.* 1996).

Aldridge *et al.* (1993) noted that a standard protocol has not been established for tests conducted by Canadian registrants. These tests include the screening of up to 30 species. Boutin and Rogers (2000) suggest that studies examining the effects of a single pesticide on a wide range of species are more useful for making risk management decisions than studies examining the response of a few species to a wide variety of pesticides. They stress that the number of species tested should represent the diversity of the system, allow for sufficient replication of each tested species to properly conduct statistical analyses, and be practical enough to conduct the experiment under costly GLP conditions.

Current EPA guidelines recommend the use of at least ten crop species representing a limited number of families in their terrestrial phytotoxicity tests. Cole *et al.* (1993) identified 14 species in 10 families that were tested in one pesticide manufacturer's baseline phytotoxicity testing. Pestemer and Zwerger (1999) identified the utility of fifteen test species (monocot and dicot crop

and weedy species) for standardized herbicide bioassays on non-target terrestrial plants under European guideline tests. Appendix 13 proposes additional test species from over forty families not already considered, with particular emphasis on non-agronomic species. Many of the species on this recommended list were identified within the British Desk Study to have distributions that encompass farmland habitats and/or they are important food/resource for vertebrate and invertebrate wildlife (Breeze *et al.* 1999). Canadian sources have also identified many of the potential test species proposed in the Appendix as both high risk for off-target exposure to herbicides and important as a resource for many bird species, occurring in upland prairies, forest margins and marshes (e.g., *Melilotus* spp., *Rosa* spp.)(Sheehan *et al.* 1987).

Many researchers have recommended the inclusion of families and genera that would allow generalizations to be made regarding phytotoxicity in woody perennials and endangered species (Fletcher and Ratsch 1991, Cole *et al.* 1993, Boutin *et al.* 1995).

An ILSI (International Life Science Institute) workshop on Low Dose, High Phytotoxicity Herbicides Impact on Nontarget Plants was held in Washington, DC on December, 1999. The workshop was attended by scientist from industry, academia, and government. A consensus was reached regarding what criteria should be used in selecting species to be tested. These criteria are as follows:

- Present in habitat/potentially affected/proximity to application site
- Important in the system's food web
- Important in some ecological process (e.g., nitrogen fixation, habitat/soil fixation).
- Little intraspecific variation in response.
- Sexually reproducing and exhibits sexual reproduction response to some herbicide(s).
- Roots into substrate (for sediment/soil exposure).
- Similar physiology as target weed/plant.
- Can be tested under laboratory or field conditions.

One of the problems identified by the ILSI participants is the difficulty of identifying enough species to fit all of the criteria for every chemical. The potential problem with selecting a baseline of species for all chemicals to be tested is that there may always be at least one sensitive species that may be overlooked.

Workshop participants also agreed that for practical considerations, a comprehensive examination of extant data should be conducted prior to the generation of new data. Particular attention should be given to evaluation of the validity of these data. This process can be used to highlight significant data gaps and identify what studies need to be repeated to validate the existing data. Data also may be rejected for various reasons in this process, such as non-reproducibility. In addition, a list might be compiled of the aquatic and terrestrial species known to be sensitive to these herbicide classes, along with the available information on sensitivities. Is floral tissue most sensitive, or seed, or reproductive endpoints, or vegetative growth? With this list, it may be possible to develop pertinent test endpoints, whether for deterministic or probabilistic analysis

Representatives from the OPPT, OPP and PMRA recently identified important plant families from

which new test species could be selected and then identified important species within these families (Appendices 13, 14). Both crop and native species comprising herbaceous and woody plants were selected. The criteria for the families and species selection were as follows:

- species that are known to be sensitive to pesticides from toxicity data or non-target incidents data.
- species that serve as important sources of wildlife food
- species that are recommended for phytotoxic testing in literature
- species that have been tested for reproductive endpoints
- species which are common and can be easily acquired
- species that represent ecologically important families

The EPA and Canada recommend testing 21 dicot species from 14 families and 5 monocots from 3 families including 2 reproductive tests (Table 4) at Level 1. A detailed discussion of the species selected follows the tables. Appendix 14 also list the species and families most frequently listed in PHYTOTOX. At this time, the PMRA /EPA team is uncertain if the number of species to be tested is scientifically appropriate or whether other species should be added to Level 1 testing.

Some recommended testing protocols are provided in Table 5.

Table 4: Recommended terrestrial species for seedling emergence and vegetative vigor, acute, partial life-cycle, and full life-cycle toxicity testing and reproductive toxicity testing

Level I

Family	Species
Monocots	
Poaceae (Grass family)	corn + ryegrass + 1 other native species
Cyperaceae (Sedge family)	purple nutsedge
Liliaceae (Lily family)	onion
Dicots	
Asteraceae (Aster family)	lettuce + 1 other
Fagaceae (Beech family)	oak
Pinaceae (pine family)	sugar or loblolly pine
Rosaceae (Rose family)	raspberry + cherry
Rosaceae (Rose family)	cherry (partial life-cycle)
Brassicaceae (Mustard family)	canola + Arabidopsis
Brassicaceae (Mustard family)	Arabidopsis or canola (full life-cycle)
Solanaceae (Potato family)	tomato + 1 other
Fabaceae (Pea family)	soybean + yellow sweet clover + 1 other
Malvaceae (Mallow family)	cotton + 1 other
Apiaceae (Carrot family)	1 species
Polygonaceae (Buckwheat family)	1 species
Lamiaceae (Mint family)	1 species
Chenopodiceae (Goosefoot family)	1 species
Scrophulariaceae (Figwort family)	1 species
Convolvulaceae (Morning-glory family)	1 species

Table 5: Recommended protocols for terrestrial plant testing at Level 1

Species/Test Type	Protocol Available	Protocol Title
Soil Toxicity Test on native and perennial grasses and forbs (Acute emergent and early plant growth only)	Yes	"Standard Guide for Conducting Terrestrial Plant Toxicity Tests" ASTM E 1963-98
Woody plant species growth and development test (Acute-short term response)	Yes	"Standard Guide for Conducting Terrestrial Plant Toxicity Tests" ASTM E 1963-98
Brassica Life Cycle Test using *Brassica rapa.*	Yes	"Standard Guide for Conducting Terrestrial Plant Toxicity Tests" ASTM E 1963-98.
Seedling Emergence Test Vegetative Vigor Test	Yes	USEPA. 1996. OPPTS Harmonized Test Guidelines, Series 850: Ecological Effects Test Guidelines. Volume II, Guidelines 850.2100 to 850-7100. Draft, April 1996. U. S. Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances. Washington, D.C.
Arabidopsis reproduction test or life-cycle test	Yes	Shimabuku, R.A., H.C. Ratch, C.M. Wise, J.U. Nwosu, and L.A. Kapustka. 1991. A New Plant Life-cycle Bioassay for Assessment of the Effects of Toxic Chemicals Using Rapid Cycling Brassica. In: Plants for Toxicity Assessment Second Volume, ASTM STP 1115. J.W. Gorsuch, W.R. Lower, W. Wang, and M.A. Lewis, Eds. American Society for Testing and Materials, Philadelphia, PA. Pp. 365-375.
Cherry reproductive test	Yes	Fletcher, J.S., T.G. Pfleeger, H.C. Ratsch, and R.Hayes. 1996. Potential impact of low levels of chlorsulfuron and other herbicides on growth and yield of nontarget plants. Environmental Toxicology and Chemistry 7: 1189-1196.

3.4.1 Annual Species

Amaranthaceae - amaranth family
Fletcher *et al.* (1988) noted that *Amaranthus retroflexus* (redroot pigweed) was the most commonly tested non-agronomic species in the PHYTOTOX database (Appendix 14). In some cases, pigweed has been shown to be more responsive to sulfonylureas than alfalfa, a potential test species considered for seedling emergence tests by Moyer 1995 and Stephenson *et al.* 1997. Pigweed species may also serve as a good reference species because there are biotypes that are both susceptible and resistant to ALS-inhibiting herbicides (Gaeddert *et al.* 1997, Sprague *et al.* 1997). Another reason to support selection of species within the Amaranthaceae for phytotoxicity tests is that seeds of these species have been shown to comprise a proportion of the dietary intake by wild quail, a test organism in avian phytotoxicity studies (Martin *et al* 1951).

Asteraceae (Compositae) - daisy family
This family contains many species that are economically valuable, including *Lactuca sativa* (lettuce) and *Helianthus annus* (sunflower). Lettuce is already recommended test species by EPA, but sunflower is not. There are several characteristics (aside from its economic value) that make sunflower a good test species to add to the current testing scheme. For example, sunflower was shown to be more sensitive than turnip and lentil in a bioassay to determine effects of herbicide leachates (Günther *et al.* 1993). In the same study, shoot growth of *H. annus* was more sensitive to the herbicide treatment than root growth and leaf weight (an easily-measured endpoint). These results support the use of sunflower as a reliable test species for determining very low level effects of a herbicide at a minimal cost and over only a few days. *H. annus* also shows sensitivity to herbicides in the field.(Derksen 1989, Wall 1994b). Breeze et. al. (1999) reports that this family is one of two families that attract the greatest diversity of nectar and/or pollen feeding insects such as bumblebees, butterflies, and solitary bees which may make this family attractive to test in terms of protecting pollinator's food source.

Brassicaceae (Cruciferae) - mustard family
This family is represented by *Brassica oleraceae* (cabbage) which is currently recommended by EPA for toxicity tests. There are, however, other species in this family (such as fast-cycling *Brassica rapa* (canola), *Arabidopsis thaliana* (thale cress), *Raphanus sativus* (radish), *B. kaber* (corn-mustard), *B. napus* (turnip), *B. campestris* (canola), *B. alba* (white mustard), and *Lepidium sativum* (pepperwort) that are easy to study, have been studied extensively in the phytotoxicity literature, or have been recommended by the OECD as surrogate test species (Fletcher 1991b). *Brassica napus* was the only crop species tested that showed high sensitivity in the Canadian phytotoxicity database (Boutin and Rogers 2000).

Chenopodiaceae - goosefoot family
Beta vulgaris (beet) and *Spinaca oleracea* (spinach) would serve as good test species within the family because they have been extensively studied in the phytotoxic and general plant physiology literature (Fletcher 1991b). Herbicide soil residues have been shown to produce injuries in *B. vulgaris* for up to two years following herbicide application. *Chenopodium album* (lamb's quarters) would be another good test species because it is a widely distributed herbaceous species

that produces many seeds (>75, 000 seeds/plant) and serves as a significant food source for wildlife (Martin *et al.* 1951, Freemark and Boutin 1995).

Fabaceae (Leguminosae) - legume or pea family
The legumes are a large, widespread family that includes numerous wild and cultivated species. This family is also ecologically important, due to the ability of many species to form associations with nitrogen-fixing bacteria. Many species in this family are important sources of nectar and/or pollen for bumblebees, solitary bees and mason bees (Breeze et. al. 1999). Many species from this family are well represented within the PHYTOTOX database, and several (e.g. soybean and beans) are currently recommended by EPA or OECD as surrogate test species (Fletcher *et al*. 1988, Fletcher 1991b). *P. vulgaris* (bean) has been proposed as a useful annual sentinel species to biomonitor chlorsulfuron drift and deposition because plants show chlorotic symptoms that are negatively related to time after spray and distance from spray source (Felsot *et al.* 1996a). Other species, such as pea and lentil, are also not currently recommended by EPA but may serve as better test species than soybean or bean (Al-Khatib *et al.* 1993b). In a study conducted to determine the use of ten plant species as bio-indicators of sulfonylureas under field conditions, onion, alfalfa and sugarbeet showed some sensitivity to sulfonylureas (e.g. reduced growth, delayed leaf development, and death), but they did not display the high sensitivity and clear diagnostic symptoms exhibited by pea, common bean, and lentil plants (e.g. leaf wilt, severe chlorosis, leaf curl, etc.).

Liliaceae - lily family
Of the Liliaceae (lily family) and Papaveraceae (poppy family) families, only one (Liliaceae) currently has a single species that is recommended by EPA for use in phytotoxicity studies, onion. All of these families contain species with high economic and ornamental resource value.

Malvaceae- mallow family
The Malvaceae contains several species (both herbaceous annuals and woody perennials) that may be particularly good test species. *Abutilon theophrasti* (velvetleaf) was used as a non-target test species to develop plant bioassay methods (Brown *et al.* 1991), and several plant characters (e.g. dense leaf hairs and large leaf area) also make this species an interesting candidate for phytotoxicity testing. Another species, *Gossypium hirsutum* (cotton), is the 4th most valuable crop in the U.S. (USDA National Agricultural Statistics Service 2000). Like velvetleaf, cotton is not a recommended test species by EPA. Cotton is one of the most frequently listed species in the PHYTOTOX database (Fletcher *et al.* 1988). In one study, quinclorac drift injured cotton seedlings in a field experiment, causing leaf strapping, malformed reproductive structures, and eventually reduced cotton yield (Snipes *et al.* 1992).

Poaceae (Gramineae) - grass family
Millet has been proposed as a good test species within the Poaceae (Gramineae) family. It is a common, small stature, wetland species that produces large quantities of seed that is easy to obtain, and it is important food for wildlife (Wang and Freemark 1995). The phytotoxic response of millet to phenolic compounds was compared to those of test species already recommended by EPA (cucumber and lettuce). Millet seeds showed consistent sensitivity to the phenolic test

compounds and proved to be better determinants of toxicity than cucumber and lettuce in root elongation tests (Wang 1986). Several other wetland species within the Poaceae family (e.g., domestic rice, prairie cordgrass and reed canary grass) have also been identified as desirable test species because they are easily cultured, readily available, and economically important (Powell *et al.* 1996).

The Poaceae (Gramineae) is the largest taxon, containing many species with significant economic and ecological value. Grasslands are major plant communities of North America, covering more area than any other plant community, and grass species constitute a significant portion of animal diets (Martin *et al.* 1951, USDA National Agricultural Statistics Service 2000). There are numerous species from Poaceae that top the list of the most frequently listed species in the PHYTOTOX database (Fletcher *et al.* 1988). Some species that are major US crops, but are not currently recommended by the EPA phytotoxicity guidelines, include *Triticum aestivum* (wheat), *Hordeum vulgare* (barley), *Setaria viridis* (green foxtail), and *Cynodon dactylon* (bermuda grass). Many of these species, with the exception of wheat, are also not recommended test species in the OECD guidelines. Many grass species are cosmopolitan in their distribution, fast growing, and easy to obtain and culture, making them good candidate species for phytotoxicity testing.

Polygonaceae - buckwheat family
Polygonum persicaria (smartweed) was consistently one of the most sensitive species tested in both the US and Canadian phytotoxicity databases (Boutin and Rogers 2000). However, it is not a regularly recommended test species. Chlorsulfuron applied to smartweed plants during the flowering stage significantly reduced seed dry weight to a greater extent than shoot dry weight (Fletcher *et al.* 1996). Furthermore, in some areas of northwest Missouri, *Polygonum sp.* may account for up to 85% of mallard duck diets, a test species in avian toxicity studies (US Forest Service 2000). High herbicide sensitivity, coupled with the fact that *Polygonum sp.* is an important food source for deer and waterfowl, supports the selection of this test species for use in phytotoxicity studies.

Solanaceae - potato or nightshade family
Many species within this family have been highly studied for their phytotoxic effects (e.g., tomato and potato). Aside from these species, many studies have been recorded in the PHYTOTOX database for tobacco (Fletcher *et al.* 1988).

3.4.2 Herbaceous Perennials

Fletcher *et al.* (1988) indicated that there were several economically important tropical perennial families with data reported in the PHYTOTOX database. Representatives from US and Canadian agencies identified numerous families containing herbaceous perennial species for species selection in guideline phytotoxicity studies (Appendix 13). Only a few of these families contain herbaceous perennial test species that are commonly accepted under FIFRA guidelines.

Campanulaceae and Caryophyllaceae families contain wildflower (bluebells) and ornamental species (carnations and pinks) respectively, and were identified by Dr. Steve McCanny (Parks Canada, personal communication) and Cole *et al.* (1993) as potential families from which to select test species. However, there is little or no data in the phytotoxicity literature to indicate that species from these families are sensitive to herbicides, which would justify their inclusion in guidelines as recommended test species (Saari *et al.* 1992).

Cyperus rotundas (purple nutsedge) would be a good herbaceous perennial test species within the Cyperaceae family. This species within the Cyperaceae family is commonly tested during pesticide development, and studies on it are well documented within the PHYTOTOX database (Fletcher 1991b; Boutin *et al.* 1995).

Trifolium pratense (red clover), in the Fabaceae family (legumes and beans), is currently recommended as a test species under OECD guidelines (Fletcher 1991b). *Medicago sativa* (alfalfa), also a member of Fabaceae, is not currently a guideline-recommended species, but has been suggested as a potential test species by Stephenson *et al.* 1997, and there have been several phytotoxicity studies conducted with this perennial species (Fletcher *et al.* 1988, Al-Khatib *et al.* 1992b).

The family Umbelliferae (carrot family) is one of two families that attract the greatest diversity of nectar and/or pollen feeding insects such as bumblebees, butterflies, and solitary bees. In addition the flowers from this family are thought to be the main food sources for the adult braconid wasps and other beneficial parasitic flies and wasps that feed on aphids and other insect pests (Breeze et. al. 1999).

