Science-Based Risk Assessment for Nontarget Effects of Transgenic Crops

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Nontarget risk assessment for transgenic crops should be case specific, depending on the plant, the transgene, and the intended release environment. We propose an ecological risk-assessment model that preserves the strengths and avoids the deficiencies of two other commonly used models, the ecotoxicology and nonindigenous-species models. In this model, locally occurring nontarget species are classified into groups according to their ecological function. Within each group, ecological criteria are used to select the species that are most likely to be affected by the transgenic crop. Initial experimental assessments are conducted in the laboratory and consist of two kinds of test: toxicity tests using purified transgene product, and whole-plant tests using intact transgenic plants. For nontarget natural enemy species, it will also be important to evaluate both direct bitrophic impacts and indirect tritrophic impacts.

Keywords: transgenic plants, risk assessment, nontarget effects, ecotoxicology, nonindigenous species

Since the 1940s, agriculture has evolved to fulfill new social roles in many developed countries. Immediately after World War II, agriculture was primarily expected to provide sufficient food and national security against food shortage. Later, agricultural products were expected to meet ever-increasing quality standards. In the 40 years since the publication of Rachel Carson's *Silent Spring*, public concerns over environmental degradation resulting from high-input, intensive agriculture have grown, stimulating the development of legislation and regulation to protect the environment (OECD 1981–1991, Bosso 1987, Hynes 1989, Kitano 1992, Forbes and Forbes 1994, Chapman 2002). In many developed countries, agriculture is now expected to produce sufficient high-quality food without harming the future productivity of the environment, and in some places it must produce food while improving the environment and meeting other cultural values. In these countries, many agricultural technologies, such as pesticides, subterranean drainage tiles, and fertilizer use, which previously had escaped public scrutiny, are now criticized for their potential adverse effects on the environment and human health.

Risk assessment has gained increasing prominence as a methodology for evaluating the environmental risks of new technologies. Risk assessment is a process by which risks are identified and the seriousness of the risks is characterized so that decisions can be made on whether or how to proceed with the technology (NRC 1996). Its prominence is due in large measure to its reliance on science to identify and characterize these risks. In many societies, the perceived value-neutrality and predictive power of science is assumed to provide the best basis for decisionmaking.

Risk assessment, however, has serious limitations. Environmental risks are most easily assessed after damage has occurred, yet risk assessment is useful for decisionmaking only when the risks are assessed before damage actually occurs. For example, the environmental risks posed by an exotic or nonindigenous species can be readily assessed after the species is established in a new environment, but until recently these risks were not assessed before the introduction of a nonnative species. Even today, early risk assessments for nonindigenous species are based on expert opinion rather than scientific fact (Orr et al. 1993), and their effectiveness is limited by large gaps in the scientific understanding of environmental and ecological processes, which create great uncertainties and undermine the predictive power of science in the analysis.

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With the advent of molecular gene technology, biologists have been able to move novel gene constructs that are coupled with novel promoters into crop plant genomes, creating transgenic plants. This enables the plants to express novel compounds, including insecticidal substances that kill certain organisms when they feed on the transgenic crop. For example, *Bt* crop plants (which can include corn, cotton, and potatoes, among others) express one of the genes from the bacterium *Bacillus thuringiensis* that produce an insecticidal crystal protein called a Cry toxin. Today's environmentally aware public has demanded a rigorous evaluation of the ecological risks of releasing these transgenic crops into the environment. This has focused public attention on the scientific basis for the environmental risk assessment that informs regulatory decisions on the release of transgenic crops (e.g., OSTP 1986, CBD 2000, EC 2001). All of the existing regulatory frameworks agree that the risk assessment should (a) be science based; (b) be conducted on a case-by-case basis, rather than automatically generalizing results from one case to the next; and (c) take into account the triad of the transgene, the organism, and the environment into which the proposed release would occur. We use these criteria to differentiate among nontarget risk-assessment models.

Although regulatory frameworks agree on these criteria, they differ in their position on using a precautionary approach to regulatory decisionmaking. A precautionary approach is fundamental to the Cartagena Protocol (CBD 2000) and to European Union regulations (EC 2001), but it is ancillary to the US oversight system (OSTP 1986). We will suggest how nontarget risk-assessment models support a scientific approach to precaution.

Given the changing scientific and social contexts for environmental risk assessment, it is inevitable that new risk-assessment models must be developed periodically. In this article, we evaluate three models for assessing environmental risks to nontarget organisms from transgenic crop plants. We first describe relevant characteristics of two models, ecotoxicology testing of chemicals and risk assessment of nonindigenous species. Both of these models have important scientific strengths and deficiencies for environmental risk assessment of transgenic organisms. Combining the strengths of both models, we describe a third model for nontarget risk assessment based on ecological principles. Although the potential applications of this model extend beyond transgenic plants, we focus our discussion only on these organisms. Our emphasis is on the scientific basis for environmental risk assessment in support of a decisionmaking process. We do not address the many important problems associated with the valuation of risk or with the decisionmaking process itself.

Some general principles for nontarget risk assessment of transgenic crops

A standard methodology for nontarget environmental risk assessment is the tiered, hierarchical ecotoxicology model (Forbes and Forbes 1994). Hierarchical schemes for ecotoxicology testing commence with simple tests on a few species and advance through a tiered sequence of tests, which increase in complexity, sophistication, cost, and duration (Cairns 1981), depending on the results from testing at the lower tiers. Tests are initiated at the second tier only if results at the first tier indicate potential adverse effects. The goals of hierarchical testing schemes are to minimize both the cost of testing and the risk of environmental harm. Cost is limited by avoiding the time and expense of the higher-tier tests, thereby concentrating effort on those cases with the greatest potential danger to the most susceptible organisms (Forbes and Forbes 1994). Risk is limited when the initial protocols minimize the adverse consequences of incomplete information and uncertainty (Hill and Sendashonga 2003). In this article, we focus on these initial protocols.

The three main challenges for a nontarget risk-assessment model are (1) to select nontarget species to test, (2) to specify an experimental end point (a response variable, or what needs to be measured), and (3) to design experimental methodologies (table 1).

Species selection criteria. From the large number of potential test species, a smaller number of species must be selected for testing using clear, scientifically justifiable selection criteria. These selection procedures are constrained by researchers' ignorance about the ecological roles of most species. For example, even in the well-studied maize monoculture in north-central North America, there are more than 600 nontarget species (Warters 1969), of which nearly half have an unknown ecological role. The state of knowledge is similar for rice in Japan (Takahashi 1989) and in the Philippines (Schoenly et al. 1996) and is likely to be worse for other crops and for nonagricultural ecosystems.

End point. The experimental end point should be clear, measurable, and relevant to the characteristics of transgenic plants. For example, existing transgenic plants produce transgene products continuously over the lifetime of the plant, so end points need to measure the outcome of events that extend over this time period. For a nontarget species with a 1-month generation time, end points probably need to measure events that extend over the entire generation time of that organism, so that reasonable measures of potential risk can be evaluated.

Test methodology. The experimental protocols must be linked to a decisionmaking process that triggers additional testing. The methodology should be repeatable and consistent and should provide results that can be extrapolated to conditions in relevant environments outside the laboratory.

In practice, these are complex issues, and suitable solutions are difficult to implement. In the brief summaries of the environmental risk-assessment models that follow, we provide suggestions for improving the risk-assessment process in some practical, constructive ways.

Table 1. Comparison of three models for assessing the risks of transgenic plants to nontarget organisms.

