Phytotoxicity testing for herbicide regulation: Shortcomings in relation to biodiversity and ecosystem services in agrarian systems

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ARTICLE INFO

ABSTRACT

The purpose of this paper is to present current knowledge on methods employed to perform phytotoxicity tests and risk assessments and to highlight shortcomings in relation to biodiversity and ecosystem services. Ecosystem services are benefits provided to humankind by a multitude of organisms present in natural ecosystems. Several studies were conducted between 2001 and 2010 aimed at investigating some of the deficiencies in phytotoxicity testing (new and existing data are presented). Herbicide toxicity responses were similar when comparing a suite of crop versus wild species. However, the validity of the evaluation was limited because of the narrow types of species tested. The number of species tested, currently set between six and ten, appears insufficient. The trait-based approach (i.e. the use of plant attributes to predict species sensitivity to toxicants) can be used to improve species selection. This approach puts more emphasis on the shared biological characteristics that affect plant function within ecological communities rather than on plant phylogeny. Results presented showed that further studies are needed. In test guidelines, protocols require that crop species be sprayed as young vegetative plants, which is assumed to be the most sensitive growth stage to herbicides. In contrast, during herbicide spray, herbicides may reach non-target plants that are at various phenological stages. Several studies demonstrated that plants may be at greater risk when contamination occurs at the reproductive stage. No data on long-term effects, plant recovery or on effects on reproductive stages are requested in current guidelines. Preliminary evidence suggests that this may be an important aspect to consider in risk assessment. In addition, herbicide impacts on plant community diversity as well as biodiversity at other trophic levels have been demonstrated in only a limited number of studies and therefore should warrant more attention in risk assessment.

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1. Introduction

Plants form the foundation of terrestrial ecosystems. The link between terrestrial primary producers, biodiversity and ecosystem services is well documented (Hooper et al., 2005). Ecosystem services are benefits provided to humankind by a multitude of organisms present in natural ecosystems. For instance, abundant floral diversity was found to be the prevailing factor related to high Lepidoptera diversity in farmland habitats (Ekroos et al., 2008; Merckx et al., 2009; Boutin et al., 2011). Some butterflies, bees and other pollinating insects are common examples of how organisms provide essential ecosystem services, as they visit and subsequently pollinate wild plants and crops (Biesmeijer et al., 2006). Additionally, birds nesting

within the vegetation of agrarian habitats provide an important means of pest control in agricultural fields (Kirk et al., 1996).

Continued conversion of natural ecosystems to different types of landscapes is a global problem. In many parts of the world, land converted into cropfields and pastures cover over 50% of the landscape (Cobham and Rowe, 1986; Pimentel et al., 1992; Foley et al., 2005). Some ecosystems have been reduced to a fraction of their original land cover not only due to agriculture but also due to urban encroachment and industrialisation (Jones, 1974). Within agricultural landscapes, field margins and other small remnant habitats constitute what remain of natural, or more correctly, semi-natural habitats. The intensification of farming practices, including larger fields, monocultures and reductions in crop diversity, has reduced the size and the diversity of habitats whilst simultaneously increasing edge effects on remaining habitats (McLaughlin and Mineau, 1995). The use of herbicides and fertilisers is further threatening biodiversity of semi-natural habitats adjacent to or interspersed within farming landscapes (Boutin and Jobin, 1998).

Field margins are linear semi-natural habitats at the edges of agricultural fields. They are by far the most common habitat type

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remaining for wild plants within farmlands. They include vegetative buffers, ditches, fencerows and woody hedgerows. Other small remnant habitats include small woodlots and wetlands. These non-crop habitats provide a suite of ecosystem services that aid in the conservation of native flora and fauna including shelters and food sources for wildlife, barriers against herbicide and fertiliser drift into adjacent land and water bodies, reduced soil erosion, and increased pollination and pest control in croplands (Altieri, 1999; Olson and Wäckers, 2007; Norris, 2008; Carvalheiro et al., 2010).

Herbicides used in agriculture are designed to kill or suppress weeds in cropland. It has been demonstrated that herbicides moving to off-target areas may affect sensitive non-target plants (Kleijn and Snoeijing, 1997; de Snoo and van der Poll, 1999). The large number of herbicides available for use, the geographical extent of their use on different crops, and the quantity applied suggest a high probability that non-target species are being exposed to herbicides. Herbicides can reach plants through spray and vapour drift, runoff, overspray and revolatilisation from soil and plants (Olszyk et al., 2004). Depending on the equipment and prevailing weather conditions during application, the quantity of sprayed herbicide that will deposit in hedgerows and other field margins from multiple consecutive spray tracks can reach 1 to 10% of the application rate within 10 m with ground equipment (Boutin and Jobin, 1998, and references therein), and much more with mist-blower (or blast) sprayers and aerial applications. Herbicides can travel considerably longer distances with aerial equipment applications with drift distances of 500 m downwind being reported (Conacher and Conacher, 1986; Davis and Williams, 1990).

Tank mixtures sprayed on cropfields may contain broad-spectrum non-selective herbicides (e.g. glyphosate, (N-(phosphonomethyl) glycine), or may consist of mixtures of herbicides aimed at controlling species from a wide taxonomic range. For instance, the sulfonyl urea herbicide metsulfuron methyl (2-(4-methoxy-6-methyl-1,3,5-triazin-2-ylcarbamoylsulfamoyl)benzoic acid) is known to control 60 species from 22 different families at doses ranging from 4 to 8 g-active ingredient ha^{-1} (Doig et al., 1983). Metsulfuron methyl is a selective herbicide with a wide spectrum of activity on terrestrial herbaceous plants, yet effects on woody species, aquatic plants or ferns and mosses are still unknown (Doig et al., 1983). Furthermore, when metsulfuron methyl is applied in mixtures with several phenoxy herbicides (e.g. 2,4-D (2,4-Dichlorophenoxyacetic acid), MCPA (2-methyl-4-chlorophenoxyacetic acid)), it extends the impacts of a single spray application to additional susceptible wild species.

Three guidelines have been sanctioned by regulatory agencies worldwide to evaluate the effects of herbicides on non-target plants. The United States Environmental Protection Agency protocol (USEPA 1996) recommends testing with ten crop species, including four monocots from at least two families and six dicots from a minimum of four different families. Test species must imperatively include corn (Zea mays L.), soybean (Glycine max (L.) Merr.) and a root crop. Although non-crop species can be used, they are rarely included. The Organisation for Economic Co-operation and Development guidelines, revised in 2006 (OECD 2006) did not specifically recommend a set number of species to be tested as the requirement is left to member countries. A list of 32 crop and 52 non-crop species was included in Annex 3 of the OECD documents (2006) as possible test species. Six to ten plant species representing as many taxonomic groups as possible were suggested in the guidance document of the European Union (European Commission, 2002). In all of these guidelines, however, experimental studies of toxicity assessment are performed under strictly controlled environmental conditions that differ markedly from the plant's natural environment. As a result, there are often shortcomings related to the results since these tests are conducted with crop species in single species trials at the seedling emergence and juvenile stages under ideal greenhouse conditions. The potential

adverse impact of herbicide use on plants growing outdoors within communities subjected to various environmental conditions is rarely investigated. It can be argued that impacts on productivity, biodiversity and ecosystem services may be considerably underestimated.

The objective of this paper is to present current knowledge on methods used to perform phytotoxicity tests used in risk assessment for terrestrial primary producers. Using existing and new data, we highlight shortcomings and consequences of current phytotoxicity assessments with respect to biodiversity and ecosystem services.

2. Material and methods

Four original experiments are presented and will be described in detail (Sections 2.1 to 2.4). In addition, a suite of already published experiments relevant to the above mentioned objectives will be briefly described (Section 2.5). All experimental work was conducted in greenhouses of the National Wildlife Research Centre of Environment Canada or Carleton University (Ottawa, Ontario) between 2001 and 2010. Average temperature ranged from 15 to 43 °C and photosynthetic active radiation (PAR) from 106 (cloudy day) to 1951 μmol m^{-1} s^{-2} (sunny day at noon). A 16 h photoperiod was maintained using artificial light when necessary. Soil composition was approximately 9.5% organic matter and consisted of soil that was 75% sand, 17% silt, and 8% clay (Dalton and Boutin, 2010). Plants were top-watered once or twice a day as needed.

During the course of all experiments, minor insect and fungi infestations were controlled using beneficial insects (predatory mites Amblyseius cucumeris Oudemans and Hypoaspis miles Berlese, aphid midges Aphidoletes aphidimyza Rondani, parasitoid wasps Encarsia formosa Gahan, lady beetles Hippodamia convergens Guérin-Méneville and nematodes Steinernema feltiae Filipjev). When biological control was necessary, all plants were treated equally.

