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Effects of herbicides on non-target plants: How do effects in standard plant test relate to effects in natural habitats?

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Title:

Effects of herbicides on non-target plants: How do effects in standard plant test relate to effects in natural habitats?

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Preface

This report presents the results of the three year project: ”Effects of herbicides on non-target plants: how do effects in standard plant tests relate to effects in natural habitats?”. The project was carried out in order to investigate the effects of herbicides on plants found in natural and semi-natural habitats within the agricultural land such as hedgerows, field borders and other small biotopes and to evaluate whether the current risk assessment represents an adequate safeguard for environmental protection of these species and habitats. The project has been carried out by Aarhus University, National Environmental Research Institute (Dept. of Terrestrial Ecology, Dept. of Atmospheric Envirnonment and Dept. of Freshwater Ecology) and Aarhus University, Faculty of Agricultural Sciences (Dept. of Integrated Pest Management). The project was financed by the Pesticide Research Programme, Danish Ministry of Environment.

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Summary

The main aim of the present project was to investigate the effects of herbicides on non-target plants occurring in natural and semi-natural habitats within the agricultural land such as hedgerows, field borders and other small biotopes and to evaluate whether the current risk assessment represents an adequate safeguard for environmental protection of these species and habitats. We use the term non-target plants in accordance with the formulation in the EU Guidance Documents for Ecotoxicology i.e. plants that are unintentionally exposed to herbicides.

When performing herbicide risk assessment in accordance with the EU Directive no. 91/414/EEC, effects are assessed on crop plants and data on effects on non-target plants are not requested. As a consequence, data on effects of herbicides on non-target plants are limited. We, therefore, both wanted to increase the general knowledge on herbicide effects on non-target plants and to assess the credibility of existing data on herbicide effects on crop plants for effects on non-target species. Furthermore, we put specific focus on a number of conditions of standard tests that differ from the conditions in natural and semi-natural habitats in order to evaluate the influence of these factors on the results – and finally on the conclusions drawn from such test regarding how well-protected non-target plants and their habitats are by the current risk assessment.

The investigations

The project included five main parts: i) analyses of existing toxicity data on terrestrial plants; ii) dose response experiments with both crops and non-target species; iii) exposure experiments in spraying chamber and agricultural field; iv) assessment of community effects of herbicides and nitrogen on experimentally established vegetation; and v) modelling of plant competition.

The project has been carried out by Aarhus University, National Environmental Research Institute (Dept. of Terrestrial Ecology, Dept. of Atmospheric Envirnonment and Dept. of Freshwater Ecology) and Aarhus University, Faculty of Agricultural Sciences (Dept. of Integrated Pest Management).

Main conclusions

We found several areas where risk assessment today is insufficient for protection of non-target species and their habitats. Our results show that conclusions based on biomass measurements are not always valid for effects on seed production. Exposure of different functional stages of the test plants should be taken into consideration as well as selection of end-point relative to time of exposure, and effect of species interactions need to be integrated in the risk assessment.

The most extensive conclusion of the present investigation is that seed production seems to be a more sensitive end-point for risk assessment of herbicides than biomass independently of plant species, life-span of the plant

(annual, biennial, perennial) and functional stage at the time of exposure (early vegetative stage and during flowering). Today effects on seed production are not a commonly used end-point for risk assessment. Presumably, risk assessment based on biomass and visual effects underestimates the sensitivity of non-target plants.

We showed that the crop species we tested, in general, were not less sensitive to herbicides (glyphosate, metsulfuron-methyl and mecoprop-P) than non-target species when dose-response experiments were run under the same conditions. Sensitivity was more dependent on the efficacy spectrum of the herbicide and whether the test species was a monocot or a dicot species. Furthermore, our results indicate that variation in test conditions may be more important for the previously observed differences in sensitivity of crops and non-target species than whether it is a crop or a non-target-species. Previous analyses are based mainly on toxicity data found in databases (PHYTOTOX and ECOTOX). Today documentation of test conditions and end-points are normally lacking in the databases. The consequence may be that wrong or misleading conclusions on species sensitivity may be drawn if these informations are not available. We therefore recommend that information concerning test conditions is included in the databases.

Finally, the results indicated that interactions between species with different sensitivity to glyphosate and different responses to nitrogen are important for species composition of experimental plots mimicking natural and semi-natural habitats exposed to these agrochemicals. Glyphosate dosages representative for spray drift resulted in decreased biodiversity and changed species composition. At present interactions between species and between herbicides and fertilizers are not part of the risk assessment even though it may render some species more sensitive to common agricultural practice than expected based on data from standard plant tests.

Sammenfatning

Det overordnede mål med projektet har været dels at undersøge effekter af herbicider på planter, der vokser i naturlige og semi-naturlige habitater i agerlandet som f.eks. levende hegn, markkanter og andre småbiotoper, dels at vurdere om risikovurderingen i sin nuværende form yder en tilstrækkelig beskyttelse af disse planter og deres habitater.

I forbindelse med risikovurderingen af herbicider, der gennemføres i følge EU Direktiv no. 91/414/EEC, undersøges effekten af herbicider på udvalgte afgrødearter. Det er således ikke et krav at levere data, for effekten af herbicider på vilde plantearter, i denne sammenhæng ofte betegnet non-target planter. Som en konsekvens af dette er data for disse planter meget begrænset. Formålet med nærværende projekt var derfor at bidrage til en øget viden om effekten af herbicider på non-target planter samt at vurdere værdien af de eksisterende data for herbicideffekter på afgrødearter i forhold til, hvor dækkende de er for effekter på non-target arterne. I projektet har vi specielt fokus på en række forhold, hvor standard plante-test adskiller sig fra betingelserne for planter i naturlige og semi-naturlige habitater.

Undersøgelserne

Projektet er inddelt i fem hovedemner: i) analyse af eksisterende toksicitets data for terrestriske planter, ii) dosis-respons forsøg med afgrøder og non-target planter, iii) eksponeringsforsøg i sprøjtekammer og mark, iv) eksperimentelle undersøgelser af effekter af herbicider og kvælstof på plantesamfund og v) modellering af plantekonkurrence.

Undersøgelserne er gennemført ved Aarhus Universitet, DMU, Afdeling for Terrestrisk Økologi, i samarbejde med to afdelinger ved DMU (Afd. for Atmosfærisk Miljø og Afd. for Ferskvandsøkologi) samt Det Jordbrugsvidenskabelige Fakultet, Afdeling for Integreret Plantebeskyttelse.

Hovedkonklusioner

Vores undersøgelser indikerer, at risikovurderingen af herbicider, som den gennemføres i dag, er utilstrækkelig på en række punkter, og dermed ikke garanterer den tilstrækkelige beskyttelse af non-target planter og deres habitater. Disse punkter inkluderer valg af effektparameter (end-points) i risikovurderingen, valg af timing for eksponeringen i forhold til relevante funktionelle vækststadier, valg af end-point sammenholdt med eksponeringstidspunkt samt betydningen af samspillet mellem planter og betydningen af samspillet mellem herbicider og kvælstof.

Den mest betydningsfulde konklusion i undersøgelsen er, at frøproduktion synes at være en mere følsom effektparameter for risikovurderingen af herbicider end biomasse uafhængigt af planteart, plantens livslængde (enårig, to-årig eller flerårig) og vækststadie på eksponeringstidspunktet (tidlig vegetativt stadium eller reproduktivt stadium). I dag benyttes effekten på frøproduktionen kun yderst sjældent som effektparameter ved risikovurderingen. Risikovurdering, der er baseret på effekter på biomassen

eller visuelle effekter, som er det normale, underestimerer sandsynligvis følsomheden af non-target planter.

Vi fandt, at de afgrødearter, vi testede, generelt ikke var mindre følsomme overfor herbicider (glyphosat, metsulfuron-methyl og mecroprop-P) end non-target planter, når dosis-respons forsøgene gennemføres under de samme betingelser. Følsomheden var mere afhængig af herbicidets effektivitetsspektrum, og hvorvidt testplanten var en-kimbladet eller to-kimbladet. Desuden indikerer vores resultater, at variationen i testbetingelserne er mere afgørende for de tidligere fundne forskelle i følsomhed hos afgrøder og non-target planter, end hvilken af disse grupper testplanten tilhører. De fundne forskelle har overvejende været baseret på toksisitetsdata fra databaser (PHYTOTOX og ECOTOX). I dag mangler oplysninger vedr. testbetingelserne og end-points sædvanligvis i databaserne. Konsekvensen af dette er, at urigtige og fejlagtige konklusioner vedrørende planters herbicidfølsomhed derved kan blive truffet. Vi anbefaler derfor, at informationer vedrørende testbetingelser og end-points inkluderes i databaserne.

Resultater fra det eksperimentelle forsøgplot, Kalø-plottet, indikerede, at interaktioner mellem planter med forskellig følsomhed overfor glyphosat og forskellig respons på kvælstof er vigtige for planternes konkurrenceevne og dermed for artssammensætningen i eksperimentelle plot, der efterligner eksponeringen af naturlige og semi-naturlige habitater over herbicider og kvælstof. Glyphosat doser, der svarer til dem planter kan blive udsat for via afdrift resulterer i nedgang i biodiversiteten og en ændret artssammensætning. Betydningen af interaktioner mellem arter og mellem herbicider og kvælstof indgår ikke ved risikovurderingen af herbicider på trods af, at de kan bevirke, at visse plantearter er mere følsomme overfor almindelig landbrugspraksis end antaget på baggrund af data fra standard plantetest.

1 Introduction

1.1 Aim

The main aim of the project is to investigate the effects of herbicides on non-target plants growing in semi-natural and natural habitats within the agricultural land such as hedgerows, field borders and other small biotopes and to evaluate whether the current risk assessment represents an adequate safeguard for environmental protection of these species and habitats.

The project focuses on three issues:

- the sensitivity of species growing in natural and seminatural habitats compared to sensitivity of annual target weeds and crops used in standard tests
- the relationship between the way the herbicides are applied and the effects on plants, and
- the combined effect of herbicides and fertilizers, specifically nitrogen, on species interactions and plant community composition.

1.2 Rationale

In agricultural landscapes, which are predominant in Europe, natural and semi-natural habitats are intermingled with agricultural fields, and natural habitats form “islands” in between the agricultural fields. In Denmark, the agricultural area covers about 60 % of the land. The natural and semi-natural habitats are, therefore, to a varying extent affected by the agricultural practice.

Biodiversity within agricultural areas including plants as well as most other groups of living organisms is declining in Denmark as well as in the rest of Europe (Green, 1990; Fuller et al., 1995; Andreasen et al., 1996; Rich and Woodruff, 1996; Chamberlain et al., 2000; Donald et al., 2000; Atkinson et al., 2002; Benton et al., 2002; Strandberg & Krogh 2011). A number of factors, often summarized as the intensification of the agricultural practice, are made responsible for the decline. Monitoring has pointed at herbicide spray drift as a major factor affecting both flora and fauna of field boundaries and hedgerows (e.g. Aude et al. 2003, Bruus Pedersen et al. 2004, Petersen et al. 2006, Bhatti et al. 1995) and application of fertilizers and pesticide usage are regarded to play an important role in the decline of species richness. However, studies of the combined effect of fertilizers and herbicide drift on non-target vegetation are virtually non-existent.

Indeed, there is a political commitment to halt biodiversity loss within the EU by 2010. The Habitat Directive (Council Directive 92/43/EEC of 21 May 1992) obligates EU Member States to ensure biodiversity through the conservation of habitats covered by the Directive. Specifically, Article 10 in the Directive emphasizes the importance of improving ecological coherence of these habitats through protecting landscape features, such as field boundaries, that are essential for migration, dispersal and genetic exchange of wild species. Moreover, the European Environmental Agency reports no progress towards

the 2010 target of halting biodiversity loss in agricultural areas (EEA 2009) and national data underpins this (Strandberg & Krogh 2011).

Before a new herbicide is approved for placement on the market, it needs to be evaluated in accordance with The Plant Protection Products Directive (Council Directive 91/414/EEC 15 July 1991). According to this Directive and the Annexes II and III, there are no specific data requirements for effects on non-target plants although the effects of herbicides, in particular, are considered to be critical for such plants. However, the data requirements and testing of effects on non-target terrestrial plants are under revision, see chapter 1. Therefore, there is an urgent need to know to what extent the vegetation of natural and semi-natural habitats is protected by the current risk assessment and if and how the assessment should be improved for an adequate protection of these habitats.

The way that natural and semi-natural plant communities become exposed to herbicides differs in many aspects from the conditions and way standard plant tests are carried out. This project aims to evaluate the importance of these differences for the protection of natural habitats on the following points:

1. The standardised plant test is normally performed with a number of annual crop species. In natural and semi-natural habitats, perennial species normally dominate, although a number of annual and biennial species may also be found.
2. In the standardised plant test, the plant is exposed to varying dosages of one herbicide. Plants in natural habitats are repeatedly exposed to sublethal dosages of a number of herbicides which have the same or different modes of action.
3. In the standardised plant test, the plants are exposed to the herbicide at an early growth stage, assuming that this stage is the most sensitive. In natural habitats, the plant species may vary in both age and functional stage at the time of exposure.
4. In the standardised plant test, the plant is directly exposed to the herbicide in the spray chamber. In natural habitats, the plant is exposed to spray droplets that might have changed in size and concentration during drift as well as evaporated herbicides originating from the spraydrift and/or field.
5. In the standardised plant test, each species is grown under optimal conditions apart from the herbicide exposure. In natural habitats, the plants are affected by many biotic and abiotic factors such as competition, herbivory, and water-, nutrient- and temperature stress that may interact with the herbicide.

In order to achieve the main aim and to determine whether standard plant test data are representative for effects on plants in natural and semi-natural habitats, the importance of the above mentioned differences have been studied by testing the following null hypotheses:

1. The sensitivity of non-target plants to herbicides measured as survival and biomass does not vary significantly from the sensitivity of crop species.
2. Selection of different end-points does not influence the outcome of the risk assessment of herbicides.
3. The effects of repeated herbicide exposure to sublethal dosages of herbicides with different modes of action do not differ from the Additive Dose Model (ADM).

4. Differences in droplet size and in herbicide concentration within droplets of spray drift and drops produced in spray chambers do not result in any differences in the effects observed on plants at a given herbicide dosage.
5. The interactions within natural habitats and the inherent complexity do not have any effects on the responses to herbicide spray drift quantified as the ecological success, i.e. biomass and reproductive allocation of the plants growing in these habitats.
6. Effects of nitrogen fertilizers do not interact with effects of herbicide spray drift in natural and semi-natural habitats.

1.3 Structure of the project related to issuses and null hypotheses

The project was a three year project (2007-2009) and consists of five main parts:

Part 1: analyses of existing toxicity data on terrestrial plants
Part 2: dose-response experiments with both crop species and non-target species
Part 3: exposure experiments in spray chamber and agricultural field
Part 4: assessment of community effects of herbicides and nitrogen on experimentally established vegetation
Part 5: modelling of plant competition

2 Background

This section presents the background for the three issuses (see p. 11) investigated in the present project.

2.1 Species sensitivity to herbicides

The herbicide sensitivity of plant species belonging to 'the natural' flora is largely unknown. In natural and semi-natural habitats, perennial plant species are dominant, but a number of annual and biennial species do occur. Therefore, the vegetation in these habitats has a large variation with respect to species composition, growth stages and plant size. The sensitivity of plants to herbicides varies between species and therefore, in general, plants present in natural habitats are expected to differ in sensitivity compaired to the annual species used in standadised plant test.

Fletcher et al. (1985) analysed the PHYTOTOX-database which was established in 1984. The aim was to assess whether any one crop species recommended by US-EPA and OECD as test species was more sensitive to herbicides than non-target species in general. Unfortunately, the amount of data present at that time was so scattered that a toxicological comparison could only be done for 6 crop species, i.e. oat, wheat, corn, sorghum, cucumber and soybean. One important conclusion drawn from the work was that a plant species that is sensitive to one class of herbicides may not be sensitive to another, suggesting that it may be necessary to test a wide variety of species to assess the risk. In 1990, Fletcher et al. (1990) made a new analysis of the PHYTOTOX database. Now the number of records was tripled. In this study, they assessed the importance of taxonomic differences for plant sensitivity to chemical treatment. They found that the more closely two plant species are related, the more similar their response is to chemical exposure. Summarizing the conclusions of the analysis of the databases, Fletcher et al. (1985 and 1990) found that:

- The data in the database (PHYTOTOX) is heavily biased towards northern-temperate agricultural species
- The difference in sensitivity between the least and the most sensitive species varied considerably among chemical groups. The largest span was found for the pesticide Picloram with a 316 times difference between the highest and lowest EC-value, whereas the difference was only 3.5 times for Linuron.
- The sensitivity of plant species is strongly correlated to taxonomic classification, which means that the more closely two plant species are related, the more similar is their response to chemical exposure.

The finding of Fletcher et al. (1990), that the more closely two plant species are related, the more similar their response is to chemical exposure, was supported by the findings of Boutin and Rogers (2000), who examined the sensitivity of plant species to various herbicides. Boutin and Rogers found that crop species were not consistently more or less sensitive to herbicides than non-crop species. Finally, based on dose–response experiments with 15 non-crop species exposed to 6 herbicides of different modes of action, Boutin et al. (2004) found that the non-crop species, in general, were more sensitive than

the crop species tested for regulatory purpose of US-EPA, and they questioned the safe-guard of the risk assessment procedure to non-target vegetation.

Except for the test by Boutin and collaborating Danish scientists (Boutin et al. 2004), only one dose-response experiment (McKelvey et al. 2002) among the very few experiments with non-target plants (Holst et al. 2008, Strandberg et al. 2006b, McKelvey et al. 2002, Asman et al. 2001, Marrs et al. 1993,) has focussed on comparison of sensitivity of crops and non-target species. In contrast to Boutin et al., McKelvey and coworkers found that crop species sensitivity to 11 selected herbicides was representative for the response of non-crop species. Among the eleven herbicides tested, only metsulfuron-methyl is in use in Denmark.

New herbicides are routinely tested in field experiments to establish their activity on weeds and the majority of the trials are conducted by the agrochemical companies. However, over the years the Institute of Integrated Pest Management, Aarhus University, has also conducted a large number of efficacy trials. These results offer an alternative source of information on the sensitivity of wild plants (weeds) to herbicides and the variability between and within plant families.