	Ecotoxicology model	Nonindigenous-species model	Ecological model
Species selection criteria	Indicator species	Species at risk and other nontarget species	Representatives of functional groups
End point	**Acute toxicity**	**Invasiveness**	**Fitness**
Clarity and measurability	Clear and measurable	Difficult to measure; estimated by expert opinion	Clear, but requires careful experimentation
Exposure within individuals	Short-term exposure	Long-term exposure	Long-term exposure
Exposure across generations	No cross-generation exposure	Considers long-term exposure across generations	Fitness can be extrapolated across generations
Test methodology	**Single-chemical dose-response assay**	**Synthesize expertise**	**Exposure to whole plant and single-chemical assay**
Repeatability and consistency	Repeatable and consistent	Possibly repeatable and consistent	Repeatable and consistent
Relevance to risk	Not very relevant	Relevant	Relevant
Relation to decisionmaking process	Linked; weak scientific justification	Often linked	Can be linked

The ecotoxicology model for nontarget risk assessment

The ecotoxicology model aims to evaluate the potential nontarget effects of chemicals released in the environment. The strategy is to expose single chemicals to the same battery of animal and plant species (universal indicator species), extrapolate estimates of nontarget effects, and recommend measures to be taken for the handling and use of the chemicals. It is a hierarchical, tiered model, and tier 1 tests are based primarily on estimating acute toxicity in the laboratory (Forbes and Forbes 1994, CEC 1996, EPA 1998).

Nontarget species selection for ecotoxicology testing. Universal indicator species are chosen because of their supposed sensitivity to chemical toxins, their wide availability, their ease of culture, and their genetic uniformity (Fent 1998, Chapman 2002). Such species are supposed to provide information on the likely effects of the chemical on a wider range of species (Kitano 1992, Steinberg et al. 1995, Elmegaard and Jagers op Akkerhuis 2000). The Organisation for Economic Co-operation and Development's minimum indicator species for premarket regulatory evaluations of chemical pesticides are an algae, a water flea, and a fish (Kitano 1992). However, this approach has weak ecological justification, because the selected species do not adequately predict the sensitivity of other nontarget species (Elmegaard and Jagers op Akkerhuis 2000, Forbes and Calow 2002) or of important population, community, and ecosystem responses (Cairns and Mount 1990, Forbes and Forbes 1994, Forbes and Calow 2002). Little can be inferred about effects on other populations, communities, or ecosystems from universal indicator species tests (Forbes and Forbes 1994).

To deal with the uncertainty associated with extrapolation from the universal indicator species to other relevant nontarget species, researchers introduce safety factors of up to 500 (a safety factor is used to extrapolate from the indicator species to all other species for the measured endpoint) (Elmegaard and Jagers op Akkerhuis 2000, Forbes and Calow 2002). For example, assuming that all nontarget species are equally important, a chemical with a safety factor of 500 is extrapolated to have no acute toxicity for at least 80% of the nontarget species, at least 50% of the time, at concentrations 500 times less than the concentration at which it shows no acute toxicity to the universal indicator species. However, the scientific basis for these factors is somewhat arbitrary, the residual uncertainty is substantial, and the predictive capability is poor (Elmegaard and Jagers op Akkerhuis 2000).

The most serious problem with this approach is that it is not consistent with the need for case-by-case risk assessment that considers the relevant transgene, crop plant, and environment. Use of indicator species is intended to transcend the details of a particular case, whereas the case-by-case approach calls for tailoring nontarget evaluations to the relevant species and environment. For example, even if algae, water fleas, and fish were good indicators of the impacts of some chemicals to aquatic habitats, any effects of a *Bt* maize on aquatic habitats would be more likely to occur through the decomposition food chains, involving aquatic arthropod shredders and filter feeders, rather than through the primary production food chain (algae and water fleas). Hence, aquatic nontarget testing for *Bt* maize might better focus on mayflies, caddisflies, and chironomids. In contrast, aquatic nontarget testing for transgenic rice, which grows in semiaquatic habitats, should probably evaluate both detritivorous and primary-production food chains. For species that are more appropriate to the case, smaller safety factors can be used.

Experimental end point for the ecotoxicology model. In the ecotoxicology model, the primary end point is mortality or some other acute response from short-term exposure to the chemical. These responses are clear, unambiguous, repeatable end points. They have become a standard largely because of historical precedence in the field of toxicology, and because tests for acute toxicity are simpler to perform and easier to

standardize and interpret than most other toxicological tests (Chapman 2002). These responses, however, reveal little about other ecological impacts at the population, community, or ecosystem level (Cairns 1981, Elmegaard and Jagers op Akkerhuis 2000, Stark et al. 2004).

To address the uncertainty associated with extrapolating from acute toxicity to all other ecological effects, researchers use uncertainty factors ranging from 10 to 10,000 (an uncertainty factor is used to extrapolate from the measured endpoint to all other possible environmental endpoints for the indicator species) (Kitano 1992, Forbes and Calow 2002). For example, a chemical with an uncertainty factor of 1000 would be considered to have no ecological effects on the indicator species if it caused no acute toxicity in laboratory tests at 1000 times the concentration expected in the environment. Unfortunately, with uncertainty factors of this magnitude, predictions from these tests are of little practical value (Forbes and Forbes 1994). Because transgenic plants release a continuous dose of the transgene product, sometimes high and sometimes low, often for the entire lifetime and sometimes across generations of the nontarget species, acute toxicity end points should be changed to end points capable of evaluating longer-term effects. This would allow the use of smaller uncertainty factors.

Methodology for ecotoxicology tests. The experimental protocol central to the first tier of the ecotoxicology model is the dose-response assay (Steinberg et al. 1995, Fent 1998). The dose-response assay exposes the selected nontarget species in the laboratory to different doses or concentrations of a single chemical and measures the response of each individual exposed. Exposure is usually for a short period of time, such as minutes to hours, through food or topical application. For example, individuals may be exposed to different concentrations of the chemical in their food for a single feeding bout and the mortality rate measured at each concentration. From these measured mortality rates, the LC_{50} (lethal concentration at which 50% of the individuals are expected to die) is estimated. If the LC_{50} exceeds the maximum expected exposure to individuals in the environment multiplied by the uncertainty factor and the safety factor, the chemical is inferred to have no significant effect on the majority of organisms in the environment (Fent 1998, Elmegaard and Jagers op Akkerhuis 2000). For example, if all nontarget species are equally important, with an LC_{50} of 5 micrograms of toxin per gram, an uncertainty factor of 100, and a safety factor of 50, then environmental exposure of 0.1 nanogram or less of toxin per gram would be inferred to have no ecological effect on at least 80% of the species at least 50% of the time.

There are several problems in adapting this methodology to transgenic plants. One problem is that the purified, microbially produced chemicals that are typically used in the tests often are not identical to the chemicals produced in the transgenic plant. For example, in lepidopteran active transgenic Cry1-maize events, the transgene products have a molecular weight of 65 to 91 kilodaltons (kDa) (table 2), whereas various microbes produce proteins ranging from 130 to 140 kDa (van Rie et al. 1989, Kumar et al. 1996), and trypsinized toxin is a 65-kDa, activated Cry1 toxin. It is crucial that the chemical that is expressed in the transgenic plant be used in toxicological testing.

