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A screening method for prioritizing non-target invertebrates for improved biosafety testing of transgenic crops

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We have developed a screening method that can be used during the problem formulation phase of risk assessment to identify and prioritize non-target invertebrates for risk analysis with any transgenic plant. In previously published protocols for this task, five criteria predominated. These criteria have been combined by our method in a simple model which assesses: (1) the possible level of risk presented by the plant to each invertebrate species (through measurements of potential hazard and exposure, the two principal criteria); (2) the hypothetical environmental impact of this risk (determined by the currently known status of the species' population in the ecosystem and its potential resilience to environmental perturbations); (3) the estimated economic, social and cultural value of each species; and (4) the assessed ability to conduct tests with the species. The screening method uses information on each of these criteria entered into a specially designed database that was developed using Microsoft® Access 2003. The database holds biological and ecological information for each non-target species, as well as information about the transgenic plant that is the subject of the risk assessment procedure. Each piece of information is then ranked on the basis of the value of the information to each criterion being measured. This ranking system is flexible, allowing the method to be easily adapted for use in any agro-ecosystem and with any plant modification. A model is then used to produce a Priority Ranking of Non-Target Invertebrates (PRONTI) score for each species, which in turn allows the species to be prioritized for risk assessment. As an example, the method was used to prioritize non-target invertebrates for risk assessment of a hypothetical introduction of *Bacillus thuringiensis* (*Bt*) Cry1Ac-expressing *Pinus radiata* trees into New Zealand.

Keywords: screening method / non-target invertebrates / transgenic crops / ecological risk assessment

INTRODUCTION

Criteria for selecting non-target species as assessment endpoints

In most countries, transgenic plants are subject to laws which require environmental risk assessment before commercial release, and most include provisions for the protection of biodiversity or minimizing harm to non-target organisms (*e.g.*, Anon, 1996; EU, 2001; USEPA, 2006). However, in any country there are likely to be hundreds of potential non-target species in the receiving environment for a transgenic crop plant, and to gather biosafety data on each before making a decision on the plant's release would be impractical and excessively restrictive. Thus, there is a need for methods to screen or rank potential non-target organisms so that subsets of species can be selected for consideration in the risk assessment of each type of transgenic plant.

A widely accepted conceptual framework for ecological risk assessment begins with a problem formulation phase, in which particular environmental entities or attributes requiring protection from harm are defined (assessment endpoints), conceptual models describing their relationships are developed, risk hypotheses formulated, and an "analysis plan" for examining these hypotheses is made (Fig. 1) (USEPA, 1998). The risk assessment then proceeds to the actual analysis and characterization of the risks posed by the stressor in question. Within this framework, the selection of assessment endpoints is a crucial early step, upon which the success of the entire risk assessment can depend (Raybould, 2007; USEPA, 1998).

Criteria for selecting assessment endpoints include ecological relevance, susceptibility to known or potential stressors (sensitivity and exposure), and relevance to management goals (usually defined in policy)

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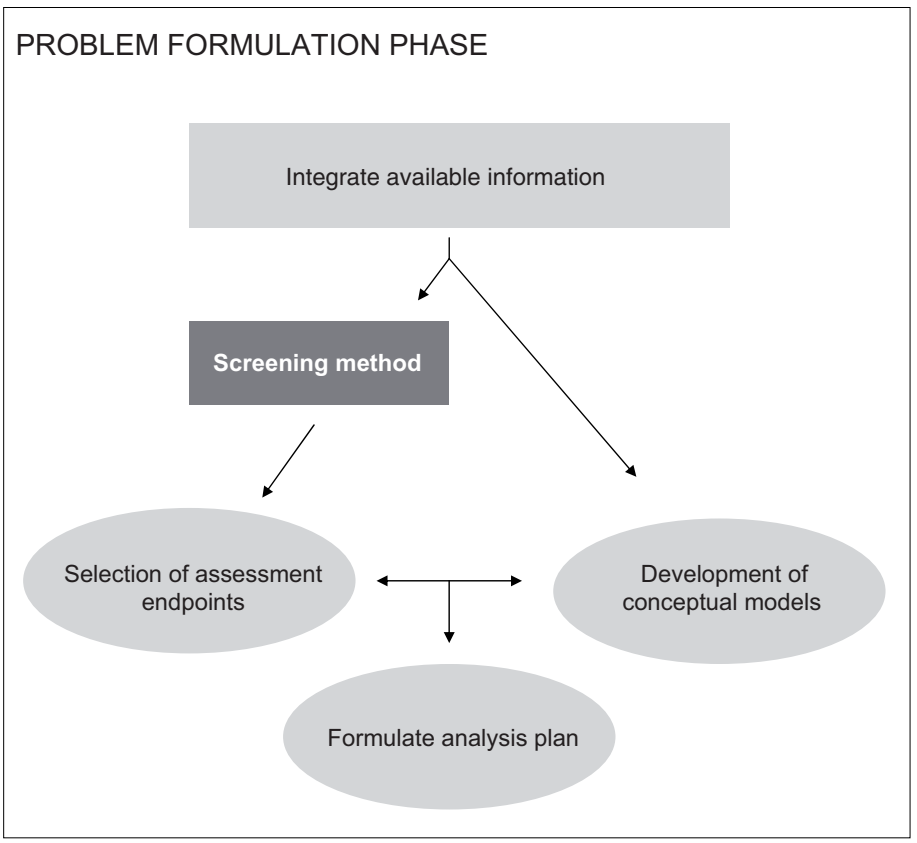

Figure 1. The screening method for prioritizing non-target invertebrates fits into the problem formulation phase of a risk assessment framework. By prioritizing the invertebrate species in the receiving ecosystem for a GMO, the screening method can be used to facilitate the selection of species as appropriate assessment endpoints for risk analysis testing. Figure is based on that produced by USEPA (1998).

(USEPA, 1998). The first two criteria are scientifically defensible; the third is subjective but essential if the risk assessment is to be of use to decision-makers and regulators (who are bound by policy). Assessment endpoints have two elements: a specific ecological entity and the characteristic about it that is potentially at risk and requires protection from harm. Assessment endpoints can be functional groups, habitats, places, or species (USEPA, 1998).

Here, we are concerned with the potential impacts of transgenic plants on non-target organisms, and we present a method for prioritizing non-target species as assessment endpoints based on five selection criteria. These five criteria have been derived from a survey of the scientific and regulatory literature and are: potential susceptibility to hazard, potential for exposure to hazard, ecological importance, anthropocentric (social/cultural) value, and testability. The first four criteria align with the accepted selection criteria for assessment endpoints (ecological relevance, potential susceptibility and relevance to management goals); the fifth is a practical consideration that is inevitably applied during the analysis planning stage of problem formulation (USEPA, 1998).

Our general hypothesis for this study was that these five criteria could be applied to a set of existing data from a large number of invertebrate species in the receiving environment, in order to produce a ranked list of non-target species with those most suitable for selection as assessment endpoints at the top of the list. Our reasons for choosing each of these five criteria are presented below, followed by a description of the approach taken to develop the species ranking method.

Potential hazard

The concept of "hazard" is central to risk assessment and transgenic plant risk assessment procedures always include a characterization of the newly-expressed protein, its mode of action and its potential toxicity to target organisms (*e.g.*, EFSA, 2005; ERMA, undated-a;

USEPA, 2004). The likelihood that non-target organisms will also be susceptible to the protein is an obvious characteristic that can be used to screen for potential test subjects. The United States Environmental Protection Agency (USEPA) lists "phylogenetic relatedness or proximity of the test insect to the target insect" as one of the criteria for choosing non-target species (Lewis and Portier, 2000; Rose, 2006; Stacey et al., 2006). This criterion assumes that the transgene product has some kind of host specificity based on the host's taxonomic position, which is a reasonable assumption for *Bacillus thuringiensis* (*Bt*) toxins, as they have reasonably well-defined specificities for particular orders of insects. The concept of phylogenetic relatedness to the target species may not be so useful for other compounds with broader insect toxicity (*e.g.*, alpha-amylase inhibitors). The USEPA also suggests that "species that are most likely to be susceptible" should be tested (USEPA, 2004). "Known susceptibility" (Dutton et al., 2003; Schmitz et al., 2003), "sensitivity to the test chemical" (Jepson et al., 1994), "potential sensitivity to the protein" (Romeis, 2006), or consideration of the protein's "activity profile" (Wolt et al., 2006) have also been proposed as means to select non-targets. Biochemical predisposition was suggested as a criterion by Cowgill and Atkinson (2003), who proposed using profiles of insects' digestive proteases as a means of predicting their sensitivity to particular protease inhibitors expressed by transgenic plants. The concept of hazard will continue to be important when considering the non-target impacts of new types of transgenic plants which have not been modified specifically for insect resistance. For example, plants with altered nutritional properties could have an effect on non-target invertebrate fitness.

Potential for exposure

Potential for exposure is an important consideration for assessing the likelihood that a potential hazard will pose a risk to an organism, and data describing potential exposure pathways can be used to help prioritize non-target species as assessment endpoints. There are many types of information available that can be used prior to embarking on the actual experimentation phase of a risk assessment to estimate exposure of a non-target organism to a transgenic plant (*e.g.*, Andow et al., 2006; Anon, 2003; DGR, undated; ERMA, undated-a; Romeis et al., 2006; USEPA, 2004). Plant characteristics, such as transgene expression levels and patterns of expression in different plant tissues may be measured. For example, pollinators may be exposed to transgene products if there is high expression in pollen, and soil biota may be exposed if root exudates contain transgene products. Thus the USEPA suggests measurement of the "concentration of the active ingredients in plant tissues, soil residues and their degradation rates" to help with exposure assessment (USEPA, 2004). The spatio-temporal availability of a transgene product can be estimated from information about the distribution of the crop plant (including weedy volunteers), the potential for hybrids between transgenic plants and their wild relatives and their distribution, and pollen and seed dispersal patterns. For example, South African authorities require information on "proximity to significant biota" (DGR, undated). This information can be combined with data on the characteristics of the non-target organisms, including their modes of feeding and feeding behavior, their ecological association with the crop, and their geographical distribution as determined by field surveys, inventories, lists and other databases to provide a measure of potential exposure to a hazard (*e.g.*, CFIA, undated).