2.2 Relationship between way of herbicide exposure and effects on plants

While plants in the standardised plant tests are exposed to varying dosages of one herbicide within a spraying chamber, plants found in natural and semi-natural habitats within the agricultural land may be exposed to repeated sublethal dosages of different herbicides or pesticide mixtures through spray drift and deposition (Siebers et al. 2003, Carlsen et al. 2006a, b, Bruus et al. 2008). The exposure to spray drift might also be affected by the change in droplet size during drift due to evaporation. Depending on the pesticide, the evaporation may increase the concentration within the droplets, leading to a stronger dosage in a smaller droplet. Further, the plant species nearby the target fields may vary in both age and functional stage at the time of exposure from spray drift and/or evaporated herbicide from the fields.

2.2.1 Dose-response: influence of longevity, growth stage and selection of end- point

In natural and semi-natural habitats, the plant species may vary in both age and functional stage at the time of exposure. In standard plant tests and most other studies as well the plants are exposed to the herbicide at an early vegetative stage. These test and studies also mainly focus on effects on plant biomass. The very few current studies with exposure of plants to herbicides during the reproductive stage have shown that the reproductive structures, i.e. flowers, pollen and fruits/seeds, seem to be particularly sensitive (Blackburn & Boutin 2003, Felsot et al 1996, Bhatti et al. 1995, Marrs et al. 1993, Christensen 2008).

2.2.2 Exposure to repeated sublethal dosages

The herbicides that plants are exposed to through spray drift may have the same or different modes of action. In previous studies on joint-action of herbicides, additive, antagonistic as well as synergistic interactions have been observed for binary mixtures of herbicides (Hatzios and Penner 1985).

Several reports have suggested the effects of a single application of a mixture of herbicides with similar mode of action to be additive (Cedergreen et al. 2007, Kudsk & Mathiassen 2005) while mixtures of herbicides with different modes of action often show antagonism (Cedergreen et al. 2007). Mathiassen et al. (2007) reported the effect of repeated applications of graminicides to be additive as long as the time period between applications was no longer than 14 days.

2.2.3 Droplet size and concentration of pesticide

A major criticism in the application of standard plant tests for prediction of effects of pesticides on plants within natural habitats is the differences in droplet size and concentration of pesticide within the droplets occurring when exposed to spray drift in the field and when exposed to the pesticide within the spraying chamber. The small droplets within the spray mist drift longer than the larger droplets, i.e. the droplets that reach the habitats outside the field, generally, are smaller than the droplets deposited within the field (e.g. Elliot & Wilson 1983, Hewitt et al., 2002, Bruus et al. 2008). Small droplets have a large surface relative to volume. For non-volatile pesticides this results in fast evaporation of water/solvent from the droplets and thus in higher pesticide concentrations within the droplets when they reach the habitats outside the field. Therefore, substantial differences in pesticide concentrations may be found with droplet size.

Based on previous studies of the importance of droplet size and pesticide concentration within the droplets for effects on plants, it is not possible to conclude whether the way of exposure, i.e. exposure in the habitats or in spraying chamber, significantly affects the outcome of the risk assessment (e.g. Hall 1997, Knoche 1994, Prasad & Cadogan 1992, Kudsk & Mathiassen 1999, Jensen 1999).

2.3 Herbicide and fertilizer interactions on species and habitats

While it is obvious that plants may be affected by herbicides at recommended application rates, little is known about impacts of lower concentrations of herbicide resulting from spray drift. Marrs et al. (1989, 1991) found that spray drift resulted in sublethal, but significant effects, such as plant damage and flower suppression on single plant species and argue that spray drift may have long-term impacts on plant community structure. Recent experiments have demonstrated that evaporated herbicides have the potential to affect plants significantly (Jensen 2006) and that low dosages of herbicides both reduced the number of species and affected the species composition (Holst et al. 2008).

In addition to pesticide exposure, plants in natural and semi-natural habitats close to the fields repeatedly receive fertilizers. Studies of the combined effect of fertilizers and herbicide drift on non-target vegetation are extremely scarce. We were only able to find three studies on effects of combined additions of glyphosate and nitrogen: one on exposure of woodland plants (Gove et al. 2007) and two on exposure of experimentally established vegetation mimicking effects on field margins (Perry et al. 1996, Bruus et al 2004). All the studies show significant effects of glyphosate concentrations equivalent to those measured in spray drift (1-25 % of full application rate) and the responses of the vegetation was affected by application of fertilizer. Gove et al. found increased mortality, reduced biomass and reduced fecundity in all six species tested, both in greenhouse experiments, where plants were grown

separately and exposed when they were six weeks old, and when transplanted into plots in woodland margins. Perry and co-workers, who only reported first year results, found that although the individual species (three monocots and three dicots) responded differently to the treatments, both fertilizer and glyphosate affected the community significantly. Concurrently, Bruus et al. found species dependent responses to glyphosate, and they showed interactions between nitrogen and glyphosate on species richness and total biomass. In addition, efficacy studies of herbicides indicate that the herbicide sensitivity of the different weed species was influenced by N level (Cathcart, Chandler & Swanson 2004).

Nitrogen addition experiments showed both no effects on botanical composition (Boatman et al. 1994, Theaker et al. 1995) and reduced species richness and changes of ecosystem composition and functioning (Clark and Tilman 2008, Gough et al. 2000, Bobbink et al. 1998, Mountford et al. 1993). The observed differences may be caused by differences in the pre-addition nitrogen level of the community. The studies by Boatman, Theaker and Froud-Williams were conducted on hedge bank vegetation, whereas the other studies were on grassland communities. Within grasslands, significant effects were found following nitrogen addition at rates of 25 kg ha-1 yr-1 or more and chronically added even lower rates had significant and negative effects on species richness (Clark & Tilman 2008).

2.3.1 Modelling of competitive interactions

Inter-specific competition is known to influence the composition of natural plant communities (e.g. Gotelli and McCabe 2002, Silvertown et al. 1999, Weiher et al. 1998), although the relative importance of inter-specific competition as a regulating factor in natural plant community dynamics has been a point of discussion (Hubbell 2001, Shmida and Ellner 1984). The role of inter-specific competitive interactions in natural plant communities has mainly been investigated by indirect methods comparing different plant communities at different points in time (Bakker et al. 1996, Barclay-Estrup 1970, Barclay-Estrup & Gimingham 1969), or by testing whether the species composition deviates from a specified null-model, where species are assumed to be independent of each other (Conner and Simberloff 1979, Wilson et al. 1996, Hubbell 2001, Gotelli and McCabe 2002).

In order to investigate the role of competitive interactions between different plant species in natural plant communities more directly, we need to investigate plant ecological data that measure important components of the ecological success of plant species and their role in competition. Historically, plant competition has been studied for annual plants, e.g. crops or weed plants, by expressing yield, biomass or fecundity of individual plants as a function of plant density (Bleasdale and Nelder 1960, Firbank and Watkinson 1985, Law and Watkinson 1987, Pacala and Silander 1990, Rees et al. 1996, Damgaard 1998, 2004). Such density-dependent competition models are less relevant for natural plant communities dominated by perennial plants, where it is often difficult to distinguish individual plants, and in the cases where individual plants can be counted, they almost always vary markedly in size so that the number of individuals is of limited value for describing the amount of competition taking place. However, the use of non-manipulative techniques in order to estimate competitive effects directly in natural plant communities is a topic of rising interest (Freckleton and Watkinson 2001), and several methods have been suggested for different types of natural herbal plant communities and ecological measures (e.g. Rees et al. 1996, Law et al. 1997, Roxburgh and

Wilson 2000b, a, Turnbull et al. 2004). For example, Rees and co-workers (1996) used the counts of annual plants in thousands of small quadrates, and Turnbull and co-workers (2004) made a neighbourhood analysis of individual plants of the same dune population of annual species, whereas Law and co-workers (1997) in a pioneering study estimated competition coefficients from spatial turnover data of four perennial grass species.

3 Material and methods

The material and method section follows the five main parts of the project:

1) Analyses of existing toxicity data on terrestrial plants (Chap. 3.1)
2) Dose-response experiments with both crops and non-target species (Chap. 3.2)
3) Exposure experiments in spraying chamber and agricultural field (Chap. 3.3)
4) Assessment of community effects of herbicides and nitrogen on experimentally established vegetation (Chap. 3.4)
5) Modelling of plant competition (Chap. 3.5).

The projects are cross-linked as e.g. the selection of plants for the experiments affects some of the dose-response experiments as well as the field experiment. The dose-response experiments are divided in three groups: dose-response of selected crop species and two non-crop species (the latter compared to earlier published results), dose-response of three pairs of test species (including selection of plant species and herbicides, timing of exposure and selction of end-point) and sensitivity of plants to repeated exposure to herbicides.

3.1 Analyses of existing dose-response data

In order to answer the main question whether the response of species used in standard tests for sensitivity of non-target species is representative, we re-analyzed existing dose-response data found in the databases PHYTOTOX and ECOTOX (http://www.epa.gov/ecotox/) and analyzed sensitivity data (weeds) from efficacy tests in the field (data derived from the Pesticide Effect Database, Department of Integrated Pest Management, Aarhus University).

3.1.1 Analyses of dose-response data for crops and non-target plants found in databases

For the purpose of comparing sensitivity of crop species and non-target plants, existing dose-reponse data from the American ECOTOX-database and from dose–response experiments with 15 non-crop plant species exposed to 6 herbicides of different modes of action (Boutin et al. 2004) were used. See Table 3.1 for data desription.

On the basis of observed EC_{25} values for crop species and non-target plants, a comparison was made in order to determine whether these two subsets of plants had different sensitivity to herbicides. A difference in sensitivity is here defined as a difference between the 5% most sensitive species of crop species and non-target plants. Since we only have a limited sample of crop species and non-target plants, the distributions of EC_{25} values, and consequently the 5% percentile in the distributions, could only be estimated with considerable uncertainty. The estimation procedure, which depends on Bayesian statistics, is outlined below.

Table 3.1. Presentation of the data used for the comparisons of sensitivity of crop species and non-target plants, respectively, to herbicides. The ECOTOX-database is the PHYTOTOX database gathered by US-EPA enlarged by new toxicity data from literature and regulatory testing. All data are obtained following protocols for standard plant tests i.e. the experiments are pot experiments and plant biomass is used as end-point in all tests.

Plant species	Herbicide	Time of exposure	Reference
15 non-crop plant species	Glyphosate Bromoxynil Dicamba Metolachlor Pendime-thalin Metsulfuron-methyl	Variable, between two-leaves and 8-leaves stage	Boutin et al. 2004
9-22 crop species dependent of chemical (*)	Metolachlor Glyphosate Dicamba Bromoxynil Pendimethalin	Variable, but always early stages	ECOTOX-database (http://epa.gov/ecotox/quick_query.htm)

(*) The number of test species varied for each herbicide in the database

The estimated EC_{25} values for the different species belonging to either non-target plants or crop plants were assumed to be log-normal distributed, and the joint posterior distribution of the two parameters in the log-normal distribution for each group was sampled by a Markov-Chain-Monte-Carlo procedure using the Metropolis-Hastings algorithm with a multinomial candidate distribution (100.000 iterations with a burn-in period of 1000). The sampling procedure was checked by visual inspections of the sampling chains as well as computing the autocorrelation and acceptance ratio (Carlin and Louis 1996). From the sampled joint posterior distribution of the two parameters, the distribution (or uncertainty) of the 5% percentile in the distribution of the estimated ED_{25} values was calculated, and the distributions of the 5% percentiles for the two groups were compared by subtracting the two samples from each other. The distribution of the difference of the two distributions of the 5% percentiles was now obtained, and statistical inferences were based on the 95% credibility interval of the distribution of the difference.

3.1.2 Analyses of data from herbicide efficacy experiments

New herbicides are routinely tested in field experiments to establish their activity on weeds. The majority of the trials are conducted by the agrochemical companies, but over the years the Department of Integrated Pest Management, Aarhus University, has also conducted a large number of efficacy trials. In most of these trials, herbicides were applied at the recommended dosage and at 50% and 25% of the recommended dosage. These results offer an alternative source of information on the sensitivity of wild plants (weeds) to herbicides and the variability between and within plant families.

All data from experiments treated with metsulfuron-methyl and mecoprop-P were extracted from the database. The data originates from experiments in both spring and winter cereals. In winter cereals, the herbicides were applied either in the autumn or in the spring. Hence, data was grouped into three groups: winter cereals – autumn application, winter cereal – spring application and spring cereals. Although the herbicides were applied at different timings relative to plant growth in the autumn and spring, e.g. when the crops were in

growth stages BBCH 11-12 and 12-13 in the autumn and BBCH 12-13 and BBCH 13-15 in the spring, this variation in timing was not considered important and data from different timings were combined for the analyses. Table 3.2 provides an overview of the data used for the analyses.

Table 3.2. Overview of data from the Pesticide Effect Database at Department of Integrated Pest Management used for estimation of ED90 dosages

Herbicide	Timing	Lowest dosage (kg a.i./ha)	Highest dosage (kg a.i./ha)	Number of weed species	Number of observations
Metsulfuron-methyl	Winter cereals Autumn	0.0015	0.030	17	209
	Winter cereals Spring	0.0005	0.030	24	1010
	Spring cereals	0.0013	0.020	21	403
Mecoprop-P	Winter cereals Autumn	133	3600	14	667
	Winter cereals Spring	200	3000	14	363
	Spring cereals	270	2400	12	97

Efficacy was assessed as percent biomass reduction on a scale from 0 to 100%. Efficacy data were subjected to non-linear regression analyses using a log-logistic dose-response model:

$$U_i = \frac{D - C}{1 + \exp[2b(\log(ED_{50i}) - \log(z))]} + C \qquad (1)$$

where $\boldsymbol{U_i}$ is percent biomass reduction, $\boldsymbol{z}$ is the dosage, $\boldsymbol{D}$ and $\boldsymbol{C}$ are the upper and lower asymptotes at zero and very high herbicide dosages, $\boldsymbol{ED_{50i}}$ is the dosage resulting in a 50% reduction in plant biomass, $\boldsymbol{b}$ is the slope around $\boldsymbol{ED_{50i}}$ and $\boldsymbol{i}$ is the different weed species. The model is similar to the one described by Streibig et al. (2008) except that b is multiplicated by 2 and consequently will be half the value in our estimations compared to Streibig et al. The reason for using the model including the factor 2 is historical as all calculations in the Decision Support System, Plant Protection Online, which is partly based on data from efficacy trials are performed with this model and omitting the factor 2 inconvenience comparisons between 'old' and future data.

As the purpose of all the field experiments was to determine the dosages required to control various weed species, the observed effects tended to be in the upper end of the scale. Rather than estimating the ED_{50} dosages, the ED_{90} dosages were estimated instead by re-parameterise equation 1:

$$U_i = \frac{D - C}{1 + \exp[2b(\log(ED_{90i}) + 1.009/b - \log(z))]} + C \qquad (2)$$

The assumption that logistic dose response curves could be fitted to the data was assessed by a test for lack of fit, comparing the residual sum of squares of an analysis of variance and the non-linear regression.

3.2 Dose-response experiments with crop - and non-target species

3.2.1 Dose-response of selected crop and two non-target species

Although all data found in the PHYTOTOX and ECOTOX databases have been obtained following protocols for standard plant tests a lot of factors that may influence the outcome of the test such as climatic conditions, soil type, pot size, number of replicates, number of plants per pot, spray equipment, watering and time of both exposure and harvest may vary. The test conditions are not documented in the databases and it could not be ruled out that these differences were not responsible for the systematic differences in sensitivity found in previous studies (Boutin et al. 2004) and also in our re-analysis of the data. We, therefore, conducted a dose-response experiment with 10 crops species under tests conditions very similar to those used by Boutin and co-workers.

Selection of plant species

The 10 crop species selected for the experiment represented grasses and cereals as well as broadleaved species: oat (***Avena sativa),*** maize ***(Zea mays),*** perennial ryegrass ***(Lolium perenne),*** oilseed rape ***(Brassica napus),*** cucumber ***(Cucumis sativus),*** soyabean ***(Glycine max),*** sunflower ***(Helianthus annuus***), buckwheat ***(Fagopyrum esculentum),*** lettuce ***(Lactuta sativa)*** and onion (***Allium cepa***). In addition, two non-crop species (***Centaurea cyanus*** and ***Papaver rhoeas***), which were also included in the study of Boutin et al. (2004), were tested in the present study in order to allow comparison of species sensitivity between the experiments. All seeds were obtained from Danish seed suppliers.

Selection of herbicides

The experiment was carried out with three of the herbicides from Boutins study: bromoxynil, glyphosate and metsulfuron-methyl. Metsulfuron-methyl was applied in mixture with 0.05% of a non-ionic surfactant (Agropol, DLA Agro, Denmark). The protocol used by Boutin et al. (2004) was followed as closely as possible, as the objective was to mimic the same experimental conditions apart from using crop species, while the previous study was only made on non-crop species. This was rather easy as the methodology is well described in the paper, and besides participants in the present project were co-authors on the paper.

Cultivation and herbicide application

A germination test and a dosage range-finding test were performed before the main experiment in order to detect species sensitivity. Depending on the germination rate, the seeds were sown either directly in 1 L pots (diameter 11 cm) (species with high germination percentage), or they were sown in trays and transplanted to the pots at an early seedling stage (species with low germination percentage). In order to syncronize spraying of the different plant species, the seeds were sown on different days. The pots were filled with a potting mixture consisting of a sandy loam soil, sand and peat (2:1:1 by weight), including all necessary micro and macro nutrients. For each treatment, six replicate pots were used with one plant per pot. The six replicates were sprayed in three independent spray events so for each run of the spray boom two replicate pots were sprayed. In addition, six control pots of each plant species were included. The herbicides were applied using an automatic laboratory pot sprayer equipped with two ISO-02-110 nozzles operating at a pressure of 2 bars and a velocity of 4.7 km/h delivering a spray volume of 196.6 L/ha. Five dosages of each herbicide were applied to each

plant species with a dosage range decided by the sensitivity test carried out before the experiment and a factor 2 between dosages. The maximum dosages and the growth stage of the individual plant species at time of exposure are shown in Table 3.3.

The pots were placed on tables in the greenhouse and watered from the bottom. Temperature was maintained between 15 and 25°C and the photoperiod was at least 16 h daylight. Three weeks after spraying, the aboveground plant parts were harvested and fresh weight recorded. The biomass was dried at 80°C for 24 h, and dry weight was measured.