An even more significant problem is that the ecotoxicology model focuses on only one chemical. The experimental protocols isolate the chemical so that its effects can be evaluated. For transgenic plants, isolating the transgene product as a pure chemical for testing also isolates it from its role in plant growth, development, and metabolism. This has been referred to as the "two-part" model (NRC 2002), in which the equivalence of the transgenic crop plant without its transgene and the unmodified plant is assessed. Actually, the transgene product is metabolized in the targeted plant and in some nontarget species; it can interact with other chemicals and alter the expression of other genes, including the production of secondary plant products. For example, for transgenic Cry1-maize events, a variety of different transgene products have been reported from single transformation events, which may be an indication that in-plant metabolic processing is occurring or that multiple products are being produced (e.g., event 176 [36-, 40-, and 60-kDa fragments], Cry9C [55- and 68-kDa fragments]; table 2). Moreover, three different insecticidal transgene proteins (snowdrop lectin agglutin GNA, jackbean lectin concanavalin A, and cowpea trypsin inhibitor CpTi) reduced glycoalkaloid content in potatoes (Birch et al. 2002), and three different *Bt*-maize events (event 176, *Bt*11, and Mon810) had higher lignin content than their nontransgenic near isolines (Saxena and Stotzky 2001). The chemical composition of transgenic plants can be substantially altered by the expression of a transgene, and these changes, in turn, can have effects on nontarget species completely independent of the transgene product itself. Hence, initial testing must be broadened beyond single-chemical testing.

Relevance of the ecotoxicology model to transgenic crops. There are many aspects of the ecotoxicology model that are relevant to assessing the environmental risks of transgenic plants, including the use of uncertainty factors, multiple lines of evidence, and tiering (Hill and Sendashonga 2003). However, although acute toxicity testing of the transgene product in the laboratory should be a part of initial testing for transgenic crops, it is insufficient to ensure accurate decisionmaking in risk assessment. It will be essential to use additional methods, relying on longer-term exposures, that consider the multiple chemical alterations occurring in transgenic plants and include end points more readily related to risk assessment. It will also be critical to abandon the use of universal indicator species and develop a species selection process that allows risk assessment to adapt on a case-by case basis to the particularities of the transgene, crop plant, and environment in which the transgenic plant will be used.

Table 2. Comparison of Bt (Bacillus thuringiensis) *protein expression in some transgenic* Bt *plants.*

Crop	Event (company)	Promoter	Transgene	Molecular weight of transgene product expressed in plant (in kilodaltons)	Reference
Maize	176 (Syngenta)	*PEPC* and *POL* (a pollen-specific promotor)	*Cry1Ab* (synthetic)	65[a]	AGBIOS (2002)
Maize	*Bt*11 (Syngenta)	*CaMV35S* (modulated by *IVS6* intron)	*Cry1Ab* (truncated, synthetic)	Possibly 65[b]	Australia New Zealand Food Authority (2001)
Maize	Mon810 (Monsanto)	*CaMV35S* (enhanced; modulated by *HSP70* intron)	*Cry1Ab* (truncated, synthetic)	91	AGBIOS (2002)
Maize	DBT418 (Dekalb)	*CaMV35S* (two copies octopine synthase enhancer and introns)	*Cry1Ac*	66	AGBIOS (2002)
Maize	CBH-351 (Aventis)	*CaMV35S*	*Cry9c* (truncated from N-, C-terminal)	68 (can be partially degraded to a 55-kDa form)	Bucchini and Goldburg (2000)
Maize	Mon863 (Monsanto)	*CaMV35S* (with four repeats of an activating sequence and rice actin intron)	*Cry3Bb* (addition of alanine residue at position 2 of protein)	74 (active form)	EPA (2001b)
Cotton	Mon531 (Monsanto)	*CaMV35S* (with a duplicated enhancer region)	*Cry1Ac* (truncated)	No information available	AGBIOS (2002)

a. Three immunoreactive proteins weighing approximately 60, 40, and 36 kilodaltons were also detected in leaves but not in pollen.

b. The *Cry1Ab* toxin extracted from corn leaf tissue displays characteristics and activities similar to those produced in *Escherichia coli* transformed to produce *Cry1Ab*. The purified tryptic core proteins from both plant and microbe were shown to be similar in molecular weight by SDS-Page (EPA 2000a).

The nonindigenous-species model for environmental risk assessment

Nonindigenous species invasions have been proposed repeatedly as a useful model for understanding the environmental effects of transgenic crops (Sharples 1983, Regal 1986, Andow et al. 1987, Tiedje et al. 1989), but little consideration has been given to the applicability of the risk-assessment methods. Present environmental risk assessment of nonindigenous species is based on a model developed by Orr and colleagues (1993). This is a nonhierarchical, nontiered model that aims to produce a complete assessment of unmitigated environmental risks associated with the unintended introduction of nonindigenous species. The risk assessment is initiated by identifying a commodity involved in international trade. High-volume or high-value commodities are often the focus of a risk assessment, because the expense of assessing them can be justified. The next step is identifying all the nonindigenous species that are associated with the commodity and may pose an environmental risk as potential pests in the country of importation. Once these species are identified, the assessment concludes with an evaluation of their potential environmental effects. Uncertainty is not explicitly incorporated into the analysis; instead, the assessment focuses on developing a best estimate of the risks. If this model were used for transgenic plants, the "nonindigenous" species would be the transgenic plant.

Nontarget species selection for nonindigenous-species risk assessment. The only nontarget species risks that are evaluated using this model are potential plant pest risks (i.e., whether nonindigenous species will directly harm economically important plants). For example, an assessment of risks associated with the importation of pine or fir logs from Mexico to the United States focused on the nonindigenous species of insects and plant pathogens associated with pine or fir that would accompany the importation. The nontarget risk was that these pest species would consume other economically important plants in the United States. These include pines and firs in forestry, natural stands of conifers, ornamental conifers, and Christmas trees (Thacz et al. 1998). The assessments are also expected to consider the potential for ecosystem destabilization, reduction of biodiversity, reduction or elimination of keystone species or of endangered or threatened species, and nontarget effects of control measures (Orr et al. 1993). However, the published assessments consider these other nontarget effects only superficially. When assessing the possible impact of transgenic crops, it will probably be insufficient to consider only potential plant pest risk.

End point for the nonindigenous-species model. Nonindigenous species risk assessment aims to determine the potential economic, environmental, social, and political impact of

nonindigenous species (Orr et al. 1993). The end point of the model is invasiveness; a species is invasive if it can cause significant alteration in community structure, such as replacing one of the more dominant species in the community, or if it can alter ecosystem function. Invasiveness is an end point that includes all the effects of the organism across its entire lifetime and across several generations. This end point, however, is ambiguous. For example, plant invasiveness may relate to nitrogen fixation, colonization of disturbances, seed size, and many other factors (Vitousek and Walker 1989, Rejmánek and Richardson 1996). Consequently, although invasiveness is a comprehensive ecological end point that relates directly to environmental risk, it is assessed only qualitatively, as low, medium, or high (Orr et al. 1993). This crude approach is case specific and takes into account both the species and its environment, but it is probably insufficient to meet the requirements for nontarget assessment of transgenic organisms.

Methodology for evaluating the end point for nonindigenous-species risk. Because of the multifaceted meaning of invasiveness, risk assessment for nonindigenous species is conducted using systematic methods for synthesizing scientific expertise (Orr et al. 1993). A team of experts is convened to identify the nonindigenous species and the native species at risk, and then to agree to subjective, qualitative assessments of the probability of establishment, and of adverse consequences resulting from establishment, for each nonindigenous species. The final assessment of risk, which combines these two assessments, tends to be dominated by considerations of the potential for economic damage. Because it is based on expertise and not on scientific data, the results of the assessment are uncertain and depend on the experts employed. Because the issues surrounding transgenic organisms are so contentious, a risk-assessment methodology based solely on scientific opinion is unlikely to satisfy anyone.