No species are currently recommended for phytotoxicity testing under FIFRA within the families Lamiaceae (Labiatae), Primulaceae and Ranunculaceae, and Scrophulariaceae. These families were recommended as containing potential test species by a number of Canadian, UK, and US authorities. Little if any phytoxicity information exists on species from these families within the literature. However, they do contain many economically and ecologically valuable species that would be useful in determining phytotoxic effects on lesser-known flora (e.g., mints, horticultural crops and ornamentals). The Lamiaceae and Scrophulariaceae families are important sources of nectar and pollen for bumblebees and large solitary bees, especially those with long tongues (Breeze et. al. 1999).

Gange *et al.* (1992) showed that germination rates of several perennial forb species were insensitive to pesticide application, indicating that seedling emergence tests may not be the most appropriate way to assess phytotoxic effects in perennial species. Few studies have been conducted in the peer-reviewed literature on the phytotoxic response of herbaceous perennials, and the majority of these have concerned effects on target weed species (Donald 1985, Bestman *et al.* 1990, Asghari *et al.* 1993). One study showed that spray drift from fluazifop-P reduced sugarcane stalk number, height, weight and development, resulting in lower sucrose levels, higher fiber (poorer cane quality) and lower sugar yields (Richard 1995).

In field tests with five different plant families, mature plants were shown to be far less sensitive than young seedlings to glyphosate spray drift when comparing vegetative and reproductive endpoints(Marrs *et al.* 1991). However, for mature monkey flower (*Mimulus rigens*) plants exposed to metsulfuron methyl, the number of capsules per plant and the ratio of reproductive to vegetative biomass was significantly reduced at 10% of the maximum spray label rate (Boutin *et al.* 2000).

3.4.3 Woody Perennials

Currently, no guideline phytotoxicity studies are conducted on woody perennial species. However, Fletcher *et al.* (1988) noted that the most commonly tested forest tree species within the PHYTOTOX database was *Pinus taeda* (loblolly pine), and that there were also many records listed for the family Fagaceae (beech family) which includes oaks, beeches and chestnuts. Potential test species also include several families containing woody perennial species (Appendices 13,14).

As mentioned before, the Pinaceae family is well-represented in the PHYTOTOX database, due to testing on *P. taeda* (loblolly pine). Total length and number of new roots of *P. taeda*seedlings were greatly reduced (32-69%) with increasing sulfometuron application in both greenhouse and field studies (Barnes *et al.* 1990). Other conifer species have also displayed sensitivity to herbicide application. For example, Cole and Newton (1989) exposed Douglas fir, Noble fir, and Grand fir trees (economically-valuable Christmas trees) to sulfometuron, atrazine and hexazinone, and found that herbicides significantly reduced the height of Douglas and Noble fir trees.

Betulaceae, including birch and alder species, is a family with great economic and ecological value, although few phytotoxic studies have been conducted with species from this deciduous tree family. There is evidence that industrial emissions in St. Louis, Missouri caused injury to several woody, deciduous tree species, ranging from the highly sensitive Chinese Elm (*Ulmus parvifolia)* to the less sensitive Ginko tree (*Gingko biloba*) (Lanphear and Soule 1970).

Woody perennials harvested for their fruit, including wine grapes, citrus, and cherry trees, have also been subject to phytotoxicity tests with herbicides, and have shown great sensitivity of vegetative and reproductive endpoints under both greenhouse and field settings (Pountney and Swietlik 1988, Beck *et al.* 1991, Al-Khatib *et al.* 1993, Bhatti *et al.* 1995).

Fagaceae (beech family) is a very important ecological species that comprises the oaks, beeches, and chestnuts. The PHYTOTOX database (Fletcher, 1988) has blackjack oak (*Quercus marilandica*) tested on several occasions. Incident reports have shown oaks to be sensitive from herbicide exposure.

Rosaceae species (rose family) is an important economic family with many fruit trees. The apple blossom also serves as valuable nectar and pollen sources for over 70 taxa of insects including various bees and wasps, beneficial parasitic hoverflies, and beneficial beetles. Most of these beneficial species also visit the hawthorn tree as well (Breeze et. al. 1999).

Rose plants were irreparably injured from low dose herbicide spray drift (e.g., chlorosis, leaf wilt and reduced growth) (Al-Khatib *et al.* 1992c). Beck *et al.* (1991) found that deformed *Citrus* fruits were associated with chlorpyrifos application in Southern California groves. In another study, wine grapes were exposed to simulated low dose spray drift from various herbicides (Al-Khatib *et al.* 1993). All herbicides in this study injured grapes at 1/100 the maximum use rate, with 2, 4-D causing the greatest injury. Adverse effects were apparent in both vegetative and reproductive structures. Grape plants in all of the concentration doses except lower rates of bromoxynil and glyphosate produced fewer and smaller berries than control plants. These studies clearly support the consideration of woody perennial species as potential test species for phytotoxicity tests.

Tissue culture testing has been recommended as a useful method to determine phytotoxic effects in slow-growing and difficult to measure woody perennials, including forest (canopy and understory) and wetland species (Fletcher and Ratsch 1991).

3.5 Reproductive Testing

EPA and PMRA are proposing reproductive testing at Level I and more focused species testing for reproductive effects at higher Levels if needed.

Although many of the studies in the literature relate to acute plant exposure, non-target plants are often located in environments of chronic exposure. There is a clear need to implement guidelines for the evaluation of pesticide and chemical toxicity on reproductive structures (e.g., flower number, fruit yield and seed set) which have significant ecological and economic value. Obrigawitch *et al.* (1998) concluded that there is a need for standardization of protocols for phytotoxicity tests. Efforts must continue toward conducting more field experiments, and that studies must be designed to expand beyond short-term response data toward an understanding of long-term consequences on reproductive yield, population and community dynamics.

According to Snipes *et al.* 1992, cotton plants exposed to less than half the application rate of quinclorac exhibited leaf strapping, elongated bracts, and malformed blooms. These injuries resulted in reduced cotton seed yield and were most pronounced in plants exposed to postemergence applications. In another study, tomato plants repeatedly exposed to small levels of quinclorac spray drift exhibited poor fruit set due to excessive bloom abortion, bloom shedding, and abnormal vegetative growth (Bansal *et al.* 1999). It is clear that phytotoxic effects on yield could have economic consequences; however, very few studies have been conducted to quantify these effects (Taylor 1999).

Fletcher *et al.* (1995,1996) showed that chlorsulfuron reduces the yield of peas, canola, cherry tree, sunflower and soybean “without causing major, easily recognized damage to foliage such as chlorosis, deformed leaves or reduced stem height.

Sulfonylureas caused a significantly higher level of foliar damage to roses at a dilution of 1/100th the label dosages than 2,4-D (Al-Khatib *et al.* 1992c). Conversely, 2,4-D caused a significantly higher level of foliar damage to grapes than sulfonylureas at 1/100 of the label dose (Al-Khatib *et al.* 1993). Chlorsulfuron sulfonylurea caused death of shoot tips and flower malformation on roses at the 1/100th dosage whereas 2,4-D did not (Al-Khatib *et al.* 1992c). Obrigawitch *et al.* (1998) reviewed field studies of the effects of sulfonylureas on yield and growth of non-target plant species. They stated that visible symptoms of injury are a more sensitive endpoint to chemical exposure than reproductive yield and therefore reductions in plant yield or quality should usually be accompanied by visible injury symptoms. The observations by Fletcher et. al. (1993, 1996, 1997) seem to contradict this hypothesis by Obrigawitch *et al.* (1998) .

In a review of Obrigawitch *et al.* (1998), Taylor (1999) concluded that the actual threshold for effects on non-target species was 0.005-0.001 times the application rate, which is several orders of magnitude lower than the threshold cited in the paper (0.01 times the application rate). Taylor concluded that adverse yield to non-target plants can occur well within the range of non-target herbicide exposure, contrary to the conclusion of Obrigawitch *et al.* (1998).

It is important to recognize long-term consequences of adverse effects on reproduction, particularly in annual plants, where reductions in seed production and viability not only impact individuals, but can greatly impact population and community dynamics. Fletcher *et al.* 1995 found that chlorsulfuron reduced flower and pod production of pea plants without adversely affecting their height or appearance Clearly, the adverse effects of this herbicide would have been overlooked if reproductive parameters had not been examined. Reproductive endpoints that can be measured in phytotoxicity tests include flower number, seed number, germination viability, and fruit yield.

More recently, research has been conducted to measure the adverse effects of the sulfonylurea herbicide metsulfuron methyl on certain wetland plants. Significant reduction in pod yield occurred to *Sinapis arvensis* L. (wild mustard) at 1% of the label dosage, while other species had significant yield reductions at 10% of label dosage (Boutin *et al.* 2000). Spray drift at the time of aerial pesticide application averages 5 to 15% of label dosage (Teske *et al.* 1997). Boutin *et al.* (2000) concluded that plants in the Fabaceae and Brassicaceae families would be most at risk from small doses of metsulfuron methyl due to off target drift.

Studies assessing adverse yield effects to cherry trees resulted in yield reductions as great as 80% at 1/500th the label dosage (nominal diluted concentrations) of chlorsulfuron herbicide (Fletcher *et al.* 1993). Cherry yield reduction was confirmed in a follow-up study at similar dosages (Bhatti *et al.* 1995). A dosage of 1/10,000 the chlorsulfuron label rate (nominal diluted concentrations) for wheat reduced yields of soybean, pea, canola, and sunflower in greenhouse studies (Fletcher *et al.* 1995, Fletcher *et al.* 1996).

Tests examining reproductive parameters are more costly than the vegetative tests (which takes up to 24 days) because of the time they take. Plants tested in the pea experiment described above were germinated from seed, and reproductive parameters were examined 45 days later. If there is

an interest in reducing costs by minimizing the experimental duration, these tests could be conducted with older plants, using initial plant weights as a covariant to account for variation in plant size unrelated to herbicide treatment. Rahman (1989) compared the results of two bioassays and found that turnip seedlings with well-developed roots were more sensitive to sulfonylurea herbicides than newly seeded plants, due to the increased herbicide bioavailability that was associated with the improved root-soil interface in developed plants. These experiments may require a greater initial time investment during setup and maintenance, but results can be obtained in a matter of weeks.

Zwerger et. al. (2000) tested canola, oat, lamb's quarters, and slender meadow-foxtail with three herbicides. Endpoints measured included fresh and dry weight, seed production, thousand-grain seed, seed viability, and germination capacity. All of these endpoints are reproductive endpoints except for the fresh and dry weight which is an acute endpoint. One of the significant problems encountered during this study was controlling aphids without the use of insecticides for a season long study. Infestation problems caused great variability in the data. EFED has approved protocols for reproductive field studies using fungicides and insecticides on crop species.

Final plant yield may provide useful phytotoxic information. Yields of sugarbeet plants were significantly reduced when exposed to 0.5 oz/A of 2, 4-D (Dexter and Fisher, 1979). Sunflower plants also experienced significant reductions in yield with increasing 2, 4-D exposure. In cases where reproductive yield is to be determined, seed germination trials can be conducted as a further step to determine the extent of any maternally-passed phytotoxic effects onto offspring.

A field population of Dyer's woad plants (weedy members of the Brassicaceae family) were exposed to herbicide treatments, and pod production, seed production, and seed germination were measured Asghari and Evans 1992b). The effects of metsulfuron methyl herbicide on seed formation and pollen viability of Dyer's woad was studied. Viable pollen was reduced by 42% 7 days and 82% 12 days after foliar exposure at 3 g/ha. At 5 g/ha, viable pollen was reduced by 25% on day one and by 94% on day 12 (Asghari and Evans 1992a). Seed development was reduced by approximately 30% at 3 g/ha and by approximately 60% at 5 grams/ha (Asghari and Evans 1992b). Development was stunted in treated plants, leaves were discolored, and fruits withered and dispersed before seeds matured. Average seed production was reduced with increasing treatment concentrations and seed viability and germination were negatively related to herbicide rate.

The 1996 SAP solicited suggestions for field testing beyond acute dose response testing to determine effects on reproduction and population-level impacts. The panel acknowledged that short-term laboratory tests on vegetative growth are not sufficient to address these changes. However, it recognized the lack of guidelines to assess tests of this nature. Some suggestions for reproductive tests include life cycle bioassays for fast cycling species, such as *Arabidopsis thaliana* (mouse-ear cress or thale cress) and *Brassica rapa* (canola) (Shirazi *et al.* 1990, Fletcher and Ratsch 1991, Smrchek *et al.* 1993). *Arabidopsis* has been shown to be an ideal test species for multi-generational studies due to its small size, short life cycle, large seed production, numerous genotypes, and ease of culture. Herbicides altered flower development, preventing

pollination in *A. thaliana.* The sensitivity of this species to several herbicides was much greater than that shown in previous studies with radish, barley, soybeans, and bush beans exposed to the same herbicides (Ratsch *et al.* 1986). There is a clear need to implement a standard protocol for a life cycle test with a fast-cycling species, such as *Arabidopsis thaliana,* which has abundant information regarding its biology and response to stress (Rodecap *et al.* 1981). A plant life-cycle bioassay was developed for EPA in 1980 to determine chemical phytotoxicity using *Arabidopsis thaliana* (Rodecap *et al.* 1980). However, the protocol has not been incorporated into current phytotoxicity guidelines. There is one disadvantage to conducting this study ---- the seeds are very small (like powder), can scatter very easy, and are difficult to handle. Multi-generational tests with fast life-cycling annuals (e.g. *Arabidopsis thaliana* or *Brassica rapa*) may provide data for assessing population dynamics. Marrs *et. al.* (1997) reported a microcosm study in which flower numbers, seed numbers and germination viability were successfully measured. In this study, seed production of *Geum urbanum* (herb-bennet) was adversely affected from MCPA exposure.

The EPA and Canada are recommending that Level I include two reproductive studies using cherry and *Arabidopsis thaliana* or canola/rapeseed and that protocols exist for these studies. If these species are found to be reproductively sensitive, EPA and Canada are uncertain about whether to ask for additional species to be tested at higher levels.

3.6 Multispecies Testing

Protocols exist for multiple-species testing and/or monitoring at Level IV. Multiple species tests typically focus on native plant populations and communities near pesticide or chemical discharge area. These studies can be small or large in scale depending on the distribution and use of the toxicants and on the total acreage impacted.

Tomkins and Grant (1974) showed that sensitivity of two monocot species, Kentucky bluegrass and timothy, to auxin herbicide treatments differed depending on the secessional status of the field where species were tested, with species showing susceptibility to treatment in pioneer communities, and resistance to treatment in mature fields.

Marrs and Frost (1997) conducted a terrestrial microcosm test where artificial communities containing eight dicot species (with and without a grass species) were exposed to drift from several herbicides. Endpoints measured were species yield, flowering, seed production, seed viability, and the occurrence of new species invasions. Results from this test provided information on relationships between plant yield, species composition, and distance from herbicide spray, which could be used in management decisions about no-spray buffer zones. The investigators in this study concluded that the microcosm test is perhaps “the most effective way of investigating the cumulative effects of plant communities to successive exposures to spray drift.”

It is well-documented that herbicide-induced changes in growth and reproduction of individual plants can have consequences at the population- and even community-level (Freemark and Boutin

1994). Tomkins and Grant (1977) found that auxin application reduced community diversity for the long term through stimulation of the number of monocot grass species. Often times, environmental heterogeneity interferes with the determination of phytotoxic effects in field studies. In an effort to overcome this variation, Pfleeger and Zobel (1995) monitored plant cover and biomass of different-sized field plots exposed to low concentrations of various pesticides. They found that percent plot cover was a better predictor than biomass of plant community dynamics.

Marrs *et al.* (1991) investigated the impact of mecocrop spray drift on the growth and yield of multiple plant species grown in a terrestrial microcosm environment. Most species, including tall buttercup (*Ranunculus acris*) (a potential terrestrial species for phytotoxicity testing), displayed damage symptoms at 4 m from the spray source. Furthermore, most species showed suppressed flowering at distances up to 2 m from the source. Approximately fifty percent of the species tested showed reduced performance one year after exposure. These changes led to differences in population size of the different species which eventually altered community structure. In another study, five species (two wetland, two terrestrial and one found in both habitats) were exposed to metsulfuron methyl (Boutin *et al.* 2000). All species were adversely affected by the herbicide, showing reductions in biomass, number of nodes and lateral branches. Reproductive yield was reduced for both wetland and both terrestrial species, but not for the mixed species. The authors concluded that all species would be adversely affected by low dose exposure to metsulfuron methyl. All of the species in this experiment - monkey flower (*Mimulus ringens*), nodding burr-marigold (*Bidens cernua*), corn-mustard (*Sinapsis arvensis*), bean (*Phaseolus vulgaris*), and barnyard grass (*Echinochloa crusgalli*) have been proposed as potential species for phytotoxicity testing (see Appendices 13,14). If these species display different degrees of herbicide sensitivity in their natural environments, population dynamics and community structure of co-occurring species could be affected. These examples stress the necessary and invaluable information that multiple species studies provide about herbicide-induced changes in population and community dynamics that is unobtainable with single-study designs.

3.7 Monitoring

The EPA and PMRA believe that post-registration monitoring of pesticides is necessary to reduce uncertainty when high potential for adverse field effects is identified.