2.1. Comparing woody and crop species

The objective of this study was to assess the sensitivity of several woody species found within a protected area in Ottawa to the herbicide PAR III® (United Agri Products Canada, Dorchester, Ontario). Seven woody species were compared to crop species that are normally used for risk assessment. Seeds were obtained from commercial seed suppliers or were collected by researchers from wild populations. PAR III® is a herbicide widely applied for domestic use to kill broad-leaved plants on lawns for aesthetic reasons. The label recommended rate is 1848 g-active ingredient ha^{-1} (6 L ha^{-1}), which includes 1140 g-active ingredient of 2,4-D (32.5%), 60 g-active ingredient of mecroprop (2-(4-chloro-2-methyl phenoxy) propionic acid, 61.6%) and 18 g-active ingredient dicamba (3,6-dichloro-o-anisic acid, 5.8%).

Seeds were planted in soil in separate trays for germination. In the case where species needed a period of stratification, trays were placed in cool environments at temperatures ranging from 1 °C to 4 °C for the required period (White et al., 2009). Once germinated, seedlings at the early cotyledon stage were transplanted into 10-cm wide by 9-cm high square plastic pots containing a 1:3 mixture of horticultural sand to potting soil.

Five herbicide doses plus a control were applied following a geometric progression with five replicates per dose. In all cases, there was one plant per pot. All plants were grouped by size across doses prior to spraying to ensure homogeneity (and to reduce size variance) between doses. Plants were sprayed at the 4–6 leaf stage. In each experiment, pots were randomly assigned a numerical identification tag to prevent bias during measurements. Herbicide application was performed using an 8-L hand sprayer (Chapin model 2103, Batavia, New York, USA) that was equipped with a flat fan nozzle and tank pressurized to 2 bar (30 psi or 204 kPa) when filled to half capacity or 4 L. The operator travelled at 1 m s^{-1} whilst spraying, delivering

0.02 L m^{-2} (200 L ha^{-1}). Plants were placed in a line outdoors and the spray event was performed: time was recorded in order to accurately maintain application rates. Following the spray operation, all plants were placed back into the greenhouse. Plants within each treatment were randomised spatially and were regularly rotated to ensure uniform exposure to greenhouse conditions. At four weeks (28 days) after herbicide application, the aboveground plant material was harvested and placed in a drying oven at 70 °C for approximately 72 h, after which aboveground biomass was determined.

2.2. Testing with ferns

Two fern species were used for the experiment: *Onoclea sensibilis* L, and *Dennstaedtia punctilobula* (Michx.) T. Moore. The objective was to assess the sensitivity of fern species to the herbicides Roundup® and Ally®. Spores were obtained from a commercial greenhouse specialised in growing native ferns (Les fougères boréales, Ste-Sophie, Québec, Canada). Plants were grown from spores and were tested at the early sporophyte stage. Spores were germinated in 10 cm pots in the greenhouse with a 16 h photoperiod with temperatures ranging from 15 °C to 35 °C. At the end of the gametophyte stage, they were transferred or thinned to nine per pot. Four replicates (pots) were used for each dose. Roundup® (Monsanto Canada Inc, Winnipeg, Canada) containing the active ingredient glyphosate was tested at 213.6 g active ingredient ha^{-1} (12% of recommended label rate) and 498.4 g active ingredient ha^{-1} (28%), and Ally® (DuPont Canada Company, Mississauga, Ontario, Canada) with the active ingredient metsulfuron methyl at 0.045 g active ingredient ha^{-1} (1%). A non-ionic surfactant Agral 90 (Syngenta Crop Protection Canada Inc, Guelph, Ontario, Canada), containing nonylphenoxy polyethyoxyethanol was added to metsulfuron-methyl concentration as recommended on the label. The intent was to use 1% (metsulfuron methyl), 10% and 25% (glyphosate) label rates. However, chemical analyses conducted on the spray solutions revealed a slight discrepancy in the highest doses (12% and 28% instead) between the nominal and measured concentrations of both herbicides.

Ferns were sprayed as in the woody experiment (Section 2.1) after which they were returned to their previous location in the greenhouse and allowed to sit 24 h before normal watering resumed. Plants were harvested at four weeks after spray and aboveground dry biomass was obtained as previously described.

2.3. Measuring recovery and reproductive outputs

The herbicide Classic 25DF® (DuPont Canada Company, Mississauga, Ontario, Canada) containing the active ingredient chlorimuron ethyl (ethyl 2-(4-chloro-6-methoxy-2-pyrimidinylcarbamoylsulfamoyl)benzoate) was used for this experiment with three annual species: *Capsella bursa-pastoris* L, *Centaurea cyanus* L. *Lobelia inflata* L, The objective was to assess the extent and time to recovery after sublethal doses of herbicides were applied on selected non-crop plant species. Herbicide application was performed using a track spray booth (de Vries Manufacturing, Hollandale, Minnesota, U.S) equipped with a TeeJet 8002E flat-fan nozzle (Spraying Systems) delivering 6.75 ml m^{-2} of prepared solutions at 206.84 kPa. The non-ionic surfactant Agral 90 was added to the herbicide as specified on the label.

Ten herbicide doses (0.09 to 9.63 g active ingredient ha^{-1}) plus controls were applied following a geometric progression and six plant replicates per dose were used. There was one plant per pot. Other experimental parameters were similar to the ones reported in Section 2.1. Dry biomass as well as other variables including height, flower and seed production were determined.

2.4. Trait-based experiment

Characteristics of 33 crop and wild plant species were assessed in greenhouse experiments. The objective of this study was to assess the influence of selected plant traits on the efficacy of a foliar applied herbicide, using Roundup Original® containing glyphosate. Traits under consideration in the experimental study included leaf characteristics and plant growth parameters.

Relative growth rates (RGR) were measured using one of two methods depending on the species. In the first method, 10–15 seeds were sown directly in small, separate pots, and then thinned to one plant per pot after germination occurred. Growth rate measurements were started after 24 seedlings from different pots had germinated, and this was taken as the zero time point. In the second method, plants were germinated in trays and later transplanted into separate pots on the day when approximately 30 seedlings had emerged. This day was taken as time zero. The latter method was only used in two situations: when the seeds required a cold stratification period or when seed germination rates were very low. For both methods, five pots were harvested at seven day intervals for four weeks plus time zero (some seedlings were discarded due to inconsistency in growth). RGR was determined as the slope of the regression of natural logarithm-transformed plant dry weight and time.

Leaves are the primary organs by which post- and pre-emergent applied herbicides will be captured by plants. Morphological characteristics can explain some of the variance in the concentration of herbicide residues on leaves and shoot samples (Baril et al., 2005). Leaf measurements included the average leaf area, average leaf length and average leaf width of all the leaves present on each control plant. These variables were determined with microscope imaging (Axiovision 4.7, Carl Zeiss Canada Ltd, Toronto, Canada) using scanned leaf material. The dry weights of the stems and leaves were obtained by cutting all mature leaves from the stem at the leaf-petiole junction when the plants had reached the growth stage appropriate for herbicide spraying. Leaves and stems were dried and weighed separately.

Other growth and development variables were calculated from the previous measurements using the following formulae from Hunt et al. (2002):

Specific Leaf Area (SLA) = (average leaf area per plant (cm^2))/ (average leaf weight per plant (g))

Leaf Area Ratio (LAR) = (average leaf area per plant (cm^2))/(average weight per plant (g))

Unit Leaf Rate (ULR) = (Ln A$_2$ − Ln A$_1$)/(A$_2$ − A$_1$)*(M$_2$ − M$_1$)(t$_2$ − t$_1$) where A$_2$ is the average leaf area at 4 weeks, M$_2$ is the average total plant weight and t$_2$ is 28 days; A$_1$, M$_1$ and t$_1$ were taken as zero (Tholen et al., 2004).

Wax content has been known to modify the absorption of herbicides through the leaves (Chachalis et al., 2001a). Cuticular wax analysis was performed on the leaves of wild plants at the growth stage deemed ready for phytotoxicity testing (3–5 leaf stage), based on the current phytotoxicity testing protocols (USEPA 1996; OECD 2006). Cuticular waxes were extracted by immersing approximately 2 cm^2 of plant leaves in chloroform for 30 s. An internal standard, 17:1 methyl ester was added to each sample to allow quantification of wax compounds. The chloroform was then evaporated to dryness under a gentle stream of nitrogen. Next, the samples were dissolved in 50 μl of *N, O*-bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane (BSTFA + TMCS; 99:1) (Sigma Aldrich, Oakville, Ontario, Canada) and derivatized at 80 °C for 90 min. The samples were then analysed using gas–liquid chromatography as described in Lü et al. (2009). Quantification was based on flame ionisation detector peak areas relative to the internal standard. Wax load was determined by dividing the total absolute wax load (obtained from adding the individual component amounts represented by each peak in the chromatograph) by the total area of the leaf sample analysed.