Data analyses

The fresh weight data were subjected to non-linear regression analyses using the log-logistic dose- response model shown in section 3.1.2. Here Ui is the fresh or dry weight, z is the dosage, D and C are the upper and lower asymptotes at zero and very high herbicide dosages, respectively, ED_{50i} is the dosage resulting in a 50% reduction in plant biomass, bi is the slope around ED_{50i} and i is the different plant species. The assumption that logistic dose response curves could be fitted to the data was assessed by a test for lack of fit comparing the residual sum of squares of an analysis of variance and the non-linear regression. For each crop species we estimated the dose-response curves of each of the three herbicides

Table 3.3. Growth stages of different plant species at time of exposure and maximum herbicide dosages (g a.i./ha) used on individual species in the dose-response experiment. The label dosages for the herbicides are: 120-400 g a.i/ha for bromoxynil (In Denmark only used in mixtures with ioxynil. The label dosage given assume that bromoxinil has an activity comparable to ioxynil), 4-6 g a.i/ha for metsulfuron-methyle and 360-720 g a.i./ha glyphosate (recommended for use on weed seedlings).

Plant species		Growth stage (number of leaves)	Bromoxynil (g a.i./ha)	Glyphosate (g a.i./ha)	Metsulfuron methyl (g a.i./ha)
Oil-seed rape	*B. napus*	2-3	47	180	1.55
Oat	*A. sativa*	4	6000	360	3.10
Maize	*Zea mays*	3	6000	360	3.10
Perennial ryegrass	*L. perenne*	3-4	6000	180	3.10
Cucumber	*C. sativus*	1	94	90	0.78
Soybean	*G. max*	2	94	180	0.78
Sunflower	*H. annuus*	2	23,5	90	0.78
Buckwheat	*F. eaculentum*	2-4	94	180	0.39
Lettuce	*L. sativa*	3	23,5	360	0.39
Onion	*A. cepa*	2	6000	360	0.39
Corn flower	*C. cyanus*	4	94	360	3.10
Corn poppy	*P. rhoeas*	2-4	47	180	0,180

3.2.2 Dose-response experiments with non-target species

In order to improve our knowledge of non-target species sensitivity to herbicides, we conducted dose-response experiments with pairs of closely taxonomically related species, representing one perennial species belonging to the 'the natural' flora and one annual weed species. The plants were exposed to three selected herbicides at different growth stages (e.g. vegetative growth versus reproductive stages). Furthermore, we examined the influence of selected end-points (biomasse versus seed production) for the outcome of the risk assessment.

Selection of plant species
Lifespan is one of the main factors that differ between the recommended test species in current guidelines and plants in natural and semi-natural habitats. Therefore, the six test species selected for the exposure and dose-response experiments form pairs of an annual weed species and a perennial species belonging to the "natural" flora, respectively. Furthermore, the following criteria have been judged when seleting the species: i) taxonomic relation; ii) existing data on sensitivity; and iii) morphological structures important for exposure and uptake i.e. plant height, leaf size and stucture, hairiness and wax layer. These should be as similar as possible for the two species belonging to a test-pair.

Based on sensitivity data in the PHYTOTOX database, Fletcher and coworkers showed that species belonging to the same genus had EC_{50}-values that showed a high degree of similarity (r_{mean} = 0.868) (Fletcher et al. 1990). They also showed that one "most sensitive species" does not exist. The sensitivity of a specific species varies with the herbicide. Therefore, the three test-pairs should be taxonomically different, but two species within each pair should be closely related and ideally belong to the same genus.

Dose-response data on perennial non-target species are rare. The Danish/Canadian dataset (Boutin et al. 2004) included dose-response data for 15 non-target species and 6 herbicides. Based on these data, the species were ranged according to sensitivity to each herbicide. Again, the data showed that no "most sensitive species" exists, but a number of species were often found among the most sensitive ones. Among these were ***Anagallis arvensis***, ***Inula helenium***, ***Prunella vulgaris*** and ***Digitalis purpurea***.

Another way of identifying species that are sensitive to herbicides is to look for weed species that have disappeared from the agricultural fields during the second half of the twentieth century. The seed bank density of a number of weed species in Danish agricultural fields decreased significantly from 1967-70 compared to 1987-89 (Andreasen et al. 1996). This applies to ***Anagallis arvensis***, ***Arenaria serpyllifolia***, ***Atriplex patula***, ***Cerastium caespitosum***, ***Galium aparine***, ***Plantago major***, ***Silene noctiflora*** and several species belonging to the genus ***Veronica***. Other factors than herbicide use may be important for the observed decline. However, studies of sensitivity indicate that for some species, like ***Anagallis arvensis***, herbicides have a major impact (Boutin et al. 2004).

Based on the list of weed species that have disappeared from agricultural fields, ***Geranium molle*** and ***Silene noctiflora*** have been selected as annual test species. To include a weed species that is still very common in the agricultural fields, we have selected ***Tripleurospermum inodorum***. As twins to these species,

we have selected closely related perennial species found in natural and semi-natural habitats that have a morphology that resembles the annual species as much as possible, ending up with the following pairs of test species (Fig. 3.1):

— ***Geranium molle*** and ***G. robertianum***

— ***Silene noctiflora*** and ***S. vulgaris***

— ***Tripleurospermum inodorum*** and ***Achillea millefolium***.

Geranium molle and ***G. robertianum*** both belong to the genus ***Geranium*** of the family Geranaceae. Both species are low growing plants with deeply divided leaves. ***Silene noctiflora*** and ***S. vulgaris*** both belong to the genus ***Silene*** of the family Caryophyllaceae (Dianthus). They have similar growth form and height. Finally, ***Tripleurospermum inodorum*** and ***Achillea millefolium*** belong to closely related genera of the composite family (Asteraceae syn. Compositae). These two species are similar with respect to plant height and leaf morphology (finely divided leaves).

Figure 3.1 Plant species selected for the dose-response experiments. The species form pairs of an annual weed species and a perennial species, respectively. (a) Indicates that the plant is an annual species and (p) indicates that the species is perennial. A: *Geranium molle* (a). B: *G. robertianum* (p). C: *Tripleurospermum inodorum* (a). D: *Silene noctiflora* (a). E: *S. vulgaris* (p). F: *Achillea millefolium* (p).

Selection of herbicides

Three herbicides were selected for the project. The criteria used to select the herbicides were their consumption, time of use and mode of action. Many years' experience with herbicide damage has revealed that systemic herbicides can cause damage at much lower dosages than non-systemic (contact) herbicides. The three herbicides were, therefore, selected among the group of systemic herbicides. Applying these criteria, we selected glyphosate, metsulfuron-methyl and mecoprop-P (MCPP), for overview see Table 3.4). Below, a description of each herbicide and its use in Denmark is presented. Figure 3.2 shows the use of the herbicides relative to the growing season.

Table 3.4 Overview of herbicides used in the experiments

Herbicide	Commercial product	Content of active ingredient (g/L)	Manufacturer
Glyhosate	Roundup Bio	360	Monsanto Crop Science
Metsulfuron methyl	Ally ST	500	DuPont Denmark ApS
Mecoprop-P (MCPP)	Duplosan	600	BASF A/S

Glyphosate belongs to the chemical group of glycines and is the most widely used herbicide in Denmark. In 2009, the treatment frequency index (TFI) for glyphosate was 0.52, i.e. more than 50% of cultivated land was sprayed with glyphosate, assuming that glyphosate was applied at 1260 g a.i./ha. Glyphosate is a non-selective herbicide that is used in non-crop situations or on mature/dormant crops. It is primarily used from August (pre-harvest application) to October (stubble treatment), but there is also some use in early spring (March-April) prior to sowing. Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate biosynthetic pathway, which is necessary for the production of the aromatic amino acids phenylalanine, histidine and tryptophan, but also auxin, lignin, plastoquinones and many other secondary metabolites in the plants. Over 30% of the carbon fixed by plants passes through the shikimate biosynthetic pathway.

Metsulfuron-methyl is one among several sulfonylurea herbicides available to Danish farmers. Sulfonylureas were used on 756, 464 ha in 2009, corresponding to a TFI of 0.34. Metsulfuron-methyl constituted approx. 20% of the total use of sulfonylurea herbicides. Metsulfuron-methyl is used for control of dicot weeds in cereals in spring (March-May). Other sulfonylurea herbicides are used both in autumn (September-October) and in spring (March-May). Some of the sulfonylurea herbicides are also active against monocot weeds. Metsulfuron-methyl inhibits acetolactate synthase (ALS), and that leads to a blockage of the synthesis of branched amino acids (valine, alanine and phenylalanine). Crop selectivity is due to metabolic conversion of the herbicide to non-phytotoxic compounds.

Mecoprop-P (MCPP) belongs to the group of phenoxyalkanoic herbicides. These used to be the most widely used group of herbicides for control of dicot weeds in cereals, but due to legislative restrictions, their use in Denmark has declined. Worldwide they are still a very important group of herbicides and data on sensitivity to mecoprop-P are numerous. We, therefore, decided to include mecoprop-P as one of the selected test herbicides. Mecoprop-P

mimics natural plant auxins causing uncontrolled growth. Sensitive plants exhibits stem twisting and leaf malformations. Metabolism and compartmentalisation are thought to be the main mechanisms providing selectivity in cereal crops. Today, MCPA is the most commonly used phenoxyalkanoic herbicide in arable crops in Denmark, primarily for control of perennial dicot weeds such as ***Cirsium arvense*** and ***Artemisia vulgaris***, but also ***Equisetum arvense***. MCPA was used on 80.417 ha in 2009. Phenoxyalkanoic herbicides are applied in autumn and early spring like metsulfuron-methyl, but also later in the growing season (mid May – mid June) targeting perennial weeds.

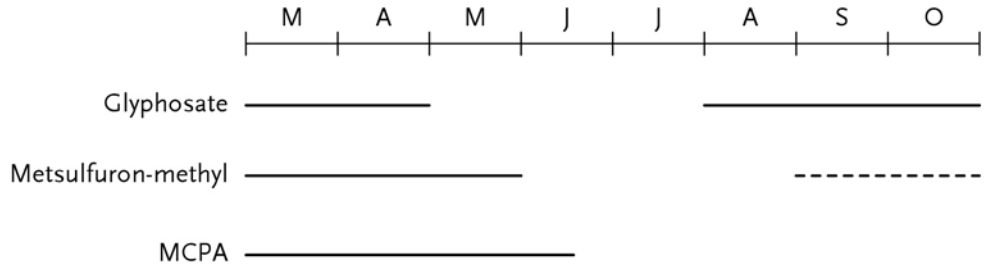


Figure 3.2. Use of glyphosate, metsulfuron-methyl and MCPA, during the growing season (May-October). The dashed line indicates use of other sulfonylurea hebicides than metsulfuron-methyl.

Selection of timing of exposure and measured end-point in dose-response experiments

As mentioned earlier it is important to know whether time of exposure or selected end-point influence the outcome of the risk assesment. Therefore, the selected the plants were exposed both at an early vegetative stage (6-10 leaves) and at the bud stage. Besides using biomass as end-point, we also examined the effect on seed production. Seed production is decisive for long-term survival in natural and semi-natural habitats and may, therefore, be regarded as a more ecologically relevant end-point than biomass.

The sensitivity of the 6 test species ***Geranium molle***, ***G. robertianum***, ***Silene noctiflora***, ***S. vulgaris***, ***Tripleurospermum inodorum*** and ***Achillea millefolium*** to mecoprop-P (MCPP), metsulfuron-methyl and glyphosate was examined in pot experiments. Seeds of ***A. millefolium, S. vulgaris, G. molle*** and ***G. robertianum*** were purchased from HerbiSeed, UK, while seeds of ***T. inodorum*** and ***S. noctiflora*** were obtained from the seed bank of Department of Integrated Pest Management, Flakkebjerg.

Cultivation and herbicide application

Seeds of ***A. millefolium, T. inodorum, S. noctiflora*** and ***S. vulgaris*** were sown in 2 L pots in a potting mixture consisting of soil(sandy loam), sand and peat (2:1:1 by weight) including all necessary micro and macro nutrients. The seeds of ***G. molle*** and ***G. robertianum*** were sown in trays and transplanted to the pots at an early seedling stage. The pots were placed on outdoor tables and watered several times a day. After germination, the number of plants per pot was reduced to the same number for each plant species. The number of plants per pot varied between 3 and 5, and the decisive factor for the number of plants was to obtain a high degree of uniformity between the remaining plants.

The herbicides were applied at the 6 to 10 leaf stage and at the bud stage using a laboratory pot sprayer. The sprayer was equipped with two ISO-02

nozzles operating at a pressure of 3 bars and a velocity of 5.6 km/h delivering a spray volume of 151 L/ha. Each herbicide was applied at 7 dosages with a factor 2 between dosages. The maximum dosages applied to each plant species and growth stages are shown in Table 3.5. The maximum dosages are within the range of the label recommendations for the herbicides except for metsulfuron-methyl (max. recommended label dosage = 6 g/ha). The experiment was carried out with 3 replicates per treatment.

Table 3.5. Maximum dosages g a.i./ha used in the dose-response experiments. *Geranium molle* and *G. robertianum* had 6 leaves, *Silene noctiflora*, *S. vulgaris*, and *Achillea millefolia* 6-8 leaves and *Triplerospermum inodorum* 10 leaves when exposed at the the vegetative stage (Veg) . At the reproductive stage (Rep) all species had flower buds.

Plant species	Mecoprop-P		Glyphosate		Metsulfuron-methyl	
Growth stage	Veg.	Rep.	Veg	Rep.	Veg	Rep.
S. noctiflora	1200	2400	720	720	2	4
S. vulgaris	1200	2400	720	720	2	16
T. inodorum	1200	2400	720	720	2	4
A. millefolia	1200	2400	720	720	2	4
G. molle	1200	2400	720	720	2	2
G. robertianum	1200	4800	720	720	2	4

After herbicide application, the plants were placed outdoors. Plants from three replicates of each treatment were harvested 3 to 4 weeks later. Thereafter, the plants used for assessing seed production were moved to the greenhouse. In order to ensure pollination of the plants, 6-8.000 honeybees were placed in the greenhouse (Figure 3.3). At maturity the seeds from each pot were harvested and cleaned. From each replicate pot 3 samples of 200 seeds were weighed and the thousand seed weight per pot was calculated based on the mean value. The mean number of seeds per treatment was calculated.

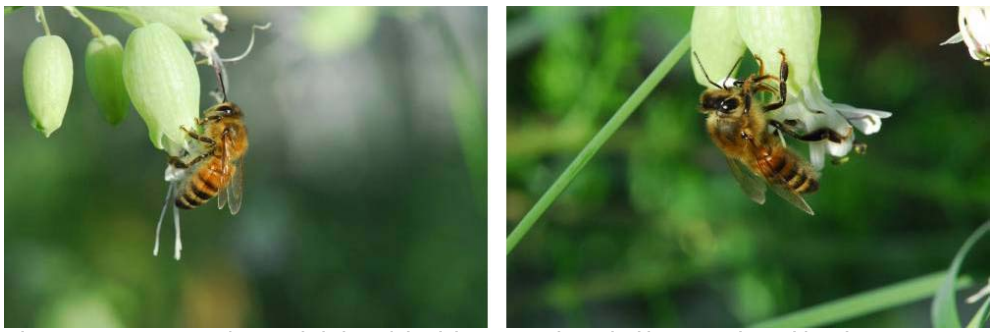


Figure 3.3. Honey bees visiting bladder campion (*Silene vulgaris*). Photos H. Rasmussen.

Data analyses

The fresh weight data were subjected to non-linear regression analyses using the log-logistic dose- response model shown in section 3.1.2. Here Ui is the fresh or dry weight, z is the dosage, D and C are the upper and lower asymptotes at zero and very high herbicide dosages, respectively, ED_{50i} is the dosage resulting in a 50% reduction in plant biomass, bi is the slope around ED_{50i} and i is the different plant species. The assumption that logistic dose response curves could be fitted to the data was assessed by a test for lack of fit comparing the residual sum of squares of an analysis of variance and the non-linear regression.

In the experiment concerning sensitivity of crop species we estimated the dose-response curve of each of the three herbicides (3.2.1) on all species. In

the experiments with non-crop species (3.2.2), we estimated 4 dose-response curves (two end-points (biomass and seed production) and two timings of exposure (vegetative and reproductive stage)) for each of the 3 herbicides on each of the six species. Data on relative fresh weight and number of seeds was subjected to non-linear regression analyses using the log-logistic dose-response model shown in section 3.4.1. The influence of growth stage and end-point (biomass, seed production) were quantified by the dosages required to obtain a specific response level (e.g. ED_{50}).

3.2.3 Sensitivity of plants to repeated exposure to herbicides

Plants in natural habitats are often exposed to several sub-lethal dosages of pesticide mixtures or different herbicides during the growth season. The most relevant scenarios for pesticide exposure of the non-target plants are repeated applications both given as simultaneous exposures and as staggered exposures, i.e. given with a period of time between the exposures to the different pesticides. This is especially relevant for perennial species. The number of pesticide combinations is numereous, and in the present project we will only examine the effect of ***staggered*** applications of herbicides with ***different modes of*** action - a combination that has not previously been examined. The aim is to introduce a method for investigating whether the effect of the total herbicide load is additive, antagosistic or synergistic. Our null hypothesis was that the effect on plants of repeated sub-lethal herbicide exposure did not differ significantly from the Additive Dose Model (ADM). ADM assumes additivity of dosages, i.e. that one herbicide can be replaced by another herbicide at equivalent biological dose rates.

Cultivation and herbicide application
Seeds of ***Silene noctiflora*** and ***S. vulgaris*** were sown in 2 L pots in a potting mixture consisting of soil, sand and peat (2:1:1 by weight) including all necessary micro and macro nutrients. The pots were placed on outdoor tables and were watered several times a day. After germination, the number of plants per pot was thinned to 4.

Mecoprop-P, glyphosate and metsulfuron-methyl were applied alone and in combinations. Two different experiments were conducted. In the first experiment, metsulfuron-methyl was applied at the 3 to 4-leaf stage (T1) followed by either metsulfuron-methyl, mecoprop-P or glyphosate 7 days later, when the plants had reached the 6 to 8-leaf stage (T2). In the second experiment, glyphosate was applied at T1 followed by metsulfuron-methyl, mecoprop or glyphosate at T2. The ratios of herbicides applied in the combinations were chosen with the aim of obtaining a contribution to the overall effect of the two herbicide applications of 25%:75%, 50%:50% and 75%:25%. All herbicide applications were carried out in a laboratory pot sprayer. The sprayer was equipped with two ISO-02 nozzles operating at a pressure of 3 bars and a velocity of 5.6 km h^{-1} delivering a spray volume of 153.5 L ha^{-1}. Each herbicide combination was applied in seven dosages and each treatment was carried out with 3 replicates.