Standards should be developed for the monitoring of phytotoxic effects during post-registration periods. There are always factors of uncertainty when assessing herbicide risk. Many phytotoxicity tests are conducted under controlled lab/greenhouse conditions and their results are generalized to more variable, field situations. For this reason, monitoring non-target plants in use areas during post-exposure periods can provide beneficial data to create more certainty in the risk assessment and to provide better mitigation based on scientifically monitored data (Kapustka *et al.* 1996).

The effects of spray drift were tested on a range of species of conservation interest at various distances downwind from the spray source (Marrs *et al.* 1989). Lethality, damage, and flowering

were assessed for plants exposed to "Finesse" and glyphosate herbicides sprayed during different seasons. Autumn-sprayed glyphosate applied at both low and high rates produced damaging effects on self-heal (*Prunella vulgaris*) (a member of the Lamaiaceae family and identified potential terrestrial species for phytotoxicity testing) up to 20 m from the spray source. High rates of glyphosate also suppressed flowering in purple foxglove (*Digitalis purpurea*) up to 10 m from the spray source. For many species, plant damage and suppression of flowering were still apparent at distances beyond where lethal effects stopped.

Bioindicators may be useful tools for assessing and monitoring the potential for damage due to spray drift and the extent of long-range transport during post-registration periods (Hall *et al.* 1996). For example, chlorinated insecticides were found as residues in mango leaves from West African trees believed to be exposed through contaminated soils and airborne drift (Bacci *et al*. 1988). Bean, lentil, and pea plants placed at various herbicide exposure sites in the field for weekly intervals were shown to be sensitive biomonitors of pesticides (Al-Khatib *et al.* 1993). Symptoms developed, including chlorosis, leaf wilt, necrosis and plant death and became more severe with exposure to higher herbicide concentrations.

Bean, corn, and pea plants were grown in pots at various locations throughout south central Washington to determine their sensitivity to herbicide residues and their utility as sentinel biomonitors to detect spray drift (Felsot *et al*. 1996a). Chlorotic spots were not only observed on these sentinel plants, but also on lilacs, roses, cherries, and apples that were growing nearby. Furthermore, symptoms persisted after spraying stopped. The authors attributed this phenomenon to the transport of secondary herbicide drift through volatilization, wind erosion, and/or precipitation and also noted that this implies that nontarget plants may experience chronic exposure to low levels of herbicide residue. This study provides evidence that exclusive reliance on atmospheric monitoring to assess risk could result in an erroneous risk assessment of spray drift for registered herbicides and that biomonitors would be a useful compliment to monitoring efforts.

Adverse plant symptoms in the field could be caused by many environmental stresses, such as drought and pests, and are often difficult to attribute only to herbicidal effects without analysis of foliar tissue (Putnam 1999, Al-Khatib *et al.* 1992a). However, a study conducted under controlled conditions in the laboratory showed that bean plants were accurate biomonitors of chlorosulfuron spray, with plants exhibiting leaf chlorosis up to 10 km from the spray source (Felsot *et al.* 1996b). Bean plants were adversely affected by chlorosulfuron spray drift to distances of 100 meters and occasionally 500 meters, through the appearance of chlorotic spots.

The persistence of ALS inhibiting herbicides can vary from a few days to years depending on soil type, soil moisture content, soil temperature, and soil pH. The soil degradation of many ALS inhibiting herbicides is slowed by low moisture, cold temperatures, and higher pH (Whitcomb, 1999). Brewter and Appleby (1983) found that chlorsulfuron herbicide reduced alfalfa growth when mixed in soil at rates as low as 25 ppt and that sugar beets were affected 26 months after application. A soil analytical detection level of 1.0 ppm is approximately 40,000 times higher than the 25 ppt adverse effect level (Whitcomb, 1999).

In another study, nine rotational crops were seeded up to seven years in a wheat field following chlorsulfuron application (Moyer *et al.* 1990). Yield of all nine crops were lower than the untreated controls one year after application. Four years following application over 50% of the crops showed reductions in yield. Legumes, potatoes, flax, and sugarbeets were susceptible to damage by very low levels of chlorosulfuron residue (0.01 ng/g soil or less).

Growth of rotational crops was determined following application of sulfonylurea herbicides and the response of alfalfa to these herbicides was also compared between field and growth chamber experiments (Moyer 1995). Alfalfa, sugarbeet, and lentil plants were injured one year following low herbicide application rates. Biomass yields were also reduced in alfalfa, lentil, canola, and corn. Growth of alfalfa was reduced 50% one year after application (<1 g/ha) in the field. Growth chamber studies confirmed the field results. All of these examples emphasize the potential for residual phytotoxic effects long after application has ceased and stress the need for post-registration monitoring efforts in native plant areas adjacent to use sites.

In a separate study, factors influencing dicamba herbicide vapor drift were examined in field and growth chamber studies. While the less volatile salt formulation of dicamba was less injurious to soybeans in the greenhouse, it was volatile and injurious to soybeans in the field. Factors such as solution pH, rainfall, temperature, and relative humidity were studied in growth chambers for their ability to enhance herbicide injury to soybeans. The researchers concluded that the poor growth chamber to field correlation for the DEOA salt of dicamba warranted further study of the effects of relative humidity on vapor pressure (Behrens and Lueschen, 1979). The researchers were able to eliminate influences such as solution pH, rainfall, and temperature affecting the volatilization of dicamba DEOA salt in the greenhouse and to focus on the influence of relative humidity in the field. The field observations in turn stimulated additional growth chamber research on relative humidity effects on formulation volatility. This study showed how greenhouse and field studies complement each other and why ecological studies need to be generated in field settings as well as greenhouses when the lab or field relationship is uncertain.

Extensive research on the effects of air pollution on plants, appropriate measurement endpoints, and the relationship of measurement to ecosystem endpoints may have direct relevance to pesticide toxicity. Taylor (1999) observed that some air pollutants can interrupt the normal flow of carbon in a plant, resulting in shifts away from root growth, shoot growth, or grain production to respiration. For example, if the relative partitioning of carbon among plant tissues remains the same, a 10% increase in total carbon lost to respiration might result in a 15 to 30% reduction in grain yield (Heck *et al.* 1988). Air pollution is also known to affect plant biodiversity and ecosystem processes (Barker and Tingey 1992).

4 APPENDICES

Appendix 1: ILSI Workshop Summary and Recommendations

In 1999, the OPPTS sponsored an international workshop titled "Impacts Of Low-dose, High Toxicity Herbicides on Unintended Plant Species". This workshop was attended by 40 scientists from academia, government, industry, and ecological groups. Five research papers were presented for discussion at the workshop. This workshop provided a forum for discussion and analysis of public concerns regarding low dosage, high toxicity herbicides. Both the manuscripts and workshop were guided by the following questions:

- What are the current methods used to quantify the exposure to unintended/nontarget plants from long-range transport of low-dose/high potency herbicides and how can they be improved?
- What needs to be done to evaluate the biological effects of chronic low-dose exposures and acute low-dose exposures?
- Are the current species and testing endpoints in laboratory and greenhouse studies sufficient for evaluating the effects of low-dose, high potency herbicides on unintended or nontarget plants?
- Is the current technology sufficient to adequately evaluate unintended or nontarget plant impacts at the individual, community and ecosystem levels?
- What data and methods are needed to better characterize the likelihood, magnitude, and severity of adverse ecological effects of these herbicides on unintended or nontarget plants
- How can we better identify and characterize risks to native plant population structure at the ecosystem level?

The workshop papers are to be published later in 2001 by The Society for Environmental Toxicity and Chemistry (SETAC).

The five papers presented at the ILSI workshop were:

1. "Low Dose, High Toxicity Herbicides: An Historical Perspective of Environmental Concerns to Frame the Issues", Anne Fairbrother and Lawrence A. Kapustka.
2. "Nontarget Aquatic Plant Effects Of Acetolactate Synthase Inhibiting Herbicides", Hans G. Peterson.
3. "Ecological Risk Characterization of Low Dosage high Toxicity herbicides", George E. Taylor, Jr.
4. "Exposure To Low-Dose, High Toxicity Herbicides - Literature Review", Donald Waite.
5. "Non-target Terrestrial Plant Effects of Low Dose, High Toxicity Herbicides", Bruce Maxwell and Rebecca Weed.

In the workshop a focus was placed on the ALS inhibiting herbicides, including imidizolinones, sulfonylureas, triazolopyrimidine sulfonamides and pyrimidyl thiobenzates, as much less data exist for other low-dose, high potency herbicides. However, the principles discussed might apply

equally to non-ALSase inhibiting compounds. The questions posed to both the authors and the workshop participants were intended to frame the discussions on the current state of scientific knowledge and provide insight and direction to future approaches to risk assessment of these compounds.

Alleged incidents of low-dose, high potency herbicide drift from application sites to distant nontarget sites are of concern. The veracity of these reports has been challenged; however few data are available to either support or refute such reports. In particular, a series of alleged incidents were reported in the state of Washington. Workshop participants were in agreement that there is no current or soon to be expected testing methodology that can prevent unforeseen incidents, such as have been reported on cherry trees in the Northwest. An uncommon combination of natural and anthropogenic events may have occurred to result in the reported impacts. Modelling and testing methodologies do not currently exist that will allow us to predict when such a confluence of factors might be expected to occur. Indeed, test methods currently used for the detection of compounds in plant tissues, soil and sediment cannot measure residues at the very low levels encountered with these herbicides.

Several new methods for detection of exposure were discussed but inherent problems with each hinder the practical application of these methods for routine testing at this time. High performance liquid chromatography with detection by mass spectrometry (HPLC/MS) is considered the most sensitive and specific methodology for detection of the low-dose, high potency herbicides. Under certain conditions, this methodology also may allow for quantification of exposure. However, HPLC/MS is expensive and issues of the tests' sensitivity remain. Although HPLC mass spectrometry costs have begun to decline, maintenance and technical skill requirement factors are high.

Immunoassay methods exist, but currently are not very specific and cross-reactivity is a confounding factor. The costs associated with the two test systems, immunoassay and HPLC/MS, are both high although the cost streams are significantly different. Immunoassay kits are relatively inexpensive on an individual basis, however very few field samples can be tested with a kit due to the necessary number of replicates and control dilutions. The high cost of reagents and controls keeps the test cost high at an application level; running a very large number of samples is expensive.

Reasonably, analytical methods should be capable of detecting the compounds at concentrations that are predicted by bioassay results to cause effects. However, ideal analytical methods also should be able to detect levels that cause persistent or significant effects under natural conditions. Ultimately, analytical methods that are able to detect very low levels will be needed to consider the function of distance from the site of application, and degradation processes and rates in plants. The general understanding from the workshop participants is that the analytical technology is not yet available for practical widespread application, but these newer methods are being refined and may provide the necessary sensitivity and specificity required in the foreseeable future, at reasonable expense.

A confounding problem with detection of exposure relates to the quick degradation by plants of the parent compound. Within hours after exposure, the plant already has significantly reduced the amount of parent compound so collecting plant tissues for analysis may not be the most efficacious approach to determining exposure to non-target and unintended plants. Although soil microbes degrade the compounds as well, concentrations more representative of exposure might be found by testing soil rather than by testing plant tissues. Metabolic by-products of these herbicides might serve as biomarkers of exposure, but some metabolites are not specific to the low-dose, high potency herbicides. Specific amino acid chains resulting from exposure to ALSase inhibitors might for example provide "signature fingerprints" of exposure to these compounds. Analytically, if an amino acid is found to be a signature metabolite, this certainly provides a more cost-effective diagnostic. Unfortunately, while signature metabolites may correlate well with exposure, they most likely will not correlate well with damage. In addition, metabolite levels in plants (e.g., alpha keta butyric acid) may increase with the environmental stresses associated with exposure under natural conditions, confounding the uncertainty in extrapolating from the laboratory to the field. Unless the rate of metabolite formation can be well characterized, it will not be possible to determine the timing of exposure. Even if signature amino acid metabolites for the ALSase inhibiting herbicides are found, these will only indicate that a plant was exposed to an ALS inhibitor. It would not indicate when, how much, or which one. It is then necessary to correlate the presence of these biomarkers with some measure, perhaps in soil or other environmental media that will correctly identify the chemical and allow the final link to causality. In terms of a forensic diagnostic, such methods may have applicability but it still is necessary to be able to actually measure the compound to confirm correlation of effects to exposure. However if signature amino acid chains are tested for and not found to be present, this is indicative of non-exposure. Chemical or biomarker tagging of the compound might provide a feasible mechanism for detection of exposure; timing of exposure and extent of adverse effects would likely not be obtainable through this approach.

A suite of plant species currently is used for laboratory, and in some case field, testing of low-dose, high potency herbicides in pre-registration risk assessment. However, this suite of species was not developed specifically for these compounds, and generally is not representative of all aquatic and terrestrial species potentially exposed under conditions of use. The current screening level testing is a simplistic approach; running through a battery of tests under conditions that maximize activity, then finding the lowest concentration for effect on the most sensitive species. The intent has been to find a point below which there is confidence that no detrimental effects will occur.

There was lengthy discussion of the representativeness, or lack thereof, of the suite of species used in the test batteries. For example, although upwards of 50 species from a variety of plant families might be used in pre-registration efficacy studies, the species used are almost exclusively crop and weed species. The endpoint of these efficacy tests typically is plant death, and is used to reflect a predicted level of control (e.g., 80%). Dose-response assessment cannot be conducted on this type of data. In addition, given that the tests are conducted for efficacy determination, they may not adhere to standardized GLP protocols. Review of efficacy data however, might aid in identifying plant families likely to be more sensitive and therefore serve as a basis for establishing

appropriate test species.

Risk assessment of ALSase inhibitor compounds has not expanded much beyond a tier 1 or screening level and there is a lack of methods to extrapolate beyond these data to other life stages, species or to the ecosystem level. Development of approaches to higher level testing and extrapolation for plant species are well behind that of avian and mammalian risk assessment. Given this, extrapolation methods for plants represent a significant void for risk assessment on plant species.

It is unclear whether more test species are necessary or warranted to evaluate adverse effects of the low-dose, high potency herbicides in laboratory or field studies, however, new and innovative methods for determining the appropriate species to evaluate were discussed. These tests are protective, but not predictive. Additional information is needed to allow for prediction of effects under natural conditions and hence protect nontarget plants and ecosystems. There may be only a few species in a community responsible for the maintenance of the structure and ostensibly the functioning of the community. If the goal is to conserve the ecosystem within which these chemicals will be used, the aim should be to protect the ecologically important species, including the primary drivers of the ecosystem. This information can be used to narrow the focus of potential species for evaluation of impacts. A broader approach is to consider potential environmental impacts on a regional basis and take advantage of new methodologies, such as GIS and drift models, to determine a priori which natural or agricultural ecosystems might be "at risk" of exposure to a pesticide based on the expected or predicted post-registration use.

For example, it is possible to examine the spatial distribution of the particular target crop on electronic maps, overlay those maps with windspeed and weather data, and again, overlay with the spatial distribution of rare endangered species. Through all these layers can be seen the area(s) potentially at risk of high exposure and the species of interest in these areas. Incident reports may be used to identify or pre-locate areas for GIS application when considering a new compound from a class similar to the incident compound. Data layering exercises like this do not reduce the uncertainty associated with the individual data used in the study, but this approach is amenable to use in probabilistic analysis. Probabilistic analysis may allow for movement away from the necessary identification of the most sensitive species. Species may instead be selected for functional and/or physiological characteristics allowing for better prediction of environmental effects.

Systems identified as "at risk" might then be grouped based on similarities of structure and function. Within groups, plant species might then be identified, such as key species, potentially more sensitive species, representatives from various trophic levels, etc., for consideration in a risk assessment. Under current approaches, these species would likely be represented in laboratory and/or field studies by the small suite of available surrogate species. Initially, this approach would generate different lists of test species for different compounds but it would take an equally immense effort to identify the 20 species that represent a one-size fits-all test for every compound; and it is highly unlikely that such a definitive list could be derived. Workshop participants discussed an approach that could be used to integrate the information from GIS site-based studies

with a generic set of criteria to determine the most appropriate suite of test species for use.

Once a list of criteria is established, the suite of test species developed has to include at least one species that satisfies each of the criteria. One criterion might be "present in habitat;" at least one species present in the habitat(s) predicted to be exposed under field application and use should be included or represented in the test. At least one species that serves as an important component of the food web in the system should be tested, as well as at least one species that serves another important process in the ecosystem, such as nitrogen fixation. A species that exhibits a reproductive response to at least one class of herbicide at concentrations encountered in the field, using models that predict concentrations if actual data are lacking, should be included. Another criteria might be a close taxonomic and/or physiologic relationship to the target weed. But one criterion is very important: it must be possible to grow the plant under practical experimental conditions. The idea here is not to identify a list of plants that each meets all criteria but rather to identify a list of plant species that collectively meets all criteria. This criteria-based exercise is not meant to exclude any of the current standard tests and might be more appropriately applied in a later tier process using the first criteria coupled with the regional or "at risk" ecosystem-based approach. For example, once an "at risk" system is identified, specific species from these habitats that meet the criteria may be considered.

A straw set of criteria for aquatic plant test species were developed during the workshop discussion for illustrative purposes:

- Present in habitat/potentially affected/proximity to application site
- Important in the system's food web
- Important in some ecological process (e.g., nitrogen fixation, habitat/soil fixation).
- Little intraspecific variation in response.
- Sexually reproducing and exhibits sexual reproduction response to some herbicide(s).
- Asexually reproducing and exhibits asexual reproduction response to some herbicide(s).
- Roots into substrate (for sediment/soil exposure).
- Similar physiology as target weed/plant.
- Can be worked with under laboratory or field conditions.