Potential for ecological impacts

Regulators in most countries require information on potential interactions between transgenic crop plants and other organisms in the ecosystem. The use of functional groups and food webs to identify potentially affected organisms is reasonably widespread (*e.g.*, Anon, 2003; CFIA, undated; DEFRA, 2002; EFSA, 2005; ERMA, undated-a, undated-b; Lewis, 2002; USEPA, 2004), and has been recommended by many researchers and regulators (*e.g.*, Andow et al., 2006; Romeis et al., 2006; Rose, 2006; Stacey et al., 2006). For example, "ecological association with the crop or target" (presumably including species that interact with, or ingest, those exposed directly to the crop, species that use the crop for shelter *etc*., and those that use it as a direct food source) is one of the criteria recommended by the USEPA for selecting non-targets for testing in the US (Lewis and Portier, 2000). The USEPA describes ecological functional groups as including non-target herbivores, secondary consumers (predators, parasitoids, parasites), pollinators, decomposers, and seed dispersers (Lewis, 2002).

Canadian regulators ask applicants to comment on "interactions with other life forms" when seeking approval to release a transgenic crop variety, or indeed any plant with a novel trait (CFIA, 2004). For selection of non-target species, applicants are referred to a series of "companion" documents with biological/ecological summaries for the major crop species (oilseed rape, flax, corn, potatoes, soybeans and wheat) and tables of "other life forms" that interact with the crop (CFIA, undated). Pathogens, symbionts, beneficial organisms, and consumers are suggested as functional groups that should be covered when selecting non-target species (*e.g.*, CFIA, 2004; EU, 2001).

In the United Kingdom, it is suggested that "other organisms in the ecosystem" be considered (Williams, 2002) and in Australia, Gene Technology Regulations require information on adverse effects on "any ecosystems". Authorities in Argentina require "exhaustive details" of the possibility of "interaction of the GMOs with other organisms other than plants, within the environment in which it usually grows" (SAGPYA, undated). In New Zealand, legislation covering the use of transgenic plants requires consideration of the "intrinsic value of ecosystems" and "safeguarding the life-supporting capacity of ecosystems" (Anon, 1996; ERMA, undated-a, undated-b).

In providing guidance on biosafety assessment of transgenic crops in developing countries, a case-specific process for selecting non-target species as assessment endpoints has been proposed that combines ecological and anthropocentric concerns (Andow and Hilbeck, 2004; Hilbeck and Andow, 2004; Hilbeck et al., 2006). A set of functional groups (including an "unknown function" group) is established and local species assigned to each group. A "risk endpoint" (*i.e.*, possible adverse effect) is then defined for each functional group and the species within each are prioritized "in relation to the likelihood of the risk endpoint associated with the functional group" using "a series of qualitative ecological characteristics" (Andow et al., 2006), including association with the crop and functional significance of the species in the cropping system (Hilbeck et al., 2006). These ranked lists allow identification of the species "most likely to cause concern" based on historical local knowledge of their significance, and their degree of association with the crop and the transgene product (Andow and Zwahlen, 2006). Several top-ranked species are then selected from each list as subjects for testing specific risk hypotheses (Andow and Zwahlen, 2006; Andow et al., 2006; Hilbeck et al., 2006). While this method produces useful, workable lists of potential endpoints, it is likely that automation of this type of selection system would greatly increase the speed and repeatability of the problem formulation process.

There has been some criticism of published risk assessment studies for a lack of information on "whole orders" of natural enemies (Lovei and Arpaia, 2005). It has been suggested that systematic diversity should be used as a criterion for species selection (Dutton et al., 2003; Romeis, 2006), in addition to testing representatives of functional groups from the ecosystem where the crop will be grown. The inclusion of species representing taxa which have not emerged from the application of other criteria could help to preserve community structures and biodiversity *per se*, both as indicators of ecosystem health (CBD, 2003) and as something of intrinsic value to humans (Lockwood, 1999). Ideally, a screening method that begins with as many invertebrates in the receiving ecosystem as possible will minimize the chances of omitting species of significance when the final selection of test species is made.

Jepson et al. (1994) suggested that the organism's potential for ecological recovery be included in ecological risk assessment by assessing information about the potential test species' life history and mobility. This concept, that recovery from ecological perturbations may be possible, and that transitory changes in ecosystems may pose lower risks to the environment than more lasting changes, is not emphasized elsewhere in the literature on transgenic plant risk assessment. However, Stark et al. (2004) have elaborated upon this concept recently in connection with pesticides and pollutants, by using models to demonstrate that equal levels of mortality and reductions in fecundity will have very different impacts on species with different life-history traits. Consequently, this concept should also be included when selecting non-target species as assessment endpoints for ecological risk assessment of transgenic plants.

Anthropocentric concerns

Impacts on non-target species with economic, social or cultural consequences may be termed anthropocentric. The USEPA lists the following examples of "anthropocentric functional groups": secondary pests, natural enemies, rare or endangered (red list), income-generating, and species of social or cultural value. Some of these overlap with the ecologically-based selection criteria, for example, where species provide ecological services that are also of value to agriculture (*e.g.*, pollination, pest control, or decomposition). European countries also acknowledge the importance of safeguarding species beneficial to agriculture (Cowgill and Atkinson, 2003; Dutton et al., 2002; Williams, 2002). Keystone, rare, endangered or threatened species are mentioned in GMO biosafety regulations in several countries, *e.g.*, USA (USEPA, 2004), South Africa (DGR, undated), India (DBT, 1998), and Europe (Cowgill and Atkinson, 2003). "Red lists" may be used to identify these (Schmitz et al., 2003). Butterflies are singled out for particular attention by Wolt et al. (2006), because of their "endangered, threatened or charismatic" status. In the United Kingdom, "Biodiversity Action Plans" for each crop identify such species (UKBAP, undated). In New Zealand, interactions and risks to "valued species" are specifically mentioned in the legislation governing the use of GMOs. These species are defined as those valued for "symbolic, spiritual, aesthetic, economic or historic reasons", and "species valued specifically by Māori (New Zealand's indigenous people)" (Anon, 1996). Particular note is made

of native flora and fauna, and "New Zealand's inherent genetic diversity" (Anon, 1996). Clearly, different societies will take different approaches to defining and prioritizing their valued species, so a screening method for selecting non-target species as assessment endpoints needs to be flexible and responsive to these different approaches.

Ability to perform tests

While the ability to perform tests with a particular non-target species (*e.g.*, the availability of standardized protocols) should not be the sole basis for the selection of an assessment endpoint, this matter is inevitably examined during the "analysis planning" phase of problem formulation (USEPA, 1998). It is during this planning phase that decisions are made about the most appropriate ways to measure the responses of each assessment endpoint to the transgenic plant. Because the method we have developed can easily handle several selection criteria simultaneously, we have added a fifth criterion of "testability" to the other selection criteria described above.

When performing experiments to investigate potential risks to non-target species prior to the release of a transgenic plant, it is preferable to use species that are available in sufficient numbers to give statistically meaningful results (Marvier, 2002). A rearing method can be a distinct advantage here, enabling the production of many individuals raised under uniform conditions as test subjects. Established bioassay systems, in which there is consistently good survival of controls, will also facilitate testing. The EPA (Kough and Vaituzis, 1999; Lewis and Portier, 2000) takes these practical considerations into account, and this approach is supported by many researchers in this field (Dutton et al., 2003; Jepson et al., 1994; Romeis, 2006; Romeis et al., 2006; Rose, 2006; Stacey et al., 2006; Wolt et al., 2006). The EPA also recommends the use of species with short life cycles (Kough and Vaituzis, 1999; Lewis and Portier, 2000), although this approach is criticized by Andow and Hilbeck (2004), who suggest that bioassays should be designed to reflect the generation time of the test species, rather than the species chosen to fit the quickest test. Stark et al. (2004) also counseled caution in extrapolating from data obtained with short-life-cycle species, such as *Daphnia*, to assess risk to other organisms with different life-history variables.

In some cases (*e.g.*, where a rare or endangered species is prioritized as an assessment endpoint), risk analysis tests with surrogate species may be advisable. Where there is good evidence to support the contention that a surrogate species can accurately represent the response of another non-target species to a biosafety test with a transgenic plant, then the use of surrogates may be more widely applicable. For example, the mechanisms of action and the host specificities of *Bt* Cry toxins are sufficiently well known in many cases to allow the conventional "surrogate species concept" (as developed for testing synthetic pesticide risk hypotheses) to be applied (Lewis and Portier, 2000). However, risk assessments conducted on transgenic plants with other modifications (*e.g.*, plants with altered nutritional properties) may be less amenable to the use of surrogates, especially where existing data on their mechanisms of action or their non-target impacts are lacking. In countries with high levels of endemic invertebrate species (such as New Zealand, where about 90% of beetle species are estimated to be endemic (Watt, 1982)), preliminary investigations may be needed to establish the adequacy of surrogate species before application of the concept. The adequacy of surrogates for testing non-target risk hypotheses is a separate case-by-case matter for consideration during the risk analysis phase of risk assessment, and is not considered further here.