Relevance of the nonindigenous-species model to transgenic crops. Several departments and ministries of agriculture, including the US Department of Agriculture, use nonindigenous-species risk as the sole or partial legal justification for regulating the release of transgenic organisms. The actual nonindigenous-species risk-assessment model, however, is too crude to be satisfactory for transgenic organisms. Its strengths are in being case specific and in relying entirely on assessments based on the organism and its potential new environment. Compared to the ecotoxicology model, the nonindigenous-species model is not as scientifically objective, but it addresses real-world environmental concerns more directly. However, it is not designed to deal with the uncertainty involved in the assessment process and does not incorporate a precautionary approach to risk assessment.

An ecological model for nontarget risk assessment

We propose a third model for risk assessment of transgenic crops, which relies on ecological principles to select species, specify an end point, and develop assessment protocols. Species selection is done case by case, considering the transgene, organism, and relevant environment; end points are concrete and relevant to environmental risk; and assessment protocols are based on transparent, scientific principles. The relevant risk comparisons are developed at the beginning of the risk analysis, which opens up its scope to include some comparisons that might not be considered under the ecotoxicology model. This method avoids some of the potential arbitrariness involved in the nonindigenous-species model. Here we focus on the initial stages of risk assessment. Costs are minimized by focusing assessment on a few nontarget species, and uncertainty is addressed by choosing relevant species, expanding the species list, and using multiple test methodologies and uncertainty factors.

Nontarget species selection for ecological risk assessment. Species selection in the ecological model is case specific, depending on the transgenic crop and its cropping context, and prioritizes species that could be adversely affected by the transgenic crop. Species selection follows four steps: (1) establishing the functional groups according to their ecological role or function in the ecosystem, (2) classifying the nontarget species found in the relevant environment into these functional groups, (3) prioritizing these species on the basis of ecological principles, and (4) selecting a number of high-priority species to test.

Establishing functional groups. By using ecological function, it is possible to avoid inappropriate conclusions associated with the indicator species used in the ecotoxicology model, to focus testing on critical ecological processes, and to limit the number of species that must be tested. Two types of functional criteria, anthropocentric and ecological, can be used (table 3). Groups whose function is anthropocentric, or related directly to human goals, include secondary pest species, natural enemies, rare or endangered species, species used to generate income, and species of social or cultural value. Ecological functions relate to ecosystem processes and are independent of human goals. Groups with ecological functions include nontarget primary consumers, secondary consumers, pollinators, decomposers, and seed dispersers. These functional groups are not mutually exclusive. For example, many species are both secondary pests and nontarget primary consumers, and others are both natural enemies and secondary consumers.

Classifying nontarget species. As a second step, the nontarget species that occur in association with the crop in the region where the transgenic crop is intended to be released are classified into functional groups using available information and expertise. Inclusion of species that actually occur in the region generates a case-specific set of potential nontarget species. Some examples of the types of nontarget species that would be classified in the functional groups are listed in table 3, although obviously these examples are not region specific.

A vast number of species found in agricultural fields probably cannot be classified into one of these functional groups. In maize in the United States, approximately 4% of the above-

Table 3. Functional classification of terrestrial nontarget organisms in or near agricultural systems for prerelease testing of transgenic plants.

Functional group	Examples
Anthropocentric	
Alternate or secondary pests	Sporadic pests, induced pests
Natural enemies	Predators, parasitoids, parasites, competitors, ants, weed-eating herbivores.
Rare or endangered species	Red-listed species or species of general value for biodiversity conservation
Species that generate income	Honeybees, silk moths
Species of social or cultural value	Monarch butterflies, honeybees
Ecological	
Competitors	Weeds
Primary consumers (excluding target species)	Plant-consuming species that are not the target of the transgene
Secondary consumers	Species that eat primary consumers (predators, parasitoids, parasites)
Pollinators	Social and solitary insects (bees, flies, beetles)
Decomposers	Scavengers, ants, collembolans, micro organisms, earthworms, mites
Seed dispersers	Birds, small mammals, ants
Species of unknown function	Nearly half the arthropod species in a habitat

ground arthropod species are maize pests, approximately 8% are natural enemies, less than 2% are pollinators, probably 20% to 30% are soil organisms (Warters 1969), and probably less than 5% are species of conservation or cultural concern. This leaves approximately 45% of the aboveground arthropod community with an unknown function. A critical precaution, therefore, is to consider a category of species with unknown function so that these species are not inadvertently overlooked.

Prioritizing species using ecological principles. Many species are classified in each functional group. Several criteria can be used to prioritize the nontarget species, including maximum possible exposure, potential adverse effects, and potential exposure (box 1).

Possible exposure can occur through many pathways. Transgenic plant material and transgene products and metabolites may affect nontarget species directly by way of plant residue (above- or belowground; Zwahlen et al. 2003); senescent leaves and sloughed root tissue; root exudates (Saxena and Stotzky 2000); pollen (Losey et al. 1999); and other plant parts that express the transgene (Hilbeck 2002), such as seeds, nectaries, guttation fluids, and phloem sap. Any nontarget organism feeding on the transgenic plant or parts of the plant would come in contact with the transgene and its product. In addition, the transgene product might interact with existing plant compounds to affect nontarget organisms. Transgenic plant material and transgene products can affect nontarget species indirectly through other organisms, such as herbivores (Hilbeck et al. 1999) or homoptera (aphids, scales, and whiteflies, whose honeydew can contain transgenic material; Raps et al. 2001). Nontarget species could therefore be affected (a) by transgene products in the original transgenic crop, in plant secretions, in herbivores, in herbivore excretions, or in other species containing transgene products; (b) by metabolites of the transgene products; or (c) by interactions with other plant or herbivore compounds that alter plant or herbivore composition or physiology (e.g., Saxena and Stotzky 2001, Birch et al. 2002). The number of possible pathways is immense; we estimate that there are more than 250 different exposure pathways by which a transgene product or its metabolites could affect a secondary consumer, of which only a few are direct effects of the transgene product. This multitude of potential pathways for exposure has important implications for test methodology (see below) and complicates the analysis of potential exposure (Hilbeck 2002).

Although many species have an unknown ecological function, this does not imply that their ecological function is insignificant. For example, the ecological significance of microbial symbionts is only beginning to be appreciated (e.g., Werren 1997). Of the species with unknown ecological function, we suggest that those with a high standing biomass or those that are found in frequent association with the transgenic crop habitat should also be selected for testing. By explicitly considering such species for initial nontarget testing, we introduce a scientifically justified precautionary approach to risk assessment.

Selecting high-priority species to test. Those species that are given the highest priority remain as candidates for testing. This final selection process is not a purely scientific one, but it should be transparent. We suggest that several species from each functional group should be tested. Clearly, testing more species improves the level of precaution in the assessment. However, the number of species tested is also likely to be influenced by other factors, including economic and political ones.

Experimental end point for the ecological model. An appropriate experimental end point for initial testing is generational relative fitness or some component of relative fitness. Generational relative fitness is the relative lifetime survival and reproduction of the nontarget species. Thus, survival experiments should last at least through one full generation, including all the immature stages of the nontarget species. Adult life-stage parameters, including age-specific mortality and female fecundity, should be measured. In principle, the duration of the test should correspond to the time the nontarget species would be exposed to the transgenic plants, plant parts, and residues and to the temporal pattern of expression and persistence of the transgene product and

Box 1. Criteria for ranking nontarget species in each functional group to facilitate selection of species to evaluate in initial nontarget tests.

All of the following criteria are consistent with annex 3 of the Cartagena Protocol (CBD 2000).

Maximum possible exposure

The maximum possible exposure of a nontarget species to a transgenic crop is based on geographic range, habitat specificity, local abundance (Rabinowitz 1981), prevalence (proportion of suitable habitat that is occupied by the species), and temporal association with the crop. These criteria can be evaluated independent of the specific transgenic crop. Species with a broad geographic range, specificity to the crop habitat, high local abundance, high prevalence, and high temporal overlap with the transgenic crop are likely to have greater exposure.