After herbicide application, the plants were placed outdoors. The plants were harvested 3 to 4 weeks later, and fresh and dry weights were recorded.

Joint action model
The Additive Dose Model (ADM) has previously been used for analysing the joint action of herbicide mixtures (Morse, 1978, Green & Streibig, 1993, Kudsk & Mathiassen, 2004) and to determine whether dosages applied at

different timings are additive, i.e. that one herbicide dosage applied at a specific time can be replaced by a dosage applied at another time at equivalent dose rates (Mathiassen et al. 2007).

The biomass data of the herbicide treatments were subjected to non-linear regression analyses using the log-logistic four parameter model shown in section 3.1.2. In the present study, we estimated the $\boldsymbol{ED_{50}}$ parameters.

Within each experiment, the non-linear regression model was fitted simultaneously to the herbicide treatments assuming similar upper (D parameter) and lower (C parameter) asymptotes. In some cases, C was not significantly different from zero, and subsequent analyses revealed that the C parameter could be omitted from the model. The assumption that logistic dose response curves could be fitted to the data was assessed by a test for lack of fit, comparing the residual sum of squares of an analysis of variance and the non-linear regression and a graphical analysis of the distribution of residuals.

The relative potency R expresses the 'biological exchange rate' between the herbicides at specific application times in single application and can be calculated as:

$$R = Z_{AT1}/Z_{BT2} \qquad (3)$$

where Z_{AT1} and Z_{BT2} are the dosages of herbicide A and herbicide B producing a 50% effect, when applied at T1 and T2, respectively. Assuming that z_{AT1} and z_{BT2} are the herbicide dosages producing the same biological response, then the isobole defining additivity according to ADM can be described at any response level as:

$$z_{AT1}/Z_{AT1} + z_{BT2}/Z_{BT2} = 1 \qquad (4)$$

The predicted ED_{50} of repeated herbicide treatments according to ADM ($\boldsymbol{ED_{50rep}}$) can be calculated on basis of the ED_{50} values of the herbicide added singly and the distribution ratios in the treatment:

$$ED_{50rep} = \frac{ED_{50T1}}{(\alpha + (1-\alpha) * R)} \qquad 5)$$

where ED_{50T1} is ED_{50} of herbicide A applied at T1, α is the ratio of the herbicide applied at T1, and R is the relative potency as defined in eqn. 3.

3.3 Exposure experiments in spraying chamber and agricultural field

In this part of the project we investigated whether exposure following spray drift in the field results in effects on the plants equivalent to the ones found following exposure to identical dosages within the spraying chamber similar to the procedure in standard tests.

Three plant species, ***Silene noctiflora***, ***Veronica persica***, and ***Tripleurospermum inodorum***, were exposed to the three selected herbicides (glyphosate, mecoprop-P and metsulfuron-methyl) as well as the dye marker brilliant sulphaflavin in i) a spray cabin or ii) in the field as herbicide drift.

3.3.1 Preliminary experiment

In order to study their growth demands and sensitivity towards the selected herbicides, a preliminary experiment was performed. The three test species were sown in 11 cm pots containing standard greenhouse peat (Stenrøgel pottemuld) and kept under greenhouse conditions. Upon germination, excessive sprouts were removed. When the plants had 2-4 leaves, they were sprayed with herbicides, and after 3-4 weeks herbicide effects on plant biomass were estimated.

3.3.2 Field experiment

In the field, variation in herbicide exposure dosages was obtained by placing plants at five different distances to the tractor during spraying (cf. Fig. 3.5). At each distance from the tractor, 2x10 pots were placed in a row, ten for measuring spray deposition and ten for estimating effects on biomass. This set-up was repeated for each of the three herbicide applications. Field dosages of 720 g/ha glyphosat, 3.75 g/ha metsulfuron-methyl, and 3.6 kg/ha mecoprop-P were chosen. Dye marker (app. 0.2 g/l) was added to the herbicide solutions. Plants for the field experiment were sown and reared in the greenhouse until they reached the 4-6 leaves stage. The number of plants per pot was 1 for scentless camomile and three for the other two species. The dye marker brilliant sulfaflavin (BSF) was added to the herbicide solutions. Ten pots of each species were brought to the field, but not exposed, in order to get field controls. Upon herbicide exposure, the plants were returned to the greenhouse, and 3 weeks later aboveground biomass was harvested.

Spraying was performed using Hardi 4110-16 flatfan nozzles at a pressure of 3 atm. resulting in a nozzle output of 1.1 l/min. Tractor speed was 7 km/h, and the resulting spray output was 200 l/ha.

Water-sensitive paper and plastic hair curlers were exposed to herbicides and dye marker by placing it in the pots next to the plants (Figure 3.4). Herbicide exposure was documented by washing herbicide off curlers by the additive DanCon F (0.1% solution) and determining the concentration in the fluid by chemical analysis. Dye exposure was estimated by measuring concentrations in the fluid used for washing curlers and leaves spectrophotometrically. Correlations between dye and herbicide exposure were obtained by comparing herbicide and dye deposition on curlers. See paragraph 3.8.4 for herbicide and dye marker analyses.

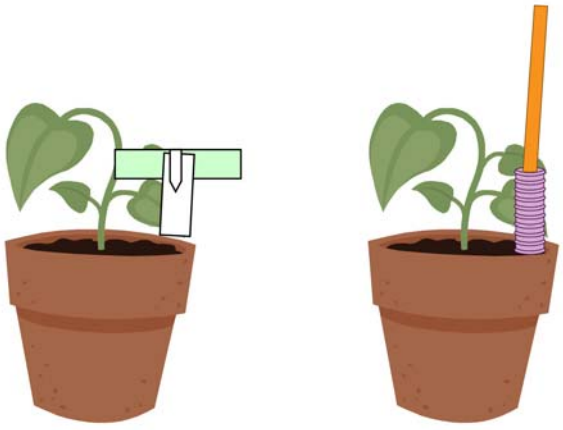


Figure 3.4. Pots with plants and water-sensitive paper (left) and curler (right).

For spray droplet larger than 20 μm, water sensitive paper was used to document differences in spray droplet size distribution at different distances from the tractor and between field and laboratory exposure. The paper works by changing colour on the moisturized parts. The papers were scanned to digitize the droplets. Droplet diameters were measured, and the number of droplets in different size classes counted automatically by computer; see Bruus et al. (2008) for methodology.

Smaller droplets were collected on special filters. Four individual filter samplers were placed on a 17 m line going South/North (171/351 degrees), behind the most distant row of plants, away from the tractor (Figure 3.5). The intakes of the filters were placed at 2 m, 7 m, 12 m and 17 m (going South-North) at a height of 10 cm. The filters used were Millipore (White RAWP, 1.2 um, 50 mm in diameter). The filters were mounted in a filter holder, having an inlet length of 80 mm and a diameter of 40 mm. During the first two experiments all four filters were exposed. Due to power failure, only two filters (2 m and 12 m) were exposed during the third spray. In order to examine the variation during a single spray, an experiment with 10 repeated sprayings was conducted. During this experiment, only two filters (2 m and 12 m) were exposed. The two samplers that weren't exposed due to power failure were used as field blanks instead. The flow was adjusted to sample droplets and aerosols of a size up to 15-20 ☒m. The flow was measured before each spraying and if necessary adjusted to 39 l/min.

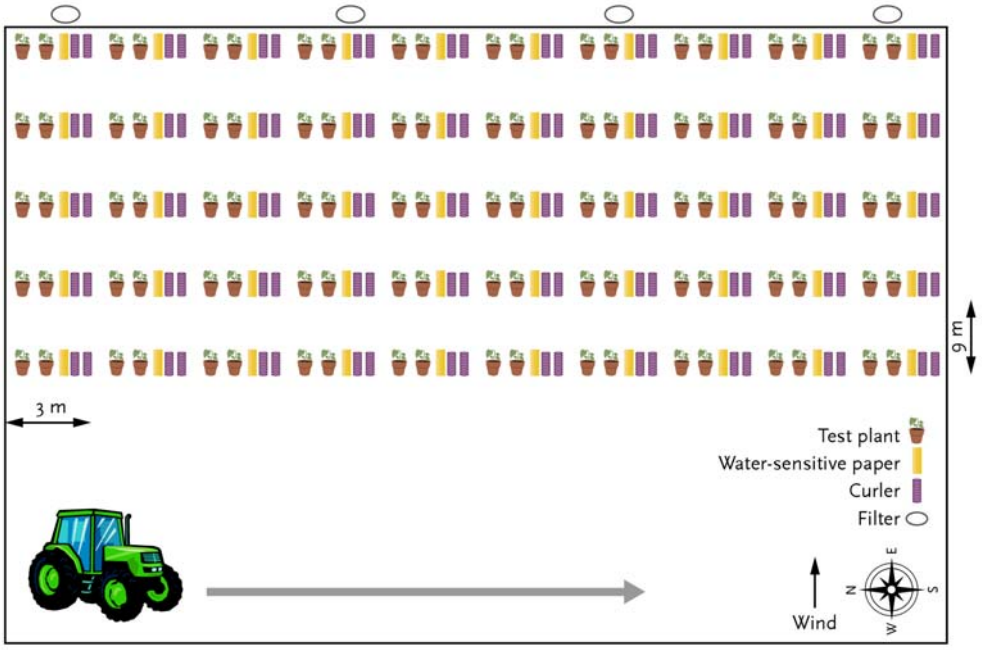


Figure 3.5. Outline of field experiment: Test plant Water-sensitive paper Curler Filter
Control plants (10 pots of each species) were placed about 100m up-wind from the tractor.

Meteorological measurements

The meteorological conditions during the field experiments have been recorded for documentation and in order to be able to assess possible major differences between the individual spray events.

The equipment consisted of a 10 Hz ultra sonic anemometer (Metek model USA-1) at 2.25 m above ground for measuring wind speed, wind direction, temperature, turbulence, and sensible heat flux.

These data were supplemented with instruments for humidity, global radiation, temperature and three wind cups just above the plants. Unfortunately, these last parameters were not recorded due to instrument failure.

Inspections of meteorological weather maps and data form nearby air ports revealed that during the day of experiments June 10th, the weather conditions were very similar in the whole region due to a cold front passage in the early morning associated with strong winds. Therefore, measurements from Skive Airport were used to partly substitute the missing parameters.

The times of the experiments and the meteorological conditions are summarised in Table 3.6 and the variation during the day of some of the parameters are shown in Fig. 3.6. The overall conditions show relatively high wind speed of around 7 m/s that at a standard observation height of 10 meters is calculated to be 9 m/s. This and higher wind speeds occur only in about 5 % of the time in Danish airports. This implicates a relatively short time of transportation and evaporation before the droplets arrive on the plants. Otherwise, the meteorological parameters (10 minute averages) are fairly constant. But during the very short period of spray release – about 15 sec. - the wind speed and direction might differ a lot from the averages.

Table 3.6. Time of experiments on June 10th 2008 and meteorological data during the experiments (10 minute averages). the first three experiments only lasted for about 15 seconds..

Exp.no.	Start - end	Wind speed m/s	Direc. deg.	u* m/s	L m	Heat flux W/m2	Temp. °C	Humidity %
1	10:51	5,9	287	0,61	-118	171	18	60
2	13:05	6,6	285	0,66	-150	166	17	51
3	15:11	7,8	293	0,70	-147	211	16	55
4	15:58 - 16:05	6,3	286	0,62	-186	113	16	54

L (Monin-Obukhov length) is a measure of atmospheric stability, u_* (the friction velocity) is a measure of turbulence.

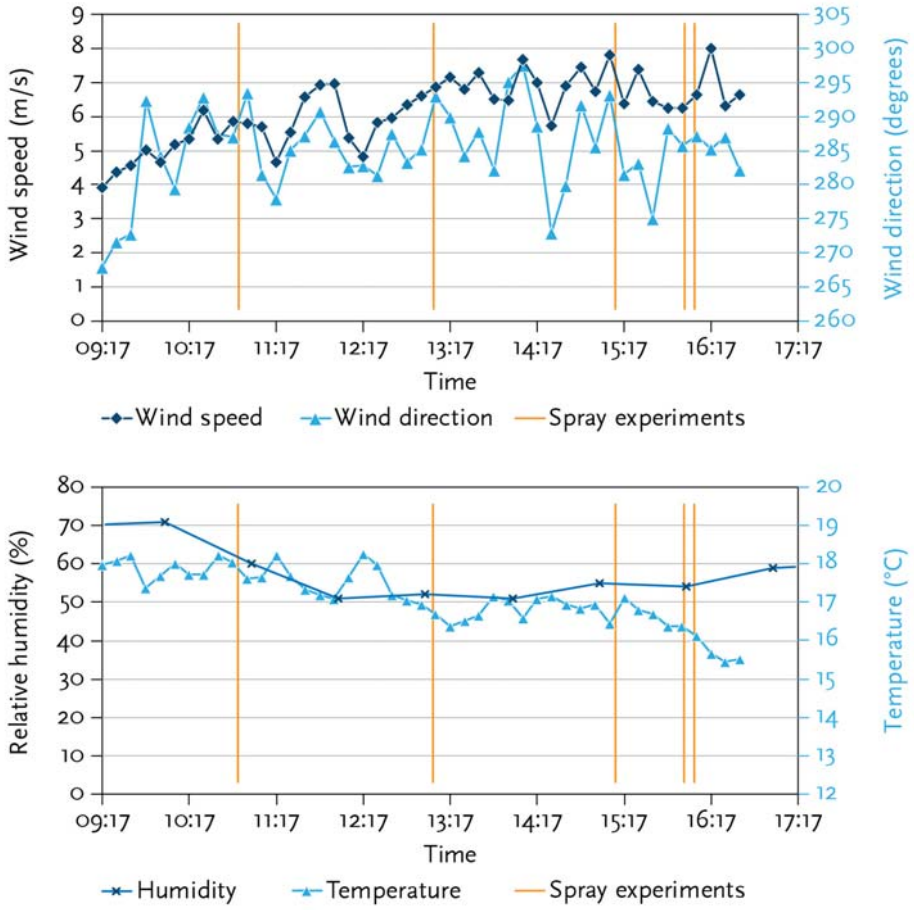


Figure 3.6. Time series of selected meteorological parameters for June 10th. The time of the experiments is marked with vertical lines.

3.3.3 Spray chamber experiment

In the greenhouse, herbicides were applied in a spray cabin, using the same nozzle and pressure as in the field. Five dosages of each herbicide were applied to separate batches of plants. The dosages corresponded to 0.5, 1, 2, 10 and 50 % label rate (label rates 720 g a.i./ha glyphosate, 3.75 g a.i./ha metsulfuron-methyl and 3600 g a.i./ha mecoprop-P). For each dosage of each herbicide, three pots of each plant species were exposed for establishment of biomass effects, and 12 pots for exposure measurements. In addition, there were six untreated control pots of each species. Exposure was estimated as described for the field experiment, except that no filter samples were taken. After exposure, the plants were treated as described above.

3.3.4 Herbicide and dye marker analyses

Extraction and analysis of metsulfuron-methyl and mecoprop-P
A common extraction method was used for the analysis of metsulfuron-methyl and mecoprop-P. The curlers were extracted two times with 250 ml deionized water. For the analysis of metsulfuron-methyl, the pesticide chlorsulfuron was used as surrogate standard, since this compound belongs to the same chemical group as metsulfuron-methyl (sulfonylurea). For the analysis of mechloprop-P, the corresponding deuterium labelled compound (D3-mecoprop-P was used as surrogate standard.

The surrogate standards were added to the water extracts, which were concentrated on solid phase extraction (SPE) columns (Oasis HLB, 250 mg).

The analytes were eluted with dichloromethane and the solvent was evaporated to dryness. The extracts were reconstituted in the LC mobile phase and then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS).

The analytes were separated by reverse phase liquid chromatography (LC) on a Hypersil-BDS C18, 5 µm, 250 mm x 2.0 mm column with a linear gradient. 5mM ammonium acetate/methanol (added 0.1% formic acid) was used as LC mobile phase. Tandem mass spectrometry (MS-MS) with electrospray ionization (ESI) was used as detection method. Metsulfuron-methyl was detected in positive mode using m/z 382/167 as transition ions, while mecoprop-P was detected in negative ionization mode using m/z 213/141 as transition ions. The analytes were quantified by linear regression using calibration standards in the range 10-250 ng/ml for metsulfuron-methyl and 10-2500 ng/ml for mecoprop. For those samples exceeding the calibration range, the extracts were diluted and analyzed again. The method detection limit (MDL) for metsulfuron-methyl and mecoprop-P was 0.02 ng/sample and 3 ng/sample, repectively.

Extraction and analysis of glyphosate

Glyphosate was extracted from curlers with 20 ml deionized water. Isotope-labelled glyphosate (13C2, 15N) was added to the water extract before derivatization. Glyphosate was derivatized by adding a solution containing FMOC-Cl in acetonitrile (12 mg/ml) and a 5% borate buffer. Excess of derivatizing agent was removed by shaking the water extract with methyl-tert-butyl ether (MTBE). The derivatization process was stopped by adding a few drops of concentrated HCl. The extract was then analyzed by LC-MS.

Reverse phase liquid chromatography (LC) coupled to mass spectrometry (MS) was used for the analysis of glyphosate derivative. A Betasil C18 5µm, 50 x 2.1 mm LC column was used with a mobile phase consisting of 5mM ammonium acetate/acetonitrile (added 0.1% formic acid). The analytes were ionized with electrospray ionization (ESI) in positive mode using the ions m/z 392/88 and 392/214. The analytes were quantified by linear regression using calibration standards in the range 25-1000 ng/ml. The method detection limit (MDL) for glyphosate was 100 ng/sample.

Extraction and analysis of brilliant sulfaflavin

Upon exposure, leaves, curlers and filters were transferred to vials containing extractant (0.1 % Dancon F) and placed in darkness in order to avoid photo degradation. A tank sample was taken after each spraying.

Concentrations of brilliant sulfaflavin in the extractant were measured by fluorescense spectrometry at a detection limit of 0.01 g/l. Brilliant sulfaflavin was excited at 410 nm and detected at 518 nm.