The need for a screening method

Given that every crop has a large number of potential non-target organisms, and applying all aspects of the five selection criteria above will involve consideration of many characteristics, a comprehensive, transparent and repeatable selection process would be difficult to achieve without the aid of a computer tool. Such a tool would be of benefit to researchers who are attempting to prioritize non-target species as assessment endpoints for risk assessments with new transgenic plants.

There are precedents for the use of screening methods in environmental risk assessment. In Europe, when assessing the environmental safety of chemicals and biocides, the initial selection of chemicals for ecotoxicity testing is performed by the European Union risk ranking method (EURAM) screening model (Hansen et al., 1999). The method ranks the chemicals based on information in the International Uniform Chemical Information Database (IUCLID) that details parameters for each chemical such as the quantity produced annually and its known environmental and human toxicity (Heidorn et al., 2003; Russom et al., 2003; Van Haelst and Hansen, 2000). A similar ranking method (the Chemical Hazard Evaluation for Management Strategies, or CHEMS-1) is used to prioritize chemicals for risk assessment testing in the USA (Swanson et al., 1997). These procedures reduce the overall time and cost involved with environmental risk assessment of chemicals, since the screening method is performed rapidly by the computer models. Ecotoxicity testing can then be performed on the prioritized chemicals in preference to lower risk chemicals.

To facilitate the process of non-target species selection, we have developed a screening method that ranks the invertebrate species in the receiving ecosystem and prioritizes the species with the highest ranks for selection as assessment endpoints in the risk analysis. The hypothesis which is used to drive the ranking process is that which has been formed in the early part of the problem formulation phase of the risk assessment. This hypothesis is likely to be a basic, generalized statement about the type of hazard the new GMO could pose to each invertebrate species in the receiving environment, and will be different for each GMO. For example, the hypothesis formed about a crop expressing a lepidopteran-active *Bt* toxin may simply state that the plants could be a toxic hazard to non-target herbivorous invertebrates and an indirect hazard to invertebrates that eat those herbivores. For other GMOs, the hypothesis may focus on structural changes to the plant or non-toxic hazards to non-target organisms. The screening method tests the generalized hypothesis by assessing the relevance of existing information on both the GMO and each non-target invertebrate species to each of the five selection criteria listed above.

The information required by the screening method should be gathered from the published literature on the attributes of each invertebrate species (or "receptor") and the transgenic plant (or "stressor") of interest. The term receptor has been chosen to cover both target and non-target invertebrate species, since it is possible that future GMOs may not have a direct target. Each receptor and stressor attribute used by the screening method can be contained in a database that has been developed using Microsoft® Access 2003. A score, on the scale of 0 to 10, can then be assigned to each attribute based on the value of that attribute in meeting each of the five selection criteria (a subjective process, utilizing our own "expert opinion"). A score of 10 should be used to indicate a high value for the criterion (*e.g.*, a receptor already known to be susceptible to the toxin would get a high hazard value), and a score of 0 a low value (*e.g.*, a receptor that is known not to be susceptible to the toxin has no hazard value). These scores can be set by the user prior to running the screening method to best reflect the requirements of the basic test hypothesis, the country's regulations, and the expert opinion of the researchers. These scores are then combined using a Priority Ranking of Non-Target Invertebrates (PRONTI) model to generate an overall score for each receptor species. These PRONTI scores allow the species to be prioritized in a final list so that those with the highest scores can be given the highest priority for risk analysis with the GMO.

The database and PRONTI model were developed using a New Zealand example as a test case. We used, as our receptor species, 80 New Zealand invertebrates from the published literature on the ecology of *Pinus radiata* (D. Don) forests in New Zealand, to test a hypothetical introduction of a new ecological stressor: *P. radiata* trees expressing the Cry1Ac lepidopteran-active *Bt* toxin. Given the size of these forests, there are often freshwater streams, rivers and lakes inside, or adjacent to, areas where *P. radiata* are grown. Our selection of invertebrates therefore included both terrestrial and aquatic species. The hypothesis being tested was that these trees could be a direct hazard to any invertebrate in the ecosystem that might be susceptible to the *Bt* toxin, and also an indirect hazard to any invertebrate that may depend on a susceptible species for food. The method was used to rank the 80 species based on the five selection criteria to determine which species would be most appropriate for ecological risk analysis should these trees be considered for introduction into New Zealand.

RESULTS AND DISCUSSION

The evaluation of the screening method by applying it to a specific example resulted in the priority listing of the 80 New Zealand invertebrate species based on their PRONTI scores (Tab. 1). Although this is only a partial list of the receptor species likely to be included in an assessment of the impacts of a real introduction of *P. radiata* expressing a *Bt* toxin, it illustrates the type of list that would be produced by the method under these conditions in New Zealand.

The list in Table 1 shows that the screening method has prioritized several herbivorous species above carnivorous species, as a result of the combination of ranks obtained for each of the selection criteria. This result is expected, since these species are likely to obtain high scores for several of the selection criteria *i.e.*, the level of hazard to these species is high since they (or closely related species) are known to be susceptible to the Cry1Ac toxin expressed by the plants (Glare and O'Callaghan, 2000); their exposure level is high since they are known to eat *P. radiata* in New Zealand (Chapman, 1999; Crowe, 2002); their status in the ecosystem is high given their large population densities (Miller, 1971; Pendergrast and Cowley, 1969; Winterbourn et al., 2006); they are valued by the people of New Zealand as endemic species with cultural, social or economic value as food items, pests or bioindicator species (Collier et al., 1997; Crowe, 2002); and there are bioassay systems available for many of these species (Clare et al., 1987; Singh, 1985). In comparison, the hazard posed by these trees to carnivorous species is much lower given that they are known to be unaffected by the Cry1Ac toxin (Glare and O'Callaghan, 2000). Although there may be indirect effects of the plants on these species through the loss of their prey species, as has been found by Pilcher et al. (2005) and Schuler et al. (2005), these indirect effects may be

Table 1. The prioritized list of 80 New Zealand invertebrate species generated by the method during the evaluation involving *Bt*-expressing *P. radiata* trees as the new stressor. Note that the PRONTI score has no biological meaning, and only provides a means with which to rank the receptor species relative to each other. The scores obtained for each of the selection criteria, however, can be used as a relative indication of the level of hazard (H), exposure (E), resilience (R), receptor status (S), testability (T) and value (V) for each receptor.

Rank	Species name	Order	Feeding guild	Criteria scores						PRONTI score
				H	E	R	S	T	V	
1	Dummy 2	Lepidopt.[1]	Ter.[9] Herbiv.[11]	20	40	209	96	80	80	982
2	*Ctenopseustis obliquana*[a]	Lepidopt.	Ter. Herbiv.	10	39	147	97	80	63	635
3	*Epiphyas postvittana*[a]	Lepidopt.	Ter. Herbiv.	10	39	160	121	80	33	568
4	*Pseudocoremia suavis*[b]	Lepidopt.	Ter. Herbiv.	9	40	157	107	80	42	526
5	*Planotortrix notophaea*[c]	Lepidopt.	Ter. Herbiv.	10	39	152	54	78	63	498
6	*Austroperla cyrene*[d]	Plecopt.[2]	Aq.[10] Decomp.[12]	13	40	168	60	50	48	482
7	*Helicoverpa armigera conferta*[a]	Lepidopt.	Ter. Herbiv.	10	39	165	85	80	28	456
8	*Aoteapsyche* spp.[e]	Trichopt.[3]	Aq. Omniv.[13]	10	36	171	89	70	58	449
9	*Cnephasia jactatana*[a]	Lepidopt.	Ter. Herbiv.	7	40	152	85	80	63	420
10	*Hydrobiosis* spp.	Trichopt.	Aq. Omniv.	13	36	179	70	40	48	408
11	*Ctenognathus cardiophorus*[f]	Coleopt.[4]	Ter. Carniv.[14]	10	27	95	51	46	45	404
12	*Hydrobiosis centralis*[g]	Trichopt.	Aq. Carniv.	13	36	168	56	40	48	394
13	*Ctenognathus crenatus*[f]	Coleopt.	Ter. Carniv.	10	27	97	57	39	45	394
14	*Triplectides obsoletus*[e]	Trichopt.	Aq. Decomp.	11	36	160	55	50	48	379
15	*Zelandoperla fenestrata*[h]	Plecopt.	Aq. Decomp.	11	40	127	32	39	38	372
16	*Psilochorema* spp.	Trichopt.	Aq. Carniv.	13	36	181	60	40	45	369
17	*Aulacopodus calathoides*[i]	Coleopt.	Ter. Carniv.	10	27	102	49	45	45	368
18	*Zelandobius furcillatus*[h]	Plecopt.	Aq. Decomp.	11	36	168	57	59	38	363
19	*Ctenognathus novaezelandiae*[j]	Coleopt.	Ter. Carniv.	10	27	110	53	55	40	363
20	*Ctenognathus bidens*[f]	Coleopt.	Ter. Carniv.	10	27	112	56	46	40	340
21	*Costachorema xanthopterum*[k]	Trichopt.	Aq. Carniv.	13	36	170	47	30	48	338
22	*Meteorus pulchricornis*[l]	Hymenopt.[5]	Parasitoid	10	27	130	66	80	15	336
23	Hydrobiosidae family	Trichopt.	Aq. Omniv.	13	36	168	43	40	35	328
24	*Orthopsyche fimbriata*[e]	Trichopt.	Aq. Omniv.	10	36	166	66	40	45	328
25	*Hudsonema alienum*[e]	Trichopt.	Aq. Omniv.	11	36	153	43	50	35	326
26	*Planotortrix excessana*[a]	Lepidopt.	Ter. Herbiv.	6	39	154	70	78	63	319
27	Dummy 1	Araneae	Unknown	10	20	112	86	40	50	314
28	*Neurochorema* spp.	Trichopt.	Aq. Carniv.	11	36	178	58	40	45	314
29	*Holcaspis brevicula*[m]	Coleopt.	Ter. Carniv.	10	27	75	10	36	35	290
30	*Acroperla trivacuata*[h]	Plecopt.	Aq. Decomp.	11	36	182	47	39	38	271
31	*Antipodochlora braueri*[n]	Odonata	Aq. Carniv.	11	27	136	29	35	58	267
32	*Rhyssa persuasoria*[o]	Hymenopt.	Parasitoid	12	27	134	61	35	13	263
33	*Liothula* spp.	Lepidopt.	Ter. Herbiv.	6	39	133	70	25	42	240
34	*Polyplectropus* spp.	Trichopt.	Aq. Carniv.	10	27	168	52	50	48	240
35	*Declana leptomera*[a]	Lepidopt.	Ter. Decomp.	6	39	135	41	45	47	231
36	*Pseudocoremia leucelaea*[p]	Lepidopt.	Ter. Herbiv.	6	40	151	58	51	35	230
37	*Peripatoides novaezealandiae*[q]	Euonych.[6]	Ter. Carniv.	11	27	121	32	25	35	225
38	*Stethaspis suturalis*[r]	Coleopt.	Ter. Herbiv.	6	39	147	66	24	45	215
39	*Ichthybotus hudsoni*[e]	Ephem.[7]	Aq. Carniv.	10	22	135	40	34	58	215
40	*Declana floccosa*[a]	Lepidopt.	Ter. Herbiv.	6	39	150	50	39	47	213
41	*Prionoplus reticularis*[s]	Coleopt.	Ter. Decomp.	5	33	123	66	30	60	209
42	*Gellonia pannularia*[t]	Lepidopt.	Ter. Herbiv.	6	40	144	51	29	40	200
43	*Zelandoptila moselyi*[h]	Trichopt.	Aq. Decomp.	10	26	139	23	40	45	199
44	*Zelandobius unicolor*[a]	Plecopt.	Aq. Decomp.	5	39	139	49	52	38	195
45	*Proteuxoa comma*[a]	Lepidopt.	Ter. Herbiv.	6	40	148	50	35	35	195
46	*Deleatidium* spp.	Ephem.	Aq. Decomp.	4	39	158	80	59	58	194
47	*Navomorpha lineata*[r]	Coleopt.	Ter. Herbiv.	5	40	127	64	24	32	188
48	*Pycnocentrodes aureolus*[e]	Trichopt.	Aq. Decomp.	5	31	169	61	70	58	173
49	*Nesameletus* spp.	Ephem.	Aq. Decomp.	5	32	151	57	45	58	170