Potential adverse effects

The potential consequences of an adverse effect on a nontarget species are considered more serious if the species has ecological or economic significance, is imperiled or rare, or has symbolic value. Ecologically significant species have significant ecological functions, such as biological control, pollination, or decomposition. Economically significant species are likely to have an economic impact if their abundance changes. Imperiled species include those that are listed on red or blue lists or are otherwise threatened or endangered; that co-occur in the same habitats as the crop; and that come into contact with the crop, with its parts, or with secretions or excretions containing its genetic material. Other rare species have some biodiversity value but are not listed. Symbolic species appear repeatedly in public in symbolic ways (e.g., flags, logos, advertisements, news); they could be species of cultural significance or species with unique attributes (e.g., social organization, mass migration, stunning and rare beauty, strength).

Potential exposure

When evaluating whether a species is likely to be exposed to the transgene product to or metabolites in the crop ecosystem, it is necessary to take into account the specific transgenic crop. Nontarget species that are not exposed directly or indirectly are less likely to be affected by the transgenic crop, and if they are affected, it will probably be through another species that was exposed to the transgene product or metabolite.

its metabolites. For example, if the transgene product is expressed through the entire growing season and the nontarget species has a 1-month generation time, it should be exposed for at least one entire generation.

Generational relative fitness is a particularly useful end point, because it relates directly to risk. If the transgenic plant adversely affects a nontarget species, its effects will come through some component of relative fitness. Hence, the results from these tests will motivate the creation and guide the design of subsequent tests by identifying the fitness components that might be affected by the transgenic plant in the environment.

To obtain useful estimates of relative fitness, true replications of the experiment should be carried out (i.e., the entire experiment should be repeated with new plants and nontarget individuals over time). At a minimum, we suggest that when uncertainty factors can be designed into experiments, the experiments should detect differences of 25% to 30% between treatments, with a P value of 0.05. The uncertainty factor probably should be greater than 10 and less than 10,000, and a higher uncertainty factor should be used when less is known about the transgene product. When uncertainty factors cannot be used, as in tests using intact plants, experiments should be designed to detect a 10% difference between treatments, with a P value of 0.05. A prospective statistical power analysis could assist in the design of these experiments, and equivalence testing could be used to analyze the results (Andow 2003). Results that exceed these minimum differences should trigger additional testing.

Methodology for evaluating the ecological end point. Two methodologies are needed to provide adequate information for nontarget risk assessment. First, the methodology of the ecotoxicology model should be modified to use long-term exposures of transgene product to the test species, mimicking potential exposure in the environment. Because it evaluates only the effects of the transgene product, this method allows the use of uncertainty factors. However, it ignores the vast majority of exposure pathways by which a transgenic plant could affect a nontarget species. Hence, such tests are ambiguous by themselves. If the transgene product affects the nontarget species, then it can be concluded that the transgenic plant might affect the species, and additional testing should be initiated. If there is no observable effect, however, it cannot be concluded that the transgenic plant has no effect on the nontarget species, because none of the indirect pathways have been evaluated. This means that these tests can be used to demonstrate potential adverse effects but cannot logically imply safety or lack of an effect.

A second methodology is therefore necessary, which we call the "whole-plant" method. This method evaluates the effects of the transgenic plant, which may be greater than the isolated effect of the transgene product. This methodology allows for the evaluation of any potential exposure pathway, but because the concentrations of chemicals cannot be increased above those that occur in the plant, it is not possible to

design uncertainty factors into the experiments. To perform whole-plant tests, it is necessary to have an appropriate experimental control for the transgenic plant and to mimic exposure as it would occur in the field. There is, however, a trade-off between these two necessities.

The ideal genetic control is a plant that is isogenic to the transgenic plant. An isogenic control is genetically identical to the transgenic plant except at the transgene locus (NRC 2002). For virtually all commercial varieties, however, such isogenic controls do not exist. Near-isogenic controls are available for some varieties, but these can differ from the transgenic variety by as much as 4% of the genome. Moreover, all transgenic varieties have had some selection for agronomic characteristics that their near-isogenic varieties have not experienced, which can result in additional systematic genetic and phenotypic differences from their near-isogenic lines, especially in agronomically important phenotypic characters.

Relying on near-isogenic controls for risk assessment is scientifically problematic. If a significant difference in ecological effect is found between the transgenic plant and its near-isogenic control, the effect could be attributed either to the transgene or to other unknown genetic differences between the varieties. If no effect is found, the lack of effect could be interpreted to mean either that the transgene has no effect or that the other genetic differences mask its effects. Thus, results from single experiments comparing transgenic and near-isogenic varieties are not very convincing. Any observed result could be caused by the transgene and its associated genetic material, by the correlated systematic differences between the transgenic variety and the near-isogenic control, or by a balancing of both effects. Perhaps the only scientifically valid approach to assessing whole-plant effects is to run multiple comparisons between several pairs of transgenic varieties and controls.

The ideal ecological control is a plant variety that could be grown in a production system in the region of interest. Such a control need not be a commonly grown cultivar. However, the variety should function adequately in a production system to be a useful ecological control. Thus, a comparison of newly regenerated transformed and untransformed plant lines would not constitute a useful ecological experiment, because results based on these newly regenerated lines are unlikely to translate to field conditions. It is also crucial that the plant and associated species are presented to the nontarget species in a way that mimics how the nontarget species would experience the plant and associated species in the field. Clearly, the ideal genetic control is unlikely to be the ideal ecological control.

Universal testing methodologies cannot be specified, because there are too many species to consider. However, an ecologically realistic experiment should meet several key criteria so that results are sound and ecologically interpretable. These criteria combine the strengths of ecotoxicological testing methods with other criteria specific to transgenic crops. At a minimum, initial tests should follow these six criteria:

1. Use the same foods in laboratory tests that are used by the test species in their relevant habitat. If a transgene product is used, it should be identical to what is produced in the transgenic plant.
2. Verify that the food offered to the species actually contained the administered material at the intended concentration or dose throughout the investigation.
3. Verify that all life stages of the species are exposed appropriately to the transgene product and actually contact the product in relevant ways.
4. Either use intact plants (or plant parts) in the experimental system, verifying that the plant parts contain the transgene product, or use the transgene product at concentrations or doses much higher than normally expressed in the plant, which incorporates an uncertainty factor into the experimental design.
5. Use a proper scientific control.
6. Screen sufficient numbers of individuals and perform sufficient replication.

Applying the ecological method to transgenic crops. We evaluated how the criteria required by the proposed ecological method for nontarget risk assessment are met in the published literature on the potential effects of *Bt* crops on *Chrysoperla carnea* (green lacewing), a nontarget secondary consumer. There are 36 species in the genus *Chrysoperla*, which occur throughout the world, except in New Zealand and Australia (Duelli 2001). Within the genus is a complex of several remarkably similar, cryptic species, within which the *carnea* group is the most important for biological control (Brooks 1994). The larvae are predaceous, whereas the adults feed on nectar, pollen, and honeydew. Because they are a common, important biological control agent for many pests that would feed on a transgenic crop, they would generally be a high-priority secondary consumer for nontarget testing (box 1). Indeed, *C. carnea* is often included as a universal indicator species in ecotoxicological testing of pesticides. We evaluated the eight published laboratory studies that assessed the potential effect of transgenic *Bt* crops on *C. carnea* by using the six key methodological criteria above (see also table 4).