3.4 Assessment of community effects of herbicide and nitrogen on experimentally established vegetation

For the investigation of effects of fertilizers and herbicide drift and the interactions of these parameters on non-target vegetation in the agricultural land, we worked at a well-established field experiment, the so-called “Kalø-experimental plot” (Holst et al. 2008, Bruus et al. 2004). The experimentally established vegetation comprises about 30 species, representing different strategies and traits, and the experimental manipulations include exposure to

glyphosate concentrations between 0 and 25 % of full application rate and three levels of nitrogen addition. Data analyses comprise traditional statistics, multivariate analyses and modelling of effects of glyphosate and nitrogen on plant competitive interactions.

3.4.1 Field experiment – the Kalø experimental plot

The field experiment was established in 2001 (Bruus et al. 2004). The area selected was a former agricultural field on dry, nutrient poor sandy soil. The field laid fallow a couple of years prior to the start of the experiment in 2001. The field is quadrangular and surrounded by small parts of forest on two sides (south and west) and separated from the neighbouring fields by 5 meter broad hedgerows on the other sides (Fig 3.7).

Figure 3.7. Air-photo of the Kalø experimental plot, early spring 2006.

In 2001, the area was deep ploughed down to 60 cm to eliminate establishment from the soil seed bank and prepared for the experiment by harrowing and rolling. Thirty-one species were sown in spring 2001. The species selected were grassland species covering different life form strategies (CRS strategies sensu Grime 1988).

Experimental manipulations

The experimental manipulations were set up as a complete randomized block design with 10 replicates of each of the twelve treatments (Fig. 3.8). The treatments included 4 glyphosate treatments (0; 14.4; 72 and 360 g a.i./ha equal to 0, 1, 5 and 25% of label rate of 1440 g glyphosate/ha) and 3 nitrogen treatments (0, 25 and 100 kg N/ha). All plots received phosphorus (53 kg/ha), potassium (141 kg/ha), sulphur (50 kg/ha and copper (0.7 kg/ha) every year. The RoundupBio® formulation of glyphosate was used for the experiment. Each plot was 7 m x7 m with a buffer zone of 1.5 m surrounding the plot. A buffer zone of 10 m separated the experiment from the surrounding vegetation. The buffer zones were also sown with the seed mixture.

For the herbicide applications, spraying equipment for experimental applications was used (Fig. 3.9). The boom was 3 m with 0.5 m between the nozzles that were Lurmark Lo-drift LD 015 Green nozzles with a pressure of 2.0 bars. The wind speed on the days selected for spraying was very low (0-2 m/s). There was no rain, neither was rain expected during the days following the day of spraying. Fertilizers were spread by hand. The plots were treated by glyphosate for the first time 24 August 2001 when the vegetation had become established at the plots. Since then, it has been treated with herbicide and fertilizer once every year in spring (Tabel 3.8).

5	1	7	11	9	3	7	5	10	2	12
4	2	3	10	3	2	11	4	4	4	11
9	4	12	9	12	9	1	1	8	10	10
10	9	4	5	11	1	9	8	6	6	9
6	3	2	2	5	10	8	2	12	9	8
2	8	6	4	1	8	3	9	7	8	7
12	7	10	6	6	11	10	3	11	11	6
11	5	1	3	7	7	2	11	9	5	5
8	11	8	12	10	5	5	7	3	7	4
7	10	9	1	2	4	12	6	1	1	3
3	6	11	7	4	12	4	10	2	3	2
1	12	5	8	8	6	6	12	5	12	1
J	I	H	G	F	E	D	C	B	A	

Treatments:

	N (kg/ha/Y)	Glyphosat (g a.i./ha)	% of field dosage		N (kg/ha/Y)	Glyphosat (g a.i./ha)	% of field dosage
1	0	0	0	7	25	72	5
2	0	14.4	1	8	25	360	25
3	0	72	5	9	100	0	0
4	0	360	25	10	100	14.4	1
5	25	0	0	11	100	72	5
6	25	14.4	1	12	100	360	25

Figure 3.8 Design of the field experiment at the Kalø experimental plot. Recommended dosage for glyphosate is 1440 g a.i./ha.

Figure 3.9 Herbicide application at the Kalø experimental plot. Photo B. Strandberg

Data sampling

Two different sampling approaches were used for assessment of community effects. During the period 2005-2007, sampling was made within 6 randomly selected 0.75m x 0.75m quadrates in each plot in order to study the effects of the treatments on vegetation composition. The focus within that period was mainly on effects of glyphosate (Holst et al. 2008). Due to that only plots receiving 100 kg N/ha were sampled systematically. The other treatments were only sampled once over the period. Thereafter, in the summer of 2007, one permanent 0.5m x 0.5m quadrat was established within each plot and during the period 2007-2009 sampling was performed within these quadrates. This was done in order to study the dynamic between the two dominant grasses ***Agrostis capillaris*** and ***Festuca ovina*** more thoroughly. These two grasses differ in sensitivity to glyphosate, the latter being the least senisitive (Holst et al. 2008). The sampling scheme is found in Table 3.7.

Table 3.7 Time of herbicide and fertilizer treatments and vegetation samplings at the Kalø experimental plot.

Treatment/Sampling	2005	2006	2007	2008	2009
Fertilizer	12 May	15 May	15 May	6 May	17 May
Glyphosate	30 May	30 May	7 June	14 May	30 May
Pre-treatment sampling	23 -25 May	24-27 May	30 May-5 June	24 – 28 April	10 – 16 May
After treatment sampling	14 – 20 June	17-22 June	21 -26 June	10 – 15 June	17-22 June
End of season sampling	26 – 31 August	25-29 August	27 – 31 August	18-22 August	24-30 August

The ecological success of the plants was measured non-destructively by the pin-point (or point-intercept) method (Levy and Madden 1933, Kent and Coker 1992). A pin-point analysis provides estimates of two important plant ecological variables: plant cover and plant biomass.

Plant cover was estimated within each quadrate using a horizontal frame with a 5x5 grid with the 25 intersections at a distance of 10 cm. The grid consisted of 25 intersections (Figure 3.10). At each intersection a sharply pointed pin

with a diameter of 0.5 mm was passed vertically through the vegetation. An estimate of percent cover of vascular plants was obtained by recording the first interception of the pin with the canopy of the different species or ground. The cover estimates were combined with a complete species list for the plot. Nomenclature follows Hansen (1991).

Aboveground biomass of selected plant species including ***Agrostis capillaris***, ***Festuca ovina*** and ***Elytrigia repens*** was estimated non-destructively using a modification of the point intercept method as described by Goodall (1952), Jonasson (1983, 1988) and Frank and McNaughton (1990). Instead of recording only the first intercept of the pin, every contact between pin and vegetation was recorded. The total number of intercepts gives an estimate of the projected plant area (PPA). The PPA correlates highly with biomass, as shown by Jonasson (1983, 1988). The method has shown very useful for estimation of biomass of individual species including grasses (Strandberg et al. 2006a). The PPA, therefore, can be used as a regression variable to predict the above ground biomass. The method is sensitive to growth form and needs separate calibrations for each. To establish the relationship between the number of intercepts and aboveground vascular biomass of the selected species, the number of intercepts between this species and the pin was registered in 15 0.5x0.5m plots in the buffer zones and the plant was harvested. The samples were oven-dried for 24 h at 80°C and weighed.

For the competition modelling, we use the total number of intercepts for each plant species as a measure for plant size or 3D-space occupancy. In Chap. 3.5 and 4.5 this sum is refered to as compactness. The point frame and the pin used for cover estimates were used also for this data collection.

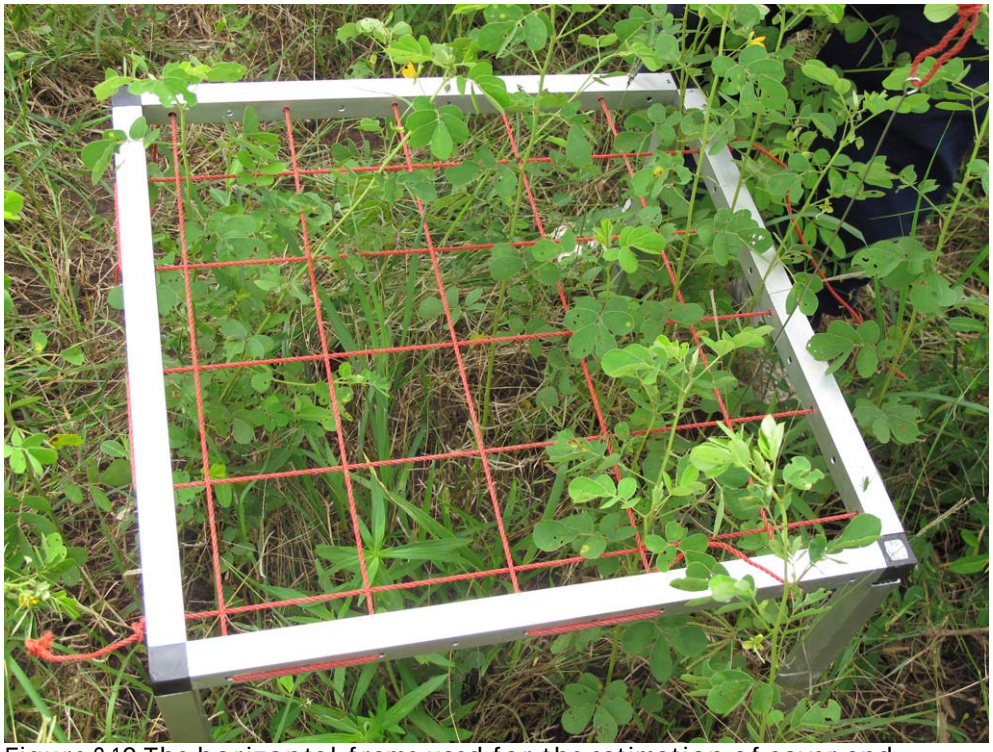


Figure 3.10 The horizontal frame used for the estimation of cover and biomass at the Kalø experimental plot. The grid consisted of 5x5 grid lines 10 cm apart. Each of the 25 intersections is used for as a sampling point. Photo B. Strandberg

3.5 Modelling of plant competition

The competitive processes are analysed in a state-space model, which allows separation of process and sampling variance. This is important because such a separation enables ecological predictions with a known degree of uncertainty (Clark, 2007). Furthermore, the estimated latent variables are less influenced by the sampling variance, and consequently the modelling of the ecological processes through time will be less biased by sampling error compared to a normal regression model where the observed values are used.

The competitive interactions in the plant community at the different treatments were analyzed by describing how cover and 3D-space occupancy (or compactness) of two selected species, ***Festuca ovina*** and ***Agrostis capillaris***, and an aggregated class of the other species found at the plots co-vary during the growing season (Fig. 3.11). The relationship between plant cover and compactness was indicated by the square boxes. The possible die back due to the herbicide treatment was investigated by the change in cover from $\boldsymbol{t}_1$ to $\boldsymbol{t}_2$. The competitive growth among the plant species was investigated by describing how plant cover at $\boldsymbol{t}_2$ influenced the compactness of the species at $\boldsymbol{t}_3$. Finally, the survival and establishment of the different species the following year were investigated by describing how plant compactness at the end of the growing season ($\boldsymbol{t}_3$) of the different species influenced the cover of the species the following year at $\boldsymbol{t}_1$. The underlying assumption of the method used here is that the species specific measure of compactness at the end of the growing season may be used as a measure of growth or the ecological success of the species over the growing season. The compactness is expected to depend on the abiotic and biotic environment and the cover of other species, which compete for resources such as light, water and nutrients. More specifically, the increase in compactness is expected to be regulated by the levels of nitrogen and herbicide through the growing season. Furthermore, it is assumed, everything else being equal, that a plant species that grows to a relatively high compactness has a relatively high cover the following year, i.e. plants allocate resources into occupying space the following year (Damgaard et al. in press).

In conclusion, both cover and compactness are assumed to be regulated by competitive growth, survival and establishment as well as by the levels of nitrogen and herbicide through the growing season (Fig. 3.11). The conceptual model in Figure 3.11 is fitted to the sampled data for cover and compactness from the permanent plots using a state-space modelling approach (Clark 2007), where the variance due to the ecological processes are separated from the variance due to sampling. The process equations (indicated by ***P***1-***P***3, below) use latent variables, which are variables that are not directly measured, but are inferred from the measured cover and compactness, to describe the development of the state of plant cover and compactness. The studied competitive interactions as well as the latent variables of cover and compactness and the associated observations through the growing season are indicated in Fig. 3.11.

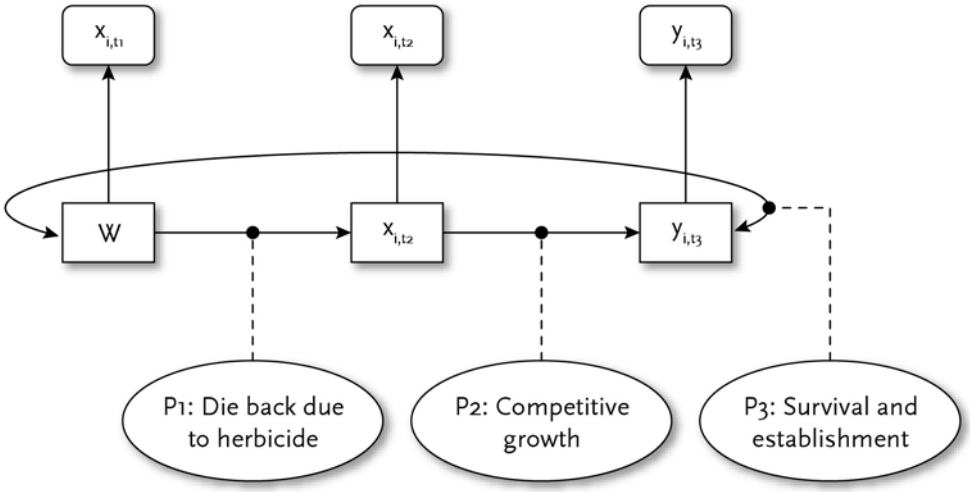


Figure 3.11. Graphical model of the studied processes at the field experiment (ellipses), the latent variables of the states of the investigated ecological success components cover (Xi,t) and compactness (Yi,t) through the growing season (square boxes), and the associated observations of cover (xi,t) and compactness (yi,t) of species i at time t (rounded boxes). Cover and compactness were measured three times during the growing season: before herbicide and nitrogen application (t1), approximately two weeks after herbicide application (t2), and at the end of the growing season (t3). +M1 and M2

The ecological processes were investigated in three process equations which were separated in time (Fig. 3.10): the first process equation investigated the direct effect of the herbicide on the cover of plant species ***i*** from $\boldsymbol{t}_1$ to $\boldsymbol{t}_2$, which was investigated in a logistic regression model,

$$\boldsymbol{P}1:\ \text{logit}(X_{i,t2,y,p}) = \text{logit}(X_{i,t1,y,p}) + \alpha_i\ h + \beta_i + \varepsilon_{i,y,p} \qquad (1),$$

where $\boldsymbol{X}_{\boldsymbol{i,t,y,k}}$ is the plant cover of species ***i*** at time ***t*** in year ***y*** in plot ***p***, ***h*** is the level of the herbicide, α measures the effect of the herbicide on the change in cover from $\boldsymbol{t}_1$ to $\boldsymbol{t}_2$, β measures the change in cover from $\boldsymbol{t}_1$ to $\boldsymbol{t}_2$ that is unrelated to the effect of the herbicide, and $\varepsilon_{\boldsymbol{i,y,p}} \sim\ Normal(0, \sigma_{12}^2)$.

It was assumed that the compactness of species ***i*** at time 3, t3, was an increasing function of the plant cover of species ***i*** at time 2 and a decreasing function of the plant cover of the other species, ***j*** and ***k***, and the competitive growth of plant species ***i*** was modelled as,

$$\boldsymbol{P}2:\ Y_{i,t3,y,p} = a_i\ (X_{i,t2,y,p})^{b_i} \cdot \exp(-c_j (X_{j,t2,y,p})^{d_j} \cdot \exp(-c_k (X_{k,t2,y,p})^{d_k} \cdot \exp(e\ n) \cdot \exp(f\ h) \cdot \exp(ef\ nh) + \varepsilon_{i,y,p} \quad (2),$$

where $\boldsymbol{Y}_{\boldsymbol{i,t3,y,k}}$ is the compactness of species ***i*** at time 3 in year ***y*** in plot ***p***, $\boldsymbol{X}_{\boldsymbol{i,t2,y,k}}$ is the plant cover of species ***i*** at time 2 in year ***y*** in plot ***p***, ***n*** is the level of the nitrogen treatment, ***h*** is the level of the herbicide, and $\varepsilon_{\boldsymbol{i,y,p}} \sim\ Normal(0, \sigma_{23}^2)$. The relationship between cover at $\boldsymbol{t}_2$ and compactness at $\boldsymbol{t}_3$ was expressed by a power function of the parameters ***a*** and ***b***. The competitive effect of the cover of species ***j*** and ***k*** on the compactness of species ***i*** were modelled by the competition coefficients $\boldsymbol{c}_j$ and $\boldsymbol{c}_k$ with modifying power functions with parameters $\boldsymbol{d}_j$ and $\boldsymbol{d}_k$. The effect of nitrogen, the herbicide, and the interaction

between the two on the compactness of species ***i*** at t_3 were measured by ***e***, ***f***, and ***ef***, respectively.

Since we expected that perennial species with a relatively large compactness in year ***y*** to have a relatively larger plant cover the following year (***y***+1), it was assumed that the plant cover of species ***i*** in year ***y***+1 is an increasing function of the compactness of species ***i*** in year ***y*** and a decreasing function of the compactness of other species ***j***, ***k*** in year ***y***, and the survival and establishment the following year of species ***i*** was modelled as,

P3:

$$\text{logit}(X_{i,t1,y+1,p}) = a_i\ (Y_{i,t3,y,p})^{b_i} + c_j\ Y_{j,t3,y,p} + c_k\ Y_{k,t3,y,p} + e\ n + f\ h + ef\ nh + g + \varepsilon_{i,y,p} \quad (3),$$

where $\boldsymbol{X}_{\boldsymbol{i,t1,y+1,k}}$ is the plant cover of species ***i*** at time 1 in year ***y***+1 in plot ***p***, $\boldsymbol{Y}_{\boldsymbol{i,t3,y,k}}$ is the compactness of species ***i*** at time 3 in year ***y*** in plot ***p***, ***n*** is the level of the nitrogen treatment, ***h*** is the level of the herbicide, and $\varepsilon_{\boldsymbol{i,y,p}} \sim Normal(0, \sigma^2_{31})$. The relationship between compactness at t_3 and the cover at t_1 the following year is expressed by a power function of the parameters ***a*** and ***b***. The effect of the compactness of the other species on the cover of species ***i*** the following year are modelled by the competition coefficients c_j and c_k. The effect of nitrogen, the herbicide, and the interaction between the two on the cover of species ***i*** at t_1 is measured by ***e***, ***f***, and ***ef***, respectively.