A potential problem with this approach is that 10 or 15 species might meet three-quarters of the criteria and the most sensitive species might still be missed. For instance, the terrestrial solanaceous plants nightshade and tomato, would likely meet the same criteria but the herbicide effects might be seen at much lower levels of exposure in the tomato than in the nightshade. However, this is a problem in the current testing protocols as well.

It may be that one or several of the current test species satisfy all of the above criteria. If not, additional species might need to be considered to provide the missing information. A potential problem with this approach is that if a species has not been used extensively, there may not be Good Laboratory Practice (GLP) protocols for its use. Adding species to testing protocols or developing new testing protocols is a nontrivial exercise. It can take two or three years to develop the protocol, ring test to get the protocol standardized, and apply GLPs. It also is

possible to conduct nonstandard tests under GLP conditions. The decision must be made whether the added information collected by testing these additional species exceeds the loss of "standardization" of protocol. Over time, GLP protocols might be developed if new species become commonly used.

Workshop participants were in agreement that the scientific community should and could improve upon the plant physiology database in this area through targeted research efforts. Questions to be addressed include: What do we know about how plants respond to different classes of chemicals and, in this case, what do we know about their ability to degrade these kinds of chemicals? What do we know about differences across plant families in terms of mechanisms of chemical uptake? What are the physiological processes in the plants that are the most sensitive to ALSase inhibitors? Basic research may be needed in order to understand how plants take up and respond to chemicals. A research program might be designed that could answer these questions in three to five years.

Workshop participants also agreed that for practical consideration, a comprehensive examination of extant data should be conducted prior to the generation of new data. Particular attention should be given to evaluation of the validity of these data. This process can be used to highlight significant data gaps and identify what studies need to be repeated to validate the existing data. Data also may be rejected for various reasons in this process, such as non-reproducibility. In addition, a list might be compiled of the aquatic and terrestrial species known to be sensitive to these herbicide classes, along with the available information on sensitivities. Is floral tissue most sensitive, or seed, or reproductive endpoints, or vegetative growth? With this list, it may be possible to develop pertinent test endpoints, whether for deterministic or probabilistic analysis.

With the development of new or additional endpoints is linked the issue of extrapolation from laboratory or field test-based endpoints to those of interest in natural or agricultural ecosystems. There is a significant lack of data and methods for extrapolation of the current testing data for adequate evaluation of the impacts of the low-dose, high potency herbicides on terrestrial or aquatic species in these ecosystems. Protocols are needed for reproductive effects studies and currently, few studies have considered early plant growth effects and effects on reproduction. Attempts to develop protocols for mature plants such as cherries and pears, as well as protocols for other vascular plants, including economic and native species should be made. In concert, methods are needed to extrapolate from: laboratory, micro- and mesocosm tests to the field; crops to native plants; seedlings to mature plants; species tested to species untested; and terrestrial plants to aquatic plants. Another important point that was brought up was whether data generated from testing terrestrial plant species can be applied to rooted aquatic macrophytes.

Finally, there is a significant need for analytical methods capable of detecting the chemical at concentrations predicted on the basis of the bio-assays to effect sensitive species. Also methods for evaluating deposition mechanisms and distribution on plants or canopies, and for comparing the amount of drift relative to the amount applied are needed. It may be possible to incorporate such methods into existing drift models. The resulting data must be coupled with information on exposure as a function of distance from the site of application with real time measurement, to

correlate exposure with effects with confidence. There was discussion but no agreement among the workshop participants that post-registration and/or post-application monitoring might provide a means by which to theoretically "validate" the safety determination made as a result of testing.

The ILSI workshop participants identified several specific basic research needs:

- Useful Residue Detection Methods - Low dose, high potency herbicides have great potential to move from the treated site to non-target areas during and after application. Current analytical methods are incapable of detecting low dose herbicides at residue levels that may injure plants, rendering foliar plant samples useless for assessment purposes. Field residue detection methods that are sensitive, specific and easily used are needed.

- Screening Methods Useful for Protection of Ecosystems - Pesticide screening tests that provide data necessary for ecological risk assessments are needed. Key plant species that represent ecoregions within Canada, the U.S., and Mexico should be identified and considered for testing purposes. GIS vegetation mapping of ecoregions that includes key ecological species, endangered and threatened plants, pristine areas, and possibly organic farms would be invaluable in this identification process.

- Probabilistic Risk Assessment Data Needs - The move from deterministic risk assessment to probabilistic risk assessment for pesticides requires an improved understanding of data uncertainties. Baseline research is needed to establish plant sensitivity ranges and confidence limits. Some uncertainty issues include: the adequacy of current test species to represent non-tested plant families and species; the predictive value of adverse effects on early plant growth (first 14-21 days) for extrapolation to adverse effects on reproduction and yield; the predictive value of adverse effects on plant growth observed in the greenhouse for extrapolation to adverse effects in the field; and the relationship of single exposures to multiple exposures or exposures to multiple toxicants. Also, baseline plant population data are needed to conduct landscape or population effects risk assessments.

- Mechanisms of Long-range Residue Transport and Plant Effects – Factors responsible for low-dose, high potency herbicide movement great distances from the target site of application must be identified and evaluated. The frequency and duration of exposures to non-target plants should be determined, and resultant acute/chronic effects on plants evaluated. Existing ozone plant toxicity research may have relevance to low-dose herbicide plant toxicity.

Appendix 2: Incident Reports And Monitoring

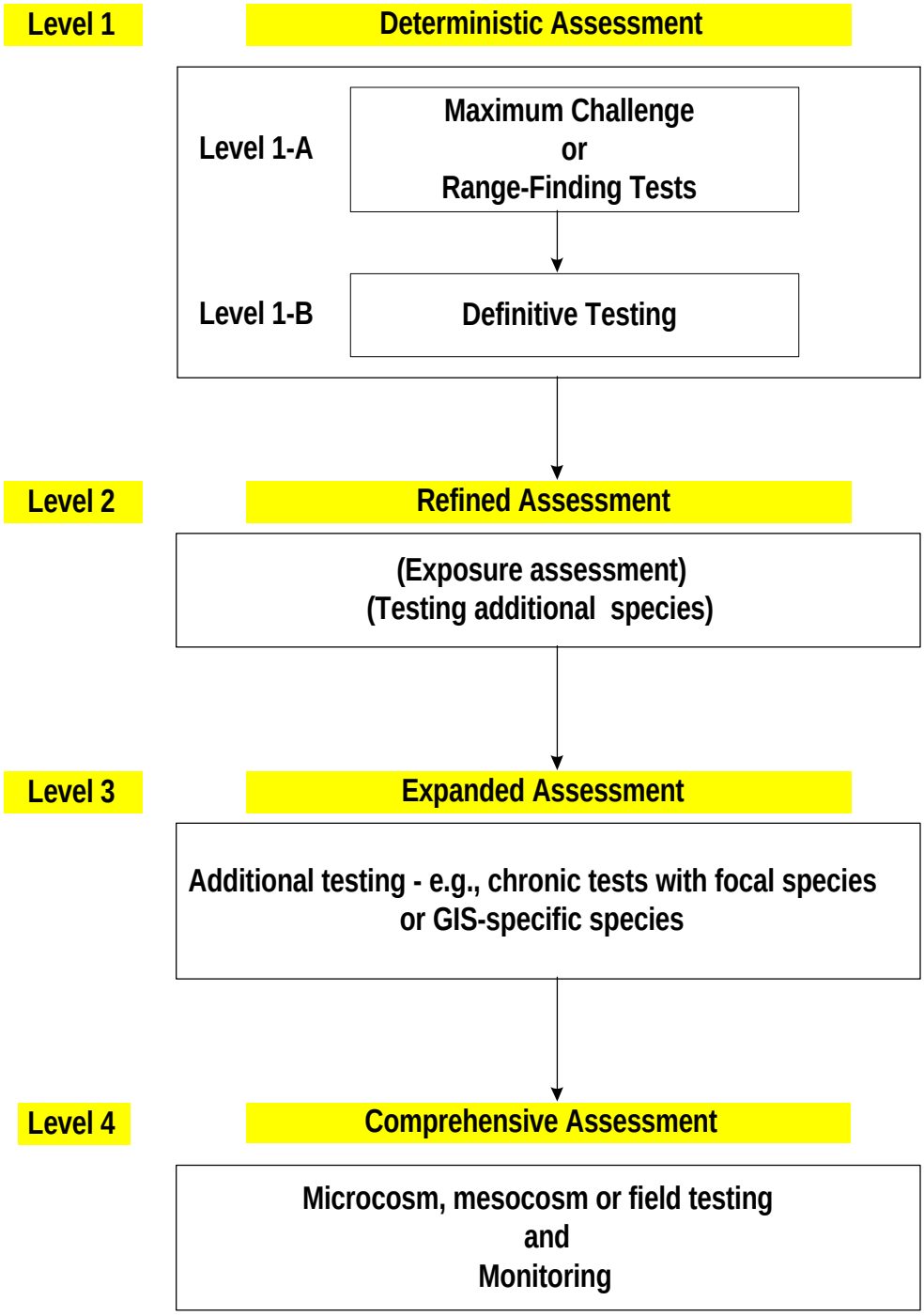
Low dose, high toxicity herbicides having herbicidal toxicity at levels below 50ppb are difficult to evaluate in the field. Both short range (adjacent land and out to 1 mile), mid range (1 to 5 miles), and long range (beyond 5 miles) transport can occur; and has been documented for some low dose herbicides. Analytical measurements have proven successful for field detections of quinclorac herbicide (Bansal *et al.* 1999). In 1998 an intensive air, soil, and plant monitoring study was initiated to determine the pesticide or pesticides responsible for reduced tomato yields miles distant from sprayed rice fields. High volume air samplers were set up to monitor a 5 mile distance between the rice fields and tomato growing area. No quinclorac applications were made within 5 miles of one of the air sampling sites. Up to 12 ppb quinclorac was found 5 miles distant. Tomato bioassay plants suffered excessive bloom abortion, poor fruit set and reduced fruit numbers or stunted fruits. Quinclorac has a low vapor pressure and is not expected to volatilize, however, movement was via vapors or wind blown soil (Bansal *et al.* 1999). Field tests using quinclorac have demonstrated 39% to 49% tomato injury at 0.01 to 0.1 the label dosage (Talbert *et al.* 1995).

Bioassay plants have been utilized to detect transport of the low dose sulfonylurea herbicides in the Horse Heaven Hills/Badger Canyon area of south-central Washington State (Fletcher, J.S. 1991c.). Sensitive bioassay plants such as lentils were grown in the greenhouse and placed at various locations distant from sprayed wheat fields because analytical methods are not available to measure these herbicides on plant tissue at levels that cause plant injury. Sulfonylurea symptoms were observed on bioassay plants placed 5 miles distant from spray sites. Injury occurred to plants placed upwind as well as downwind suggesting non-point air deposition mechanisms other than spray drift at the time of application. Further, the timing of bioassay exposures to sulfonylureas did not correlate with their application to wheat. The SU's have low vapor pressure, however, non-point source deposition may involve volatilization or condensation via air or soil particle transport (Felsot *et al.* 1996b).

A combination of visual inspection and experimentation was conducted by the ORD/WED Corvallis, Oregon laboratory following complaints of suspected sulfonylurea herbicide transport from wheat fields on the Heaven Hills plateau to cherry and apricot orchards 2 to 5 miles down wind in Badger Canyon, south-central Washington (Fletcher1991c). The Corvallis EPA research laboratory has conducted research to assess effects of sulfonylurea herbicides on annual and perennial plant species following application of 1/500th the SU label dosage of 1/8th oz./acre when applied at or within 2 weeks of flowering. The results of this study showed that at very low levels (as low as 1/500th the label dosage) the reproduction of cherry trees was reduced without visible disruption of vegetative organs (Fletcher *et al.* 1993). In a follow up study by Washington State University, the authors concluded that multiple exposures of a susceptible cherry cultivar to low levels of chlorsulfuron at full bloom and post bloom stage can reduce fruit yield and delay maturity of cherries while increasing fruit firmness (Bhatti *et al.* 1995). In further studies by the EPA/WED Corvallis, Oregon laboratory on crop yields, the low dosage sulfonylurea herbicide chlorsulfuron was compared at comparable label dosages with 2,4-D and atrazine herbicides.

Chlorsulfuron was found to be 100 times more toxic to the vegetative growth of plants than atrazine or 2,4-D. In addition, the sulfonylurea was also more toxic to plant reproduction when exposure occurred during flowering, seed set, or fruiting. (Fletcher *et al.* 1995, Fletcher *et al.* 1996). The author concluded that the yield reduction that chlorsulfuron may cause in crops such as soybean or canola would not be accompanied by visible injury or noticeable growth reduction. The cause of the yield reduction may go unnoticed in collected vegetation samples due to the inability to analytically find the sulfonylurea at such a low concentration in the environment. At similar dilutions (based on EPA registered labels), atrazine, 2,4-D, and glyphosate herbicides did not cause significant soybean or canola yield reductions (Fletcher *et al.* 1996).

Appendix 3: Overview of Level Progression for Plant Toxicity Testing



Appendix 4: Comparative toxicity of 16 herbicides to *Selenastrum capricornutum* (Fairchild *et al.* 1997).

Herbicide	Chemical class	96-h EC50 (µg/L)	95% C.I. (µg/L)
Alachlor	Acetanilide	6	36989
Metolachlor	Acetanilide	77	70-84
Atrazine	Triazine	235	189-281
Cyanazine	Triazine	27	25-30
Metribuzin	Triazine	43	40-46
Simazine	Triazine	1240	1088-1393
Chlorsulfuron	Sulfonylurea	135	109-161
Metsulfuron	Sulfonylurea	190	137-243
Diquat	Pyridine	80	64-95
Paraquat	Pyridine	559	471-646
EPTC	Thiocarbamate	6451	5455-7446
Triallate	Thiocarbamate	47	41-49
Bromoxynil	Benzonitrile	7762	6863-8662
Dicamba	Benzoic acid	36375	31309-41440
trifluralin	Dinitroaniline	673	594-751
2,4-D	Phenoxy	41772	37352-46192

Appendix 5: Comparative toxicity of 16 herbicides to *Lemna minor* (Fairchild *et al.* 1997)

Herbicide	Chemical class	96-h EC50 (µg/L)	95% C.I. (µg/L)
Alachlor	Acetanilide	198	80-316
Metolachlor	Acetanilide	343	187-872
Atrazine	Triazine	153	89-217
Cyanazine	Triazine	705	577-834
Metribuzin	Triazine	37	22-47
Simazine	Triazine	166	102-230
Chlorsulfuron	Sulfonylurea	0.7	0.5-0.9
Metsulfuron	Sulfonylurea	0.4	0.3-0.5
Diquat	Pyridine	18	37099
Paraquat	Pyridine	51	25-77
EPTC	Thiocarbamate	7512	1736-13,288
Triallate	Thiocarbamate	>10,000	
Bromoxynil	Benzonitrile	8065	3783-12,348
Dicamba	Benzoic acid	>100,000	
Trifluralin	Dinitroaniline	170	10-330
2,4-D	Phenoxy	>100,000	

Appendix 6: Test conditions for toxicity tests with bioluminescent marine dinoflagellates

A toxicity test using two species of marine dinoflagellates (*Gonyaulax polyedra* or *Pyrocystis lunula*) determines the effect of toxicants upon their bioluminescent capabilities. A cuvette containing the test material, medium and cells is placed into a darkened test chamber attached to a photomultiplier tube. The contents of the cuvette are stirred, the dinoflagellates bioluminesce and the generated light is measured by the photomultiplier tube (ASTM, 1997c). The testing conditions are summarized in the table below.

Parameter	Conditions
Test species	Unialgal culture of *Gonyaulax polyedra* or *Pyrocystis lunula*
Test type	Static
Test duration	4 to 7 days or longer for *Gonyaulax polyedra* or 4 hours for *Pyrocystis lunula*
Culture medium	Clean enriched natural seawater or artificial seawater
Incubation chamber	Constant temperature incubator
Temperature	19 ± 1 ˚C for *Gonyaulax polyedra* or 20 ± 2 ˚C for *Pyrocystis lunula*
pH	7.8 to 8.2 for *Gonyaulax polyedra* or 7.6 to 8.0 for *Pyrocystis lunula*
Salinity	33 ± 2 g/kg
Light quality	Cool-white fluorescent
Light intensity	1075 lux for *Pyrocystis lunula* and 4000 lux for *Gonyaulax polyedra* measured at the height of the test solution
Photoperiod	12 hr light and 12 hr dark
Test vessels	Optical grade disposable spectrophotometric cuvettes or clear borosilicate sample vials
Nutrient/test solution volume	100 ml
Timing of test	Measurements are conducted 3 to 5 hours into the dark phase
Number of cells per test vessel	2000 cells/ml
Number of replicate test vessels/concentration	5 or more
Measured water quality parameters	Salinity; pH in highest, middle, lowest test concentrations and controls
Measured endpoints	Change in light output
Statistical endpoints	IC50

Appendix 7: Test conditions for sexual reproductive tests with the red alga, *Champia parvula* (ASTM, 1998a)

For the reproductive test with marine red algae, female and male gametophytes are exposed to various concentrations of the toxicant in test chambers for two days under static or renewal conditions. At the end of the exposure period, female gametophytes are removed and incubated (if necessary) for an additional period of time in toxicant-free medium to allow development and germination of the zygote. At the end of the development period, the number of sexually produced structures is determined (ASTM, 1998a; Steele and Thursby, 1983; Thursby and Steele, 1984; 1986; Thursby *et al.* 1985). The testing conditions are outlined in below.