Criterion 1: Test food used. None of the studies that used microbially produced activated Cry toxin verified that the toxin was identical to that produced in the transgenic plant (Sims 1995, Hilbeck et al. 1998a, 1999). Dutton and colleagues (2003) used Dipel, which contains multiple Cry toxins not found in the transgenic plant. The remaining studies used transgenic plants as the source of Cry toxin (Lozzia et al. 1998, Hilbeck et al. 1998b, Dutton et al. 2002). Pilcher and colleagues (1997) used *Bt*-maize pollen, which is not typically consumed by *C. carnea* larvae.

Criterion 2: Toxin present. All of the studies, except that of Lozzia and colleagues (1998), demonstrated that Cry toxin was present at the beginning of the experiments. Lozzia and colleagues (1998) exposed *C. carnea* to the aphid *Rhopalosiphum*

Table 4. Review of published studies investigating the impact of Bt (Bacillus thuringiensis) *proteins on* Chrysoperla carnea.

			Exposure system					
Reference	**Test system**	**Concentration of toxin**	**Criterion 1: Test food used**	**Criterion 2: Toxin present (substance or organism tested)**	**Criterion 3: Exposure (stage exposed)**	**Criterion 4: Use of whole plants or plant parts (or high concentrations of transgenic material)**	**Criterion 5: Control system**	**Criterion 6: Replication—number of trials (sample size)**
Sims 1995	Microbial, activated Cry1Ac toxin	17 µg per gram eggs	Eggs dipped in *Bt*-solution (bitrophic)	Yes (Cry solution)	Probably not (early instars; experiment terminated on day 9)	No	*Bt*-free solution	1 (30)
Pilcher et al. 1997	*Bt* maize, event 176, activated Cry1Ab toxin	As in pollen (2.57–2.94 µg per gram dry weight)	Eggs, pollen (bitrophic)	Yes (pollen)	Probably not (24 hours during each larval stage	Yes (pollen)	Non-*Bt* maize pollen	1 (90)
Lozzia et al. 1998	*Bt* maize, event 176, activated Cry1Ab toxin	As in plant phloem	*Bt*-maize-fed aphids (tritrophic)	No (aphids)	No[a] (trial 1, second and third instar; trial 2, all instars)	Yes (excised leaves)	Non-*Bt* maize plants	2 (unknown)
Hilbeck et al. 1998b	*Bt* maize, event 176, activated Cry1Ab toxin	As in plant	*Bt*-maize-fed lepidopteran larvae	Yes (plant)	Yes (neonate larva to adult)	Yes (excised leaves)	Non-*Bt* maize	4 (50 each)
			O. nubilalis (susceptible)	Yes (*O. nubilalis*)	Yes (neonate larva to adult)			
			S. littoralis (resistant; tritrophic)	Yes[b] (*S. littoralis*)	Yes (neonate larva to adult)			
Hilbeck et al. 1998a	Microbial, activated Cry1Ab toxin	100 µg per milliliter artificial, encapsulated diet	*Bt*-incorporated artificial diet (bitrophic)	Yes (diet)	Yes (neonate larva to adult)	Yes (high concentration)	*Bt*-free diet	5 (30 each)
Hilbeck et al. 1999	Microbial, activated Cry1Ab toxin, Cry1Ab protoxin, or Cry2A protoxin	Toxin, 25, 50, or 100 µg per gram diet; protoxin, 50, 100, or 200 µg per gram diet; Cry2A, 100 µg per gram diet	*Bt*-incorporated artificial diet	Yes (diet)	Yes (neonate larva to adult)	Yes (high concentration)	*Bt*-free diet	4 (30 each)
			S. littoralis (resistant; tritrophic)	Yes[b] (*S. littoralis*)	Yes (neonate larva to adult)			
Dutton et al. 2002	*Bt* maize, *Bt*-11, activated Cry1Ab toxin	As in plant phloem	*Bt* maize-fed insects	Yes (plant)	Yes (neonate larva to 2-day-old third instar)	Yes (intact leaves)	Non-*Bt* maize	2 (30 each)
			S. littoralis (resistant)	Yes (*S. littoralis*)	Yes (neonate larva to 2-day-old third instar)			
			R. padi	No (*R. padi*)	No (neonate larva to adult)			
			T. urticae (resistant)	Yes[c] (*T. urticae*)	Yes (neonate larva to 2-day-old instar)			

Table 4. Review of published studies investigating the impact of Bt (Bacillus thuringiensis) proteins on Chrysoperla carnea.

Reference	Test system	Concentration of toxin	Exposure system: Criterion 1: Test food used	Criterion 2: Toxin present (substance or organism tested)	Criterion 3: Exposure (stage exposed)	Criterion 4: Use of whole plants or plant parts (or high concentrations of transgenic material)	Criterion 5: Control system	Criterion 6: Replication—number of trials (sample size)
Dutton et al. 2003	Dipel, four Cry1 and Cry2 protoxins[d]	Approximately 0.41 mg active ingredient per plant	Dipel-exposed insects	Probably	No (neonate larva to 2-day-old third instar)	No	Nonsprayed maize	2 (30 each)
			S. littoralis (resistant)	Yes (*S. littoralis*)	Yes (neonate larva to 2-day-old third instar)	Yes (intact leaves)		
			R. padi	No (*R. padi*)	No (neonate larva to adult)			
			T. urticae (resistant)	Yes[c] (*T. urticae*)	Probably not (neonate larva to 2-day-old third instar)			

O. nubilalis, Ostrinia nubilalis (European corn borer); *R. padi, Rhopalosiphum padi* (corn leaf aphid); *S. littoralis, Spodoptera littoralis* (armyworm); *T. urticae, Tetranychus urticae* (two-spotted spider mite).
a. Raps and colleagues (2001) found no *Bt* toxin in maize phloem, in aphids feeding on maize, or in aphid honeydew; Dutton and colleagues (2002) found minor quantities of *Bt* toxin in aphids feeding on *Bt* maize.
b. Raps and colleagues (2001) confirmed the presence of *Bt* toxin in *S. littoralis* larvae.
c. ELISA reactive moiety was detected, but it may not be an active toxin.
d. See Feitelson and colleagues (1992).

padi, an obligate phloem feeder. Neither the phloem in *Bt* maize nor the honeydew of phloem-feeding aphids contains any detectable Cry toxin (Raps et al. 2001), so it is unlikely that *C. carnea* larvae would have been exposed to any Cry toxin in their contact with the aphids (Dutton et al. 2002). None of the studies confirmed that Cry toxin was maintained at the desired concentration throughout the experiment, although the food-renewal protocols probably maintained concentration levels in the experiments of Hilbeck and colleagues (1998a, 1998b, 1999) and Dutton and colleagues (2002).

Criterion 3: Insect exposed. Larvae have piercing, sucking mouthparts, with which they consume the liquid contents of eggs or prey; they do not ingest the shells or exoskeletons (or any other dry food). Hence, Cry toxin applied externally to food (Sims 1995, Dutton et al. 2003) and *Bt* pollen (Pilcher et al. 1997) will result in low, uncontrolled rates of exposure to Cry toxin. The other studies exposed larvae appropriately to transgenic material in food, although, as already mentioned, the aphids used by Lozzia and colleagues (1998) were unlikely to contain any Cry toxin.

Criterion 4: Level of exposure. Of the three studies using purified Cry toxin, one (Sims 1995) did not control exposure levels, but even their maximum reported concentration corresponded to an uncertainty factor of only 2. The other two studies used concentrations corresponding to uncertainty factors of 10 to 15 (Hilbeck et al. 1998a, 1999). Values for exposure in the study by Dutton and colleagues (2003) could not be calculated. Uncertainty factors of 10 to 10,000 are recommended (Kitano 1992), so experiments using even higher toxin concentrations can be readily justified. Of the studies using *Bt* maize, one (Pilcher et al. 1997) used intact pollen, two used excised plant tissue (Lozzia et al. 1998, Hilbeck et al. 1998b), and one (Dutton et al. 2002) used intact leaves attached to the plant.