Note that the parameters in process equations (2) and (3) have the same notation; this does not mean that the parameters of the two processes are identical, but only that the parameters with the same notation have an analogous interpretation.

In the measuring equations, the likelihood of the cover of species ***i***, $\boldsymbol{X}_{\boldsymbol{i,t,y,p}}$, is measured by a binomial process as the number of grid points, $\boldsymbol{x}_{\boldsymbol{i,t,y,p}}$, where species ***i*** is hit by the pin out of ***n*** grid points:

M1: $\text{p}(x_{i,t,y,p}) = Bin(n, X_{i,t,y,p})$ (4),

and the likelihood of the compactness of species ***i***, $\boldsymbol{Y}_{\boldsymbol{i,y,p}}$, is measured by the number of pin-point hits per pin, $\boldsymbol{y}_{\boldsymbol{i,y,p}}$ using the normal distribution where the variance, σ_i^2, is assumed to differ among species but not among years or plots:

M2: $\text{p}(y_{i,y,p}) = Normal(Y_{i,y,p}, \sigma_i^2)$ (5).

The process equations and the measurement equations are linked by a combined likelihood function,

$$p(z,u \mid \theta,\pi) = \prod_{i=1}^{n}\left(p_1(z_0)\prod_{t=1}^{t}\left(p_1(z_t \mid z_{t-1},\theta)\prod_{j=1}^{nt} p_2(u_{j,t} \mid z_t,\pi)\right)\right) \quad (6),$$

where $p_1(z_t,\theta)$ describes the likelihood of the latent variables ***z*** in year ***t*** with parameters θ, and $p_2(u_t, z_t, \pi)$ describes the likelihood of the observations ***u*** in year ***t*** with parameters π, ***n*** is the number of years, ***nt*** is the number of observations in year ***t***, and $p_1(z_0)$ is a prior of the latent variables z_1. In the combined likelihood function (6) it was used that,

$$p_1(z_1, z_2, ..., z_n) = p_1(z_n \mid z_{n-1}) \cdot ... \cdot p_1(z_2 \mid z_1) \cdot p_1(z_1) \qquad (7)$$

i.e. the Markov properties of the process equations.

3.5.1 Estimation and statistical inference

In order to check the fitting properties of the three process equations (***P***1 – ***P***3), the residuals of the three models were investigated independently using the observed values of cover and compactness from the samplings in 1997-1999. The maximum likelihood estimates of the parameters were obtained using the NMaximize procedure in Mathematica (Wolfram 2007), and the fit of the models was checked by visual inspection of i) plots of expected values vs. observed values and expected values vs. residuals; ii) histograms of residuals; and iii) fractile diagrams of the residuals. Based on the plots, it was concluded that it was necessary to square root transform the compactness in ***P***2 (both observed and expected compactness). Furthermore, the possible addition of a power function to modify the effect of compactness on cover the following year in ***P***3 was not supported by the data.

The joint Bayesian posterior distribution of the parameters, as well as the latent variables for cover ($\boldsymbol{X}_{i,t}$) and compactness ($\boldsymbol{Y}_{i,t}$) were calculated using a MCMC (Metropolis-Hastings) run of 100,000 iterations with a burn-in period of 30,000 iterations and a multivariate normal candidate distribution (Carlin and Louis, 1996). The prior distributions of all parameters and latent variables were assumed to be uniformly distributed in their specified domains ($0.0001 < X < 0.999,\ 0.5 < b, d < 2,\ 0.01 < \sigma_{12}, \sigma_{23}, \sigma_{31}$), except σ_i, which was assumed to inverse gamma distributed with parameters (0.001, 0.001). Plots of the deviance and the sampling chains of all parameters and latent variables were inspected in order to check the fitting and mixing properties of the used sampling procedure.

The statistical inferences of the different treatments were assessed using the calculated 95% percentiles of the marginal posterior distribution of the parameters of interest (credibility intervals).

3.5.2 Model interpretattions

The analysis of data on cover and compactness of two species by the competition model allows an understanding of the various ecological processes that occur during and between growing seasons as well as estimates the effect of glyphosate and nitrogen on the ecological processes. Furthermore, it enables the testing of precisely formulated hypotheses on the effects of nitrogen and glyphosate on plant community dynamics. For example, was the observed decrease in an herbicide sensitive species in plots, that were sprayed with glyphosate, due to i) an immediate die back of the species caused by the toxic effect of glyphosate, ii) caused by a reduced competitive ability during the growing seasons or iii) a result of reduced survival or establishment during winter and early spring?

4 Results

The results are presented according to the five main parts of the project:

1) Analyses of existing toxicity data on terrestrial plants (Chap. 4.1)
2) Dose-response experiments with both crop species and non-target species (Chap. 4.2)
3) Exposure experiments in spray chamber and agricultural field (Chap. 4.3)
4) Assessment of community effects of herbicides and nitrogen on experimentally established vegetation (Chap. 4.4), and
5) Modelling of plant competition (Chap. 4.5).

The results from the dose-response experiments are presented in four sub-chapters: Reprocucibility of earlier published results (Chap. 4.1.1), species sensitivity (Chap. 4.2.1), selection of end-point (Chap. 4.2.2) and repeated exposures (Chap. 4.2.3).

4.1 Analyses of existing toxicity data on terrestrial plants

4.1.1 Analyses of dose-response data for crops and non-target plants found in databases

The assessment of the effects of herbicides on non-target plants relative to the effects on crop species is based on a limited set of surrogate species. Non-target plant species are here defined as those plant species occurring outside but in the vicinity of agricultural fields. In a risk assessment of a specific herbicide, it is assumed that the sensitivity of the selected test species, which typically are annual crop plants, is representative for all species that are found in habitats surrounding sprayed fields. In the existing analyses of differences in sensitivity between crop plants and wild plants testing, conditions were variable giving rise to uncertianity of the EC estimates. Therefore, this report re-analyses the sensitivity of crop plants and several non-target plants by comparing species sensitivity distributions (SSDs) and calculate hazardous concentration thresholds for 5% of species (HC5). The data for non-target wild plants came from Boutin et al 2004, and the data for the crop species came from either the ECOTOX database created by USEPA or from the dose – response data for crop species reported in chapter 4.3

The two different crop databases reflect different experimental circumstances: the data in the ECOTOX database does not specify how exposure levels were calculated, under which abiotic conditions the test were conducted and the experimental conditions in general (for example how many plants were present in the pot etc.), whereas the experimental conditions for the “new crop data” were comparable to experimental data for non-crop species presented by Boutin et al (2004).

If the mean or the median effects of the herbicides are compared, then both the work of Boutin et al. (2004) and the calculations performed in this report on ECOTOX-data suggest that crop plants may be less sensitive than non-

crop species. However, when the estimated HC5 values are compared, then the sampling size is generally to low to provide sufficient power for detecting any difference. Never the less, it was found that for glyphosate, the crop plants were less sensitive than the wild plants (2.5-97.5% confidence interval did not intercept with 0) (Figure 4.1).

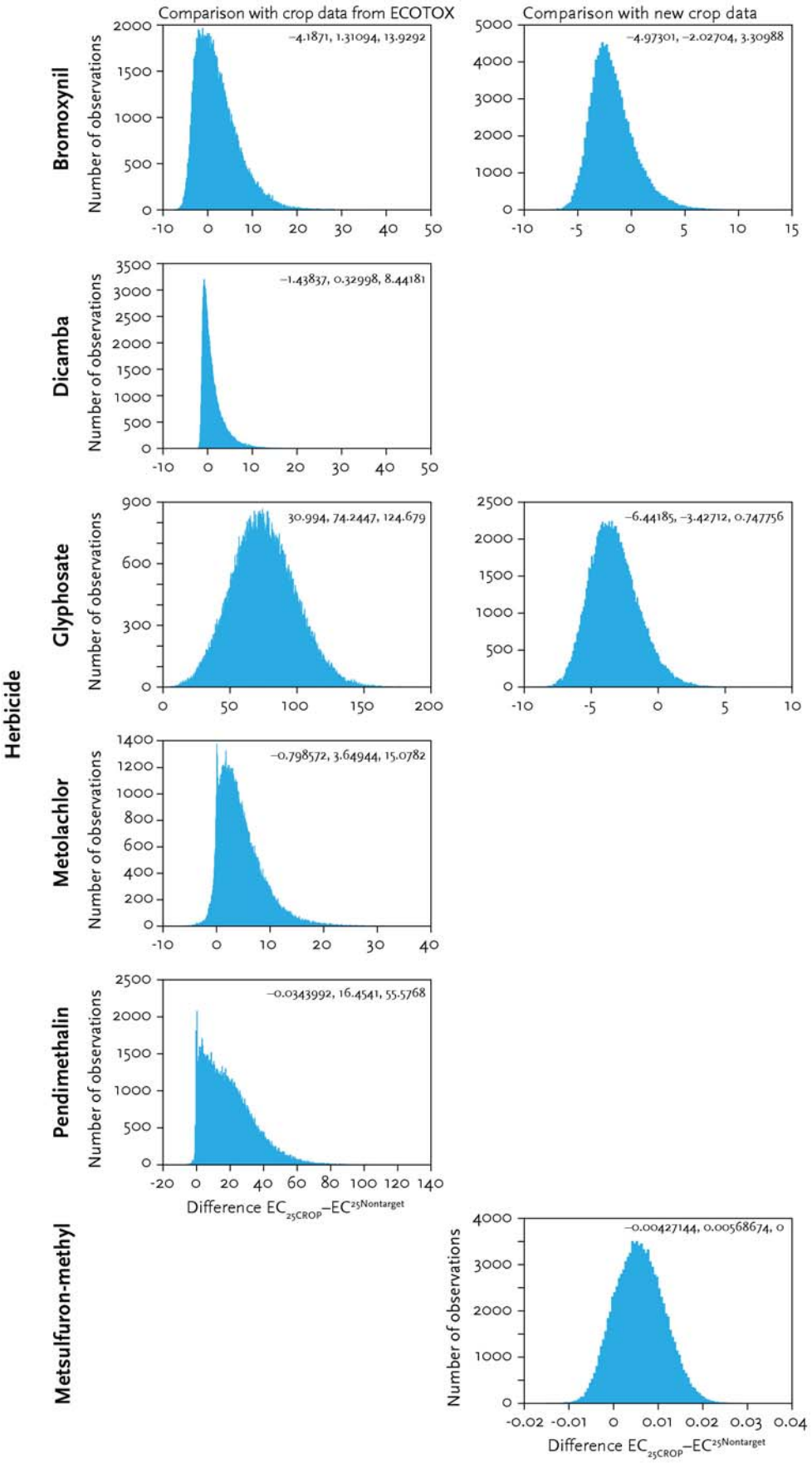


Figure 4.1. Graphic presentation of the difference in sensitivity (HC5) between the effect of herbicides on non-target plants species and the effect on crop species. The numbers in the upper left of the graphs are 2.5%, 50% and 97.5% percentiles. If the lower limit is positive or the upper limit is

negative a difference in sensitivity between the two groups exists. The difference is reported by the estimated 95% credibility interval, and if the 2.5%-percentile is positive then the non-target plant are significantly more sensitive than the crop plants, and opposite if the 97.5%-percentile is negative then the non-target plant are significantly less sensitive than the crop plants. If the 2.5%-percentile is negative and the 97.5%-percentile is positive, then there are no significant difference crop plants and non-target plants. The columns represent the comparisons of the non-target plant data (Boutin et al 2004) with the ECOTOX crop data (left column) and with crop data obtained in the present project (right column, see Chap. 4.1.3).

4.1.2 Analyses of data from herbicide efficacy experiments

The purpose of herbicide efficacy trials is to demonstrate effective control on a wide range of weed species, i.e. the effect level observed in the trials with the recommended dosage tend to be close to 100%. Inclusion of the two lower dosages (25 and 50% of the recommended dosage) makes it possible to classifiy the weed species according to sensitivity, but even the lowest dosage very often produces effects in the range between 75 and 100%. Thus, for some weed species it is not possible to estimate dose response curve and ED_{90} dosages based on efficacy tests. The efficacy experiments are carried out in different years and on different locations, i.e. experimental conditions can vary significantly. Sometimes this is reflected in pronounced variations in the effects of particularly the lower herbicide dosages which may also cause problems when fitting dose response to the the data. Consequently the standard errors of some of the ED90 dosages are quite high and in some cases the ED90 dosages are not significant different from 0. However due to the high number of trials the results can give an indication on differences in sensitivity of wild plants between and within families.

Of the 38 weed species that occurred in one or more of the efficacy trials, ED_{90} dosages could be estimated for 5 and 10 weed species, respectively, for metsulfuron-methyl applied to winter cereals in the autumn or in the spring, and for 13 weed species for metsulfuron-methyl applied in spring barley (Table 4.1). The corresponding figures for mecoprop-P were 10, 6 and 7 (Table. 4.2).

Metsulfuron-methyl

The ED_{90} dosages of metsulfuron-methyl varied from 0.23 g/ha on ***Papaver rhoeas*** to 16.6 g/ha on ***Polygonum aviculare***, i.e. the most sensitive species was killed by a dosage 72 times lower than the least sensitive species (Table 4.1). Some species were present both in winter and spring cereals and a comparison of the ED_{90} dosages within species also revealed large differences. For example, the estimated ED_{90} dosages of ***Stellaria media*** varied from 0.39 g/ha in spring barley to 3.94 g/ha in winter cereals (sping application), while the ED_{90} dosages of ***Myosotis arvensis*** varied from 0.87 in spring barley to 8.98 g/ha in winter cereals (autumn application), i.e. the variations within species were up to a factor 10. Most likely these differences could be ascribed to growth stage, but differences in climatic conditions may also have contributed to the observed differences.

Table 4.1. Estimated ED90 dosages of metsulfuron-methyl expressed as g/ha metsulfuron-methyl applied in winter cereals in the autumn or spring and in spring cereals. Figures in parantheses are standard errors

Winter cereals Autumn application		Winter cereals Spring application		Spring cereals	
Species	ED_{90} dose (g a.i./ha)	Species	ED_{90} dose (g a.i./ha)	Species	ED_{90} dose (g a.i./ha)
T. inodorum	1,44 (0,54)	B. napus	0.96 (0,30)	B. napus	1,91 (1,12)
C. bursa-pastoris	5,40(1,78)	G. tetrahit	0,38 (0,14)	C. bursa-pastoris	1,90 (1,14)
M. arvensis	8.98 (4,06)	G. aparine	2,62 (0,75)	C. segetum	10,06 (5,20)
S. media	1,02 (0,43)	L. communis	0.95 (0,34)	G. aparine	2,46 (1,73)
V. arvensis	6,70 (1.96)	M. arvensis	5,02 (2,29)	L. communis	0,47 (0,65)
		P. rhoes	1,18 (0,26)	T. inodorum	0,43 (0,28)
		P. convolvulus	2,26 (0,59)	M. arvensis	0,87 (0,71)
		S. media	3,94 (1,04)	P. rhoeas	0,23 (0,30)
		V. persica	6,04 (1,43)	P. aviculare	16,60 (11,04)
				P. convolvolus	8,06 (3,54)
				S. media	0,39 (0,29)
				V. persica	1,04 (0,47)
				V. arvensis	3,44 (2,22)

Mecoprop-P

The variation in ED_{90} mecoprop-P dosages between weed species varied from 61.6 g/ha (***Chenopodium album*** in spring barley) to 3521 g/ha (***Lamium purpureum*** in winter cereals in the autumn) (Table 4.2), i.e. the magnitude of variation between species (a factor of 57) was in the same range as found for metsufuron. Similar to metsulfuron-methyl, the variation within weed species was less than between species. For example, the ED_{90} dosage of ***Stellaria media*** only varied from 219 g/ha in spring cereals to 1205 g/ha in winter cereals treated in the spring, i.e. a factor of 5.5.

Table 4.2. Estimated ED90 dosages of mecoprop-P expressed as g mecoprop-P/ha applied in winter cereals in the autumn or spring and in spring cereals. Figures in parantheses are standard errors.

Winter cereals Autumn application		Winter cereals Spring application		Spring cereals	
Species	ED_{90} dosage (g a.i./ha)	Species	ED_{90} dosage (g a.i./ha)	Species	ED_{90} dosage (g a.i./ha)
B. napus	365,4 (141,8)	G. aparine	1464,6 (447,1)	C. album	61,6 (95,1)
C. bursa-pastoris	149,9 (71,4)	L. communis	2224,5 (832,5)	G. tetrahit	479,9 (524,2)
G. aparine	3419,6 (722,9)	T. inodorum	2999,8 (1067,1)	G. aparine	273,1 (228,3)
L. purpureum	3521,1 (731,6)	S. media	1204,6 (375,1)	M. noctiflora	269,1 (265,2)
T. inodorum	1065,4 (251,9)	V. hederifolia	1083,9 (405,6)	P. concolvolus	139,4 (154,3)
P. rhoeas	662.0 (202,2)	V. arvensis	2933,2 (1035,3)	S. media	219,0 (183,6)
S. media	472,4 (104,5)			V. arvensis	1239,1 (1017,7)
T. arvense	837,6 (152)				
V. persica	1952,4 (435,5)				
V. arvensis	3164,6 (852,5)				

For both herbicides, it can be concluded that variation between species were larger than within species. This suggests that differences in the inherent sensitivity of weed species are more pronounced than differences in sensitivity within species causes by different experimental conditions.

4.1.3 Summarizing analyses of existing toxicity data on terrestrial plants

In accordance with Boutin et al. (2004), the re-analyses of existing data from the PHYTOTOX and ECOTOX databases on sensitivity of crops and non-target plants to a number of commonly used herbicides suggest that crops are less sensitive than non-target plants. The sensitivity of the two groups of plants, however, was only significant for glyphosate. For the rest of the herbicides, including bromoxynil, dicamba, metolachlor and pendimethalin, the two groups did not differ significantly in sensitivity, but a nonsignificant trend of crops being less sensitive than wild plants were found. The sensitivity data, however, originated from many different experiments. Although the experiments have been carried out in accordance with standard procedures for toxicity tests, the experimental conditions may vary considerably and it could not be excluded that differences in experimental conditions render the differences in sensitivity.