Parameter	Conditions
Test species	Unialgal culture of *Champia parvula* (or other marine macroalgae)
Test type	Static or renewal
Test duration	2 days in test material solutions, plus an incubation period (5 to 7 days) in toxicant free medium
Culture medium	Clean enriched natural seawater, artificial seawater or a 50 – 50 mixture
Incubation chamber	Constant temperature incubators or water bath
Temperature	22 to 24 ˚C
pH	7.8 to 8.2
Salinity	28 to 32 g/kg
Light quality	Cool-white fluorescent
Light intensity	40 to 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$
Photoperiod	16 hr light and 8 hr dark
Test vessel size	125 ml Erlenmeyer flasks or 200 ml polystyrene cups
Nutrient/test solution volume	100 ml
Life stage	Gametophytes
Length of plants	7 to 10 mm branches of female plants and 3 cm long branches of male plants
Number of plants per test vessel	5 females and 1 male
Number of replicate test vessels/concentration	3 or more
Measured water quality parameters	Salinity
Measured endpoints	Number of cystocarps per female, presence of necrotic tissue and morphological changes
Statistical endpoints	IC50, NOAEC or LOAEC

Appendix 8: Test conditions for toxicity tests with golden-brown algae

The marine golden brown alga, *Phaeodactylum tricornutum*, is recommended for toxicity testing. This species can be tested in the standard flask method (see Table 8), so long as either enriched saltwater medium or complete saltwater medium is used (ASTM 1997a, OECD 1984, US EPA 1971)

Parameter	Conditions
Test species	Uni-algal cultures of *Phaeodactylum tricornutum*
Test type	Static
Test duration/renewal	96 hours
Culture medium	AAP medium; axenically subcultured into fresh medium on a weekly basis
Temperature	24 ± 2 °C
pH	7.5 ± 1 for AAP medium
Light quality	Cool-white fluorescent
Light intensity	As measured adjacent to each test container at the surface of the test solution, 60 µmol•m^{-2}•s^{-1}
Photoperiod	Continuous (24 hr)
Test vessel size	250 ml Erlenmeyer flask
Nutrient/test solution volume	100 ml
Age of test plants	3 – 7 days
Number of cells per test vessel	1-2 • 10^4 cells/ml
Number of replicate test vessels/concentration	3
Measured water quality parameters	pH at start and end of each experiment
Measured endpoints	Cell number and growth rate
Statistical endpoints	EC25 and EC50

Appendix 9: Test conditions for toxicity tests with submersed vascular aquatic plants

The axenic aquatic macrophyte toxicity test with *M. sibiricum* is a replicable and repeatable system. Features of this toxicity test that enhance standardization include a chemically defined medium (Roshon *et al.* 1996), an artificial rooting substrate and inoculation of each tube with an axenic macrophyte segment. The artificial rooting substrate is extremely easily to prepare and provides consistent test results. It is a static, partial life cycle laboratory toxicity test that determines the toxicant effect over fourteen days. This species is easily cultured in test tubes in the laboratory. Every second day, plant shoot height can be measured to allow for the development of growth curves. Endpoints that can be measured include total plant height, root number and length, fresh weight, dry weight, plant area, amount of oxygen produced, change in membrane integrity, chlorophyll *a*, chlorophyll *b* and carotenoid content (Roshon, 1997) and chlorophyll fluorescence (McCann, 1997). Test parameters are summarized below.

Parameter	Conditions
Test species	Axenic *Myriophyllum sibiricum* Komarov
Test type	Static
Test duration/renewal	14 days
Culture medium	Modified Andrew's medium; axenically subcultured into fresh medium on a weekly basis
Incubation chamber	One cabinet for culturing and one for testing
Temperature	25 ± 2 ˚C
pH	5.8 ± 1 (can be higher if required)
Light quality	Cool-white fluorescent
Light intensity	100 – 150 µmol•m^{-2}•s^{-1}
Photoperiod	16 hr light and 8 hr dark
Test vessel size	50 ml test tubes
Nutrient/test solution volume	40 ml
Age of test plants	Less than two weeks
Height of plant per test vessel	3 cm
Number of replicate test vessels/concentration	5
Measured water quality parameters	pH at start and end of each experiment
Measured endpoints	Plant height, root number and dry weight based on start and end measurements
Statistical endpoints	IC25 and IC50

Appendix 10: Brief description of the methodology for exposure to sprayed glyphosate to *Lemna minor* (Lockhart *et al.*, 1989).

For application of a spray to the foliar surface of *Lemna minor* plants, a laboratory sprayer was used consisting of a spray nozzle which moved along a rigid track at a rate and pressure selected by the operator. The sprayer was calibrated using dyes to allow delivery of any desired quantity of material to the surface of the *Lemna* cultures.

The application rate of glyphosate was that recommended for the control of annual weeds up to 15 cm high. The rate was 2.25 L of Roundup per hectare which is equivalent to 800 g a.i./ha. This rate of application to the surface of the test dishes would produce a concentration of 3.96 g a.i./L.

Exposures were conducted by placing sufficient *Lemna* fronds in deep Petri dishes with a surface area of 0.00785 m^2 and containing 117.8 ml of culture medium. After spraying the exposed plants were allowed to stand for 6, 12 or 24 hours without disturbance. The, 10 normal fronds were selected from each dish and transferred to 125 ml Erlenmeyer flasks where they were grown in clean culture medium. Preliminary experiments showed that the removal of sprayed cultures immediately after spraying resulted in loss of toxicity, presumably by wash-off of the glyphosate from the *Lemna* fronds. Controls were treated in the same manner except that the dish covers were left in place.

Cultures were grown in a controlled environmental room at 25°C. Light was provided by Gro and Sho lights (GE) at an intensity of about 60 mE $^{-2}$ sec^{-1} with a photoperiod of 16 hours light and 8 hours dark.

The number of fronds were counted several times over a 2-week period following exposure. At termination of the test, cultures were drained, blotted, weighed then air dried to constant weight at 95°C and re-weighed.

Appendix 11: Test conditions for growth and development toxicity tests with emergent vascular aquatic plants

Details of the growth test with emergent aquatic macrophytes (ASTM, 1996) are summarized in the table below.

Parameter	Conditions
Test species	Recommended monocot: *Oryza sativa.* Alternative monocots: *Spartina pectinata, Scirpus acutus, Phalaris arundinacea*. Alternative dicot: *Polygonum muhlenbergh*
Test type	Renewed 3 times/week
Test duration/renewal	14 days
Culture medium	50% Hoagland's medium
Incubation chamber	Incubated in greenhouse or growth chambers; one cabinet for culturing and one for testing
Temperature	20 to 30 ˚C
pH	6.5
Light quality	Natural sunlight, fluorescent lights, incandescent lights or a combination of both types of lights
Light intensity	150 – 200 $\mu mol \bullet m^{-2} \bullet s^{-1}$
Photoperiod	16 hr light and 8 hr dark
Sediment Type	Standardised sediment should be used (e.g., Walsh *et al.* 1991a). A natural sediment maybe used if plant growth and chemical response are not affected.
Test vessel size	Plastic pots maintained in trays partially filled with water or nutrient solution
Plant portion	Tubers, rhizomes or seeds of selected emergent macrophytes
Age of test plants	Two weeks for *O. sativa*; 3 to 6 weeks for native species
Height of plant per test vessel	8 to 10 cm for *O. sativa*; > 10 cm of first to third leaf blades for monocot species; 5 to 10 leaves for dicots.
Number of replicate test vessels/concentration	5 (minimum)
Measured water quality parameters	pH in highest, middle, lowest test concentrations and controls at beginning of the test and in both fresh and used solutions at renewal. Also other physical parameters, such as water hardness, conductivity, dissolved oxygen and salinity may also be measured.
Measured endpoints	Chlorophyll content extracted from leaf material. Dry weight maybe used for *O. sativa*
Statistical endpoints	ECx (such as EC50), NOAEC

Appendix 12: Test conditions for germination and seedling emergence toxicity tests with emergent vascular aquatic plants

An alternative method was designed to evaluate the effects of contaminants in water on the germination and seedling growth (root elongation, root and shoot dry weight) of emergent plants (APHA, 1998c). Inhibition of germination or seedling growth will affect the ability of the plants to compete and survive. *Echinochloa crusgalli* (Japanese millet), *Leersia oryzoides* (rice cutgrass), *Nelumbo lutea* (American lotus), *Oryza sativa* (domestic rice), *Rorippa nasturtium-aquaticum* (watercress), and *Zinania aquatica* (wild rice) are the recommended species. These tests may be either static, renewal or flow-through systems. Test system details (APHA, 1998c) are provided below.

Parameter	Conditions
Test species	*Echinochloa crusgalli*, *Leersia oryzoides*, *Nelumbo lutea*, *Rorippa nasturtium-aquaticum*, and *Zinania aquatica*
Test type	Static, renewal or flow-through
Test duration	4 days (depends on test species, but control root growth should be at least 20 mm)
Culture medium	Reconstituted freshwater
Incubation chamber	Seed germinator or other growth facility
Temperature	25 ± 1 °C (depends on test species)
pH	6.4 to 8.4 (depends on type of reconstituted freshwater prepared)
Photoperiod	Light or dark (depends on test species)
Test vessel size	100 x 15 mm culture dish or 47 mm test tube
Plant portion	Seeds of selected emergent macrophytes
Number of seeds per test vessel	10 to 15 seeds
Number of replicate test vessels/concentration	4 (minimum)
Measured endpoints	Seed germination (radicle 5 mm or longer), root length, shoot and root dry weight, abnormal appearance
Statistical endpoints	IC10, IC50, IC90 and SC20

Appendix 13: Potential species for terrestrial phytotoxicity testing

Below are terrestrial plant species that have potential for phytotoxicity testing. The table reflects the various attributes for testing gleaned from references that describes the importance of the species for testing purposes. The taxonomic classification is from: Kartesz, J.T., and C.A. Meacham. 1999. Synthesis of the North American Flora, Version 1.0. North Carolina Botanical Garden, Chapel Hill, NC.

Family	Species	Common Name	Importance
Apiaceae - (Umbelliferae) - carrot family	*Daucus carota*	carrot Queen Anne's Lace	wildlife food item - Boutin EC25 value <0.009 lb ai/A in EPA database sensitivity #2 - Environmental Canada
	carrot family		Significant family in Canada - Gold One of top 10 families - Cole
Amaranthaceae - amaranth family	*Amaranthus retroflexus*	redroot pigweed	PHYTOTOX database - Fletcher Sensitive to various herbicides - Boutin
Annonaceae - Pawpaw family	*Asimina*	paw paw	wild plants of economic import - Catling
Araliaceae - ginseng family	*Panax*	ginseng	Wild plants of economic import - Catling
Asteraceae - (Compositae) aster family	Aster family		Signif. Plant Families in Canada. - McCanny, Gold One of top 10 families - Cole
	Artemisia filifolia	silver sagebrush or sand sage	PHYTOTOX database - Fletcher Sensitive to various herbicides - Boutin
	Achilla millefolium	yarrow	PHYTOTOX - Fletcher
	Bidens cernua	bur-marigold	SU effects on growth and reprod - Boutin
	Centaurea cyanus	Cornflower	wildlife food item - Boutin,
	Bellis perennis	Lawn daisy	wildlife food item - Boutin,
	Chrysothamnus viscidiflorus	rabbit brush	PHYTOTOX database - Fletcher
	Echinacea	coneflower	wild plants of economic import - Catling
	Fragaria	strawberry	wild plants of economic import - Catling

Family	Species	Common Name	Importance
	Xanthium strumarium	Rough Cockleburr	EC25 value <0.009 lb ai/A in EPA database
	Inula helenium	Elecampane	wildlife food item - Boutin,
	Cirsium arvense	Thistle	PHYTOTOX database - Fletcher
	Helianthus annuus	sunflower	SU effects on growth and reprod - Fletcher wildlife food item - Boutin EC25 <0.009lb ai/A in EPA database
	Lactuca sativa	lettuce	Sensitive to various herbicides - Boutin PHYTOTOX database- Fletcher EC25 value <0.009 lb ai/A in EPA database sensitivity #12 - Environmental Canada
	Rudbeckia hirta	black-eyed Susan	wildlife food item - Boutin
	Solidago canadensis	Canadian Goldenrod	wildlife food item - Boutin
	Ambrosia psilostachya	western ragweed	PHYTOTOX database - Fletcher
Betulaceae - birch family	*Betula nigra*	river birch	wild plants of economic import - Catling
	Corylus americana	American hazelnut	wild plants of economic import - Catling
	Betula occidentalis	water birch	wild plants of economic import - Catling
Brassicaceae - (Cruciferae) mustard family	mustard family		Signif. Plant Families in Canada. - McCanny, Gold One of top 10 families - Cole
	Arabidopsis thaliana	thale cress	protocol and sensitivity to reproductive test - Ratsch
	Brassica oleracea	Cabbage	Sensitive to various herbicides - Boutin EC25 value <0.009 lb ai/A in EPA database
	Brassica rapa ssp. *Campestris*	Chinese cabbage	Sensitive to various herbicide, wildlife food item - Boutin
	Barbarea vulgaris	Yellow-Rocket	wildlife food item - Boutin
	Hesperis matronalis	Mother-of-the-Evening or Dame's Rocket	wildlife food item - Boutin

Family	Species	Common Name	Importance
	Sinapis arvensis	Wild mustard	wildlife food item - Boutin EC25 value <0.009lb ai/A in EPA database
	Brassica rapa	canola	SU effects on growth and reprod - Fletcher wildlife food item - Boutin sensitive to various herbicides - Boutin EC25 value <0.009 lb ai/A in EPA database
	Brassica napus	turnip	wildlife food item - Boutin EC25 value <0.009 lb ai/A in EPA database
	Isatis tinctoria	Dyer's Woad	Asghari & Dyer - sensitive reproductive endpoint
	Raphanus sativus	radish	sensitive from alleged mom-target incidents - EPA EC25 value <0.009 lb ai/A in EPA database sensitivity #6 - Environmental Canada
	Sinapis arvensis	corn-mustard	Wetland plant Sensitive to SU - Boutin
	Sinapis alba	white mustard	PHYTOTOX database - Fletcher
Caryophyllaceae - pink family	*Cerastium fontanum*	Common mouse-ear chickweed	Signif. Family in Canada - McCanny One of top 10 families - Cole
Chenopodiaceae - goosefoot family	*Beta vulgaris*	sugar beet	PHYTOTOX database - Fletcher sensitive from alleged mom-target incidents - EPA EC25 value <0.009 lb ai/A in EPA database
	Chenopodium album	Lamb's-quarters	wildlife food item - Boutin sensitive to various herbicides - Boutin
Convolvulaceae - morning glory family	*Ipomoea purpurea*	morning glory	PHYTOTOX database - Fletcher EC25 value <0.009 lb ai/A in EPA database
Cucurbitaceae	*Cucumis sativa*	cucumber	sensitivity #4 - Environmental Canada PHYTOTOX database - Fletcher EC25 value <0.009 lb ai/A in EPA database
Cyperaceae - sedge family	*Cyperus rotundus*	purple nutsedge	wildlife food item - Boutin PHYTOTOX database - Fletcher Signif. Plant Families in Canada. - McCanny One of top 10 families - Cole EC25 value <0.009 lb ai/A in EPA database

Family	Species	Common Name	Importance
Fabaceae - (Leguminosae) pea family	Pea family		Signif. Plant Families in Canada. - McCanny, Gold One of top 10 families - Cole
	Glycine max	Soybean	wildlife food item - Boutin PHYTOTOX database - Fletcher EC25 value <0.009 lb ai/A in EPA database sensitive from alleged mom-target incidents - EPA sensitivity #13 - Environmental Canada
	Acacia farnesiana	huiache/mealy wattle	PHYTOTOX database - Fletcher
	Baptisia	wild indigo	wild plants of economic import - Catling
	Cercis	redbud	wild plants of economic import - Catling
	Gymnocladus	Kentucky coffee tree	wild plants of economic import - Catling
	Phaseolus vulgaris	garden bean	PHYTOTOX database - Fletcher sensitive to SU - Boutin EC25 value <0.009 lb ai/A in EPA database
	Psoralea	California tea	wild plants of economic import - Catling
	Lens culinaris	lentil	Reproduction sensitive to SU -Fletcher Sensitive to various herbicide - Grealy
	Pisum sativum	pea	PHYTOTOX database - Fletcher sensitive from alleged non-target incidents -EPA EC25 value <0.009 lb ai/A in EPA database Sensitive to various herbicide - Grealy
	Medicago sativa	Alfalfa	PHYTOTOX database - Fletcher sensitive from alleged mom-target incidents - EPA sensitivity #4 - Environmental Canada
	Medicago lupulina	black medic	wildlife food item -Boutin
	Vicia cracca	vetch	wildlife food item -Boutin
	Melilotus alba or M. officinalis	sweet clover	wildlife food item -Boutin
	Trifolium ornithopodioides	fenugreek	PHYTOTOX database - Fletcher
	Trifolium pratense	red clover	PHYTOTOX database - Fletcher sensitivity #8 - Environmental Canada
	Trifolium repens	white clover	PHYTOTOX database - Fletcher