Table 1. Continued.

50	*Graphania ustistriga*[a]	Lepidopt.	Ter. Herbiv.	5	40	150	56	35	32	163
51	*Pycnocentria funerea*[e]	Trichopt.	Aq. Decomp.	5	32	157	51	60	45	159
52	*Zephlebia dentata*[u]	Ephem.	Aq. Decomp.	4	32	144	61	59	58	158
53	*Hylastes ater*[v]	Coleopt.	Ter. Herbiv.	6	40	145	44	29	22	157
54	*Austroclima sepia*[w]	Ephem.	Aq. Decomp.	4	32	146	56	56	58	149
55	*Orthopsyche thomasi*[x]	Trichopt.	Aq. Omniv.	4	36	151	62	50	45	149
56	*Sirex noctilio*[r]	Hymenopt.	Ter. Herbiv.	5	30	122	66	30	23	147
57	*Oniscigaster wakefieldi*[e]	Ephem.	Aq. Herbiv.	5	31	146	37	44	58	146
58	*Zephlebia borealis*[w]	Ephem.	Aq. Decomp.	4	29	135	47	56	58	137
59	*Zelandobius illiesi*[y]	Plecopt.	Aq. Decomp.	5	32	147	49	51	25	136
60	*Spaniocerca zelandica*[h]	Plecopt.	Aq. Decomp.	5	32	140	41	39	38	134
61	*Calliprason pallidum*[z]	Coleopt.	Ter. Decomp.	5	29	135	60	40	22	131
62	*Coloburiscus humeralis*[a]	Ephem.	Filter-feeder	5	32	157	44	25	58	129
63	*Oeconesus maori*[e]	Trichopt.	Aq. Decomp.	5	32	160	34	50	45	128
64	*Zephlebia* spp.	Ephem.	Aq. Decomp.	4	29	141	44	54	58	128
65	*Amarygmus* spp.	Coleopt.	Ter. Decomp.	6	32	162	52	46	10	128
66	*Neozephlebia scita*[a]	Ephem.	Aq. Decomp.	4	29	155	52	61	58	128
67	*Zelandoperla agnetis*[y]	Plecopt.	Aq. Decomp.	5	32	117	16	39	38	127
68	*Zephlebia spectabilis*[u]	Ephem.	Aq. Decomp.	4	29	151	52	56	58	127
69	*Triplectides cephalotes*[a]	Trichopt.	Aq. Herbiv.	5	32	145	19	50	45	126
70	*Beraeoptera roria*[aa]	Trichopt.	Aq. Decomp.	5	29	151	49	30	48	122
71	*Zelolessica cheira*[k]	Trichopt.	Aq. Herbiv.	5	31	151	33	50	35	121
72	*Hexatricha pulverulenta*[bb]	Coleopt.	Ter. Decomp.	5	32	143	54	29	22	117
73	*Hydrobiosella* spp.	Trichopt.	Filter-feeder	5	32	169	49	40	35	117
74	*Arhopalus ferus*[cc]	Coleopt.	Ter. Herbiv.	5	30	177	68	35	30	113
75	*Megaleptoperla grandis*[dd]	Plecopt.	Aq. Carniv.	5	27	138	36	39	35	108
76	*Mitrastethus baridioides*[ee]	Coleopt.	Ter. Decomp.	4	33	130	43	24	25	94
77	*Heliothrips haemorrhoidalis*[ff]	Thysan.[8]	Ter. Herbiv.	3	39	144	35	40	25	81
78	*Helicopsyche* spp.	Trichopt.	Aq. Herbiv.	5	10	151	30	40	48	39
79	*Oxyethira albiceps*[e]	Trichopt.	Aq. Herbiv.	4	10	155	47	50	48	37
80	Dummy 3	Trichopt.	Aq. Herbiv.	0	15	84	3	10	10	0

[a] Walker; [b] Butler; [c] Turner; [d] Newman; [e] McLachlan; [f] Chaudoir; [g] Ward; [h] Tillyard; [i] Broun; [j] Fairmaire; [k] McFarlane; [l] Wesmael; [m] Butcher; [n] Fraser; [o] L.; [p] Meyrick; [q] Hutton; [r] Fabricius; [s] White; [t] Guenee; [u] Eaton; [v] Paykull; [w] Phillips; [x] Wise; [y] McLellen; [z] Pascoe; [aa] Mosely; [bb] Westwood; [cc] Mulsant; [dd] Hudson; [ee] Redtenbacher; [ff] Bouché. [1] Lepidopteran; [2] Plecopteran; [3] Trichopteran; [4] Coleopteran; [5] Hymenopteran; [6] Euonychophoran; [7] Ephemeropteran; [8] Thysanopteran; [9] terrestrial; [10] aquatic; [11] herbivore; [12] decomposer; [13] omnivore; [14] carnivore.

mitigated somewhat by the species' resilience (Holling, 1973). For example, their generalized diet and high mobility may allow them to find alternative prey items. Consequently, while there are bioassay systems available for many of these species, and their value to the people of New Zealand can be high, the lower level of risk posed to these species results in their lower priority ranking than many herbivorous species. These results are consistent with the findings of several field studies on the impacts of *Bt*-expressing plants on herbivorous and carnivorous invertebrates (Candolfi et al., 2004; Duan et al., 2004; Glare and O'Callaghan, 2000; Pilcher et al., 2005).

This screening method may also help to identify species that are not as obviously expected to be affected by the plant. In our evaluation, the freshwater species that have been prioritized near the top of the list may not have been seen as non-target organisms by the developers of a *Bt*-expressing pine tree, but our method clearly shows that these species are worthy of further investigation. This is by virtue of their unknown responses to *Bt* toxins, their dependence on pine material and herbivores of pine for food in this ecosystem, their high biomass in New Zealand streams, and their value both as native invertebrates and food for native and introduced fish (such as trout).

The list in Table 1 includes three "dummy" species as examples of the internal consistency checks used in the evaluation of the method. These are example species with attributes that are designed to define their places in the list: Dummy 1 is a fake spider about which very little is known, and consequently it receives a score of 5 for each unknown attribute; Dummy 2 is a fake lepidopteran with attributes that provide it with the highest scores for each of the selection criteria; and Dummy 3 is a fake caddisfly

to which the Bt-expressing *P. radiata* poses no hazard. The placing of Dummy species 2, 1 and 3 at the top, middle and bottom of the list, respectively, shows that the screening method is ranking these species correctly based on their attributes. The placement of Dummy 1 in position 27 on the list is particularly interesting, given that it represents a species about which very little is known. This suggests that species for which there is little information are not penalized or prejudiced against by our method, which is desirable given that these species are just as likely to be affected by the introduction of the transgenic plant as species about which much is known.