4.2 Dose-response experiments with crops and non-target species

4.2.1 Dose-response on selected crop species and two non-crop species

The sensitivity of 10 crop species (see Table 3.3, p. 23) and two non-crop species (***C. cyanus*** and ***P. rhoeas***), the latter in common with the study of Boutin ***et al.*** (2004), to the three herbicides bromoxynil, glyphosate and metsulfuron-methyl was tested.

Comparison of results on two non-crop species

The estimated ED_{50} dosages for the two non-crop species are shown in Table 4.3. For both plant species, the ED_{50} dosages of metsulfuron-methyl are significantly lower in the present study compared to the ones reported in the study by Boutin and co-workers, while ED_{50} dosages for the other herbicides are similar.

Table 4.3. ED50 dosages (g a.i./ha) for corn flower (C. cyanus) and corn poppy (P. rhoeas) in Boutin et al. (2004) and in the present study. Figures in parentheses are 95% confidence intervals

Herbicide	Plant species	ED_{50} (g a.i./ha)	
		Boutin et al. 2004	Present study
Bromoxynil	C. cyanus	17.2 (14.0-21.5)	14.9 (11.9-17.9)
	P. rhoeas	56.9 (39.2-78.0)	26.8 (16.9-36.7)
Glyphosate	C. cyanus	29.2 (23.3-27.3)	32.7 (26.5-38.8)
	P. rhoeas	18.5 (13.1-25.1)	24.8 (20.3-29.3)
Metsulfuron methyl	C. cyanus	1.62(0.68-2.83)	0.13 (0.05-0.17)
	P. rhoeas	0.04 (0.04-0.06)	0.02 (0.01-0.02)

In conclusion, the results of the two studies are comparable for bromoxynil and glyphosate, whereas the sensitivity of the species to metsulfuron-methyl can be expected to be higher in the present study than in the study by Boutin et al. (2004).

ED_{50} for ten crop speices and two non-crop species

The estimated ED_{50} dosages of the different herbicides and plant species are shown in Figure 4.2 (A-D). For bromoxynil, the ED_{50} dosage varied from 7.5 to 38 g a.i./ha on the broadleaved species with lettuce, cucumber and buckwheat being the most sensitive species (Fig. 4.2B). The tolerance of the monocot species was much higher with ED_{50} dosages, ranking from 300 g a.i./ha on onion to 4066 g a.i./ha for maize (Fig 4.2A)**.** The high tolerance of monocot species is not surprising, as bromoxynil is registered for control of broadleaved weeds in monocot crops such as maize, cereals and onions. Boutin et al. (2004) did not include any monocot species in their study and reported ED_{50} dosages from 8.7 to 78 g a.i./ha for bromoxynil on broadleaved non-crop species. In conclusion, the interval of ED_{50} for broadleaved crop species did not deviate significantly from the ED_{50} dosages on broadleaved non-target species in the study by Boutin et al.

The ED_{50} dosages of glyphosate ranked from 1.6 g a.i./ha for sunflower to 84.6 g a.i./ha for onion (Figure 4.2 C). In the study by Boutin et al. (2004) on non-target species, the ED_{50} dosages of glyphosate laid in the interval from 14 to 65 g a.i./ha. Onion was the only crop species in the present study that was less sensitive to glyphosate than found by Boutin et al. for the non-crop species.

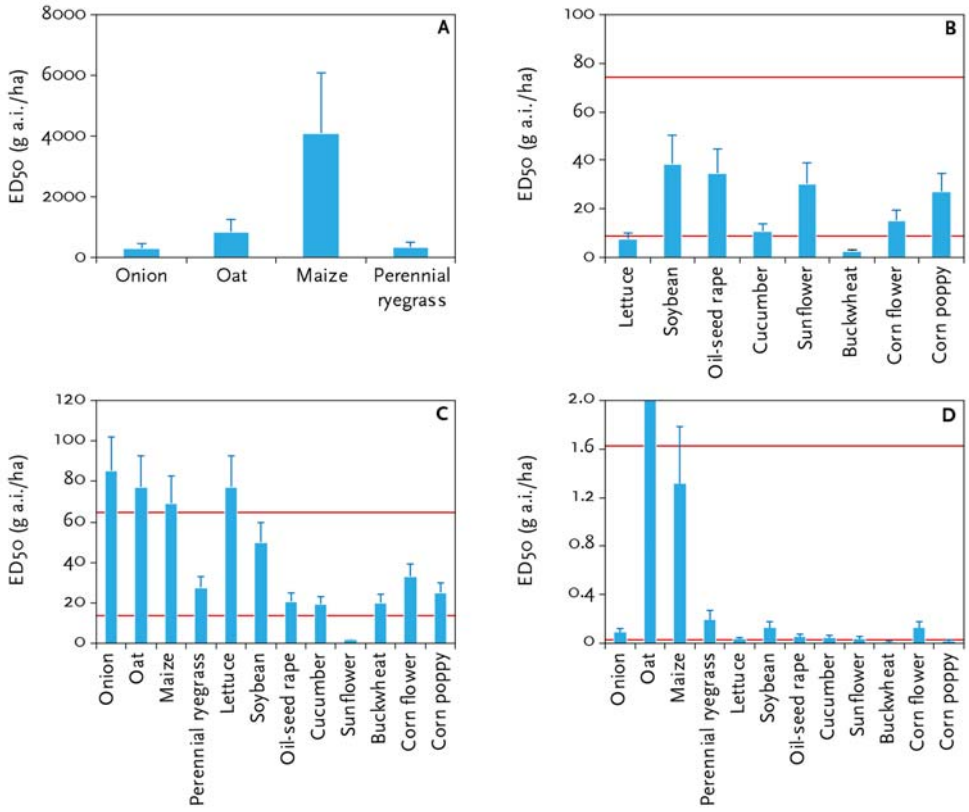


Figure 4.2. ED50 dosages (g a.i. ha-1) of bromoxynil on monocot crops (A) and dicot crop and non-crop species (B), and of glyphosate (C) and metsulfuron-methyl (D) on both crop species and two non-crop species. Lines show 95% confidence intervals. Horizontal red lines indicate the minimum and maximum values obtained in the study of Boutin et al., 2004.

Figure 4.3 Test of the sensitivity of oat (top), lettuce (middle) and *P. rhoeas* (bottom) to bromoxynil. The dosages on oat from left to right: 0, 375, 750, 1500, 3000 and 6000 g a.i./ha. On lettuce the dosages are: 0, 3.5, 7, 14 and 28 g a.i./ha and on *P. rhoeas*: 0, 3, 6, 12, 23.5 and 47 g a.i./ha.

Buckwheat and lettuce were the most sensitive crop species to metsulfuron-methyl with an ED_{50} of 0.012 and 0.037 g a.i./ha. In the opposite end of the scale was oat and maize, found to be the most tolerant crop species. The ED_{50}

of oat was 8.4 (out of scale in the figure) and for maize the ED_{50} was 1.3 g a.i./ha. The minimum and maximum ED_{50} dosages obtained by Boutin et al. for non-target species were 0.0236 and 1.625 g a.i./ha, respectively. Taking into account that the responses of ***C. cyanus*** and ***P. rhoeas*** to metsulfuron-methyl indicated a higher sensitivity in the present experiment compared to Boutin et al., the results indicate that crop species are less sensitive to metsulfuron-methyl than the non-crop species.

4.2.2 Dose-response experiments with non-crop species

Comparison of ED_{50} dosages on test pairs using biomass as end-point

The ED_{50} dosages were used to compare the sensitivity of the plant species. The relationship between biomass and dosages was described using the dose-response model shown in section 3.1.2. The model was fitted to the datasets of each herbicide and plant combination and the ED_{50} dosages were estimated for each combination of herbicide, plant species and growth stage (Table 4.4). In some cases, it was not possible to estimate the dose response curve due to a too low efficacy of the applied dosages. Consequently, it was not possible to estimate an ED_{50} dosage. In these cases, the ED_{50} dosages are denoted as larger than (>) the maximum applied dosage. In other cases, the plants were fully developed when sprayed at the reproductive stage and the untreated plants did not increase their biomass from the time of application until harvest (***G. robertianum***). This shows that although biomass reduction is highly relevant from an ecological viewpoint, it can be difficult to use in dose-response experiment at late growth stages.

Figure 4.4. Efficacy of mecoprop-P on *S. vulgare* (back row) and *S. noctiflora* (front row). From left to right: 0, 18.9, 37.5, 75, 150, 300, 600 and 1200 g a.i./ha. Photo: S. Mathiassen.

Mecoprop-P: The ED_{50} dosages were in the interval from 25 to 705 g a.i./ha at the vegetative growth stage and from 180 to more than 4800 g a.i./ha at the reproductive stage. ***A. millefolium*** was the most sensitive species at both growth stages. ***G. robertianum*** was very sensitive at the early growth stage, but it was the most tolerant species at the reproductive development stage mainly because the plants were fully developed when they were sprayed and, consequently, the dose-response curve was very flat.

Glyphosate: The sensitivity of the species to glyphosate differed less than for mecoprop-P. The min/max of the ED_{50} dosages were respectively 29 and 130.9 g a.i./ha at the vegetative stage and 67 and >2800 g a.i./ha at the reproductive stage. The most sensitive species were ***A. millefolium*** and ***G. molle*** and the least sensitive species was ***G. robertianum*** at the early as well as the late growth stages.

Metsulfuron-methyl: Similar to the responses to mecoprop-P and glyphosate ***A. millefolium*** was the most sensitive species at both development stages. The ***Geranium*** species were also moderately sensitive to metsulfuron-methyl, while ***S. vulgaris*** was the most tolerant species.

Table 4.4. Sensitivity of 6 plant species (3 annuals and 3 perennials) to mecoprop-P, glyphosate and metsulfuron-methyl . The table shows the dosages that are needed for a 50% reduction in biomass (mean ED50 dosages (g a.i./ ha) and range given within the brackets) when applied on the vegetative (Veg) and reproductive (Rep) stages, respectively.

Herbi-cide	Growth stage	Annual plant species	ED_{50} (g a.i./ ha)	Perennial plant species	ED_{50} (g a.i. /ha)
Mechlorprop-P	Veg	Tripleurospermum inodorum	705.0 (214.7-1195.3)	Achillea millefolium	24.9 (8.0-41.7)
		Silene noctiflora	77.3 (48.1-106.6)	Silene vulgaris	178.4 (105.1-251.8)
		Geranium molle	139.0 (78.2-199.8)	Geranium robertianum	57.8 (20.6-95.1)
	Rep	Tripleurospermum inodorum	>2400	Achillea millefolium	179.0 (113.7-244.3)
		Silene noctiflora	699.3 (370.9-1027.8)	Silene vulgaris	451.5 (239.8-663.3)
		Geranium molle	819.6 (-84.6-1723.0)	Geranium robertianum	>4800*
Glypho-sate	Veg	Tripleurospermum inodorum	76.8 (52.1-101.4)	Achillea millefolium	36.4 (24.4-48.5)
		Silene noctiflora	74.7 (52.6-96.7)	Silene vulgaris	67.4 (47.1-87.7)
		Geranium molle	28.6 (6.9-50.3)	Geranium robertianum	130.9 (60.7-210.0)
	Rep	Tripleurospermum inodorum	357.9 (234.9-480.7)	Achillea millefolium	67.5 (41.7-93.2)
		Silene noctiflora	155.0 (92.9-217.1)	Silene vulgaris	83.3 (50.6-115.9)
		Geranium molle	67.6 (32.2-102)	Geranium robertianum	>2880.0*
Metsul-furon	Veg	Tripleurospermum inodorum	0.44 (0.17-0.71)	Achillea millefolium	0.14 (0.09-0.19)
		Silene noctiflora	0.76 (0.55-0.97)	Silene vulgaris	>2.00
		Geranium molle	0.04 (-0.10-0.20)	Geranium robertianum	0.34 (0.24-0.44)
	Rep	Tripleurospermum inodorum	>4.00	Achillea millefolium	0.67 (0.34-1.00)
		Silene noctiflora	>4.00	Silene vulgaris	>16.00
		Geranium molle	0.99 (0.70-1.30)	Geranium robertianum	>8.00

* This species was fully developed at the time of application and for biomass of the plants did not increase in the period from exposure to harvest. For that reason dose-response curves could not be estimated.

Sensitivity of annual weed species and perennial non-target plants using biomass as end-point

The biomass results support our hypothesis that the sensitivity of non-target plants does not vary from the sensitivity of annual weed species from the same family (Table 4.4). For one of the three groups of plant species, we found that the perennial non-target plant was more sensitive than the annual weed species to all three herbicides (***A. millefolium*** compared to ***T. inodorum).*** For the ***Silene*** species, the perennial non-target species (***S. vulgaris***) was more sensitive to mecoprop-P and glyphosate than the annual weed species (***S. noctiflora***) and, in contrast, the annual weed was more sensitive than the perennial plant species to metsulfuron-methyl. ***G. molle*** was more sensitive than ***G. robertianum*** in 4 cases, they were equally sensitive in 1 case and ***G. robertianum*** was the most sensitive in one case**.**

In order to get an overview of these results and compare the sensitivity of annual weed species and perennial non-target plants, the number of cases where i) the annual weeds were significantly more sensitive than the perennial non-target plants, ii) the sensitivity was similar and iii) the perennial non-target plants were significantly more sensitive than the annual plant species were listed (Tabel 4.5). The sum of cases in each category was almost equal, indicating that overall there was no difference in sensitivity of annual weeds and perennial non-target plants for the plants tested here.

Table 4.5. Number of cases where the sensitivity of annual species ($ED_{50(annual)}$) was significantly higher, similar to and significantly lower than the susceptibilty of the perennial plant species $ED_{50(perennial)}$ belonging to the same family.

	ED_{50}(annual) > ED_{50}(perennial)	ED_{50}(annual) = ED_{50} (perennial)	ED_{50}(annual)< ED_{50} (perennial)
Sensitivity to mecoprop-P	2	3	1
Sensitivity to glyphosate	2	2	2
Sensitivity to metsulfuron-methyl	1	2	3

Figure 4.5 shows the sensitivity of the species graphically. In general ***A. millefolium*** was the most sensitive species at both growth stages and ***G. robertianum*** was the most tolerant species at the reproductive stage. Except for these two species the ranking of species varied between herbicides showing that even for a non-selective herbicide like glyphosate the sensitivity of species differ.

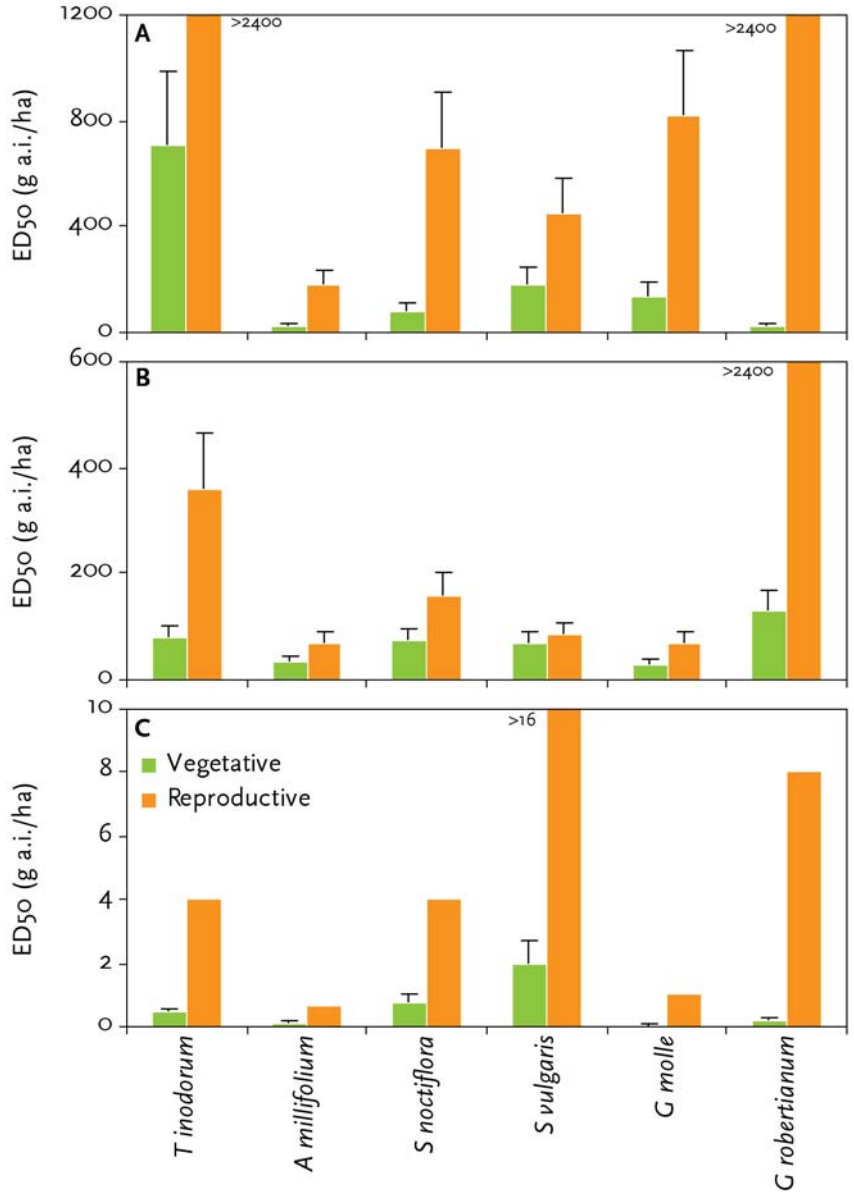


Figure 4.5 Sensitivity of different plant species at two development stages to MCPP (A), glyphosate (B) and metsulfuron-methyl (C). Efficacy measured on biomass.

In conclusion, plant sensitivity seems to be more species specific than dependent on grouping in annual weed species and perennial non-target species**.** However, in this study the herbicide application was carried out in the first year of growth of the perennial plants and the response may change when plants are exposed in the second or later years.

Species sensitivity at different growth stages using biomass as end-point
Regardless of plant lifespan, the species were frequently more sensitive to herbicide treatments at the vegetative compared to the reproductive development stage when using biomass as end-point (Table 4.6). This is in accordance to the common recommendation of increasing the dosage when controlling larger weeds.