It was suggested that the uptake of herbicides through stomata may be more important for some species than others (Wang and Liu, 2007). Stomata parameters of the adaxial side of leaves were measured using nail polish impressions of the epidermis made at the point of maximum leaf width near the leaf midrib according to a modified version of Hilu and Randall (1984). Replica impressions were taken from five different plants per species by applying a clear film of nail polish directly to the leaf surface. The type of nail polish used varied with the leaf type. For the more delicate plant species (e.g. *Papaver rhoeas* L.), a very light watery (i.e. low viscosity) nail polish was used (Revlon® nail varnish) so as not to aggressively irritate or physically damage the leaf surface — which caused ripping. A thicker (i.e. more viscous) nail polish (Chick Advisor® Sally Hansen — hard as nails) was applied on more hardy leaves, such as *Zea mays* L.. The film was allowed to dry for up to 5 h in a well-ventilated room, after which it was peeled off of the leaf and placed on a slide. The impression was kept immobile with the use of clear tape. Light microscopy was used to determine the stomata frequency and the microscope imaging facility was used to measure stomatal size parameters.

Trichomes may exert various effects on herbicide uptake (Benzing and Burt, 1970; Hull et al., 1982; Hess et al., 1974). Trichomes present on leaves were measured by visual estimates using a microscope taking into account the proportion of leaf area covered by the trichomes. This percentage was then converted to a code where 0 indicated that no trichomes were present on the adaxial leaf surface and 4 indicated that >90% of the leaf was covered by trichomes. Additional details are available in Aya (2011).

2.5. Other studies: single species and microcosm experiments

Four studies recently published are discussed. Three of the studies were conducted as single species tests. The objectives of these three experiments were numerous: to compare crop and non-crop species in phytotoxicity testing, to establish response variability between crop varieties and wild plant ecotypes and to determine uncertainties associated with phytotoxicity testing. The experimental design and methodology for these tests are as described in Section 2.3. Specific additional details can be found in White and Boutin (2007), Boutin et al. (2010) and Carpenter and Boutin (2010).

A fourth study was conducted with plants growing in microcosms (Dalton and Boutin, 2010) with the objective of comparing species growing singly in pots with the same species growing within plant communities in the greenhouse in short- and long-term experiments or outdoor. Nine terrestrial and seven wetland species were tested separately with AAtrex Liquid 480® (Syngenta Crop Protection Canada Inc, Guelph, Ontario, Canada) containing atrazine (6-chloro-N-ethyl-N0-(1-methylethyl)-1,3,5-triazine-2,4-diamine) and Roundup original® containing glyphosate. All microcosm experiments were conducted separately for each herbicide and each plant type. Seedlings of each species were transplanted into single pots or in 5 L round plastic microcosm pots according to a standardised planting arrangement. Plants in long-term studies were fertilised with a 20–20–20 mix of nitrogen, phosphoric acid and soluble potash. Further details can be found in Dalton and Boutin (2010).

2.6. Statistical analyses

Inhibition concentration (IC25 or IC50), defined as the dosage that results in a 25% or 50% reduction in biomass respectively, was calculated for species tested in the different herbicide experiments. The inhibition concentration was determined using non-linear regression models (Environment Canada, 2005) available in Systat (2004, 2009). In the cases where the assumptions of normality of residuals (Shapiro–Wilk test) or homogeneity of variance (Levene's test) were not met, even after data transformation, the interpolation method of Norberg-King (1993) was used.

In the fern experiment (Section 2.2), analyses of variance were used to test for differences between the glyphosate treatments (control and two doses). A Student *t*-test was conducted between the controls and the 1% dose of metsulfuron methyl. The assumptions of homogeneity of variance and normality were tested and were met in all cases. SAS (1988) was used for these analyses.

To test for differences between vegetative and reproductive endpoints of the recovery experiment (Section 2.3), analyses of variance (ANOVA) were conducted. In cases where assumptions of normality of residuals or homogeneity of variance were not met, non-parametric Kruskal-Wallis tests were performed. Tukey's Honestly-Significant-Difference test (ANOVA) or Conover–Inman test (Kruskal-Wallis) for all pairwise comparisons was used to uncover significant differences amongst groups. Systat (2009) was used for these analyses.

Simple regression analyses were conducted to assess relationships amongst the 18 variables measured or calculated in the trait-based approach (Section 2.4). Multiple regression statistics were completed using SAS (2010). The presence of colinearity amongst traits was investigated using variance inflation factors (VIF). Using a 2.0 cut off value, we were able to reduce our list of 18 factors to seven. The tested model became: IC25 = Relative growth rate*Total dry weight*Leaf Area Ratio*Leaf length/Width ratio*Total wax loading*Stomata frequency (adaxial side)*Trichome coverage. IC25 values were Log_{10} transformed in order to normalise the residuals of the subsequent regressions.

We tested all possible combinations of the seven parameters selected in the traits based approach. By using the information theoretic model comparison (ITMC) technique, an Akaike's Information-tion Criterion (AIC) value for each model was calculated from the log likelihoods obtained from fitting linear regressions to the data. In lieu of using the standard AIC, we used the small-sample bias correction form (AIC_c) which has been shown to converge to the standard AIC value as you increase sample size (Burnham and Anderson, 2004). Calculations and model selection followed the AIC methodology presented in Thomas et al. (2011).

3. Results and discussion

Table 1 describes characteristics of the phytotoxicity tests in relation to the goals of risk assessment. The table shows important gaps between data produced by current tests and the types of data required for risk assessment; not only in species selection but also in the manner in which tests are conducted. The results and discussion section is organised around Table 1.

3.1. Effects on native species

Two studies comparing crop and non-crop species grown under analogous conditions have demonstrated that the toxicity level was equivalent between crop and non-crop species (White and Boutin, 2007; Carpenter and Boutin, 2010). In other instances, native species were more sensitive than crop species (Olszyk et al. 2008; Clark et al. 2004). However, these four studies were limited to a narrow taxonomic range in line with the crop species usually tested. This raises the question as to whether or not other types of plants, such as woody species will be protected.

Fig. 1 presents the range of species sensitivity for 13 crops and seven woody species tested with the herbicide mixture PAR III®. The results reveal that this herbicide mixture was not very toxic to five of the woody species when sprayed at the young vegetative stage (4–6 leaves). In contrast, *Ulmus americana* L. and *Populus grandidentata* (Michx) were very sensitive to the herbicide PAR III® and would be affected at less than 10% of recommended label rate. The three grass crop species (*Lolium perenne* L., *Z. mays* L. and *Avena sativa* L.) were very resistant to the herbicide with little effect at 100% of the

Table 1

Characteristics of phytotoxicity tests conducted contrasted with the purpose of risk assessments performed for the protection of plant diversity, habitat biodiversity and ecosystem services.

Purpose of risk assessment	Characteristics of tests conducted	Shortcomings
Effects on native species	Crop species used	Crops are introduced and have traits highly altered by directed breeding. Large intraspecific crop variety exists
Effects on different plant types	Mostly annual species used	Semi-natural habitats dominated by perennial species. Woody and fern species, key and rare species not considered
Effects on hundreds of species	Six to ten crop species tested	Species richness worldwide and in non-target habitats reaches several hundreds. Plants show affinity in toxicity at low taxonomic level. Species selection based on traits not considered
Effects on vegetative, regenerative parts, and on survival	Effects recorded at juvenile stage (2–4 weeks after spray)	Recovery over time and effects on reproduction are not recorded
Effects when sprayed at various phenological stages	Crops sprayed at 3–5 leaf stage	Pesticides are sprayed in fields when non-target plants are at various phenological stages with sometimes important effects
Effects of biotic and abiotic factors	Crops tested in greenhouses with absence and/or control of wind, drought, disease, predators, interactions with other trophic levels	Plant sensitivity under ideal greenhouse conditions differs from plants growing under outdoor natural conditions
Effects on communities and ecosystems	Crops tested singly in pots	Effects on biodiversity, cascading effects at different trophic levels, ecosystem functions and services are not or only indirectly considered

recommended label rate (1848 g-active ingredient ha^{-1}). *Rhamnus cathartica* L. and *R. frangula* L. were not sensitive to the herbicide PAR III®. They are both introduced species that are considered invasive in forested areas and which inhibit the growth of native understory herbaceous plants as well as the establishment of native shrubs and trees. These species would be at an advantage if spray drift reached a forested community adjacent to a spray area or if overspray occurred. This study revealed that current tests with crop species may be sufficient for the assessment of some woody species when tested at the seedling stage, except for *U. americana* if only the ten least sensitive crop species was used as surrogate species.

The use of crop species is based on their ease of testing and on their economic importance. Guidelines were first developed to assess unwanted effects on neighbouring crops. Additionally, in the evaluation of efficacy and crop margin safety, screening tests on a large number of species are routinely conducted for herbicides (and other pesticides) to be registered and thus data on crops are readily available (Boutin et al., 1995). Crops are non-native species in most

countries in which they are grown. However, emphasis has shifted over the years with research undertaken both in Europe and North America clearly demonstrating that herbicides, and other agrochemicals, can have adverse impacts on wild plants, habitats and wildlife.