Criterion 5: Control system. All of the studies used appropriate scientific controls.

Criterion 6: Replication. Two of the studies had no true experimental replication (Sims 1995, Pilcher et al. 1997), and Lozzia and colleagues (1998) did not report sufficient information to determine whether their study included replication. Three studies (Hilbeck et al. 1998a, 1998b, 1999) had four to five true replicates, with total sample sizes of more than 100 larvae with appropriate statistical analysis. Dutton and colleagues (2002, 2003) had two true replicates, with sample sizes of 60 larvae.

Overall, we found that the methodological criteria facilitated clear, unambiguous interpretation of the published literature on nontarget risk assessment. Of the published laboratory trials, three reported no effect of Cry toxin on *C. carnea* larvae (Sims 1995, Pilcher et al. 1997, Lozzia et al. 1998). In all three studies, it was likely that *C. carnea* larvae were not exposed to any Cry toxin (table 4). The other studies demonstrated that exposure to Cry toxin had significant toxic effects on *C. carnea* larvae (Hilbeck et al. 1998a, 1998b, 1999, Dutton et al. 2002, 2003). These experiments demonstrate the susceptibility of immature *C. carnea* to Cry1Ab toxin

through direct or indirect bitrophic and indirect tritrophic pathways. The results of Dutton and colleagues (2002, 2003) suggest additional complexity; the effects of tritrophic exposure pathways on *C. carnea* may depend on the prey species involved. Together, these five experiments suggest that transgenic *Bt* maize might cause increased mortality to *C. carnea* in the field and should trigger additional testing (Hilbeck 2001, 2002).

Conclusions

Transgenic plants possess several attributes that require modification of existing models for assessing the environmental risks to nontarget species. We propose a new ecological model for nontarget risk assessment of transgenic plants, focusing on initial assessment methodologies. We have discussed the methods for selecting nontarget species, the designation of appropriate end points for assessment, and the scientific criteria for judging the adequacy of the experimental protocols. This model addresses the deficiencies of the ecotoxicology and nonindigenous-species models while preserving the strengths of each.

Transgenic plants express the transgene as an integral part of their growth and development. This implies that the transgene is likely to interact with the plant's physiology and with the expression of its other genes. In addition, the transgene product is metabolized into other products in the plant or in associated organisms, and these products in turn could exert effects on nontarget species. Thus, the effect of a transgene, which may include pleiotropic and epistatic responses as well as potentially complex physiological interactions, is likely to be greater than the isolated effect of the transgene product. Consequently, risk assessment cannot rely solely on single-chemical toxicity tests; it must include tests that use intact plants or plant parts. We suggest that a whole-plant methodology be incorporated as a part of nontarget risk-assessment protocols.

Published experimental methodologies are extraordinarily variable, so we have proposed six criteria by which the adequacy of an experiment can be evaluated. We applied these criteria to studies examining the effect of Cry toxins on the green lacewing *C. carnea*. In almost all of the experiments that reported no effect of Cry toxin, we found that lacewings probably did not ingest any significant amount of toxin; hence, the negative findings are inconclusive. In contrast, many of the experiments that met the criteria found that ingestion of foods with Cry1Ab toxin increased the mortality of lacewings. Moreover, these experiments affirmed the need for tritrophic studies and for the use of whole organisms to assess the risks of transgenic insecticidal plants to nontarget natural enemies. These results suggest that additional testing should be done for the effects of *Bt* maize on *C. carnea* in the field.

Ecological theory can be used to improve environmental risk assessment and tailor it to specific environments. Local species can be classified functionally and prioritized using risk-based ecological criteria to quickly identify potential test species, assessment end points, and experimental methodologies. This should improve the scientific basis for decisionmaking about transgenic crops and, potentially, about other possible environmental risks.

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References cited

AGBIOS. 2002. Essential Biosafety. 2nd. ed. CD-ROM. Merrickville, Ontario (Canada): AGBIOS. (7 June 2004: *www.essential.biosafety.com*)

Andow DA. 2003. Editorial: Negative and positive data, statistical power, and confidence intervals. Environmental Biosafety Research 2: 75–80.

Andow DA, Levin SA, Harwell MA. 1987. Evaluating environmental risks from biotechnology: Contributions of ecology. Pages 125–144 in Fowle III JR, ed. Application of Biotechnology: Environmental and Policy Issues. Boulder (CO): Westview.

Australia New Zealand Food Authority. 2001. Draft Risk Analysis Report, Application A386, Food Derived from Insect-Protected, Herbicide-Tolerant Bt-11 Corn. (28 June 2004; *www.agbios.com/docroot/decdocs/2001079-A.pdf*)

Birch ANE, Geoghegan IE, Griffiths DW, McNicol JW. 2002. The effect of genetic transformations for pest resistance on foliar solanidine-based glycoalkaloids of potato (*Solanum tuberosum*). Annals of Applied Biology 140: 143–149.

Bosso CJ. 1987. Pesticides and Politics: The Life Cycle of a Public Issue. Pittsburgh (PA): University of Pittsburgh Press.

Brooks SJ. 1994. A taxonomic review of the common green lacewing genus *Chrysoperla* (Neuroptera: Chrysopidae). Bulletin of the British Museum (Natural History), Entomology 63: 137–210.

Bucchini L, Rebecca Goldburg R. 2000. Comments from Environmental Defense on EPA's "Cry9C Food Allergenicity Background Document," 29 February 2000. (7 June 2004; *www.biotech-info.net/Comment3.pdf*)

Cairns J Jr. 1981. Sequential versus simultaneous testing for evaluating the hazards of chemicals to aquatic life. Marine Environment Research 4: 165–166.

Cairns J Jr, Mount DI. 1990. Aquatic toxicology. Environmental Science and Technology 24: 154–161.

[CBD] Secretariat of the Convention on Biological Diversity. 2000. Cartagena Protocol on Biosafety to the Convention on Biological Diversity: Text and Annexes. Montreal: Secretariat of the Convention on Biological Diversity.

[CEC] Commission of the European Communities. 1996. Technical Guidance Document in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC) No. 1488/94 on Risk Assessment for Existing Substances. (4 June 2004; *http://ecb.jrc.it/php-bin/reframer.php?B=/TGD*)

Chapman PM. 2002. Integrating toxicology and ecology: Putting the "eco" into ecotoxicology. Marine Pollution Bulletin 44: 7–15.

Duelli P. 2001. Lacewings in field crops. Pages 158–171 in McEwen P, New TR, Whittington AE, eds. Lacewings in the Crop Environment. Cambridge (United Kingdom): Cambridge University Press.

Dutton A, Klein H, Romeis J, Bigler F. 2002. Uptake of *Bt*-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. Ecological Entomology 27: 441–447.

———. 2003. Prey-mediated effects of *Bacillus thuringiensis* spray on the predator *Chrysoperla carnea* in maize. Biological Control 26: 209–215.

[EC] European Community. 2001. Directive 2001/18/EC of the European Parliament and of the Council, 12 March 2001, on the Deliberate Release into the Environment of Genetically Modified Organisms and Repealing Council Directive 90/220/EEC. (4 June 2004; *http://europa.eu.int/eur-lex/pri/en/oj/dat/2001/l_106/l_10620010417en00010038.pdf*)

Elmegaard N, Jagers op Akkerhuis GAJM. 2000. Safety Factors in Pesticide Risk Assessment: Differences in Species Sensitivity and Acute-Chronic Relations. Silkeborg (Denmark): National Environmental Research Institute. NERI Technical Report 325.