The evaluation of the method included another 40 dummy species which had identical receptor attributes except for single changes where a known receptor attribute was changed to an unknown data gap. In most cases, these changes had very little impact on the final PRONTI score, with the majority resulting in an increase or decrease of less than 30 points. However, changes to attributes that were included in the measurement of criterion 1 or 2 (*i.e.*, hazard or exposure) resulted in much bigger changes to the PRONTI score. This is expected, since the H and E parameters are the main drivers of the PRONTI model. Consequently, any change to a receptor attribute that resulted in an increase in the scores for hazard or exposure resulted in a large increase in the PRONTI score, while any change that reduced the hazard or exposure scores to zero resulted in a PRONTI score of zero. This result is obviously desirable, since a receptor for which there is no risk from the stressor (*i.e.*, zero hazard or exposure scores), does not need to undergo biosafety testing. Conversely, those species for which there is a high risk should receive a high PRONTI score and be prioritized for testing. Some of the changes to attributes that contributed to the measurement of the exposure parameters also resulted in a change to the measurement of the resilience parameter. For example, a receptor that is known to eat the stressor will be exposed to the hazard, but will also have a chance of developing resistance to the hazard, as might be the case for a lepidopteran species exposed to a Cry1-type *Bt* toxin. In these cases, a change made in the resilience parameter was found to have a much lesser influence on the overall PRONTI score than changes in the exposure scores. This outcome is desired, since the high level of uncertainty surrounding the measurement of species' resilience reduces the level of certainty around the ability of these measures to influence the level of risk posed to each receptor, and therefore, their level of influence on the PRONTI score should be smaller. The only other attribute found to have a large impact on the PRONTI score was the species' population density. Dummy species with a high population density scored more highly than species with unknown or low population density, with a difference of up to 65 points in the final PRONTI scores. Although this could result in the marked movement of a species up or down the final list, this movement is preferred, since species with a high population density are considered to be more important to the ecosystem in which they occur than species of low population density (Hilbeck et al., 2006; Silby et al., 2005). Species with large population densities should, therefore, be prioritized for risk assessment. Conversely, rare species that are at high risk from the stressor should also be prioritized, despite their low density. This is achieved in our method by the high scores generated for these species under criterion 4 (receptor value) and their low resilience scores. For example, *Holcaspis brevicula*, a rare coleopteran, is placed at number 29 on the list because of these criterion values.

An illustration of how each screening criterion acts to produce the final priority listing can be found in Table 2. While the criteria that give an indication of the potential risk of the stressor to each receptor (*i.e.*, H and E) are the main drivers in the PRONTI model, the scores obtained by each species for the other criteria can have an impact on the final placement of each receptor in the priority list. Table 2 shows that the priority placement of species in the list differs when different combinations of criteria are applied, with only one species (*Austroperla cyrene*) occurring in all three lists. For example, when only hazard and exposure are used as criteria, *A. cyrene*, a plecopteran aquatic decomposer with an unknown direct response to Cry1Ac, a high potential for exposure to Cry1Ac-pine material, and a heavy reliance on species which may be directly affected by the toxin, ranks at number one. When resilience (R) is added as a denominator, the extremely rare beetle *H. caspis* ranks first, as its scarcity and restricted range gives it a very low R value. When ecological status, value to humans and testability are added to the criteria used, the native lepidopteran foliage-feeder *Ctenopseustis obliquana* becomes number one. We suggest that such lists should be generated and checked each time the screening method is used to ensure that all the criteria considered to be important in the prioritization process are involved in the construction of the final PRONTI list.

The ranking system used in the screening method is extremely flexible, given that the ranks assigned to each receptor attribute are easily modified as required for each new stressor, or to suit the requirements of the basic test hypothesis. For example, the rankings can be modified to reflect a change in the value of different receptor attributes in the presence of different stressors. Alternatively, the weight of each parameter may be altered if these are seen to be more or less important under different hypotheses, simply by increasing or decreasing the value assigned to the attributes of which the parameter

Table 2. Lists of the top ten of 80 possible non-target species as they would be ranked if only some of the method's screening criteria were used in a risk assessment of Cry1Ac-expressing *P. radiata* in New Zealand. Shading shows where the same species occurs in more than one list. The first list shows the species that would be prioritized if only the potential risk posed by the stressor was used (*i.e.*, potential hazard (including potential loss of prey) × potential exposure; H × E). The second list is that produced once each species' potential resilience has been used to modify the potential risk (*i.e.*, (H × E)/Resilience). The final list is that produced by the full PRONTI equation, indicating the change in the list produced by adding the species' scores for ecological status (S), value (V) and testability (T).

	Priority lists of non-targ et invertebrates produced by:		
Rank	**H x E only**	**(H x E)/R**	**[(H x E)/R] x (S + V + T)**
1	*Austroperla cyrene*	*Holcaspis brevicula*	*Ctenopseustis obliquana*
2	Hydrobiosidae family	*Zelandoperla fenestrata*	*Epiphyas postvittana*
3	*Hydrobiosis* spp.	*Austroperla cyrene*	*Pseudocoremia suavis*
4	*Hydrobiosis centralis*	*Ctenognathus cardiophorus*	*Planotortrix notophaea*
5	*Psilochorema* spp.	*Ctenognathus crenatus*	*Austroperla cyrene*
6	*Costachorema xanthopterum*	Hydrobiosidae family	*Helicoverpa armigera conferta*
7	*Zelandoperla fenestrata*	*Hydrobiosis centralis*	*Aoteapsyche* spp.
8	*Triplectides obsoletus*	*Costachorema xanthopterum*	*Cnephasia jactatana*
9	*Zelandobius furcillatus*	*Ctenopseustis obliquana*	*Hydrobiosis* spp.
10	*Acroperla trivacuata*	*Aulacopodus calathoides*	*Ctenognathus cardiophorus*

is comprised. For example, if the conservation value of a species was deemed to be the most important parameter for criterion 4, the ranks obtained for each attribute of this parameter could be doubled, thus lending more weight to this parameter. The value placed on data gaps is perhaps a particularly important decision to make early in the process of using this screening method. In building the method, we assigned a mid-point value of five to each unknown receptor attribute, since it was not known whether the receptor's actual attribute would be of greater or lesser value to the selection criteria. It may be decided *a priori*, however, that a more conservative approach is needed such that attributes for which there is no information should be treated as a worst-case scenario for the receptor species and, therefore, should be given the maximum rank. Conservative values are often used in ecological risk assessment models (*e.g.*, Fan et al., 2005; Hilbeck et al., 2006; Hope, 1995). When considering the impact of a stressor that is less well-known than plants expressing *Bt* toxins, it will be particularly important to state any assumptions made, and to be clear and consistent when determining the value of each receptor attribute and selection parameter. There are likely to be fewer extant data on the non-target impacts of newer types of GMOs, such as plants with altered levels of secondary metabolites (*e.g.*, Kim et al., 2006). The generalized hypothesis that should be used to drive the ranking process for each new GMO will need to take into account the different hazard possibilities presented by the plant. Thus, all risks presented by the stressor should be considered when the values of the receptor attributes are set, so that the screening method can be as effective as possible.

The generation of this list using our automated system is rapid, transparent and repeatable, reducing the time, cost and sources of bias that can be involved with the selection of organisms for biosafety testing. While the assignment of ranks to each of the species' attributes is necessarily a subjective process, the actual prioritized listing of the species is done using the PRONTI model, which treats all of the species equally. Thus, the species at the top of the list have been placed there following an unbiased comparison with all the other species in the list, and have not been prioritized simply because they are well-known or better understood by the researcher. However, the accuracy of the final list is somewhat uncertain, as is the case with other, similar ranking selection methods

(*e.g.*, Hansen et al., 1999; Lu et al., 2003; Russom et al., 2003). Although the use of only published sources of information to confirm each receptor attribute should reduce the level of uncertainty around the criterion values, the screening method still only provides a very basic measure of the possible non-target impacts of the transgenic plants. Consequently, the scores attained by each species for each of the selection criteria are only approximate, and the final placement of each species on the PRONTI list is not absolute. For example, new information on a species may fill data gaps that could alter its final score, and therefore change its priority placement. In addition, information obtained for a species may be used as surrogate attributes for related species in the same taxonomic family, which may also alter the position of these related species on the list. The uncertainty in the final placement of each species by the PRONTI model may be offset by the use of expert knowledge when selecting the sub-set of prioritized species from the PRONTI list that should undergo biosafety testing. For instance, the list produced from the evaluation of our method has placed seven lepidopteran species in the top ten places. When formulating a risk analysis plan, risk assessors may, therefore, decide that it would be a better use of resources to select only one or two of these species so that a wider range of other taxa could also be tested. The other species selected for testing may be selected in terms of their taxonomy (*e.g.*, the top ranking Plecoptera, Trichoptera, and Coleoptera), or ecological function (*e.g.*, the highest ranking aquatic and terrestrial herbivore, carnivore, decomposer and omnivore). Alternatively, those with the highest anthropocentric value, or the highest level of uncertainty about their risk status, may be chosen if that better meets the regulatory requirements of the receiving country. Closer examination of the list may reveal significant information gaps that could be usefully filled by further research. The use of expert opinion to select from a ranked list is commonly used to reduce the uncertainty inherent in this form of model (*e.g.*, Hansen et al., 1999; Hilbeck et al., 2006; Padovani et al., 2004; Van Der Werf and Zimmer, 1998).

Following the selection of species as assessment endpoints from the PRONTI list, impacts on the chosen species can then be determined by following the risk assessment framework. Dutton et al. (2003) and Garcia-Alonso et al. (2006) suggest the next step in the assessment process should involve worst-case toxicity testing with the selected species in a laboratory environment. If no impacts are found, then it can be concluded that the transgenic plant will pose little risk to this species when grown in the field; if the selected species is affected by the new stressor, further risk assessment testing can be conducted involving detailed field studies. Addition of the data generated by these studies to the selection database outlined here may allow for the generation of an updated PRONTI list with more certainty around the species prioritized for related stressors. In addition, ecological population modeling can be used to assess potential effects on each individual test species in considerable detail (*e.g.*, Fan et al., 2005; Macintosh et al., 1994; Pastorok et al., 2003; Topping et al., 2005). The resulting increase in information obtained from these tests should increase the certainty with which decisions are made on the likely ecological impacts of a new stressor.