Family	Species	Common Name	Importance
Fagaceae - beech family	*Quercus marilandica*	blackjack oak	PHYTOTOX database - Fletcher wild plants of economic import - Catling sensitive from alleged mom-target incidents - EPA
	Castanea spp.	chestnut	wild plants of economic import - Catling
Hippocastanaceae - horsechestnut family	*Aesculus glabra*	Ohio Buckeye	wild plants of economic import - Catling
Iridaceae - iris family	Iris	Iris	wild plants of economic import - Catling
Juglandaceae - hickory family	*Carya* spp.	Hickory	wild plants of economic import - Catling
	Carya illinoinensis	pecan	wild plants of economic import - Catling sensitive from alleged mom-target incidents - EPA
Lamiaceae - (Labiatae) - mint family	mint family		One of top 10 families - Cole
	Leonurus cardiaca	Motherwort	wildlife food item - Boutin
	Mentha spicata	Spearmint	wildlife food item - Boutin
	Monarda	monarda	wild plants of economic import - Catling
	Nepeta cataria	Catnip	wildlife food item - Boutin
	Prunella vulgaris	Self-heal	wildlife food item - Boutin
Limnanthaceae - meadow-foam family	Limnanthes	meadow-foam	wild plants of economic import - Catling
Liliaceae -lily family	lily family		One of top 10 families - Cole
	Allium cepa	onion	PHYTOTOX database - Fletcher sensitive from various herbicides - Boutin sensitive from alleged mom-target incidents - EPA EC25 value <0.009 lb ai/A in EPA database
	Chamaelirium	fairy-wand	wild plants of economic import - Catling
	Aletris	colicroot	wild plants of economic import - Catling
Linaceae - flax family	*Linum usitatissimum*	flax	wild plants of economic import - Catling PHYTOTOX database - Fletcher sensitivity #11 - Environmental Canada
Magnoliaceae - magnolia family	*Magnolia*	magnolia	wild plants of economic import - Catling

Family	Species	Common Name	Importance
Malvaceae	*Gossypium hirsutum*	Cotton	PHYTOTOX database - Fletcher sensitive from alleged mom-target incidents - EPA EC25 value <0.009 lb ai/A in EPA database
Moraceae - mulberry family	*Morus*	mulberry	wild plants of economic import - Catling
Oleaceae - olive family	*Fraxinus americana*	white ash	PHYTOTOX database - Fletcher wild plants of economic import - Catling sensitive from alleged mom-target incidents - EPA
Orchidaceae - orchid family	orchid family		Signif. Plant family in Canada - McCanny
Papaveraceae - poppy family	*Papaver rhoeas*	corn poppy	wildlife food item -Boutin Signif. Plant family in Canada - McCanny
Poaceae - grass family (Gramineae)	grass family		Signif. Plant Families in Canada. - McCanny, Gold One of top 10 families - Cole
	Agropyron	wheatgrass	sensitivity #1 - Environmental Canada wild plants of economic import - Catling
	Avena sativa	oat	PHYTOTOX database - Fletcher wildlife food item - Boutin sensitive to various herbicides - Boutin sensitive from alleged mom-target incidents - EPA EC25 value < 0.009lb ai/A
	Cynodon dactylon	bermuda grass	PHYTOTOX database - Fletcher
	calamagrostis canadensis	bluejoint	sensitivity #3 - Environmental Canada
	Lolium perenne	Perennial ryegrass	PHYTOTOX database - Fletcher wildlife food item - Boutin sensitive to various herbicides - Boutin EC25 value <0.009 lb ai/A in EPA database sensitivity #15 - Environmental Canada
	Beckmannia syzigachne	American sloughgrass	sensitivity #9 - Environmental Canada
	Zea mays	corn	PHYTOTOX database - Fletcher wildlife food item - Boutin sensitive from alleged mom-target incidents - EPA EC25 value <0.009 lb ai/A in EPA database

Family	Species	Common Name	Importance
	Setaria viridis	green foxtail	PHYTOTOX database - Fletcher sensitive to various herbicides - Boutin wildlife food item - Boutin SU reduces survival and reproduction - Khan
	Setaria glauca	yellow foxtail	wildlife food item - Boutin SU reduces survival and reproduction - Khan
	Alopecurus myosuroides	slender meadow-foxtail	sensitive to various herbicides - Boutin
	Panicum dichotomiflorum	fall panic grass	sensitive to various herbicides - Boutin
	Panicum miliaceum	millet	PHYTOTOX database - Fletcher wildlife food item - Boutin
	Phleum pratense	timothy	sensitivity #14 - Environmental Canada
	Echinochloa crusgalli	barnyard grass	sensitive to various herbicides - Boutin PHYTOTOX database - Fletcher EC25 value <0.009 lb ai/A in EPA database
	Triticum aestivum	wheat	PHYTOTOX database - Fletcher sensitive from alleged mom-target incidents - EPA EC25 value <0.009 lb ai/A in EPA database
	Sorghum bicolor	sorghum	PHYTOTOX database - Fletcher EC25 value <0.009 lb ai/A in EPA database
	Digitaria ischaemum	Crab Grass	PHYTOTOX database - Fletcher
	Bromus secalinus	bromegrass	sensitive from various herbicides - Boutin
	Festuca arundinacea	tall fescue	PHYTOTOX database - Fletcher
	Festuca rubra	Red Fescue	PHYTOTOX database - Fletcher
	Hordeum vulgare	barley	PHYTOTOX database - Fletcher
	Imperata cylindrica	Cogon grass	PHYTOTOX database - Fletcher
	Sporobolus indicus	smutgrass	PHYTOTOX database - Fletcher
	Poa pratensis	Kentucky Blue Grass	PHYTOTOX database - Fletcher wild plants of economic import - Catling

Family	Species	Common Name	Importance
Pinaceae pine family	*Pinus resinosa*	Red Pine	wild plants of economic import - Catling
	Pinus lambertiana	sugar pine	PHYTOTOX database - Fletcher wild plants of economic import - Catling
	Pinus taeda	Loblolly Pine	PHYTOTOX database - Fletcher wild plants of economic import - Catling
Polygalaceae - milkwort family	*Polygala*	snakeroot	wild plants of economic import - Catling
Polygonaceae - buckwheat family	buckwheat family		One of top 10 families in North America - Cole
	Fagopyrum esculentum	Buckwheat	wildlife food item - Boutin EC25 value <0.009 lb ai/A in EPA database
	Polygonum convolvulus	wild buckwheat	wildlife food item - Boutin
	Polygonum punctatum or P. lapathifolium	Smartweed	sensitive to SU - Fletcher
	Polygonum persicaria	lady's thumb	sensitive to various herbicides - Boutin
	Convolvulus arvensis	field bindweed	sensitive to various herbicides - Boutin
	Rumex crispus	Curly Dock	sensitive to various herbicides - Boutin
Primulaceae - primrose family	*Anagallis arvensis*	pimpernel	wildlife food item - Boutin
Ranunculaceae - buttercup family	buttercup family		One of top 10 families - Cole
	Hydrastis	goldenseal	wild plants of economic import - Catling
Rosaceae - rose family	rose family		One of top 10 families - Cole Signif. Plant Families in Canada. - McCanny, Gold
	Amelanchier spp	Service-Berry	wild plants of economic import - Catling
	Fragaria spp.	Strawberry	wildlife food item - Boutin sensitive from alleged mom-target incidents - EPA
	Malus spp.	Apple, Crabapple	PHYTOTOX database - Fletcher sensitive from alleged mom-target incidents - EPA
	Prunus spp.	Almond, Cherry, Peach, Plum	sensitive from alleged mom-target incidents - EPA wild plants of economic import - Catling Sensitive to SU - Fletcher

Family	Species	Common Name	Importance
	Rubus spp.	Blackberry, Dewberry, Raspberry	wild plants of economic import - Catling sensitive from alleged mom-target incidents - EPA wildlife food item - Boutin
	Pyrus communis	pear	wild plants of economic import - Catling sensitive from alleged mom-target incidents - EPA
	Rosa multiflora *Rosa* spp.	Wild Rose cultivated rose	sensitive from alleged mom-target incidents - EPA
	Cydonia oblonga	Quince	wild plants of economic import - Catling
Rubiaceae - madder family	*Galium aparine*	cleavers	wildlife food item - Boutin
Scrophulariaceae - figwort family	figwort family		One of top 10 families - Cole Signif. Plant Families in Canada. - McCanny
	Digitalis purpurea	foxglove	wildlife food item - Boutin
	Mimulus spp.	Monkey-Flower	sensitive wetland plant to SU - Boutin
	Veronicastrum	Culver's root	wild plants of economic import - Catling
	Veronica persica	Speedwell	wildlife food item - Boutin
Salicaceae - willow family	willow family		Signif. Plant Families in Canada. - McCanny, Gold
	Salix spp.	Willow	sensitive from alleged mom-target incidents - EPA
Solanaceae - potato family	*Lycopesricon esculentum*	tomato	PHYTOTOX database - Fletcher sensitive from alleged mom-target incidents - EPA sensitive to various herbicides - Boutin EC25 value <0.009 lb ai/A in EPA database sensitivity #10 -Environmental Canada
	Datura stramonium	Jimsonweed	Sensitive to various herbicides - Boutin
	Nicotiana tabacum	tobacco	PHYTOTOX database - Fletcher
	Solanum nigrum or *S. dulcamara*	Nightshade	Wildlife Food Item - Boutin
	Solanum carolinese	horse nettle	PHYTOTOX database - Fletcher

Family	Species	Common Name	Importance
	Solanum tuberosum	Irish Potato	PHYTOTOX database - Fletcher sensitive from alleged mom-target incidents - EPA sensitive to various herbicides - Wall
Taxaceae - yew family	*Taxus*	yew	wild plants of economic import - Catling
Valerianaceae - valerian family	*Valeriana*	valerian	wild plants of economic import - Catling
Vitaceae - grape family	*Vitis vinifera*	grape	sensitive from alleged mom-target incidents - EPA wild plants of economic import - Catling

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Appendix 14: Frequently listed species in PHYTOTOX[2]

Order of frequently listed species in PHYTOTOX with one being the most frequent species tested.

1 - wheat (Triticum aestivum) - Grass family
2 - pea (Pisum sativum) - Pea family
3 - tomato (Lycopersicon esculentum) - Potato family
4 - oat (Avena fatua) - Grass family
5 - garden/field/dry/green bean (Phaseolus vulgaris) - Pea family
6 - Apple (Malus spp.) - Rose family
7 - soybean (Glycine max) - Pea family
8 - corn (Zea mays) - Grass family
9 - barley (Hordeum vulgare) - Grass family
10 - flax (Linum usitatissimum) - Flax family
11 - cucumber (Cucumis sativa) - Cucumber family
12 - tobacco (Nicotiana tabacum) - Potato family
13 - pigweed (Amaranthus retroflexus) - amaranth family
14 - rice (Oryza sativa) - Grass family
15 - potato (Solanum tuberosum) - Potato family
16 - cotton (Gossypium hirsutum) - Mallow family
17 - lettuce (Lactuca sativa) - Aster family
18 - radish (Raphanus sativus) - Mustard family
19 - barnyard grass (Echinochloa crusgalli) - Grass family
20 - sugar beet (Beta vulgaris) - Goosefoot family

Order of most frequently listed genera represented with most frequently listed species of the genera for old-field/wild-grown plants in PHYTOTOX.

1 - Kentucky bluegrass - Grass family
2 - red fescue - Grass family
3 - horsenettle - Potato family
4 - blackjack oak - Oak family
5 - western ragweed (Ambrosia psilostachya) - Aster family
6 - huiache/mealy wattle (Acacia farnesiana) - Pea family
7 - peach - Rose family
8 - Smutgrass (Sporobolus poiretti) - Grass family
9 - thistle (Cirsium arvense) - Aster family

[2] Fletcher, J., Johnson, F, and McFarlane, J. 1988. Database Assessment of Phytotoxicity Data Published on Terrestrial Vascular Plants. Environmental Toxicology and Chemistry, Vol. 7, pp. 615-622.

10 - yarrow (Achilla millefolium) - Aster family
11 - cogon grass (Imperata cylindrica) - Grass family
12 - crabgrass - Grass family
13 - tall fescue - Grass family
14 - bindweed - morningglory family
15 - bermuda grass - Grass family
16 - silver sagebrush or sand sage (Artemisia filifolia) - Aster family
17 - red maple (Acer rubrum) - Maple family
18 - winged elm - Elm family
19 - white ash - Olive family

Appendix 15: Uncertainty Factors: Experience From Use In Hazard Assessment Of Industrial Chemicals Under the Toxic Substances Control Act (TSCA)

Introduction

As an introduction to discussing the topic of uncertainty factors as they are used by the Office of Pollution Prevention and Toxics (OPPT), involvement by OPPT in this proposal is first discussed.

The Office of Pollution Prevention and Toxics (OPPT) is the "sister" office of OPP, both being part of the larger office, the Office of Prevention, Pesticides and Toxic Substances (OPPTS). OPPT has been interested and involved in this Proposal to Update Non-Target Plant Toxicity Testing Under NAFTA, since the initiation of this work with the Canadian PMRA in 1997. The main focus of this project has been on pesticides, in contrast to industrial chemicals; the latter are the interest and regulatory focus of OPPT. There are a number of reasons, relevant to the topic of uncertainty factors, why OPPT has participated and been interested in this project.

1. OPPT expertise used in this project in hazard/risk assessment of industrial chemicals.

OPPT is the lead office for implementing the Toxic Substances Control Act (TSCA), the Pollution Prevention Act (PPA), several provisions of the Federal Food, Drug and Cosmetic Act (FFDCA), and the residential Lead-Based Paint Hazard Reduction Act. Specifically, the office is charged with reducing the risk of existing and new (premanufacture or PMN) industrial chemicals in the marketplace. The Risk Assessment Division (RAD), which is involved in this project, is responsible for assessing the health and environmental hazard/risk of chemicals. Since the passage of TSCA in 1976 a whole new hazard assessment approach had to be developed (ASTM 1993a, 1993b; Nabholz 1991; Smrchek and Zeeman 1998; Smrchek et al. 1995). Hazard/risk assessment methods and procedures were developed for pesticides by OPP in the 1970s under FIFRA. Many of these procedures were used by OPPT as the basis or starting point to assess industrial chemicals and to implement TSCA requirements. Other concepts and procedures, for example uncertainty factors, were developed with input from other Agency offices and sources. Thus, with this project we have come full-circle; there has been a valuable exchange of expertise, ideas and information between OPPT and OPP, which has resulted in agreement in OPPTS on a harmonized plant testing scheme or design for all chemicals.

2. Commonalities in OPPT and OPP regulatory activities.

As part of OPPTS both OPPT and OPP are required to work together to carry out their missions. Development of the series 850 harmonized ecological effects test guidelines is an important example. This activity began in 1990; after a lengthy process draft harmonized test guidelines

were published in 1996 (Smrchek and Morcock 1999). At the same time participation in OECD development of new test guidelines and revision/updating of existing guidelines became increasingly important because it was concluded that participation in OECD activities was important and because both offices would, under the MAD (Mutual Acceptance of Data) principle, have to accept and conform to what was approved by OECD. At present OPPTS remains heavily involved in many OECD ecotoxicological activities.

An inert ingredient of a pesticide formulation registered with OPP may also be submitted to OPPT for approval to be marketed as a TSCA Section 5 new industrial chemical. Thus, in order to avoid unnecessary duplication and to maximize available resources, as well as to minimize redundant ecotoxicity testing, harmonization of regulatory activities is encouraged between the offices as much as possible. Decision making for both industrial chemicals and pesticides must be consistent and uniform as far as is possible; similar hazard/risk decisions should theoretically at the least, be reached when the same chemical is reviewed separately by each office.

3. NAFTA impact on OPPT.

While currently there is little impact of NAFTA on OPPT activities, this is not to imply that this situation will not change in the future. If and when the situation does change, both offices will have gained valuable experience and insight, in the meantime, by actively participating in the current plant harmonization activities with the PMRA.

4. A “unified” and consistent position must be presented by OPPTS.

It is important to have a unified, consistent position and obtain agreement between OPPT and OPP on issues of importance to both offices, especially when discussing these issues in the international arena (e.g., OECD, EU, UNEP). Sometimes the U.S. position or OPPTS position on some ecotoxicity issues is at variance with that of other countries. Such differences must be supported by the “best” science (e.g., support from valid, scientifically sound test guidelines and ecotoxicity-related activities). Participation in common activities such as the NAFTA plant work will also go a long way toward developing unified, consistent positions. Under NAFTA, the U.S. and Canada must develop and present harmonized positions also based on the “best” science; as U.S. and Canada are interested an in developing a joint position.

5. Impact of Probabilistic Risk Assessment (PRA) activities on OPPT.

Currently, OPPT uses mainly a deterministic hazard/risk assessment process, e.g., use worst-case scenarios, concentrate on most sensitive tested species, rank toxicity and hazard, determine quotient of expected environmental concentration compared with the concern concentration. In contrast OPP (and the PMRA) are moving toward a PRA approach. This difference is due to several reasons. For example, there are currently few industrial chemicals with a large toxicity

test data bases and test values conducted on a variety of species, which are optimal for PRA (for a single chemical, at least 7 for a single group such as plants and sometimes as many as 45 or more test values). Furthermore, there is no office directive or overriding reason for OPPT to go to a PRA approach at this time. Also, there is still a reluctance to conduct even a limited number of toxicity tests for PMN chemicals. Nevertheless, in the long term, PRA may become more common and important as more testing is completed for more industrial chemicals. PRA may well be useful, applicable, and offer definite advantages over the current deterministic approach to assessing the hazard/risk of industrial chemicals.