Another aspect to consider in phytotoxicity testing is intraspecific variability. Any given crop species encompasses many different varieties exhibiting varying levels of sensitivity to a particular herbicide (White and Boutin, 2007, and references therein). White and Boutin (2007) showed, using three herbicides, that the range in sensitivities amongst varieties of the same crop can be quite extensive and that conclusions regarding phytotoxicity of any given herbicide may differ depending on the variety included for risk assessment. Similarly, it was found that ecotypes of native species originating from different parts of the world (North America and Europe) responded differently to atrazine and glyphosate (Boutin et al., 2010).

The studies presented above showed that native species can be successfully germinated and grown for use in phytotoxicity testing (see also Boutin et al., 2004; Olszyk et al., 2006; OECD, 2006; White
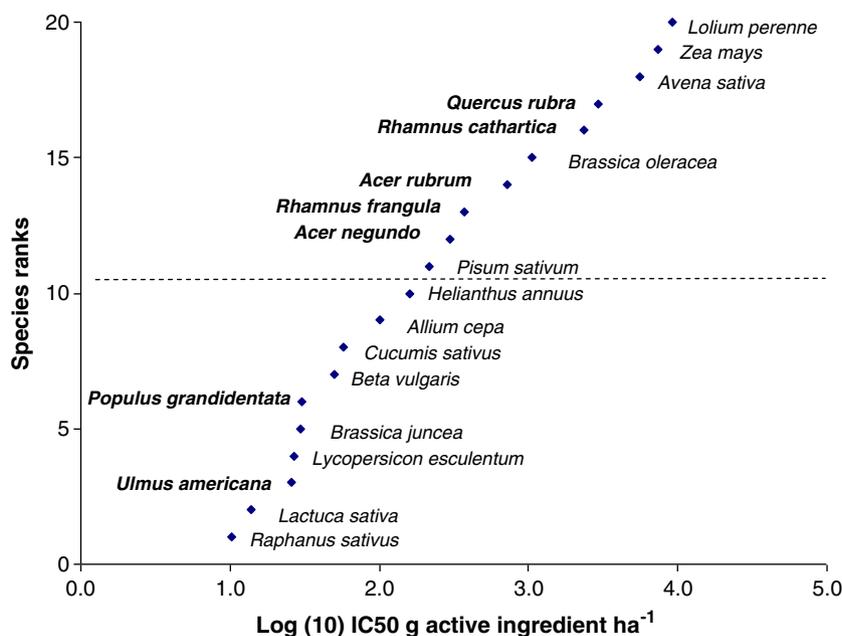

Fig. 1. Ranks of species sensitivities plotted for 20 species tested with the herbicide PAR III®. The seven woody species, in bold, were found in a protected area of Ottawa, Ontario, Canada, and were compared to crop species normally used for risk assessment. Species below the dashed line were affected at doses 10% below the recommended label rate of 1848 g active ingredient ha^{-1}.

et al., 2009). Results revealed that crop species may be used as surrogates for native species in the limited framework of regulatory guidelines. However, the link between crop sensitivity to any given herbicide and the effects on biodiversity of natural or semi-natural habitats is tenuous at best due to the discrepancy between the limited crop types tested, which contrasts with the large variety of wild species to be protected.

3.2. Effects on different plant types

Species selection in phytotoxicity testing is an unresolved dilemma in terms of representativeness (Table 1). Most test species selected are annual crop species. This contrasts with natural and semi-natural habitats, which are dominated by perennial species (Boutin and Jobin, 1998). Annual species differ from perennial species in the allocation of resources to belowground and aboveground systems as well as to reproductive parts. Effects on annual species can be immediate and dramatic if seed output is reduced or inhibited by herbicides. It can lead to reductions in seedling recruitment in habitats of interest in the short-term. Conversely, perennial species can carry over effects to following years. Lasting effects during the course of the growing season following spray events become an important factor to consider in herbicide risk assessment.

Fern species are never used in phytotoxicity testing despite the fact that some species constitute important components of the moist and shaded habitats adjacent to cropfields in North America. *D. punctilobula* (Michx.) T. Moore is a common fern species found in forested areas whilst *O. sensibilis* L. grows in a wider range of habitats, including ditches and hedgerows adjacent to field margins. Tests conducted with two herbicides (Fig. 2) revealed a high sensitivity to metsulfuron methyl and to a lesser extent to glyphosate in both species when tested at the early sporophyte stage. Glyphosate caused a significant reduction in biomass of the two fern species at 12% and 28% of the label rate whilst metsulfuron methyl significantly decreased biomass at 1% label rate. In addition, the sensitivity of several herbicides on pteridophytes, bryophytes and lichens was clearly demonstrated in North America (Newmaster and Bell, 2002) and on lichens in Europe (Juuti et al., 1996). These findings suggest that the taxonomic range used in phytotoxicity testing may be too limited.
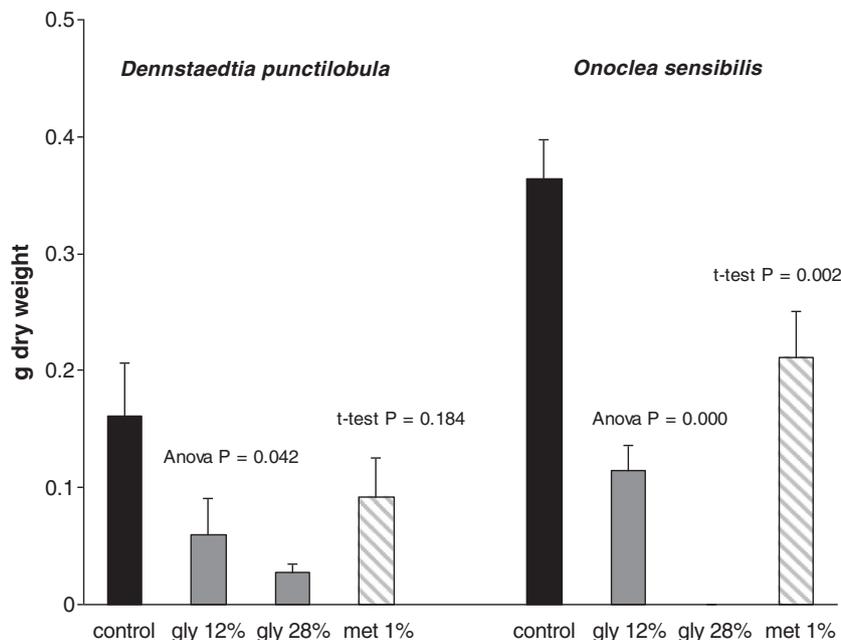
In a study by Pfleeger et al. (2006), the authors used Geographic Information System technology (GIS) to identify areas of the United States most at risk of herbicide exposure based on wind speed, frequency of wind directions, herbicide application rates and spatial location of crops. Using existing databases, native species were chosen as a test case for phytotoxicity testing for the Illinois area that was potentially at risk to herbicide drift (Olszyk et al., 2008). Plant species selection was based on plant life form as well as on species abundance in different habitats, genus representation, seed availability and ease of germination and growth under experimental conditions. This experimental work was a very elegant demonstration that it is possible to include ecologically relevant plant species in regulatory testing with minimal effort. A combination of the above technique with the trait-based approach (see below) may make it possible to extrapolate across regions (Baird et al., 2008). Yet the loss of key species or genera, e.g. milkweed plants (*Asclepias* spp.) that are essential for the survival of the Monarch butterfly (*Danaus plexippus* L.) for example, may not be assessed and thus, important effects will go undetected even with the systematic procedure just described. However, there are existing databases that could be used as well as new ones being developed that could in turn be incorporated into a GIS system to address this concern and further improve this promising method.

3.3. Effects on hundreds of species

There is an ongoing debate about the appropriate optimal number of species to be tested in phytotoxicity trials (Boutin and Rogers, 2000). The number of species currently required in guidelines varies from six to ten. This may seem like a large number of species when compared to the requirements for other organisms such as invertebrates, birds and mammals. The actual number of flowering plants around the world approximates 450,000 (Convention for Biological Diversity, 2010). In comparison, the estimate for insects approximates one million species, whilst other estimates include 28,000 for living fish species, 10,000 for birds and 5400 for mammals. However, in habitats that are susceptible to the effects of herbicide drift, the number of plant species recorded can be in the hundreds. In a recent survey of field margins undertaken around 60 cropfields near

Fig. 2. Effects of glyphosate (gly) and metsulfuron methyl (met) on two fern species, *Onoclea sensibilis* and *Dennstaedtia punctilobula*. Average biomass ± error bars are shown. None of the plants of *O. sensibilis* treated with 28% of glyphosate survived.