The database specially designed for the PRONTI method may be a useful source of information for ecological models used in the next phases of the ecological risk assessment protocol, reducing the time taken to gather further information. The database has been designed to be as user-friendly as possible, so that new information can easily be added to the database by inexperienced users. Future developments may include provision of the database in an internet-based format, allowing for easier access by researchers elsewhere. Although it can be time-consuming initially to enter each receptor attribute into the database, the information needs to be entered only once, and the small amount of information pertaining to the impact of a new stressor on that species can be entered very quickly (see the attribute lists in Appendix I). Thus, once the information for a species has been entered, it is available for ranking in any ecosystem in which it occurs as a step in determining the impact of any new plant that may be introduced to that ecosystem.

It is possible that the flexibility of the ranking system and the simplicity of the PRONTI method will allow this screening method to be used for a variety of ecological stressors, not just transgenic plants. Researchers wishing to determine the impacts of a new pesticide or biological control agent on the invertebrates in the receiving ecosystem may be able to use the database and method presented here. Further research and evaluation of the method is required before this can be confirmed. It is also essential that the method is validated by applying it to a real situation where the introduction of a new ecological stressor can be studied to verify the assumptions made by the method in the prioritization of the receptor species. Ideally this work should be done as a cross-country comparison using the same ecological stressor. In addition, a study into the validity of the parameters used by the PRONTI method, particularly those used to measure the ecological impact of the stressor on the receptor species, is also planned.

MATERIALS AND METHODS

Selection criteria

Each of the five selection criteria encompasses a large data set in the literature. There is, however, considerable

variation in the quality and quantity of the data available for each criterion and each receptor species. We have, therefore, selected a set of parameters to provide a measure of each criterion based on both the closeness of the relationship between the parameter and the criterion, and the availability of data for that parameter in the literature (Tab. 3). Despite this, there are still several data gaps for these parameters in the peer-reviewed literature on non-target invertebrates. This is probably not surprising, considering the large number of existing invertebrate species and the relatively small amount of research that has been conducted on each one. There is also little complete research on the effects of transgenic plants on invertebrates, reducing the data available for each parameter still further. As a consequence, we selected a number of receptor attributes to provide data for each parameter. The attributes were again chosen for their ability to provide a measure of the parameter and their availability in the literature (see Appendix I).

The receptor attributes for each parameter were contained in a specially designed database developed using Microsoft® Access 2003. This program has been used previously to store information on receptors for ecological risk assessment studies (*e.g.*, Fan et al., 2005; Lu et al., 2003; Nute et al., 2004), suggesting that the database developed for use by this screening method may also be useful for interrogation by other models used for ecological risk assessment, since the same data storage system has been used.

Selection criterion 1: Potential hazard (H)

Three parameters were used to define this selection criterion (Tab. 3). The first parameter (H1) was used to identify the type of hazard the new stressor might pose to each invertebrate receptor species present in the receiving ecosystem. The hazard posed by a new transgenic plant is likely to be due to a change in protein expression or the expression of a new protein. This change could pose a hazard to those receptors that eat the plant, or use the plant during their life cycle for shelter or reproduction, *etc*. It is also possible that the change in protein expression may result in a change in the plant's physiology or morphology, which could be an additional hazard to a receptor. Consequently, parameter H1 was used to identify all possible new hazards posed by the stressor, so that predictions could be made regarding the type of hazard to which each receptor could be exposed, and the level of exposure that could occur.

The second and third hazard parameters were used to predict the way in which each receptor could be affected by the stressor. Direct effects (parameter H2) were measured using receptor attributes that indicated the possible susceptibility of the receptor to the hazard. For example, any published information on the receptor's susceptibility to the new, or changed, protein expressed by the plant was gathered. Where no information was available on the susceptibility of the receptor to the protein in question, surrogate attributes such as the known susceptibility of the receptor to related proteins, or the susceptibility of other members of the receptor's taxonomic family to the new protein, were used to provide a hazard estimate where possible. Attributes that related directly to each receptor were used in preference to surrogate attributes, to ensure the most robust information available was used to predict the effects of the plant. Since direct effects may also include the loss of a receptor's shelter or reproduction sites, information pertaining to these receptor attributes was also added to the database. Parameter H3 was used to obtain a measure of the indirect hazards posed by the stressor. For example, for a carnivore, indirect effects are most likely to occur through a decline in the receptor's prey species. Consequently, information on each receptor's diet was used to predict whether the species was likely to eat a prey species that was susceptible to the new stressor.

Selection criterion 2: Potential for exposure (E)

Three parameters were selected to measure whether or not each receptor was likely to be exposed to the hazard, and to predict the level of exposure that could occur (Tab. 3). Parameter E1 required data on the receptor's population distribution, primarily gained from publications on invertebrate surveys. This parameter ensured that only those receptors that were found in the ecosystem into which the stressor was to be introduced were included. Parameter E2 was measured using each receptor's dietary attributes, including data on which trophic level the receptor belonged to in the ecosystem food web, and its main dietary intake, down to species level where available. In the case of herbivores and omnivores, the proportion of the diet that was likely to be composed of the new plant was assessed, along with whether or not the parts of the plant that expressed the transgenic protein were likely to be consumed by the receptor. The prey consumed by omnivorous and carnivorous receptors were assessed, to determine whether each receptor was likely to consume invertebrates that lived in the ecosystem, especially those which may have eaten the new transgenic plant. Information on how the receptor could be exposed to a stressor through other uses of the plant (*e.g.*, through the loss of shelter or egg laying substrates) was also included (parameter E3).

Selection criterion 3: Ecosystem impact (I)

The estimated level of hazard and exposure, measured for each receptor using criteria 1 and 2, could result in changes in the receptor population which could have

multiple ensuing effects on the ecosystem. Although these effects are difficult to predict, it is likely that the level of impact will depend on how integral the receptor is to the maintenance of the ecosystem. Thus, in the simplest terms, a change in the population of a species with large biomass and several links to other parts of the ecosystem food web is likely to have a larger impact on the ecosystem than a change in the population of a less connected species with lower biomass. A change in the population of a species that has the only existing links to another species in the ecosystem (such as the only pollinator of a particular plant species) could also have consequential ecological impacts.

Accordingly, parameters I1, I2 and I3 were used to provide a measure of the importance of each species to the ecosystem. Parameter I1 was used to determine a rough estimate of the biomass of each receptor in the receiving ecosystem prior to the introduction of the transgenic plant. Biomass estimates were obtained from known dry weight and density measurements for each receptor, using Biomass = dry weight × density. Where the dry weight was unknown, estimates were obtained using equation (1):

$$\text{dry weight} = A(L)^B \quad (1)$$

where L is the length of the final larval instar or adult form of each receptor, and A and B are factors obtained from the literature (Hódar, 1996; Meyer, 1989; Rogers et al., 1976, 1977; Schoener, 1980; Stoffels et al., 2003; Towers et al., 1994). Since these factors have been determined for a number of different taxonomic levels, the lowest available taxonomic level was used preferentially (*i.e.*, the A and B factors used to determine the dry weight of the species were used in preference to those determined for the genus, family or class).

Where the actual density of a species in a given ecosystem was known, this value was added to the database. Where it was unknown, estimates were obtained using the average of the receptor's known densities in other ecosystems, or, where this was unavailable, the average of the known densities for other receptors in the same feeding trophic level. Obviously there was quite a high level of uncertainty around these average density values and calculated dry weights. Consequently, the resulting biomass values were log transformed to reduce the weight of these estimates in the final PRONTI model. In addition, the final biomass scores were multiplied by 10, to bring the values into a similar range to that obtained for the other ecosystem impact parameter scores.

The number of links that each receptor had to other parts of the ecosystem's food web (parameter I2) was estimated using published information on known food webs in New Zealand ecosystems. As with parameter I1, the estimate for parameter I2 was only an approximate measure, and not a real assessment of the ecosystem's invertebrate community structure. The estimate for I2 consists of a measure of downward links as well as upward links: that is, links to both lower and upper trophic levels for each receptor.

Links to lower trophic levels were obtained from the data on each receptor's trophic level and diet. Again, data for each of these attributes were not available for all receptors in the ecosystem, and some surrogate attributes were required. For instance, for herbivorous or omnivorous receptors, actual known links to plant species were counted, but if none were known, it was assumed the receptor ate at least one plant species. For a carnivorous or omnivorous receptor, unless the literature clearly showed that it only ate certain known species, probable links for its feeding guild and taxonomic order were added to the actual, known links. To estimate probable links, a reference food web of 114 generalized species was constructed to represent the New Zealand invertebrate ecosystem. The generalized species were generated from the published literature, and were developed to represent each feeding guild in each invertebrate order and for several families in the larger insect orders. Links between the resulting 114 generalized species were represented as a 1, 0.5 or 0 indicating that the link was definitely, likely or unlikely to exist respectively. The number of links for each of the generalized species was then totaled to provide an estimated reference value for each of the actual species in the database. Decomposers were differentiated by their preference for plant material, animal material, or both, wherever possible. Links between each receptor and other species in higher trophic levels were gained from the available data on the diets of invertebrates and vertebrates known to be present in the receiving ecosystem.

In addition to the food web links measured by I2, parameter I3 determined whether or not the receptor had a special function in the receiving ecosystem. This may include functions such as biological control, pollination, mite transfer, disease transmission, or seed dispersal. These attributes were seen as being a measure of the importance of the receptor to the ecosystem, with the magnitude of the measure being greater than a single food web link, and therefore were included as a separate parameter.