Definition, Background and History of Uncertainty Factors

Uncertainty factors have been called assessment factors, extrapolation factors, and safety factors. OPPT favors the use of either the terms uncertainty factors or assessment factors, depending on whether existing chemicals (including pesticides) or new chemicals are being assessed, respectively (see below). The term "safety factor" is discouraged as using this term gives a false sense of security and is misleading. In general, it is impossible that a chemical will be "safe" to all organisms, and there always will be some risk to some organisms at a particular concentration or dose.

Uncertainty factors have been defined in a number of ways, but most are related to two concepts, 1) addressing uncertainty due to variability in testing and extrapolating from testing, and 2) addressing uncertainty due to the amount of available ecotoxicity testing.

A certain amount of uncertainty in hazard/risk assessments will always be present, especially when evaluating industrial chemicals, because it is impossible to test more than a limited number of species within a group and more than a limited number of groups. Moreover, uncertainty, by definition, is an integral part of risk assessment (Suter 1993) and this explains the large number of references, research and interest in this topic. In assessing industrial chemicals it is important to determine high or highest levels of exposure and toxic effect where the chemical may be at least hazardous to organisms in the environment. Risk mitigation, risk reduction, pollution prevention measures (e.g., source reduction, modification of manufacturing/production processes), and other risk management actions are taken as a result of this determination.

Often decisions on hazard and risk must be made and be based on incomplete or limited information. This is especially true in OPPT when reviewing most PMN chemicals and many existing chemicals. Thus, it is only logical that OPPT has relied on uncertainty factors to a much greater extent than OPP where the latter deals with chemicals of better defined toxicity having a greater number of available toxicity tests.

Furthermore, uncertainty has been of great concern to ecotoxicologists (see, for example, Slooff et al. 1986; Greig-Smith 1992; Calabrese and Baldwin 1993; Suter 1995). In fact, uncertainty analysis in ecological risk assessment was the topic of a SETAC Pellston Workshop held in 1995 (Warren-Hicks and Moore 1998).

Uncertainty has also been an important ecotoxicological topic at the international level. Uncertainty factors in Europe are usually called assessment factors. The Organization for Economic Cooperation and Development (OECD) held a workshop in 1990 on extrapolation of laboratory aquatic toxicity data to the real environment (OECD 1992). Working groups were divided into one considering procedures for extrapolating from small data sets, and one considering procedures for extrapolating from relatively large data sets. Uncertainty factors were discussed in the first working group, while species sensitivity distributions were considered by the second working group. Thus, the topic of the first working group is more applicable to OPPT and relates to how this office deals (see below) with uncertainty in deterministic hazard/risk assessment. Other important references considering uncertainty and extrapolation procedures include EC (1994), ECETOC (1993), OECD (1989, 1995a, 1995b), and Pederson et al. (1992).

Uncertainty factors have an historical background derived from other concepts and calculated factors, mainly in the field of water pollution biology. These include application factors (AFs), acute to chronic ratios (ACRs), and uncertainty factors as used in mammalian regulatory toxicology (Chapman et al. 1998).

Application Factors (AFs): The slope of the time-mortality curve was used to estimate cumulative toxicity; an AF of 0.3 was tentatively proposed by Hart et al. (1945) and used to calculate, from acute toxicity data, the presumed "harmless" concentration of the chemical. Other factors were considered and these included, for example, species tested, exposure time, life history stages likely to be exposed, and quality of test organisms. AFs were considered by Henderson (1957) and Henderson and Tarzwell (1957) to be decimal fractions multiplied against an acute LC50 to predict (Chapman et al. 1998) "the concentration which will have no detrimental effect on aquatic life..." These factors were considered to be universally applicable and convenient to use in the absence of data. Warren and Doudoroff (1958) and Mount (1977) determined AFs by dividing the maximum acceptable toxicant concentration (MATC) by the LC50. Later, the MATC was estimated from partial or complete life cycle toxicity tests, and in OPPT the MATC is calculated as the geometric mean of the no-observed-adverse-effect-concentration (NOAEC) and the lowest-observed-adverse-effect-concentration (LOAEC). AFs were standardized at 0.1, 0.05, and 0.01, depending on whether the substance was persistent or cumulative, or both. However, it is difficult to use AFs to predict chronic toxicity from acute toxicity information because of variability among chemicals and test species. The AFs were to be refined in the intervening years since they were first developed, but in many cases this did not occur (Chapman et al. 1998). Thus, many AFs may be "out of date."

Acute-to-Chronic Ratios (ACRs): These are numerical values that are the inverse of AFs (US EPA 1991). The lethal test endpoint is divided by the sublethal (chronic) test endpoint. An ACR of 10 is generally used when data are lacking or an ACR may be calculated for each chemical. ACRs for a variety of chemicals have been calculated and an ACR of 40 has been suggested for general use (ECETOC 1993). However, ACRs have been found to vary from 1 to 20,000 (Personne and Janssen 1994). Different ACRs are needed for different chemicals (OECD 1992).

Uncertainty Factors (UFs) In Mammalian Regulatory Toxicology: A number of years ago it was proposed that UFs could be used in mammals (Lehman and Fitzhugh 1954). A 100-fold factor was proposed to derive acceptable daily intakes relative to food additives and contaminants to account for sources of uncertainty and variability, for example, inter- and intraspecies differences. Toxicological Profiles have been developed for the Agency by the Agency for Toxic Substances and Disease Registry, U.S. Public Health Service. An uncertainty factor in these documents has been defined as: a factor used in operationally deriving the RfD from experimental data. UFs are intended to account for the variation in sensitivity among of the human population, the uncertainty in extrapolating animal data to the case of humans, the uncertainty in extrapolating from data obtained in a study that is of less than lifetime exposure, and the uncertainty in using lowest-observed-adverse-effect level (LOAEL) data rather than no-observed-adverse-effect level data (NOAEL). Usually each of these four factors is set equal to 10. LOAELs and NOAELs are similar to LOAECs and NOAECs. An RfD is an estimate of the daily exposure of the human population to a potential hazard that is likely to be without risk of deleterious effects during a lifetime.

These three concepts and factors lead to the development of UFs in OPPT.

Use of Uncertainty Factors in OPPT

OPPT deals with variable biological systems, which sometimes act randomly, and must rely on imperfect, variable test methods. All or most species of concern (or the most sensitive) cannot be tested, and it is impossible to determine “safe” levels or truly, and precisely or accurately assess the hazard of industrial chemicals to all or even most aquatic and terrestrial organisms. In addition, it is difficult to estimate field concern levels. Thus, uncertainty is unavoidable and is present at every step in the hazard/risk assessment process; extrapolation up or down in the process is difficult.

Uncertainty factors were devised by OPPT in the early to mid-1980's to help reduce uncertainty, deal with variability, and improve extrapolation capabilities. This was done, in part, to avoid or minimize false negative hazard calls or results (Type II error in hypothesis testing), or in other words to consider a chemical to be “safe” when in reality it will be toxic, and to estimate field concern levels (Forbes and Forbes 1994).

There are several sources of variability that are considered (Calabrese and Baldwin 1993, Chapman et al. 1998, Personne and Janssen 1994, US EPA 1991):

-Variability due to the range of sensitivities of species to chemicals (intra- and inter species or taxa variations), including variability due to life stage or in test conditions,

-Variability due to estimation of chronic effect levels from acute test data,

-Variability due to extrapolations from the laboratory to the field or to natural ecosystems,

Dealing effectively with uncertainty is important because the ultimate goal of OPPT TSCA regulatory activities is to obtain a simple, conservative, bottom-line single number, or a range of numbers, which can be used as an indicator of hazard or risk and as the basis for making scientifically credible, defensible risk management or policy decisions.

When evaluating PMNs, levels of a chemical are estimated, which if met or exceeded in the environment, could cause adverse effects. The data set from which such concentrations (level of concern or concern concentrations) are estimated and range from both acute and chronic test data, to only acute data, to no test data. In 1984 a position document was completed that described the procedure and rationale followed to estimate concern levels for concentrations of chemicals (US EPA 1984). The methodology described should identify at least 95% of the time concern levels for TSCA chemicals that may cause adverse environmental effects. The number used to adjust toxicity test endpoints (e.g., LC50s, EC50s, MATCs) to arrive at environmental concentrations of concern were called assessment factors. Since the mid-1980s the latter have been used interchangeably with uncertainty factors, however, there are differences. Assessment factors were originally developed to account for limited available toxicity test data for PMN chemicals and to identify those chemicals which should be tested under TSCA Section 5 if little or no data are available or to test further if some test data are available, to more fully characterize their inherent toxicity and hazard. The assessment factors used for PMN chemicals are listed below.

Toxicity Derivation Method or Available Data	Assessment Factor
QSAR-calculated LC50/EC50, Acute LC50/EC50 from a single test species for the actual chemical or for an analog	1000
Two LC50s/EC50s for actual chemical or for the same analog (e.g., 1 algae, 1 fish)	1000
Three LC50s/EC50s for actual chemical (fish, invertebrate, algae)	None used: This is a data-based decision on need for chronic testing
Three LC50s/EC50s for same analog (fish, invertebrate, algae)	100
Five LC50s/EC50s for actual chemical or for same analog (e.g., 2 fish, 2 invertebrates, 1 algae; 2 fish, 3 algae)	100

MATC single value from chronic test for analog	10
MATC single value for PMN	None used: This is a data-based decision on need for further testing or to reach a regulatory decision by risk mangers

The lowest toxicity value is divided by the assessment factor to give the level of concern (LOC), concentration of concern (COC), or concern concentration (CC) for the PMN chemical. An additional uncertainty factor may be agreed to by OPPT risk managers and applied to the concern level. This will serve to further lower the level of concern and give an increased level of protection to organisms in the environment.

However, since the assessment factors concept was completed in 1984, the scope of this concept has been widened to existing chemicals and merged with the concept of uncertainty factors. European countries have adopted the use of the term assessment factors, but in actuality they are really using uncertainty factors because multiples of 1 to 1000 are applied to the lowest toxicity test value only once (see, for example, EC 1994, Nabholz 1991, OECD 1989, 1991a and b). Thus, the result used by OPPT for existing chemicals is described below. These uncertainty factors are based on multiples of 10. Lower tiered acute tests (see the second to-the-last figure in this Appendix) should result in more uncertainty and wider confidence limits than higher tiered chronic or field tests. See the classic Figure 1 in ASTM (1978) illustrating this relationship, and see also Cairns et al. 1992. Also, the reliability of the toxicity estimate should progressively increase. Given the sources of variability listed above, field tests would have little or no uncertainty in the context used above. Experimental variability, however, may be high in field tests and this may make interpretation of complex field test data very difficult. As additional, valid toxicity test results are obtained for a chemical, uncertainty in general and the uncertainty factors themselves progressively decrease. If there is a choice, higher tier chronic tests with sublethal (more sensitive) endpoints (e.g., growth, reproduction) are emphasized over lower tier lethality tests. Uncertainty factors are listed below.

Source of Variability or Available Data	Uncertainty Factor
QSAR-calculated LC50/EC50 or Acute LC50/EC50 from 1-2 test species for the actual chemical or for an analog	1000
Acute LC50/EC50 with 2-3 species (use most sensitive tested species), or QSAR LC50/EC50 based on 2-3 species	1000-100

Acute LC50/EC50 with several species (use most sensitive tested species), or QSAR LC50/EC50 based on several species	100
Chronic toxicity value (MATC) from 1 tested species	100-10
Chronic toxicity value (MATC) from 2-3 tested species (use most sensitive)	10
Toxicity value derived from microcosm or mesocosm tests	10-1
Toxicity value derived from field test(s)	1

Criticism and Support of Uncertainty Factors

Criticism has been raised over the use of uncertainty factors (Zeeman 1995). Some ecotoxicologists are of the opinion that these factors are suitable for use only in a preliminary effects assessment, and that a more involved extrapolation method using statistical models, with acute and chronic test data from several species, is needed for completing a detailed effects or hazard assessment that would be protective of 95% of species occurring in ecosystems. The factors are viewed as being too simple, with little or no scientific or theoretical foundation (Okkerman et al. 1991, 1993; van den Berg 1992/1993; Balk et al. 1993; Emans et al. 1993). An attempt has been made in this Appendix to explain that in OPPT one is forced to assess hazard/risk, based on limited test data and therefore must use something like UFs to address uncertainty and variability. Moreover, uncertainty factors do have some scientific basis in application factors, acute to chronic ratios, and in mammalian toxicology. When the factors used for industrial chemicals by OPPT are compared with an alternative statistical method neither was clearly better than the other (Okkerman et al. 1993). Moreover, Calabrese and Baldwin (1993) and Forbes and Forbes (1994) found that the statistical methods were neither more accurate nor more conservative than the simple assessment factor approach. The more complex statistical methods should only be used if they are clearly more useful and predictive, but this has not yet been shown (also see Belanger 1994). Sometimes the particular statistical model cannot be used because there are not the specified minimum number of chronic NOAEC values available for different representative species (Warren-Hicks and Moore 1998). Chapman et al. (1998) found the risk assessment schemes (for example that used by OPPT, see below) where safety or uncertainty factors are gradually reduced as more data become available, to be useful and based on good science. It was further concluded that safety or uncertainty factors do have a place in risk assessment. A workshop was conducted in 1995 to assess the use of uncertainty analysis in ecological risk assessment (Warren-Hicks and Moore 1998). Participants concluded that the current use of conservative, deterministic methods is both practical and appropriate when those methods result in a conclusion of very low or very high risk. When a worst-case approach like uncertainty factors results in determining a significant risk then other methods such as quantitative

uncertainty analysis may be useful. The latter would be particularly appropriate at Levels 2-4 of the proposed plant design. Thus, the simple uncertainty factor approach remains a useful and effective tool in addressing sources of uncertainty and ecotoxicological variability.

Use of Uncertainty Factors in the OPPT Hazard Assessment Process

The OPPT hazard assessment process is shown in the following diagram. This process conforms with the U.S. EPA ecological risk assessment framework (US EPA 1998). This framework was first published by the Agency in 1992 (US EPA 1992). The 1998 guidelines for ecological risk assessment expanded upon and replaced the previous framework. The purpose of the latest framework is to improve the quality of ecological risk assessments at EPA while increasing the consistency of assessments among the Agency's program offices and regions. Figures labelled 1-1 and 1-2 illustrate the framework.

Very briefly, the OPPT deterministic hazard assessment process is part of Characterization of Ecological Effects (Step 2b of the Framework) and consists of a multi-tiered testing scheme. This Testing Scheme I, shown in the next un-numbered Figure, was developed over a number of years and is described in Smrchek and Zeeman (1998), Zeeman and Gilford (1993), and in Smrchek et al. (1993). OPPTS harmonized testing guidelines are used to perform toxicity testing at each tier. Uncertainty factors are one estimation method that will result in concern levels being determined. These concern levels relate directly to hazard to organisms.

Hazard ranking criteria are shown in the next Table. These have been developed by OPPT to rank concern (high, medium or moderate, and low) in assessing industrial chemicals. These criteria are based on the results of valid toxicity tests.

The steps in the OPPT deterministic hazard assessment process may now be outlined.

1. Conduct toxicity testing (at Tiers I, II, III, or some combination)

2. Validate all test methods (before testing begins) and test results (after testing concludes)

3. Concentrate on most sensitive tested group, select most sensitive tested species, verify with further testing; determine lowest (worst-case) effect value

4. Use hazard ranking criteria (Table) to determine level of concern. Low concern: stop; medium or moderate concern: continue review or stop; high concern: continue review

5. Complete toxicity testing; for all high and medium concern chemicals, use uncertainty factors to determine concern concentration or a predicted no-effect concentration

6. Complete hazard assessment or ecological effects characterization. Compare predicted environmental concentration (derived from the exposure characterization, step 2a) or

expected environmental concentration to the concern concentration

7. Begin Risk Characterization (Risk Estimation). Determine magnitude, probability of occurrence, and ratio (Quotient Method):

-If predicted environmental concentration is greater than the concern concentration, begin risk management activities (e.g., risk reduction, pollution prevention)

-If predicted environmental concentration is "near" the concern concentration, risk management activities may or may not be needed. Other factors come into play: for example, how near are the values, annual production volume

-If predicted environmental concentration is lower than the concern concentration, few or no risk management activities are needed

Application of Uncertainty Factors to Plants

OPPT Testing Scheme I is again presented as the last Figure, with the aquatic/terrestrial plant portion of the scheme highlighted. A long-term office goal is to revise and expand this portion by incorporating new plant test species and assessment procedures. Work on the proposal and the results of the SAP meeting will greatly help in meeting this goal.

With respect to the possible development and use of uncertainty factors with plants there are many unanswered questions. The existing uncertainty factors have been developed for aquatic organisms, including algae. There is some question whether these same factors can be applied to aquatic higher vascular plants such as duckweeds, rooted submersed plants, and emergent plants. Another related question that must be first answered is whether or not aquatic uncertainty factors can be applied to terrestrial plants. Does one size fit all, or are modifications necessary? Are entirely different UFs needed?