Table 2
Species, family and lifespan of wild and crop plants used in the trait-based experiment. The lC25 (g active ingredient /ha^{-1}) defined as the dosage that resulted in a 25% reduction in aboveground biomass are presented with confidence intervals (Cl). Plants are arranged by increasing lC25s. A = annual, B = bienniad, P= perennial. n/a = not available.

Species	Family	Life span	Type	IC$_{25}$ (g ai/ha^{-1})	95% Cl (g ai/ha^{-1})
Brassica oleracea L.	Brassicaceae	P	Crop	43.1	28.2–65.5
Helianthus strumosus L.	Asteraceae	P	Wild	52.2	42.1–78.4
Phalaris arundinacea L.	Poaceae	P	Wild	58.0	47.0–72.0
Bellis perennis L.	Asteraceae	P	Wild	59.7	45.5–78.3
Eupatorium maculatum L.	Asteraceae	P	Wild	60.0	47.0–77.0
Helianthus annuus L.	Asteraceae	A	Crop	60.9	20.1–90.0
Andropogon gerardii Vitman	Poaceae	P	Wild	62.0	n/a
Solanum dulcamara L.	Solanaceae	P	Wild	904	57.9–141.2
Zea mays L.	Poaceae	A	Crop	92.0	73.0–111.0
Lactuca sativa L. var. Tom Thumb	Asteraceae	A/B/P	Crop	112.0	94.0–155.0
Nicotiana rustica L.	Solanaceae	A	Wild	114.1	91 .3–142.2
Melilotus officinalis (L.) Pallas	Fabaceae	A/B/P	Wild	118.1	68.5–203.2
Papaver rhoeas L.	Papaveraceae	A	Wild	128.7	96.3–1724
Capsella bursa-pastoris (L.) Medik.	Brassicaceae	A	Wild	135.5	108.1–169.6
Digitalis purpurea L.	Scrophulariaceae	B/P	Wild	156.0	131.7–1844
Phytolacca americana L.	Phytolaccaceae	P	Wild	156.9	117.9–180.8
Lactuca sativa L. var. oakleaf	Asteraceae	A/B/P	Crop	1704	129.6–2244
Panicum clandestinum L.	Poaceae	P	Wild	178.3	120.9–201.3
Bouteloua gracilis (HBK.) Lagasca	Poaceae	P	Wild	182.7	155.7–214.8
Fagopyrum esculentum Moench	Polygonaceae	A	Crop	196.1	169.0–304.8
Lolium perenne L.	Poaceae	P	Crop	206.0	161.2–263.9
Prunella vulgaris L.	Lamiaceae	P	Wild	214.8	150.3–305.9
Centaurea cyanus L.	Asteraceae	A	Wild	234.6	152.9–330.09.9
Polygonum pensylvanicum L.	Polygonaceae	A	Wild	240.7	193.1–3244
Solidago canadensis L.	Asteraceae	P	Wild	246.2	199.5–304.1
Lycopus americanus L.	Lamiaceae	P	Wild	250.0	187.0–334.0
Vicia americana Muhl	Fabaceae	P	Wild	304.5	146.6–6314
Cerastium fontanum L.	Caryophyllaceae	B/P	Wild	390.7	301.0–507.2
Verbena hastata L.	Verbenaceae	B/P	Wild	449.8	2854–708.6
Campanula americana L.	Campanulaceae	A	Wild	468.9	333.2–661 .2
Chrysanthemum leucanthemum L.	Asteraceae	P	Wild	633.13[a]	965.1 (692–1345) 113 (43–296) 821 (630–1073)
Inula helenium L.	Asteraceae	P	Wild	761.1	577.1–1005.9
Rumex crispus L.	Polygonaceae	P	Wild	974.0	872.0–1088.0
Rudbeckia hirta L.	Asteraceae	A/B/P	Wild	1042.8	401.5–1545.7

[a] Average of three toxicity tests taken from Boutin et al. (2010).

Barcelona, Spain, 517 different plant species were identified including many of high conservation value (Bassa et al., 2011). The question arises of how it is possible to estimate the risk from herbicide use when only a maximum of ten crop species are assessed. Boutin and Rogers (2000) estimated that at least 40 species should be tested by showing that the greater the number of species evaluated, the broader the observed sensitivities for a given chemical. This is in accordance with other published findings for organisms such as birds (Baril et al., 1996).

Several factors must be taken into account to determine the appropriate number of species to test. First, the number of species actually tested must adequately represent the diversity of responses that will be observed amongst all non-target plants. Plants show comparative sensitivity to herbicides at the species level (i.e. species within genus) and comparative sensitivity decreased considerably when comparing genera within a family and family within an order (Fletcher and Johnson, 1990). Likewise, Boutin and Rogers (2000) demonstrated that patterns of chemical sensitivity were more similar for taxonomically related plants than for distant plant species. This is quite different from results obtained for other organisms. Suter (1983) reported that fish sensitivity was comparable at all levels, from species to order. In birds, the limited dataset analysed showed that the separation in sensitivity to cholinesterase insecticides seemed to occur at the family level, e.g. between the Icteridae and the Phasianidae (Baril et al., 1994). These findings demonstrated that the level of plant variability is very high compared to other organisms, thus necessitating the need for more plant species to be tested.

Other factors include methodological aspects such as the statistical power of the test. However, the number of species and replicates must be manageable given the expense, space and time involved in testing several representative plant species. At present, the limited numbers and life forms (lifespan, growth types, taxa) used in regulatory testing do not encompass the wide range of plant species to be protected in agrarian systems (Table 1). For this reason, obtaining information on the attributes in plants that may influence responses to herbicides would assist in the process of species selection. The trait-based approach has recently been proposed as a way to circumvent this variability problem in toxicity testing to better extrapolate from one species to another.

Several experiments analysing single plant traits versus herbicidal efficacy have been successfully conducted (Chachalis et al., 2001b; Huangfu et al., 2007); however, understanding the influence that an assemblage of traits has on efficacy remains an important factor for improving species selection in phytotoxicity testing. A study of the morphological, ecological and physiological traits of 33 crop and wild species (Table 2) was conducted to assess the influence of plant traits on the efficacy of the foliar applied herbicide glyphosate (formulation Round-Up Original®). It was hypothesized that herbicidal efficacy can be greatly influenced by multiple plant characteristics. Traits observed included stomata frequency and length side of leaves, trichome coverage, total cuticular wax load and various leaf and growth traits (Table 3). IC25 values calculated based on the dry biomass of plants grown singly in pots under greenhouse conditions are shown in Table 2. Results of simple linear regressions conducted between the different plant traits and the calculated IC25s revealed no significant relationships.

The AIC performed using seven of the variables measured (see Section 2.6) revealed that the top most parsimonious model included

Table 3
Minimum and maximum values of traits measured or calculated on the 33 species used in the trait-based experiment. Abbreviations of selected variable used in the multiregression analyses are presented (see Table 4).

Variables	Selected variables	Range of values	
		Minimum	Maximum
Relative growth rate (g/day)	RGR	0.05	0.30
Aboveground dry weight (g)	Totdw	0.01	0.63
Leaf dry weight (g)		0.01	0.43
Stem dry weight (g)		0.00	0.20
Ratio leaf dry weight to stem dry weight		1.56	12.49
Ratio leaf dry weight to total plant dry weight		0.60	0.90
Ratio stem dry weight to total plant dry weight		0.07	0.39
Total leaf area (cm^2)		3.03	62.42
Leaf Area Ratio (cm^2/g)	LAR	34.96	1105.35
Specific Leaf Area (cm^2/g)		51.56	1450.30
Unit leaf rate (g/cm^2/day)		10.42	315.82
Leaf width (cm)		0.13	3.92
Leaf length (cm)		2.37	14.24
Ratio length/ width	L/W	1.36	43.36
Stomata frequency (adaxial)	Stofr	2.00	48.17
Stomata length(μm)		14.85	49.94
Trichome coverage (adaxial)(%)	Trich	0.00	100.00
Wax load (μg/cm^2)	Wax	0.24	30.35

Leaf Area Ratio (LAR) only, but with a low coefficient of determination (AIC = −65.55, Δ_2 = −0.8, R^2 = 0.07) (Table 4). The first ranked model combined LAR and trichome coverage (AIC = −66.35, Δ_1 = 0, R^2 = 0.14); however, the R^2 value was still quite low. Even if this model had a higher coefficient of determination and a lower Δ value, it was not the preferred model because it was not the simplest model with a Δ value <2 (Anderson et al., 2000). Our best model could then be represented by:

$$\text{Log}_{10}(\text{IC25}) = -0.00044(\text{LAR}) + 2.46.$$

However, an ANOVA revealed that the regression was not statistically significant ($F_{1,31}$ = 2.25, p = 0.14). Our first regression model ranked by AIC$_c$ was also not significant ($F_{2,30}$ = 2.51, p = 0.1) but could be considered marginal at the α = 0.1 level.