Parameter I4 was used to determine the ecological resilience of each receptor population to environmental changes (Gunderson, 2000; Holling, 1973; Walker, 1992). Ecosystems are dynamic, and biological or manmade perturbations can occur relatively frequently during an invertebrate's lifetime. To withstand these natural perturbations, species have evolved mechanisms, behaviors, and characteristics that reduce the impact of the environmental change on the receptor population. Since a receptor may use these "resilience factors" to reduce the effect of a new hazard, or decrease the level of exposure to a

Table 3. List of selection criteria, and the parameters used to define each of these criteria, used by the Priority Ranking of Non-Target Invertebrates (PRONTI) method. "Stressor" refers to the new GMO, while the term "receptor" is used to encompass both target and non-target invertebrates in the receiving ecosystem.

Selection criteria	Defining parameters
1) Could the stressor pose a hazard to the receptor? (H)[1]	H1. Identification of the stressor/s
	H2. Potential direct effects of stressor on receptor
	H3. Possible indirect effects of stressor on receptor
2) Could the receptor be exposed to this stressor? (E)	E1. Receptor found in receiving area
	E2. Receptor's diet
	E3. Receptor's use of plant
3) Could there be an impact on the ecosystem if this receptor is affected? (I)	I1. Receptor's biomass (S)
	I2. Food web links from receptor to other organisms in the ecosystem (S)
	I3. Receptor's special ecological function (S)
	I4. Receptor's resilience (*i.e.*, ability to avoid the hazard or reduce its exposure level) (R)
4) Do people value this receptor? (V)	V1. Value of the receptor to indigenous human cultures
	V2. Conservation value of the receptor
	V3. Value of the receptor to society
	V4. Economic value of the receptor
	V5. Links from the receptor to higher levels in the food web (including human diets)
5) Can researchers perform tests with this receptor? (T)	T1. Accessibility of the receptor
	T2. Generation time of the receptor
	T3. Rearing protocols available for the receptor
	T4. Bioassay protocols available for the receptor

[1] Representative symbols used in the text.

new stressor, a measurement of each receptor's resilience is needed to obtain a more accurate prediction of the impact of the new stressor on the receptor population.

The receptor's resilience attributes were divided into four factors: (1) resistance (the likelihood of the receptor developing a genetic resistance to the hazard); (2) behavior (the likelihood of individual receptors using learned or innate behaviors to avoid exposure); (3) migration (the likelihood of the receptor moving out of the unfavorable environment); and (4) recovery (the likelihood of the receptor population increasing following implementation of the other three factors). In each case, the amount of information available on these four resilience factors was severely limited, and surrogate data were often used. To this end, information on a number of attributes was obtained for each of the factors, and combined to provide the most accurate prediction of resilience possible for each receptor (Tab. 4).

Selection criterion 4: Estimated receptor value (V)

The measurement of this criterion involved determining whether the receptor species might be of value to humans. Five parameters were selected to represent the ways in which societies may value a receptor. Parameter V1 dealt specifically with the value of the species to the country's indigenous cultures. Measurement of this parameter included gaining information on whether the receptor had been named, and therefore recognized, by the culture, and whether the receptor appeared in the culture's ceremonial, dietary or mythological records. Parameter V2 looked at the conservation status of each receptor (since rare species are often more highly valued), and whether or not the species was endemic or native. The third and fourth parameters (V3 and V4) were related, in that they dealt with present-day attitudes towards the receptor: V3 measured whether or not the species was of aesthetic or symbolic value, or provided a free, useful service to society (*e.g.*, as a natural enemy or indicator species); V4 dealt specifically with the economic value of the receptor, which could be beneficial (*e.g.*, income-generating species such as pollinators) or detrimental (*e.g.*, pest species which must be controlled). Parameter V5 provided a measurement of the dietary value of the receptor to species at higher levels in the food chain (including humans). For example, humans often place

a value on colorful bird species, edible fish species and native mammals, and it is likely that people would be concerned if the diets of these animals were affected by the new transgenic plant. This parameter was therefore used to measure the possible importance of each receptor to vertebrate species that may be valued by humans.

Selection criterion 5: Receptor testing (T)

The final selection criterion pertained to the ability of researchers to conduct tests on each receptor. Four parameters were selected: T1 assessed the accessibility of each receptor, and whether or not it could be collected for laboratory tests; T2 assessed the length of time a test would take, determined by the generation time of each individual receptor; T3 assessed the availability of rearing information for the receptor; and T4 assessed the availability of bioassay protocols for each receptor. The four parameters were then combined to provide a measure of the ease with which research could be performed with each receptor species.

Ranking method for measurement of parameters

In order to transform the receptor attribute data into values that could be used to provide a measure for each parameter, we used a ranking system similar to that used by Hansen et al. (1999), Lu et al. (2003) and Russom et al. (2003). The initial, generalized risk hypothesis that has been formulated was used to direct the ranking process. For example, in the case of Cry1Ac-expressing pine trees, we hypothesized that most lepidopteran species that use pine as a food source would be harmed by ingestion of the toxin, and other species, such as predators and decomposers, that rely on these for food would also be affected. Each receptor attribute was given a rank on a scale of 0 to 10, with 10 representing the best receptor attribute, and 0 representing attributes that were viewed as having no value to the parameter in question under the hypothesized conditions. The values from 1 to 9 were assigned to attributes that provided a measure of the parameter but were either a less robust measure (*i.e.*, there was some uncertainty as to their value), or were deemed to be of less value under the test hypothesis. All data gaps were given a ranking of 5, since the real, but unknown, attribute may have been of more or less value to the parameter being measured. An example of the ranking system is given in Table 5. The table shows that each receptor attribute may have been ranked differently when it was used to provide a measure for more than one parameter. The ranks were assigned using expert knowledge, but the system used allows the ranks to be changed should the value of an attribute be viewed differently under a different hypothesis. In addition, the weight of each selection criterion or parameter could be increased or decreased depending on the researcher's requirements. For example, the ranks assigned to parameters T3 and T4 were doubled in the PRONTI model, as the ability to rear and carry out bioassays with the receptor were seen as more important parameters than the receptor's generation time and accessibility, for the measurement of selection criterion 5.

Priority ranking of non-target invertebrates (PRONTI) model

Three interconnected steps were used to produce a priority list of non-target invertebrates under the conditions stipulated by the test hypothesis. In Step 1, the ranks for each receptor attribute were combined to produce a value for each parameter. In almost all cases, the ranks for each relevant receptor attribute were simply added together to produce a total score for each parameter. There were, however, some situations where this was not appropriate, and the following two rules were used:

Rule 1: Where the ranks assigned to a combination of attributes resulted in undue weight being given to those attributes in the measurement of a parameter, the ranks from those attributes were averaged to produce a more realistic attribute value. For example, for the measurement of the resistance factor of parameter I4, the ranks that had been assigned to the receptor's attributes of potential number of offspring and number of yearly generations, were averaged to produce a single rank that more accurately represented the receptor's reproductive rate. Summing the rankings for these two attributes would have exaggerated the value of the receptor's reproductive rate in estimating the likelihood of the receptor developing resistance to the hazard.

Rule 2: Where multiple rankings were obtained for a single attribute (*i.e.*, there was more than one measure in the literature for that receptor attribute), the maximum ranking was used. For example, part of the measurement of the migration factor of parameter I4 involved the combination of ranks assigned to each of the receptor's mobility attributes. Where the receptor was able to disperse using more than one method, the one that achieved the highest rank was used (*e.g.*, a receptor's ability to fly was used in preference to its ability to crawl). Where these attributes were also subject to Rule 1, an average of the maximum rankings was obtained.

In Step 2, values were obtained for each of the selection criteria. For criteria 1 (H), 2 (E), 4 (V) and 5 (T), the total scores obtained for each parameter in Step 1 were simply added together to produce criterion values for each receptor species. For example, criterion 2

Table 4. Receptor attributes used to measure species' population resilience. The attributes were used to inform four resilience factors which may be used by the species to mitigate the effects of the stressor: resistance, behavior, migration and recovery. Surrogate attributes, listed in the third column, are used by the Priority Ranking of Non-Target Invertebrates (PRONTI) method when the receptor attributes listed in the second column are unknown.

Resilience factor	Receptor attributes likely to result in this resilience factor	Surrogate attributes used to estimate receptor resilience
Resistance	Published reports of the receptor showing resistance to the stressor	Published reports of taxonomic family members showing resistance to the stressor
	Published reports of the receptor showing resistance to related stressors	Published reports of the receptor showing resistance to different, unrelated stressors
	Receptor is known to be exposed (*e.g.*, known to eat the stressor, or to prey on species that eat the stressor)	Information that indicates the receptor may be exposed (*e.g.*, may eat the stressor, or prey on species that may eat the stressor)
	The stressor is known to have an effect on the receptor (*e.g.*, the receptor is known to be susceptible to the change in protein expression in the plant giving an opportunity for resistance to arise)	Information that indicates the receptor may be affected by the stressor (*e.g.*, is susceptible to a related hazard, or taxonomic family members are known to be susceptible to the hazard)
	Density of the receptor population in the ecosystem is known to be large, so that it is more likely that resistant individuals may arise	Receptor is not known to be rare or threatened in the ecosystem of interest, but actual population size is unknown
	Reproduction rate is high	Reproduction rate of other species in the same genus is high
	Number of generations per year is high	Number of yearly generations of other species in the same genus is high
Behavior	Feeding stage coincides with presence of the stressor allowing individuals to display avoidance behaviors	Feeding stage may coincide with the presence of the stressor
	Known to be a generalist feeder with a flexible diet that may allow it to avoid eating the new stressor	May be a generalist feeder
	Known to have mechanisms to detect food quality and can avoid eating parts of the stressor or prey that are unpalatable	Information indicates the receptor may be able to detect the change in the stressor or identify prey that have eaten the stressor
	Life stages that are exposed to the stressor are highly mobile, allowing them to find alternative foods	Information on mobility and dispersal distances indicates the exposed life stages may be able to disperse to find other food sources
	Receptor's diapause known to reduce exposure to the stressor	Receptor's diapause may reduce exposure to the stressor

Table 4. Continued.