[EPA] US Environmental Protection Agency. 1998. Guidelines for Ecological Risk Assessment. Washington (DC): EPA. EPA/630/R-95-002F.

———. 2001a. Biopesticides Registration Action Document—*Bacillus thuringiensis* Plant-Incorporated Protectants, 15 October 2001 (7 June 2004; www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm)

———. 2001b. Pesticide Products; Bt Corn Registration Application. Federal Register 66: 15435–15436. (7 June 2004; *www.epa.gov/fedrgstr/EPA-PEST/2001/March/Day-19/p6720.htm)*

Feitelson J, Payne J, Kim L. 1992. *Bacillus thuringiensis:* Insects and beyond. Bio/Technology 10: 271–275.

Fent K. 1998. Ökotoxikologie. Stuttgart (Germany): Thieme Verlag.

Forbes VE, Calow P. 2002. Extrapolation in ecological risk assessment: Balancing pragmatism and precaution in chemical controls legislation. BioScience 52: 249–257.

Forbes VE, Forbes TL. 1994. Ecotoxicology in Theory and Practice. London: Chapman and Hall.

Hilbeck A. 2001. Implications of transgenic, insecticidal plants for insect and plant biodiversity. Perspectives in Plant Ecology, Evolution and Systematics 4: 43–61.

———. 2002. Transgenic hostplant resistance and nontarget effects. Pages 167–185 in Letourneau D, Burrows B, eds. Genetically Engineered Organisms: Assessing Environmental and Human Health Effects. Boca Raton (FL): CRC Press.

Hilbeck A, Moar WJ, Pusztai-Carey M, Filippini A, Bigler F. 1998a. Toxicity of the *Bacillus thuringiensis* Cry1Ab *toxin* on the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae) using diet incorporated bioassays. Environmental Entomology 27: 1255–1263.

Hilbeck A, Baumgartner M, Fried PM, Bigler F. 1998b. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environmental Entomology 27: 480–487.

Hilbeck A, Moar WJ, Pusztai-Carey M, Filippini A, Bigler F. 1999. Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea.* Entomologia Experimentalis et Applicata 91: 305–316.

Hill RA, Sendashonga C. 2003. General principles for risk assessment of living modified organisms: Lessons from chemical risk assessment. Environmental Biosafety Research 2: 81–88.

Hynes HP. 1989. The Recurring Silent Spring. New York: Pergamon Press.

Kitano M. 1992. Updating of OECD guidelines for the testing of chemicals. Water Science and Technology 25: 465–472.

Kumar PA, Sharma RP, Malik VS. 1996. The insecticidal proteins of *Bacillus thuringiensis.* Advances in Applied Microbiology 42: 1–43.

Losey JE, Rayor LS, Carter ME. 1999. Transgenic pollen harms monarch butterflies. Nature 399: 214.

Lozzia GC, Furlanis C, Manachini B, Rigamonti IE. 1998. Effects of Bt-corn on *Rhopalosiphum padi* L. (Rhynchota Aphididae) and on its predator *Chrysoperla carnea* Stephens (Neuroptera Chrysopidae). Bollettino di Zoologia Agraria e di Bachicoltura, Series II 30: 153–164.

[NRC] National Research Council. 1996. Understanding Risk: Informing Decisions in a Democratic Society. Washington (DC): National Academies Press.

———. 2002. Environmental Effects of Transgenic Plants: The Scope and Adequacy of Regulation. Washington (DC): National Academies Press.

[OECD] Organisation for Economic Co-operation and Development. 1981–1991. Guidelines for the Testing of Chemicals. Paris: OECD.

Orr RL, Cohen SD, Griffin RL. 1993. Generic Non-indigenous Pest Risk Assessment Process (for Estimating Pest Risk Associated with the Introduction of Non-indigenous Organisms). Washington (DC): US Department of Agriculture.

[OSTP] US Office of Science and Technology Policy. 1986. Coordinated framework for the regulation of biotechnology. Federal Register 51: 23303–23350.

Pilcher CD, Obrycki JJ, Rice ME, Lewis LC. 1997. Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology 26: 446–454.

Rabinowitz D. 1981. Seven forms of rarity. Pages 205–217 in Synge H, ed. The Biological Aspects of Rare Plant Conservation. New York: Wiley.

Raps A, Kehr J, Gugerli P, Moar WJ, Bigler F, Hilbeck A. 2001. Immunological analysis of phloem sap of *Bacillus thuringiensis* corn and of the nontarget herbivore *Rhopalosiphum padi* (Homoptera: Aphididae) for the presence of Cry1Ab. Molecular Ecology 10: 525–533.

Regal PJ. 1986. Models of genetically engineered organisms and their ecological impact. Pages 111–129 in Mooney HA, Drake JA, eds. Ecology of Biological Invasions of North America and Hawaii. New York: Springer-Verlag.

Rejmánek M, Richardson DM. 1996. What attributes make some plant species more invasive? Ecology 77: 1655–1660.

Saxena D, Stotzky G. 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn in vitro and in situ. FEMS Microbiology Ecology 33: 35–39.

———. 2001. Bt corn has higher lignin content than non-Bt corn. American Journal of Botany 88: 1704–1706.

Schoenly K, Cohen JE, Heong KL, Litsinger JA, Aquino GB, Barrion AT, Arida G. 1996. Food web dynamics of irrigated rice fields at five elevations in Luzon, Philippines. Bulletin of Entomological Research 86: 451–466.

Sharples FE. 1983. Spread of organisms with novel genotypes: Thoughts from an ecological perspective. Recombinant DNA Technical Bulletin 6: 43–56.

Sims SR. 1995. *Bacillus thuringiensis* var. kurstaki [CryIA(c)] protein expressed in transgenic cotton: Effects on beneficial and other non-target insects. Southwestern Entomologist 20: 493–500.

Stark JD, Banks JE, Vargas R. 2004. How risky is risk assessment: The role that life history strategies play in susceptibility of species to stress. Proceedings of the National Academy of Sciences 101: 732–736.

Steinberg C, Klein J, Brüggmann R. 1995. Ökotoxikologische Testverfahren. Landsberg (Germany): Ecomed.

Takahashi F. 1989. From an Exclusive Pest Management towards a Harmonized One [in Japanese]. Tokyo: Nobunkyo.

Thacz BM, Burdsall HH Jr, DeNitto GA, Eglitis A, Hanson JB, Kliejunas JT, Wallner WE, O'Brian JG, Smith EL. 1998. Pest Risk Assessment of the Importation into the United States of Unprocessed *Pinus* and *Abies* Logs from Mexico. Madison (WI): US Department of Agriculture. General Technical Report FPL-GTR-104.

Tiedje JM, Colwell RK, Grossman YL, Hodson RE, Lenski RE, Mack RN, Regal PJ. 1989. The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. Ecology 70: 298–315.

van Rie J, Jansens S, Höfte H, Degheele D, van Mellaert H. 1989. Specificity of *Bacillus thuringiensis* δ-endotoxins. European Journal of Biochemistry 186: 239–247.

Vitousek PM, Walker LR. 1989. Biological invasion by *Myrica faya* in Hawaii: Plant demography, nitrogen fixation, ecosystem effects. Ecological Monographs 59: 247–265.

Warters ME. 1969. A faunistic and ecological study of the insects found on the corn plant in Minnesota. PhD dissertation. University of Minnesota, St. Paul.

Werren JH. 1997. Biology of *Wolbachia.* Annual Review of Entomology 42: 587–609.

Zwahlen C, Hilbeck A, Howald R, Nentwig W. 2003. Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris.* Molecular Ecology 12: 1077–1086.