Although only marginally significant, there is a trend suggesting that LAR and trichome coverage may play a role in determining herbicide sensitivity. LAR is a measure of the net assimilation rates by plants and of the photosynthetic capacity. The results showed a negative trend between LAR and sensitivity to the herbicide glyphosate. Since glyphosate is a contact herbicide, greater leaf surface area means more points of entry for the herbicide thus increasing efficiency.

The relationship between herbicidal efficacy and trichome density is complex due to the variety in leaf epidermal trichome forms. Trichomes are an especially interesting trait since they can either decrease or increase herbicidal efficacy. Trichomes increase herbicide tolerance when they hinder the wetting and spreading of herbicide droplets (Hull et al., 1982), create air pockets that inhibit contact between the chemical and the leaf surface and/or cause droplets to shatter or bounce away from the leaf epidermis (Hess et al., 1974). Conversely, they may also decrease plant resistance to herbicides by providing an entry site for foliar-applied herbicides (Benzing and Burt,

1970). The positive trend between trichome coverage and the IC25s suggests that trichomes may prevent herbicides from reaching and penetrating the cuticle, thus minimising potential uptake by the plant. It was observed that the majority of resistant species had trichome coverage on more than 75% of their leaf surface. For example, *Rudbeckia hirta* L. (1043 g active ingredient ha^{-1}), *Inula helenium* L. (IC$_{25}$ = 761 g active ingredient ha^{-1}), *Verbena hastata* L. (450 g active ingredient ha^{-1}), *Cerastium fontanum* Baumg. (391 g active ingredient ha^{-1}) and *Centaurea cyanus* L. (235 g active ingredient ha^{-1}) have the highest percentage of leaf area covered by trichomes and also have correspondingly high IC$_{25}$ values (Table 2).

Stomata frequency and cuticular wax did not exert any influence on plant sensitivity to glyphosate potentially revealing the lack of importance of these modes of entry for this herbicide; however, these results may vary for other formulations and herbicides. Stomata were considered a minimal route for foliar uptake in *Brassica juncea* (L.) Czern (Huangfu et al., 2009), but Wang and Liu (2007) suggested that the stomatal uptake of herbicides varies greatly with plant species. Nonetheless, quantitative information on the role of stomata is still lacking. Cuticular wax deposition may affect the efficiency of herbicide uptake in a variety of ways. It is expected that the quantity and composition of the cuticular wax may be relevant in phytotoxicity testing (Chachalis et al., 2001a,b). Changes in cuticle and wax deposition may affect the interception, retention, penetration and/or translocation of glyphosate to its site of action. It is important to note that although wax load was taken into consideration, the actual wax composition was not determined due to the high level of complexity involved in assessing multiple plant species. A number of studies have indicated that wax composition may be a significant indication of herbicidal efficacy (Mayeux and Jordan, 1980; Wilkinson, 1980; Wilkinson and Mayeux, 1987; Chachalis et al., 2001a) and thus this trait should be taken into consideration in future studies.

In the above study, traits were selected for their possible importance in the uptake of the contact herbicide glyphosate by plants when grown individually in pots. Whilst a trait-based approach has some potential in evaluating herbicide efficacy for different species, our study did not indicate this. Other additional traits not assessed may have been more appropriate, such as belowground root characteristics, leaf angle, metabolic rates or biochemical pathways. Furthermore, the traits measured may not have been the most appropriate for plants growing within communities receiving sublethal doses of herbicide and did not take into consideration such factors as competitive ability, size or growth form. The trait-based approach has the advantage of linking organisms with traits that are related to functions within communities including nutrient affinity, drought tolerance, competitive ability, time and resource allocation to reproduction or photosynthetic rates, but that are more complicated to measure. This approach also takes advantage of the convergence in plant growth form and function, e.g. succulent plants occur in several families including Cactaceae, Euphorbiaceae or Crassulaceae (Keddy, 1990).

3.4. Effects on vegetative, regenerative parts and on survival

Most herbicides used in farmlands are selective products that affect species differently. Long-term effects of herbicides on plants

Table 4
Summary (Akaike's Information Criterion) AICc results for the top five candidate models ranked by AIC value (from smallest to largest) for 33 plant species. AICc value, delta value (Δ), error sum of squares (SSE) as well as the coefficient of determination (R^2) are presented after a Log10 transform of the IC25 values. See Table 3 for abbreviations of variable names.

	RGR	Totdw	LAR	L/W	Wax	Stofr	Trich	AIC$_c$	ΔAIC	SSE	R^2
1	.	.	✓	.	.	.	✓	−66.35	0	169	0.14
2	.	.	✓	.	.	.	.	−65.55	−0.8	4.01	0.07
3	.	.	✓	.	.	✓	.	−65.2	−115	82	0.11
4	.	.	.	.	.	.	✓	−65.12	−1.23	4.06	0.06
5	.	.	✓	.	.	✓	✓	−65.03	−1.32	61	0.16

and recovery of the vegetative and reproductive parts following the application of sublethal doses of herbicides were recently assessed using annual and perennial species tested with Ignite SN® containing 150 g L^{-1} active ingredient of glufosinate ammonium (DL-phosphinothricin, Bayer Crop-Science Inc.) sprayed at the juvenile plant stage (Carpenter and Boutin, 2010). Of the 12 crop and non-crop species for which reproduction could be measured, no species exhibited full recovery at the end of the reproductive period (between 38 and 139 days after spray depending on the lifespan of the species). Reproduction was the most sensitive endpoint for seven species. In the case of the perennial grass species *Elymus canadensis* L., there was almost total recovery when considering total aboveground biomass; however, seed output was negatively affected by increasing herbicide dosages. Similarly, Riemens et al. (2008) found that the seed production of *Stellaria media* (L.) Vill. was more negatively affected than the aboveground weight at the same doses when sprayed with glufosinate ammonium.

The time required for plants to recover following herbicidal impacts would likely play an important role in plant community dynamics. This was investigated on three annual species sprayed with the sulfonyl urea herbicide chlorimuron ethyl. All three species reacted very differently. *Lobelia inflata* L. showed signs of recovery over time (IC25 of aboveground biomass was 0.342 and 1.305 g-active ingredient ha^{-1} at 28 and 91 days after spray, respectively; Table 5), although the reproductive output (capsule production) was lowered at doses above 14% of the label recommended rate. Additionally, capsule production was substantially reduced at doses of 55% and 107% of the label rate (Fig. 3a; p<0.001 for capsule production at 91 days). In contrast, the IC25 calculated using the total aboveground biomass of *Capsella bursa-pastoris* L. Medik. declined over time (Table 5), probably because growth of the controls and lower dose plants was completed before affected plants could recover (Fig. 3b). Yet the reproductive output (number of pods) of doses at 3.8% of the recommended label rate and higher had not reached the levels observed in the control plants at the time of harvest (n=261 versus 450 pods for control plants 49 days after spray; p<0.001). It seems that although plants sprayed at doses between 3.8% and 14.5% label rate recovered in terms of their height (Fig. 3b), they did not recover in terms of reproduction. Lastly, the vegetative parts of *C. cyanus* L. had completely recovered 98 days after chlorimuron ethyl application (Table 5); however, the reproductive output (seed head production) was diminished with increasing doses (Fig. 3c; p=0.047). In a natural setting, these three annual species could likely be outcompeted by less affected species within the plant community, with the exception of individuals exposed to very low doses.

Zwerger and Pestemer (2000) measured the phytotoxic effects of herbicides in a greenhouse study where they grew four plant species in separate experiments, applying herbicides with different modes of action once to plants at the second or third leaf stage. Plant biomass, seed production, seed viability, germination capacity and seed weight were measured. At sublethal doses, a reduction of growth was initially observed; however, plants recovered and there was no reduction in seed production. In accordance with the findings above, at the end of the growing period, only plants subjected to small doses of herbicide were well developed and had fully recovered.

Results from studies conducted to assess long-term effects and recovery of vegetative and reproductive parts of plants exposed to various doses of herbicides are sparse. Undoubtedly, short-term phytotoxicity tests performed following current guidelines fail to assess long-term herbicide effects and more studies are required to fully address this problem.