Migration	Receptor known to be highly mobile and able to disperse into refuge areas or other ecosystems	Receptor has several modes of dispersal and the dispersal distances may be quite large
	Receptor's population density in the receiving ecosystem is high, increasing the likelihood that some individuals may migrate out of the receiving environment	Receptor is not known to be rare or threatened, but actual population density is unknown
	Receptor's population density in other ecosystems is high, suggesting the receptor will survive in areas to which it disperses	Receptor has been found in several other ecosystems, or has a widespread distribution
Recovery	Receptor's reproduction rate and number of generations per year will allow it to quickly re-populate an area	Estimates of receptor's reproduction rate and number of generations per year suggest it might be able to re-populate the area
	Populations in other ecosystems are large, providing a source for re-introductions	Receptor is not known to be rare or threatened or has been found in several other ecosystems or has a widespread distribution
	Receptor known to be highly mobile and able to move into vacated areas	Receptor has several modes of dispersal and the dispersal distances may be quite large

(E) = parameter E1 + parameter E2 + parameter E3. For criterion 3, three of the parameters (I1, biomass; I2, food web links; and I3, special function), were summed to produce a value for the receptor's status (S) in the ecosystem. The fourth parameter of criterion 3 (I4, resilience (R)) was used separately since summing I4 with the other criterion 3 parameters would not produce a realistic estimate of the ecosystem impact. Each of these criterion values may be used individually to make comparisons between different receptors and may, therefore, also be useful during later phases of the risk assessment protocol.

In Step 3, two equations were used to combine each of these criterion values to produce a Priority Ranking of Non-Target Invertebrates (PRONTI) list. The first equation assumes that where the stressor (k) does not represent a hazard to the receptor, or the receptor is not exposed to the hazard (*i.e.*, criteria 1 (H) or 2 (E) values are 0), then the level of risk (A_k) for the receptor is zero. In addition, the level of risk posed to each receptor is assumed to be reduced by the receptor's resilience (R) (*i.e.*, the value of parameter I4), such that:

$$A_k = \frac{H \times E}{R} \quad (2)$$

The resulting value for A_k is then used in the second equation to calculate the receptor's PRONTI score:

$$\text{PRONTI score} = A_k \times (S + V + T) \quad (3)$$

where S is the status of the receptor in the ecosystem, V is the receptor value and T is the testability of the receptor. Receptors were then listed in the order of their PRONTI scores, from highest to lowest, to identify the priority non-target invertebrate species for biosafety testing.

Evaluation of the method

The described method was tested using a hypothetical test introduction of Cry1Ac-expressing *P. radiata* to New Zealand as the new stressor. The test hypothesis was that this plant could have a direct impact on species that might be susceptible to the *Bt* protein, and could also have an indirect impact on species that may use susceptible species as a food source. *P. radiata* are grown to supply paper, pulp, and wood over 1.6 million hectares of New Zealand (Grace et al., 2005). The resulting forests are found in both the North Island and South Island, and cover a large percentage of New Zealand's total arable land mass. It is possible that genetically modified versions of this tree will be planted in New Zealand in the future, given that there is already some transgenic research being conducted (*e.g.*, Grace et al., 2005; Henderson and Walter, 2006). Consequently, invertebrates living in this environment may be exposed to new plants in the future, and the impact of this exposure could potentially be large, given the substantial land area over which the trees are grown. The insect toxins produced by *Bt* are commonly used to modify plants to confer insect resistance. The actions of these proteins are well known, and there is much literature available on the impacts of the various toxins on a number of invertebrate species, genera and families.

Table 5. An example of the ranking system used to transform the receptor attribute data into values for the measurement of each parameter in the Priority Ranking of Non-Target Invertebrates (PRONTI) method. In this example, the ranking obtained by each receptor depends on the number of generations the receptor undergoes each year, and the potential value of that attribute to the parameter being measured. All data gaps received a ranking of 5, since the actual value may lie anywhere between 0 and 10.

Receptor attribute		Rank for parameter T2 (receptor testing)		Rank for parameter I4 (resistance factor)		Rank for parameter I4 (recovery factor)
> 1 generation per year	10	Attribute allows tests to be performed very quickly	10	Attribute allows for resistant individuals to reproduce quickly	10	Attribute allows receptor population to recover quickly
1 generation per year	5	Attribute allows tests to be performed relatively quickly	1	Attribute is less useful for production of resistant individuals	5	Attribute allows receptor population to recover relatively quickly
< 1 generation per year	0	Attribute makes testing difficult	1	Attribute is less useful for production of resistant individuals	1	Attribute suggests receptor population will have less ability to recover
Unknown number of generations per year	5	Attribute is unknown – receptor gets middle rank value	5	Attribute is unknown – receptor gets middle rank value	5	Attribute is unknown – receptor gets middle rank value

We therefore chose to use *P. radiata* trees expressing Cry1Ac protein, a lepidopteran-active *Bt* toxin, as the hypothetical stressor, and 80 of the invertebrates living in the *P. radiata* ecosystem as the potential receptors.

Eighty species of invertebrates were selected from the published literature on the ecology of *P. radiata* forests in New Zealand. Given the size of these forests, there are often freshwater streams, rivers and lakes inside, or adjacent to, areas where *P. radiata* are grown. Our selection of invertebrates therefore included both terrestrial and aquatic species. If an individual was not identified to species level, the lowest taxonomic level to which it was identified was used. For example, the *Deleatidium* mayflies are a species complex that has not yet been fully characterized, so these were included in the database as a single entry at the genus level.

The attributes for each of the 80 receptor species were entered into the database, including attributes regarding the hazard and exposure levels posed by the transgenic trees (Appendix I). In addition, some "dummy" species were included in the database. The attributes of these dummy species were modified in order to evaluate the method. For example, "Dummy 1" was entered into the database as if there were no data available (*i.e.*, all receptor attributes were unknown, each returning a rank of 5). Other dummy species were created with varying numbers of unknown attributes so that the impact of these data gaps on the receptor's final PRONTI score could be determined. The PRONTI model was then run to obtain the prioritized list of invertebrate species for non-target testing of *Bt*-expressing *P. radiata* trees in New Zealand.

CONCLUSION

The evaluation of the PRONTI screening method shows that this is a useful tool to assist in problem formulation at the beginning of a risk assessment, providing a systematic, repeatable method of prioritizing receptor species for risk analysis tests. The use of the Microsoft® Access database allows all species in the receiving ecosystem to be assessed simultaneously, and the use of an automated model reduces the time taken to produce priority lists of non-target organisms. Although the method necessarily uses a subjective ranking scheme, each species is treated in the same way, ensuring consistent application of subjective criteria. This allows the user to select species from this list as assessment endpoints for the risk analysis with confidence that all species in the ecosystem have been considered equally. The use of expert judgment and the application of local regulatory requirements to make the final selection of species from this list also increases the confidence with which the selection of endpoints is made. The flexibility of both the ranking method for each receptor attribute, and the final species selection process, should allow this method to be used for many different GMOs in many different ecosystems and countries.

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Appendix I: List of receptor attributes contained in the database used by the PRONTI model

Attributes that remain the same for all stressors:

Receptor genus and species name
Taxonomy (phylum to subfamily)
Common or alternate names
Status in the country (endemic, native, introduced, self-introduced)
Conservation status (rare, threatened, common)
Population range in the country or region of interest
Locations of previous field collections
Ecosystems that are known to contain receptor populations
Receptor density within each ecosystem
Receptor dry weight
Length of final instar larva, or adult
Primary feeding guild
General diet in any ecosystem
Known dietary species (name of plant and/or prey species)
Mechanisms to detect food quality
Special function (*e.g.*, pollinator, seed disperser, disease carrier, *etc.*)
Known predators (including predator's name)
Dispersal mechanisms (fly, crawl, hitch-hike, *etc.*)
Dispersal life stages
Dispersal distances
Fecundity
Number of yearly generations
Name given by indigenous culture
Value of the receptor to indigenous cultures
Value of the receptor to society
Value of the receptor to agriculture
Value of the receptor to the country's economy
Presence of the receptor in the human food chain
Rearing information for the receptor
Bioassay information for the receptor
List of useful references and authors used to obtain data.

Attributes that need to be entered for each new stressor:

Plant/stressor genus and species name
Identification of changes to the stressor (new protein or change in protein expression, change in plant morphology or physiology, *etc.*)
Identification of the parts of the stressor in which the new protein is expressed and levels of expression
Identification of the parts of the plant with new morphology or physiology
Presence of the receptor in the stressor's target ecosystem
Use of the stressor by the receptor (*e.g.*, as food source, shelter, reproduction sites, *etc.*)
Timing of receptor's presence in the ecosystem compared with time of plant presence
Receptor's diet in the receiving ecosystem (*i.e.*, does the receptor eat the stressor, or something that eats the stressor)
Information on receptor's diapause stages
Life stages of the receptor that eat the stressor, or eat another species that eats the stressor
Susceptibility of the receptor to the stressor's new protein/change in protein expression
Susceptibility of the receptor to related proteins
Susceptibility of related receptors to the new protein/change in protein expression in the stressor
Susceptibility of the receptor to other changes in the plant
Published reports of the receptor's resistance to this change in the stressor
Published reports of resistance to other stressors
Benefits to the plant from the loss of the receptor
List of useful references and authors used to obtain data.

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