If new factors need to be developed they may have to account for other sources of variability unique to plants. For example, are UFs needed to account for variability and extrapolate from crop species to non-crop species, from terrestrial plants to rooted emergent plants, and from seedlings to mature plants? Also, as is obvious from the hazard ranking criteria table, it is critical that specific criteria be developed for semi-emergent and terrestrial plants. Can the aquatic criteria simply be converted and used for terrestrial criteria? If new criteria are developed, how will these differ from criteria for aquatic organisms?

There is yet another source of variability in developing plant uncertainty factors: taxonomic distance to explain variability. Fletcher et al. (1990) studied the wide range of sensitivity to herbicides expressed by plants. Extrapolations within a genus can be done without using uncertainty factors as with aquatic animals. Extrapolations between genera within the same family can be done by using a UF of 2 (80% of the variability will be captured with this UF). They found

that extrapolations across families within or across orders within the same class should be discouraged. However, if this were performed a UF of 15 should be used for intraorder extrapolations and a UF of 300 or greater should be used for intraclass extrapolations to capture 80% of the variability. Variability for aquatic animals also increases with taxonomic distance, but this relationship has been "disguised" by or merged with the other sources of variability and the amount of available plant toxicity tests.

Fletcher et al. (1990) also studied another source of variability: laboratory-to-field extrapolations. Surprisingly they found good agreement between laboratory and field determinations of EC50s for terrestrial plants. A UF value for this source of variability need be no greater than 5 or 10. Thus, plants may be very different from animals where there can be large differences in lab-to-field comparisons for the latter. Other sources of variability remain to be studied.

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Appendix 16 - Environmental Monitoring and Incidents

In response to numerous complaints of phenoxy herbicide damage to non-target plants from the states and regions, the EPA in conjunction with several states such as Arkansas and Louisiana have enacted strict regulations and label requirements to require use of low-volatile formulations, to restrict aerial applications to early morning and late evening flights, to require aircraft nozzle inspections and flight plans, to check for air inversions prior to application, and to require application at specific dates to reduce drift damage to cotton and soybeans at sensitive growth stages. The state of Washington has imposed a strict buffer zone on aerial applicators in the Horse Heaven Hills wheat growing areas to reduce off-target movement of herbicides to cherry tress, alfalfa, and other sensitive crops many miles distant from the application site.

In a prairie wetland study, surface waters were monitored for herbicide residues in wetland areas that are on or adjacent to pesticide use sites vs untreated wetland or pristine areas (Donald *et al.* 2001). In this study, 2,4-D, MCPA, bromonynil, dicamba, mecoprop, and dicloprop accounted for 87% of detections of the 10 herbicides analyzed. The 10 herbicides assessed account for 34% of all herbicides used in the study area. The mean concentration of 2,4-D in water on the four land types ranged from 0.12 to 0.26 ug/l, and MCPA residues ranged from 0.08 to 0.17 ug/l. The authors found similar detection frequencies and concentrations of individual herbicides in all sampling sites. The authors concluded that atmospheric transport via volatilization and/or plant evapotranspiration with redistribution by rainfall are mechanisms responsible for the occurrence of herbicide residues in pristine wetlands.

Other commonly used pesticides chlorpyrifos, chlorothalonil, metholachlor, terbufos, and trifluralin have been found in marine arctic fog samples collected in the remote and pristine Chukchi and Bearing Seas (Rice and Chernyak 1997). The authors believe that fogwater plays an important role in recycling pesticide residues within the ecosystem, and that fog can act as a natural concentrator of contaminants, especially when it begins to evaporate. Fog can then serve as a carrier of the concentrated chemicals to receptors such as plants. The sample areas were located “several thousand miles from likely usage areas”. The levels of different pesticides in fog ranged from 0.08 to 12 ng/L.

Atrazine, the most commonly used herbicide in the US, has become ubiquitous in most soil, water, and air samples collected by the United States Geological Survey (USGS). Atrazine residues and those of other herbicides and metabolites have been documented as occurring in stream surface waters, the Great Lakes, the Chesapeake Bay, the Mississippi River, and the Gulf of Mexico (USGS 1995, USGS 1999, Goolsby *et al.* 1997, Scholtz and vanHeyst 2000). Atrazine in surface water samples has exceeded the Canadian aquatic life criteria of 2 ug/l at 17 sampling sites, with some peak samples as high as 27 ug/l (Larson *et al.* 1999). The 2 ug/l criterion was exceeded for a period of 35 days or more at 14 sites with the longest period being 91 days at a site in Indiana. Long range pesticide transport is well documented and can be traced several hundreds of miles from the initial application site via air, rainfall and fog samples (Gotfelty *et al.* 1987, Richards *et al.* 1987, Sieber *et al.* 1989, USGS 1995, Majewski and Chapel 1995, Rice and Chernyak 1997, Majewski *et al.* 1998, Goolsby *et al.* 1997). Majewski and Chapel

(1995) reported peak atrazine concentrations of 40 ppb in rain, 0.82 ppb in fog, 0.03 ppb in snow, and 0.02 ppb in air. The 99th percentile concentration out of 6,100 rainfall samples averages 1.1 ppb which is slightly below the Canadian aquatic life criteria reported by Larson *et al.* (1999). The large land area treated annually with atrazine, plus its persistence in cool, Northern US soils may contribute to its prevalence in most media samples.

Recently, the USGS has begun sampling for low-dose, high toxicity herbicides in surface and groundwater (Battaglin *et al. 2000).* In the first year of the study (1998), the USGS found at least one acetolactate synthase (ALS) inhibitor herbicide (sulfonylurea, sulfonamide, imidazolinone) above the limit of detection of 0.01 ug/l in 83% of stream samples and imazethapry in 71% of samples. Flumetsulam was found in 63% of stream samples and nicosulfuron in 52%. Acetochlor, alachlor, atrazine, cyanazine, and metolachlor were detected in 90% of stream samples. The sum of herbicide concentrations exceeded 50 ug/l in 10% of the samples. At least one low dose herbicide was detected above the limit of detection (0.01 ug/L) in 24% of 25 groundwater samples and 86% of seven reservoir samples. The sum of ALS inhibitor herbicide concentrations exceeded 0.5 ug/l in less than 10% of stream samples. Battaglin *et al.* (2000) assessed the literature, and the EPA and DuPont Chemical Co. data bases for data on aquatic plant toxicity on sixteen ALS inhibiting herbicides and concluded that duckweed (*Lemna gibba*) may be adversely impacted at 0.1 ug/l. This would suggest that duckweed may be adversely impacted by ALS inhibiting herbicides in about 10% or more of the streams.

There have been numerous reported incidents of non-target plant injury resulting from off-target movement of pesticides and chemicals. These incidents are reported to EPA from states, pesticide companies, private citizens and EPA regions.

Based on incident reports, non-target plants may be injured from pesticides that have moved off the targeted site. Of the alleged non-target plant incidents reported to EPA from 1990 to 1999, more than 90% occurred as a result of herbicide movement via air. The remaining alleged incidents occurred as a result of runoff or contaminated irrigation water. Much of the data reported to EPA is of a sparse nature. Of the total (1010) alleged non-target plant incidents reported, 638 did not identify the specific non-target plant injured. The 372 incidents which did identify injury to non-target plants may be grouped as follows:

- 31.9% trees (of all the trees injured, 23.2% = fruit trees, 24.4% = nut trees, and 52.4% = other trees)
- 28.8% field crops (19.2% corn, 20.5% soybean, 13.9% wheat, 7.3% oat, and 39.1% other)
- 12.2% vegetables
- 11.1% ornamentals
- 4.2% gardens
- 3.8% berries
- 3.3% fruit vines
- 1.9% turf
- 2.8% other

Details of some recent incidents can be found in Appendix 2.

The 1999 AAPCO pesticide spray drift enforcement survey found that agricultural crops accounted for 34% of the confirmed spray drift complaints of adverse effects, trees and ornamentals accounted for 23%, and lawns and gardens accounted for 22% compared to 2% or less for domestic animals/wildlife, aquatic ecosystems, livestock, and endangered/threatened species.

Appendix 17 - Valued Resources

According to some estimates, there are approximately 27,000 algae species, and 248,000 vascular plant species living today (Stiling 1996). Based on a 1995 United Nations Report, since the 1600's, 654 plant species are recorded as extinct and 26,000 currently face extinction. Only 25% of all vascular plants have edible properties and only 3,000 species are actually used for food. Wheat, rice, corn and potatoes account for one-half the calories consumed by humans. Over 25,000 plant species are used in medicine and 80% of the top prescription drugs are formulated directly from plants or from synthetic or semi-synthetic molecules modelled from plant compounds (Watson *et al.* 1995).

Frequently the same plants that serve as the food source also serve as cover. Cover conceals animals from predators and provides shelter from cold, rain, snow, wind, and heat. Plant cover needs vary with the animal species. For example, deer, bear, and wild turkey must have large acreages of certain types of plant cover to survive. Many farmers maintain plots of dual purpose plants (both food and cover) to encourage the survival of wildlife on farmlands. Hedgerows are boarders of native plant species that grow along fence rows and field edges. The hedgerows contain valuable food producing plants for wildlife such as wild cherry, wild rose, berries, honeysuckle, and poison ivy. Types of wildlife that occupy hedgerows include songbirds, upland game birds such as pheasant and quail, rabbits, opossums, raccoons, foxes and small rodents. A study of the food habits of more than 300 species of birds and mammals has been conducted. Of the 4 plant groups, the top 5 plants of importance to wildlife in the U.S. are: WOODY PLANTS - oak, pine, blackberry, wild cherry, and dogwood; UPLAND WEEDS AND HERBS - bristlegrass, ragweed, pigweed, panic grass, and wild oats; MARSH AND AQUATIC PLANTS - pondweed, bulrush, smartweed, widgeon grass, and spikerush; and CULTIVATED PLANTS - corn, wheat, oats, barley, and sorghum (Martin, 1951).

Although, economic resource driven, studies of the Great Lakes and the Chesapeake Bay have both concluded that the commercial and recreational resources will not return to these water bodies until plant life is renewed to previous (historical) levels. Submersed aquatic vegetation (SAV) has declined from an historic level of 200,000 acres to 41,000 acres in 1978. The absence of SAV translates to a loss of food and habitat for many Chesapeake Bay species (Reshetiloff 1997).

Valued plant resources include individual species such as a crop or endangered plant species; plant populations or communities such as those found in prairie potholes and wetlands; or combinations of numerous species at varying stages of growth and development in aquatic or terrestrial ecosystems.

Different types of plant habitat include:

Managed Agricultural
Unmanaged Agricultural
Managed Nonagricultural

Unmanaged Nonagricultural.

The vegetation in these habitats can be identified as having:

- Human Value
  managed food/fiber crops, plants for erosion control via hedgerows, windbreaks, vegetative buffers, cover crops, intentional plantings to enhance hunting, fishing, and bird watching; forestry products; golf courses; and managed/unmanaged nonagricultural areas for fishing, hunting, and bird watching, aesthetics (natural beauty) and recreational activities.

- Fish/Wildlife Value
  prairie potholes, conservation reserve areas, irrigation ditches, levees, and ponds in managed agricultural areas; riparian zones, swamps, wetlands, bogs, shelter belts in unmanaged agricultural areas; nature preserves, lakes, reservoirs and forested areas in managed nonagricultural areas; and wetlands, swamps, riparian zones, and forested areas in unmanaged nonagricultural areas.

- Shared Value (Human and Fish/Wildlife)
  all of the above plus: endangered plant/animal species in managed agricultural areas; endangered plant/animal species and pollution filtration in unmanaged agricultural areas, Government managed nonagricultural land (rangeland, forests, rights of way, wetlands, parks, etc.) for pollution filtration and preservation of endangered plant/animal species; and pollution filtration and preservation of endangered plant/animal species on unmanaged nonagricultural land.

The EPA/OPP is further charged with the protection of 706 plant species listed as endangered or threatened by the US Fish and Wildlife Service. Endangered/threatened plant species are often located in non-agricultural areas such as highway and powerline rights-of-way, forests, shelterbelts and wetlands and include 672 flowering plants, 26 ferns and allies, 6 conifers, and 2 lichens (US FWS 1998). These areas can receive direct pesticide applications. If a risk assessment identifies specific endangered or threatened plants at very high risk, it may be possible to propagate and test certain endangered plants in the greenhouse. A sensitivity analysis comparing the endangered plant and others within it's Family and Genus to various phytotoxicants might identify better surrogate test species and would reduce the uncertainty in risk assessments.

Breeze *et al.* (1999) described field margins that contain hedgerows, windbreaks and fences as having useful functions including: promotion of ecological stability in crops, exploitation of pest predators and parasitoids to help reduce pesticide use, enhancement of crop pollinator populations, reduction of weed ingress, buffer for pesticide spray, reduction in runoff of pesticides and fertilizer, reduction in soil erosion, promotion of bio-diversity and farm wildlife conservation, maintenance of landscape diversity, promotion of game species, encouragement of "countryside" enterprises, maintenance of historical features and maintenance of heritage or "sense of place".

Obrigawitch *et al*. (1998) state that "damage to native plants cannot always be expressed in economic terms, their value can be diminished by inadvertent exposure to herbicides affecting their use in many ways such as: (1) altering species composition; (2) lessening the value as a wildlife habitat, recreational area, or aesthetic vista; (3)reducing timber or wood pulp production; (4) impacting lifestock-carrying capacity; and (5) creating undesirable effects on the environment such as soil erosion, emergence of noxious plant species, and formation of vegetative barriers." Therefore, there comes a need to determine the potential risk to non-target plants from pesticide exposure by using phytotoxicity data.

The EPA Office of Wetlands, Oceans, and Watersheds within the Office of Water has estimated that there are approximately 353,000 acres of wetlands in the entire US. Coastal and inland wetlands protected under Section 404 of the Clean Water Act. The 2002 Farm Bill may contain wetland protection initiatives and incentives. Wetlands support aquatic and terrestrial plant species. Inland wetlands are common along rivers and streams (riparian wetlands) and some are isolated depressions surrounded by dry land such as playas, basins, and potholes. Areas of groundwater interface or saturated soils include marshes, vernal pools, bogs, wet meadows, and swamps. Benefits of wetland plants include: wildlife habitat; flood water retention; nutrient and sediment filtering; reduced pesticide and fertilizer leaching and runoff; ground water recharge; reduced shoreline erosion; natural products such as blueberries, cranberries, timber, and wild rice; recreation - hunting - fishing opportunities; and contribution to aesthetics for bird watching, hiking, and boating (www.epa.gov.OWOW wetlands). It is estimated that $15 billion are spent by the public annually on outdoor marine and estuarine recreational activities (Summers 2001).

Coastal zone areas include terrestrial and marine systems such as mangrove swamps, estuaries (bays, lagoons, tidal rivers, bayous), and wetlands (tidal and non-tidal) that contain a variety of brackish and salt water plants such as mangroves, reeds, grasses, shrubs, and trees. The most biologically diverse coastal zone areas are estuaries since their primary productivity is greater than other sectors of the marine environment (Rand and Carriger 2001). Estuaries serve as critical feeding and nursery grounds for important fish and shellfish species, provide buffers for erosion control, and provide the primary source of energy for food webs. Estuaries are particularly vulnerable to pollutants from upland sources as millions of pounds of pesticides are applied in coastal watersheds each year. In South Florida approximately 1,415 tons of atrazine, 36 tons of endosulfan, and 622 tons of chlorpyrifos are applied to crops each year. Exposure of pesticides, their degradates, and mixtures are expected to adversely affect aquatic life in the everglades estuary of Florida (DeLorenzo *et al.* 2001). Estuaries of the Atlantic and Gulf Coasts were monitored by the EPA for the time period 1990 to 1997. Water, sediments, and estuarine biota were sampled. Approximately 75% of all sediment samples contained pesticides. Sublethal effects included reductions in growth, changes in community structure (biodiversity), and changes in abundance. Poor light penetration can result in the reduction and/or loss of submerged aquatic vegetation. Low dissolved oxygen can result in large algal blooms.

Much work has already occurred that identifies plants species, habitats, communities, and ecoregions of importance to geographers, botanists, ecologists, and Federal Agencies. Terrestrial and aquatic eco-regions in the US and Canada can be determined and key plant species, habitats, communities and ecosystems within these eco-regions can be identified. Bailey (1996) describes 14 distinct ecoregion divisions within the United States (including Hawaii, Alaska, and Puerto Rico). The ecoregion divisions are: tundra, subarctic, warm continental, hot continental, subtropical, marine, prairie, Mediterranean, tropical/subtropical steppe, tropical/sub-tropical desert, temperate steepe, temperate desert, savanna, and rainforest. Risk assessments can become more refined by focusing on specific areas impacted by the chemical.

Plant geographers have used the appearance and general nature of plants to describe plant communities, which can be defined as: "....an aggregation of living organisms having mutual relationships among themselves and to their environment." The US has approximately 116 different native plant communities (Kuchler 1964). Size and form (evergreen vs deciduous or herbaceous vs woody), the position, size and shape of buds and ligules have been used to classify plants. Climate, soil types, and competitive ability are all important considerations when identifying plant communities (Oosting 1956). Given the geographical location and composition of these native plant communities, it is possible to identify those within or in close proximity to pesticide use areas. Recently developed tools that aid in the study of plant communities and distribution patterns include a combination of supercomputing technologies, remotely sensed satellite imagery, and Geographic Information System (GIS) mapping (Gosz 1993).

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