3.5. Effects when sprayed at various phenological stages

Studies conducted to assess the effects of herbicides on phenological stages other than the juvenile period revealed interesting findings. Glufosinate ammonium, a widely used herbicide, reduced the seed production and seedling emergence of *S. media* plants that had been sprayed four weeks after emergence whilst seedling emergence of plants sprayed at an earlier stage was not affected (Riemens et al., 2008). Conversely, applications of seven different herbicides in separate experiments with various modes of action yielded similar responses whether they were applied 14 or 28 days after seedling emergence on potatoes (*Solanum tuberosum* L.), with tubers being greatly affected at sublethal doses (Pfleeger et al., 2008). Glyphosate sprayed on plants at maturity resulted in alterations in subsequent seed germination and seedling development (Blackburn and Boutin, 2003). Gange et al. (1992) found that two insecticides (chlorpyrifos, O,O diethyl-O-(3,5,6-ytivhloto-2-pyridinyl) phosphorothiate and dimethoate, O,O-Dimethyl S-(methylcarbamoylmethyl) phosphorothioate) and one fungicide (iprodione, 3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide), and various combinations of these pesticides were found to reduce the germination number and/or germination rates of nine of the 20 weed species investigated at the recommended field application rates. This surprising finding revealed that pesticides other than herbicides can have adverse effects on non-intended plant targets. The impact on other organisms can be substantial, hence the importance of studying the aftermath on plants.

In the 1990s, it was observed in greenhouse experiments that a sharp reduction in reproduction was attributable to the use of sulfonyl urea herbicides at doses expected in drift situations (Al-Khatib et al., 1993; Felsot et al., 1996; Fletcher et al., 1993, 1996; Boutin et al., 2000). Under field conditions, it was found that the reproductive endpoints (e.g. green and mature hawthorn, *Crataegus monogyna* Jacq., berries) were severely affected by average spray drift concentrations higher than 2.5% of the label rate of the sulfonyl urea metsulfuron methyl, and that the effect was still observed one year after the spray event (Kjær et al., 2006a, 2006b). Reducing flowering and fruiting of plants could have a dramatic effect on ecosystem services provided by local pollinators and subsequently on fruit or seed eating animals. It was also reported that foliar applications of sulfonyl ureas at or shortly before the flowering period caused a complete inhibition of plant reproductive output without any negative effects on their vegetative parts (Olszyk et al., 2009). This effect on reproduction may have severe, negative repercussions for species that have only one opportunity to reproduce during their life cycle such as annuals and other monocarpic species.

Table 5
Inhibition concentration (IC25 in g active ingredient ha^{-1}) defined as the dosage that resulted in a 25% reduction in short-term biomass, long-term biomass and reproductive output of three plant species tested with chlorimuron ethyl herbicide. Short-term aboveground biomass was measured when half the plants were harvested 28 days after spray. Plants for the long-term biomass were harvested at 91, 49 and 98 days after spray for *L. inflata*, *C. bursa-pastoris* and *C. cyanus,* respectively. Reproductive output includes capsule production (*L. inflata*), number of pods (*C. bursa-pastoris*) and seedhead production (*C. cyanus*).

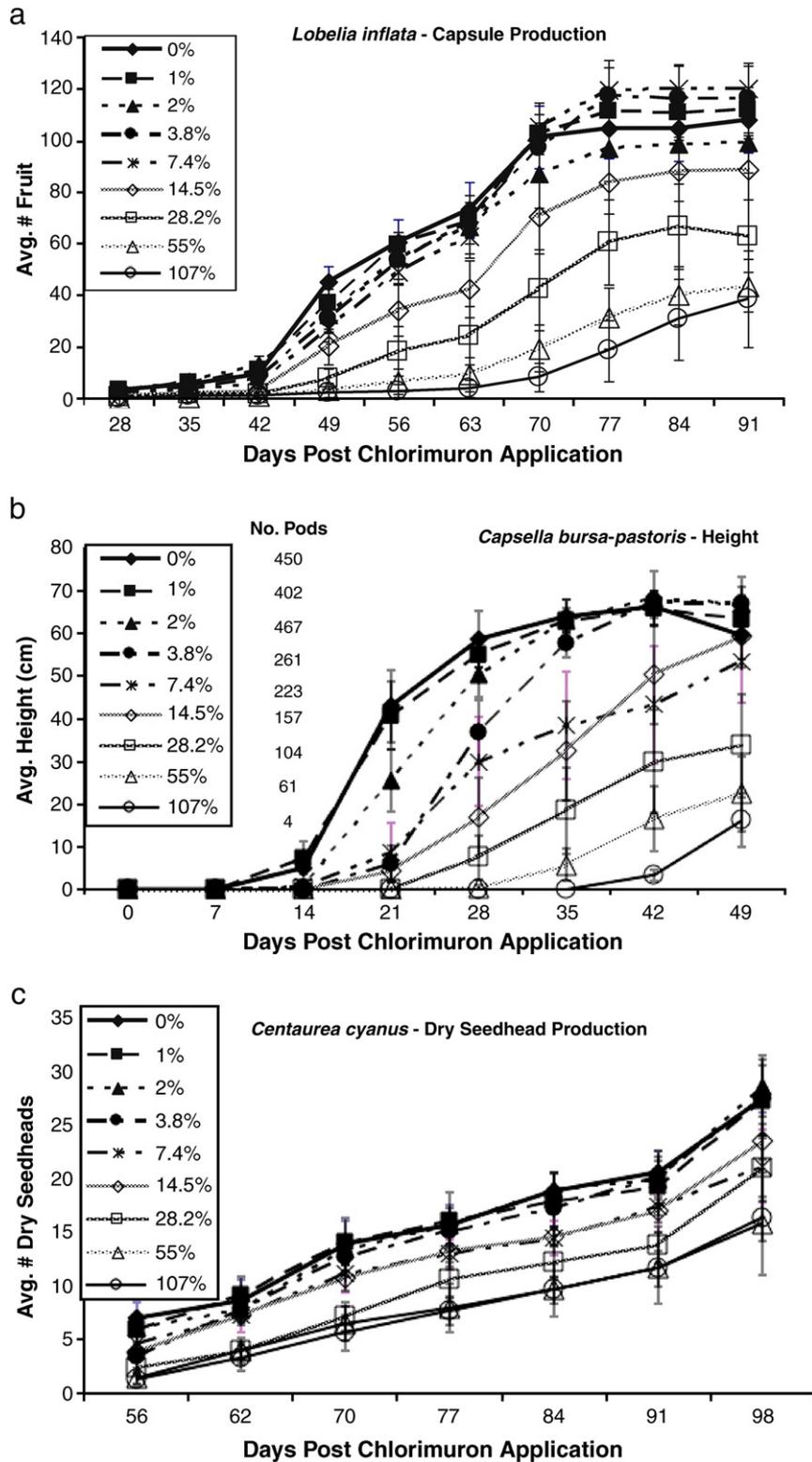| Species tested	Short-term biomass IC25 (g ai ha^{-1})	Long-term biomass IC25 (g ai ha^{-1})	Reproductive output IC25 (g ai ha^{-1})	Effects over time
Lobelia inflata L.	0.342	1.305	1.305	Some recovery over time
Capsella bursa-pastoris (L.) Medik.	0.675	0.242	0.285	Decline over time
Centaurea cyanus L.	1.51	No effect	2.51	Recovery of vegetative parts but not of reproduction

Fig. 3. Measures of reproductive outputs collected during the course of phytotoxicity tests conducted with the herbicide chlorimuron ethyl. Plants were harvested at the beginning of natural senescence of the controls. Doses span 1% to 107% of the label recommended rate of 9.0 g active ingredient per hectare (i.e. 100% dose). a) Capsule production of *L. inflata*, b) height and pod production of *C. bursa-pastoris* and c) seed head production of *C. cyanus*. Averages and standard error bars are shown.

Life cycle tests may be the obvious means of measuring effects over the entire lifespan of plants. Experimental work has been reported for a few species although for practical reasons only short-lived species have been studied, including *Arabidopsis thaliana* (L.) Heynh. and *Brassica rapa* L., greatly limiting the value of this approach (Ratsch et al., 1986; Shimabuku et al., 1991; Zwerger and Pestemer, 2000). It

has been demonstrated that morphological deformations of the flowering parts in *A. thaliana* induced by some herbicides prevented pollination and seed production in this species (Ratsch et al., 1986). Resource partitioning amongst vegetative and reproductive parts was greatly altered and in many cases, the seed output was reduced whilst no effect was observed on leaf and stem development. Likewise, in

B. rapa, higher sensitivity to the herbicide dalapon (2,2-dichloropro-pionic acid) was observed at the reproductive stage thus emphasising the importance of measuring several endpoints of ecological significance (Shimabuku et al., 1991). In sharp contrast, greater negative effects were observed on the vegetative parts than on the reproductive parts on four plant species tested at the 2–4 leaf stage with three different herbicides (Zwerger and Pestemer, 2000).

The impacts of herbicides on terrestrial primary producers can be very subtle and difficult to detect. Clearly, changes in the local flora have been detected in non-target habitats (Boutin and Jobin, 1998) that would not have been predicted with current simplistic phytotoxicity tests. Impacts on biodiversity of plant communities and on ecosystem services cannot be assessed using the current testing scheme (Table 1).

3.6. Effects of biotic and abiotic factors

Single-species tests are conducted under ideal greenhouse conditions and results are used to assess effects on plant communities growing outdoors where a multitude of stressors may be present. Contradictory results have emerged from the few studies that have been performed comparing greenhouse and field experiments. Effects of herbicides in the field have been shown to be reduced when compared to plants grown under greenhouse conditions (Garrod, 1989). This has been attributed to a multitude of confounding variables, including environmental factors (e.g., wind, temperature, rainfall conditions), plant anatomy (e.g., cuticle thickness), and the physiological states of the plant (e.g., more active growth in the greenhouse) (Clark et al., 2004). In contrast, Fletcher et al. (1990) reached the opposite conclusion from their literature review, showing that plants were more sensitive under field conditions. Kleijn and Snoeijing (1997) did not find a good correspondence between field and greenhouse experiments when testing 18 forb and grass species with the herbicide fluoxypyr (1-methylheptyl ester).

Additional interacting factors such as high levels of fertilisers or the presence of phytophagous insects and diseases are also common under field conditions but uncommon and controlled under greenhouse conditions. It has been suggested that when plants are stressed, weakened, or injured by toxicants, air pollution or predators, they are likely to succumb to disease or be out-competed by more tolerant species, thus further confounding field responses (Wang and Freemark, 1995).

3.7. Effects on communities and ecosystems

Plants tested in the greenhouse are at uniform growth stages when sprayed and may represent a worst-case scenario. In the field, species and even individuals within a species are often at markedly different growth stages and hence may differ in susceptibility; this introduces variability into the results that makes interpretation difficult. Furthermore, studies with a large number of species are complex because of the normal heterogeneity of natural sites. This problem may be circumvented by using microcosms (several species growing in pots) or mesocosms (small experimental plots). Studies involving only a few plant species provide a means to investigate interactions amongst species whilst attempting to avoid the inherent difficulties of natural sites with elaborate plant communities (Davies and Blackman, 1989; Marrs and Frost, 1997). Dalton and Boutin (2010) initiated a study aimed at comparing single-species tests with the same species growing within microcosms placed both in the greenhouse and outdoors. For terrestrial species, single-species tests were usually not the most sensitive when compared to plants growing in greenhouse microcosms indicating that they did not represent the worst-case scenario. There was more variability with the wetland species but in some cases, single species tests underestimated sensitivity. When comparing greenhouse and outdoor microcosms with the same plant

species, it was shown that although outdoor plants were generally less sensitive than plants grown in the greenhouse, the latter did not capture the large variability encompassed in microcosms subjected to semi-natural outdoor conditions (Dalton and Boutin, 2010). The obvious conclusion reached was that for an accurate risk assessment, the experimental design should be able to accommodate this natural variability (Cousens et al., 1988). Riemens et al. (2008, 2009) found that plants growing in the greenhouse were more sensitive than those growing outdoors but these results could not be extrapolated to species growing in mixtures.

There have been limited attempts to study the effects of herbicides on whole communities or ecosystem levels through the food web. Biodiversity in agrarian systems has been reduced by herbicide use as well as other stressors, including fertiliser input (Marrs et al., 1989; Kleijn and Snoeijing, 1997). Past studies have demonstrated that biodiversity has positive effects on ecosystem processes and functioning (Balvanera et al., 2006). In a very elaborate experiment, Scherber et al. (2010) recently demonstrated that changes in terrestrial grassland plants negatively affected herbivores and pollinators, with cascading effects on other trophic levels. Interestingly, decreasing biodiversity enhanced the negative effects of invasive plants.

Plant responses to agricultural intensification have repercussions on other trophic levels and may be more pronounced in simplified ecosystems. A suite of very comprehensive studies was conducted in Britain demonstrating that herbicide drift (and to a lesser extent insecticide use) was found responsible for the decline of the grey partridge (*Perdix perdix* L.) (Potts, 1970; Rands, 1986; Sotherton, 1990). It was established that floral diversity was greatly reduced in field margins that make up the primary habitats for this bird species. A decline in plant diversity led to a reduction in arthropods on which the chicks (a precocial species) relied upon during the first 3–4 weeks of their lives. Birds that had greater distances to travel in search of their food were more prone to predation and had to rely heavily on aphids (*Aphidoidea sp.*) as a food source (Green, 1984). Over the course of 30 years, the grey partridge population had declined by 80%. The mediated impact of herbicides through the food web is well illustrated in this example. The crash in preferred food was partly compensated by aphids, which were the most abundant insect remaining that could fill the niche left by the decline in sawfly (*Symphyta sp.*), some coleopteran and ant diversity. Unfortunately, aphids are very sensitive to low temperatures and chicks were left with a shortage of food supply during cold springs.

The impact of disturbances (or herbicide use in this case) illustrates well the importance of maintaining high biodiversity in order to ensure the resistance and recovery of ecosystems (Elmqvist et al., 2003) and the maintenance of ecosystem services. The loss of some species in a diverse ecosystem appears largely compensated because of existing ecological redundancy due to species having similar effects on some ecosystem processes or similar responses to environmental conditions (Hooper et al., 2005). Conversely, in a low diversity system, the loss of key species may threaten the functioning of ecosystem processes. In landscapes where intensive agriculture prevails, field margins tend to become simplified and dominated by grasses because herbicides and fertilisers tend to suppress broad-leaved species. Grasses are wind pollinated and, as a consequence, pollinators are reduced or eliminated. A complete ecosystem function becomes marginal in these habitats with possible consequences on crop pollination.

The use of statistical techniques may be a way to improve risk assessments and extrapolation to community level testing. The species-sensitivity distribution (SSD) method is gaining increasing popularity in ecological risk assessments (Newman et al., 2000; Baird and Van den Brink, 2007). The SSD method is based on data obtained from single-species tests used to calculate an IC25 or IC50 corresponding to the 25% or 50% inhibition concentration of a substance.

Since ecotoxicologists are concerned with the protection of organisms at the population or community level, single-species test data are combined and the various species are fitted into a distribution used to calculate a hazardous concentration at which a certain percentage of all species is assumed to be affected (Aldenberg and Slob, 1993). This method presupposes that species' sensitivities to a toxicant approximate a log-logistic or a log-normal distribution, so that a restricted number of species can represent the universe of species sensitivities to a toxicant. Some uncertainty is incorporated into the calculation of the parameters of the distribution that are determined for each herbicide because it is logistically impossible to test all species. This is set as a function of the sample size (Aldenberg and Slob, 1993). Therefore, as the number of species tested increases, the error associated with the calculation of the parameters decreases.

There are legitimate concerns associated with the use of the SSD method and the underlying dose-response data on which it heavily relies. First, there are uncertainties associated with a single measure of IC25 or IC50 for any given crop or wild species (White and Boutin, 2007; Olszyk et al., 2009; Boutin et al., 2010). Second, it has long been noted that there are significant deficiencies in correlating these single-species data to predict the effects at a population or community level (see above). Therefore, any deficiencies, uncertainties or errors in the data will be directly transferred to the hazardous concentration. Furthermore, many data sets deviate from the expected log-normal or log-logistic distribution and may not be appropriate for modelling into specific distributions (Newman et al., 2000).

Another concern is that the data set is usually biased due to the subset of test species traditionally used (usually crop species that are easy to manipulate in a greenhouse setting (Table 1)). Moreover, it is assumed that the loss of species is equal at the community level. This assumption does not hold since the loss of a dominant, rare or keystone species has particular implications at the ecosystem level. Additionally, a fairly large sample size is generally needed for the SSD method. Newman et al. (2000) suggests that an optimal sample size for this method ranges from 15 to 55 species, which is much greater than the six to ten species recommended in herbicide regulatory guidelines. Finally, there is a paucity of field data showing that the SSD method actually provides evidence that organisms are adequately protected.

4. Conclusions

Ecological risk assessment for terrestrial primary producers is based on toxicity data obtained in single species tests. This scheme has been criticised in terms of the number and types of species assessed as well as in the very narrow set of experimental conditions explored. Ways to improve phytotoxicity tests and risk assessments have been proposed, but have been so far inconclusive for numerous reasons. First, difficulties arise when attempting to extrapolate from simple tests to community level assessments. Secondly, more complex tests such as microcosm or mesocosm studies are time-consuming, costly and have not been standardised for terrestrial primary producers. Likewise, the use of ecologically relevant species depending on the geographical area of concern is an alternative technique that needs to be validated for plants and other organisms. Lastly, the trait-based approach which is emerging as a promising avenue to improve species selection and risk assessment requires refinements to prove its usefulness with regards to terrestrial primary producers.

Much progress has been achieved since phytotoxicity tests were designed in the early 1980s. Current phytotoxicity tests are useful to inform regulators and conservationists on the inherent toxicity of a given herbicide to a narrow range of species (usually crops) grown under standard laboratory conditions. Unfortunately, risk assessment tools are limited by the types of tests presently provided and lack the means to assess and extrapolate effects on wild species diversity, biodiversity, ecosystem services and functions.

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