Table 4.6. Number of cases where the sensitivity at the vegetative growth stage was significantly lower, similar to and significantly higher than the sensitivity at the reproductive growth stage for annual and perennial plant species belonging to the same family

	ED_{50}(vegetative) > ED_{50}(reproductive)	ED_{50}(vegetative) = ED_{50}(reproductive)	ED_{50}(vegatative)< ED_{50}(reproductive)
Sensitivity at different growth stages of annual plant species	0	2	7
Sensitivity at different growth stages of perennial plant species	0	3	6

Effects on seed production

Herbicides may influence seed production by reducing the number of seeds or by reducing seed weight. A low seed weight (=small seeds) again may influence germination negatively. In order to examine the influence of herbicide dosages on seed production, we recorded the number of seeds produced per pot and the 1000-seeds weight. From these parameters, the number of seeds per pot was calculated. Table 4.7 shows the 1000-seeds weights and number of seeds per plant in the control (untreated) pots.

Unfortunately, it was not possible to collect seeds of all species. ***A. millefolium*** did not produce any seeds and neither did the ***T. inodorum*** plants that were sprayed at the reproductive development stage. Seed production from ***T. inodorum*** sprayed at the vegetative growth stage was very low, and for this reason the results were not reliable. The most reasonable explanation for the lack of seed setting is that the experiment with these two species was conducted during the autumn without artificial light supply and the species need a long day for initiating the reproductive stage. An additional explanation could be the lack of pollinators, as the experiment was carried out in a glasshouse. In later experiments, we ensured pollination by placing a family of bees in the glasshouse from the beginning of flowering which improved the seed production on the Geranium species.

Table 4.7 Number of seeds per plant and 1000-seeds weight in control pots

	S. noctiflora	S. vulgare	G. molle	G. robertianum
1000-seeds weight	1.6-2.0	1.3	0.9	2.0
No of seeds/plant	990	750	1500	600

Figure 4.6 shows the mean seed weight of *S. noctiflora*, *S. vulgaris*, *G. molle* and *G. robertianum* after exposure to mecoprop-P, glyphosate and metsulfuron-methyl at different growth stages. The seed weight of *G. molle* had a high variability. With a few exceptions (*S. vulgaris* and *G. robertianum*, after late exposure) the seed weights of the other plant species were reduced by increasing dosages The results did not indicate a general trend in seed productivity being more affected by application at early or late growth stages.

The germination rate was examined on ***S. vulgaris*** and ***G. molle***. Seeds of ***S. vulgaris*** germinated by 88 to 100 % and none of the treatments had a significant effect on the germination rate. Seeds of ***G. molle*** had a very low germination rate, however, this was probably more related to seed dormancy and not to herbicide treatments as seeds from untreated plants also had a low germination. We were not able to show that a low seed weight influenced germination rate.

In general, the seed production data of all species showed a high variability between replicates, and in some cases the dosages were too high (MCPP on ***S. vulgaris*** and all herbicides on ***G. molle*** at the reproductive stage) and it was not possible to describe the dose response curves. Due to the high variability, we could only fit the dataset of seed production of ***S. noctiflora*** and ***S. vulgaris*** to the dose response model. The ED_{50} dosages are shown in Table 4.8. This table also shows the approximate estimated intervals for ED_{50} dosages on ***G. molle*** and ***G. robertianum*** that were estimated from the data shown in Figure 4.6.

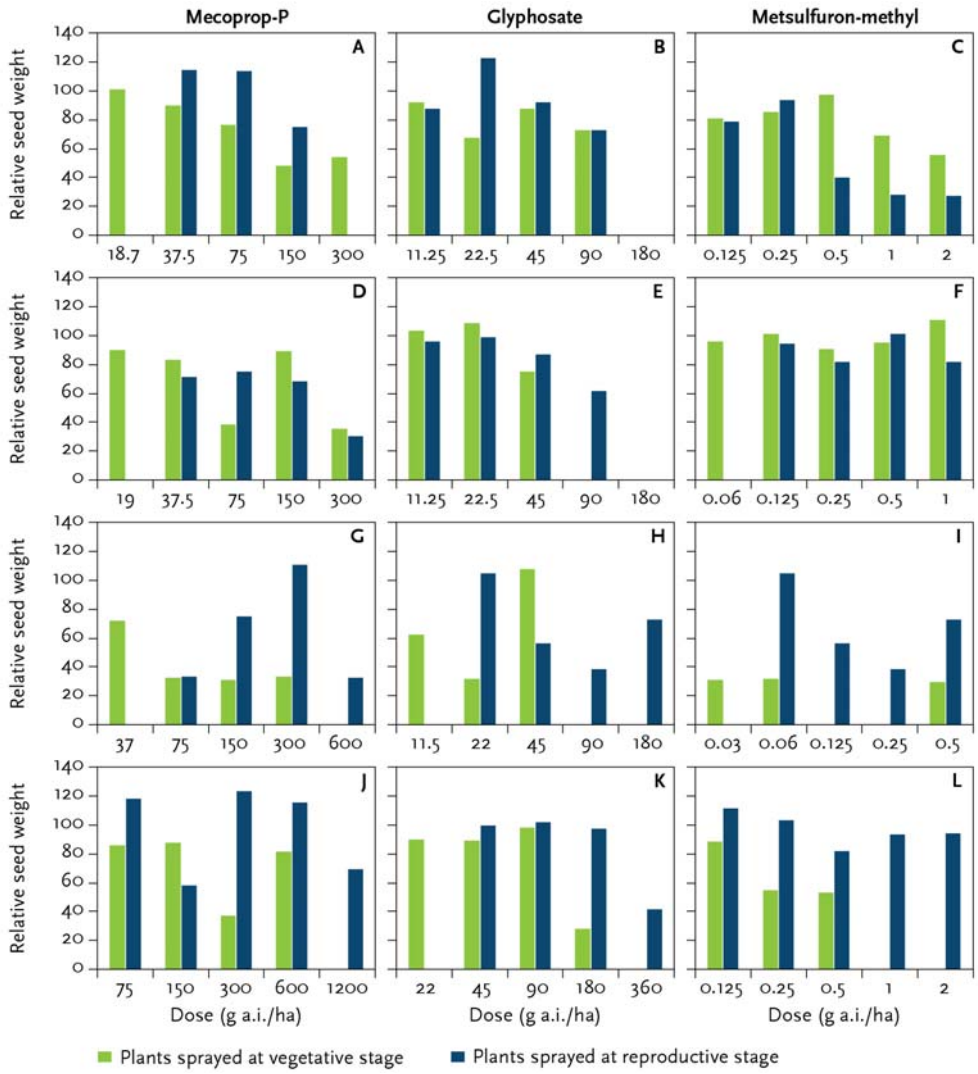


Figure 4.6 Efficacy of Mecoprop-P, glyphosate and metsulfuron-methyl on relative seed weights of Silene noctiflora (A-C), S. vulgaris (D-F), Geranium molle (G-I) and G. robertianum (J-L) after application at different development stages. The seed weight for untreated plants of each species and growth stage is set to 100.

Table 4.8. Sensitivity of 4 plant species (2 annuals and 2 perennials) to mecoprop-P, glyphosate and metsulfuron-methyl shown as the dosage needed for a 50% reduction in seed production (ED_{50} dosages (g a.i. /ha)) applied on the vegetative (Veg) and reproductive (Rep) stages, respectively.

Herbicide	Plant stage	Annual weeds	ED50 (g a.i. ha^{-1})	Perennial plants	ED50 (g a.i. ha^{-1})
Mecoprop-P	Veg	*Silene noctiflora*	38.1 (21.2-54.9)	*Silene vulgaris*	<20
		Geranium molle	App. 37.0-75.0	*Geranium robertianum*	App 150
	Rep	*Silene noctiflora*	48.8 (1.3-96.2)	*Silene vulgaris*	49.2 (16.0-82.4)
		Geranium molle	App. 37.0-75.0	*Geranium robertianum*	App. 300
Glyphosate	Veg	*Silene noctiflora*	87.2 (51.7-122.7)	*Silene vulgaris*	37.6 (17.7-57.4)
		Geranium molle	App. 22.0-45.0	*Geranium robertianum*	App. 110
	Rep	*Silene noctiflora*	43.1 (20.9-65.4)	*Silene vulgaris*	App. 11-45
		Geranium molle	App. 0-22.0	*Geranium robertianum*	App. 120
Metsul-furon	Veg	*Silene noctiflora*	0.3 (0.2-0.5)	*Silene vulgaris*	0.95 (0.54-1.36)
		Geranium molle	App. 0-0.03	*Geranium robertianum*	0.20
	Rep	*Silene noctiflora*	0.31 (0.14-0.47)	*Silene vulgaris*	5.76 (1.47-10.10)
		Geranium molle	App. 0.06-0.13	*Geranium robertianum*	App. 1.20

Selectivity of annual weed species and perennial non-target plants at different growth stages using seed production as end-point

Table 4.9 compares herbicide selectivity of annual and perennial plants species and Table 4.10 compares the sensitivity of different growth stages using seed productivity as an end-point. The tables complement table 4.5 and 4.6 which compare the same factors using biomass as end-point.

Table 4.9. Comparison of herbicide sensitivity of taxonomically closely related annual and perennial species indicated by the number of cases where the sensitivity the annual species was significantly lower, similar to and significantly higher than the sensitivity of the perennial species when seed production per pot was used as end-point.

	ED_{50}(annual) > ED_{50}(perennial)	ED_{50}(annual) = ED_{50} (perennial)	ED_{50}(annual)< ED_{50} (perennial)
Sensitivity to Mecoprop-P	0	2	2
Sensitivity to glyphosate	0	2	2
Sensitivity to metsulfuron-methyl	0	0	4

Table 4.10. Comparison of herbicide sensitivity at the vegetative and the reproductive stages of taxonomically closely related annual and perennial plant species indicated by the number of cases where sensitivity at the vegetative stage was significantly lower, similar to and significantly higher than the sensitivity at the reproductive growth stage when seed production per pot was used as end-point.

	ED_{50}(vegetative) > ED_{50}(reproductive)	ED_{50}(vegetative) = ED_{50} (reproductive)	ED_{50}(vegetative)< ED_{50} (reproductive)
Sensitivity at different growth stages of annual plant species	2	3	1
Sensitivity at different growth stages of perennial plant species	0	2	4

In contrast to the analysis based on effects on biomass (se Tabel 4.4), we found small or no differences in sensitivity among species exposed to the herbicides at the vegetative and the reproductive stages, respectively, using seed production as end-point (Tabel 4.8). Likewise, there were only small or no differences in sensitivity between taxonomically closely related annual and perennial species.

We found that in most cases the ED_{50} dosages of biomass was higher than the ED_{50} on seed production for both annual and perennial species (Tabel 4.11; Fig. 4.7) indicating that seed production is a more sensible end-point than biomass.

Table 4.11. Comparison of the two end-points biomass and seed production indicated by the number of cases where the sensitivity using biomass as end-point was significantly lower, similar to and significantly higher than the sensitivity using seed production as end-point for taxonomically closely related annual and perennial species.

	ED_{50}(biomass) > ED_{50}(seed prod.)	ED_{50}(biomass) = ED_{50} (seed prod.)	ED_{50}(biomass)< ED_{50} (seed prod.)
Sensitivity on annual plant species	8	4	0
Sensitivity on perennial plants	8	3	1

This result contrasts to previous studies (Asman et al., 2001) and may be highly important for the performance and fitness of specifically plant species that need to produce viable seeds every year to persist in the vegetation and in addition go through the sensitive early stages to stay as part of the flora. Furthermore, it makes these species more sensitive to herbicides than can be expected from the outcome of a risk assessment based on biomass data. See general discussion, p. 87, for a thorough discussion of the implications of the result.

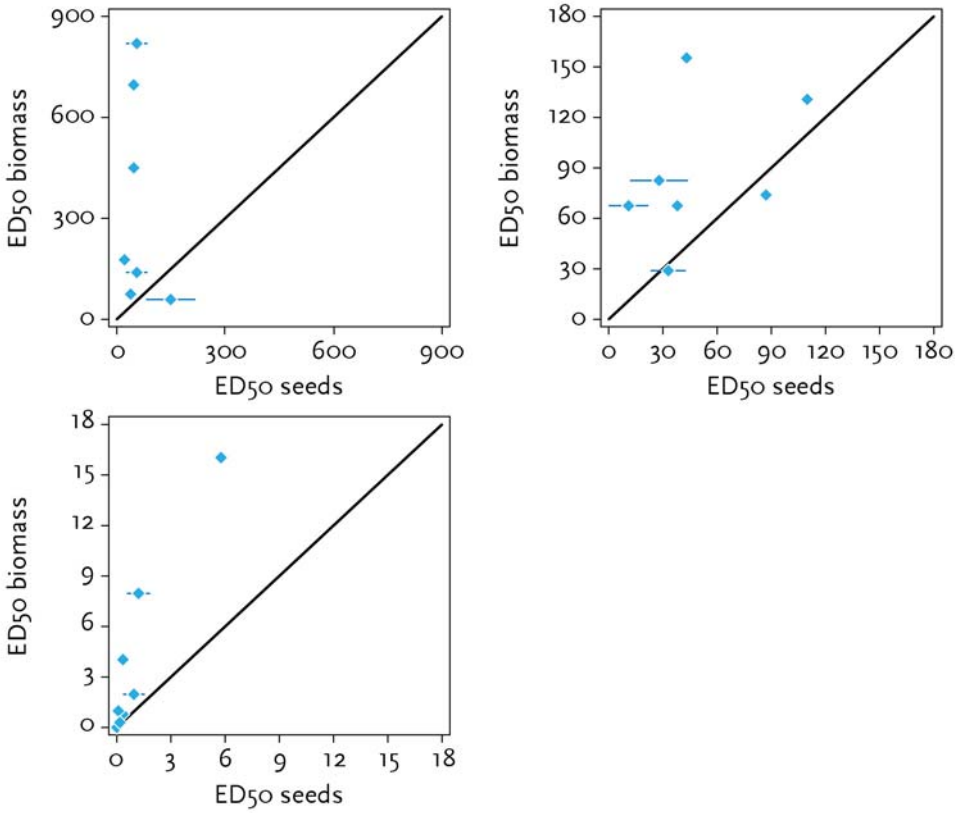


Figure 4.7. Comparison of biomass and seed production as end-points for sensitivity of *Geranium molle, G. robertianum, Silene noctiflora* and *S. vulgaris* to three herbicides. Top, left = mecoprop-P, top, right = glyphosate and bottom = metsulfuron-methyl. Each point represents a specific combination of weed species and growth stage. The straight line represents the isobole where the sensitivity of the paramaters is similar. In cases where dose-response curve could not be estimated approximate values are indicated by a line from minimum to maximum through the data point.

Figure 4.8 *Geranium robertianum* at the early and late flowering stage. Photos: S. Mathiassen.

4.2.3 Sensitivity of plants to repeated exposure to herbicides

We tested the hypothesis that the effects of repeated herbicide exposure to sublethale dosages of herbicides with different modes of action do not differ from the Additive Dose Model (ADM) in two experiments carried out on ***S. noctiflora*** and ***S. vulgaris***. In one experiment, the effects of repeated treatments following a metsulfuron-methyl treatment were studied. The second experiment dealt with effects of repeated treatments following exposure to glyphosate.

Figure 4.9 shows a schematic illustration of possible interactions between two repeated exposures to herbicides. The ED_{50} of each application is plotted on the x- and y-axis and a straight line is drawn between them. This line is the ADM isobole of predicted responses – i.e. the dose composition of repeated applications that produce 50% effect provided that the responses follow ADM. If the observed ED_{50} points lie above the isobole, it shows that the efficacy of the repeated exposures is lower than predicted (antagonism). If the data points lie below the isobole, the efficacy of the repeated exposure is higher than predicted and the applications are synergistic.

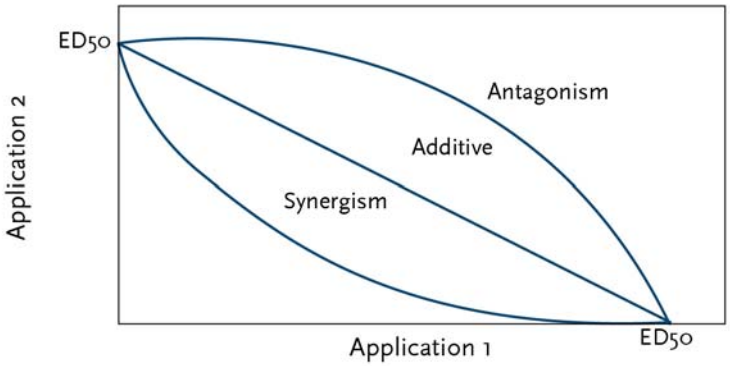


Figure 4.9 Schematic illustrations of additive, synergistic and antagonistic responses.

The results of our experiments are shown as isobolograms at the 50% effect level in Figure 4.10 and 4.11. The ADM isobolograms were constructed by plotting the ED_{50} values of application of herbicide 1 (=glyphosate or metsulfuron-methyl) at T2 (=timing 2) at the x-axis and the ED_{50} values of the 3 herbicides at T2 (=glyphosate, metsulfuron-methyl or mecoprop-P) along the y-axis and drawing a straight line between them. To accommodate the results of the various treatments in the same plot, the x- and y-axis were standardized so that the ED_{50} value of the herbicide applied singly at a specific growth stage was always fixed to 1. In the experimental set-up we have designed the repeated treatments in a way that ensures having different combinations of effect-rates from the two individual treatments. The observed ED_{50} dosages of the repeated treatments were plotted on the graph and compared to the ADM isobole.

No generally accepted procedure exists for testing for statistical significant deviations from ADM. In the present study, we examined whether the predicted ED_{50} dosages of the repeated treatments calculated by the model were contained in the 95% confidence interval of the estimated ED_{50} dosages derived from the model. This approach inevitably overestimates the number of significant deviations because it does not incorporate the variation around the isobole. Significant deviations were termed antagonism if higher and synergism if lower than the corresponding estimated ED_{50} dosages.

Figure 4.10 shows the isobolograms of repeated applications with metsulfuron-methyl as the first treatment. On ***S. noctiflora,*** five of the treatments had a higher effect than predicted by ADM (synergistic), while four treatments did not deviate from ADM, i.e. they were additive. The additive treatments included the combined treatments in which the T1 metsulfuron-methyl treatment had a high ratio. Five of the treatments were also synergistic on ***S. vulgaris***, one followed ADM and 3 were antagonistic. Two of the antagonistic treatments were composed of a high ratio metsulfuron-methyl at T1. In summary, most of the treatments were additive or synergistic. The results indicate that plants that are already affected by a low metsulfuron-methyl dosage can be more sensitive to later herbicide treatments. On the other hand, higher dosages of metsulfuron-methyl reduce the sensitivity to later treatments, probably by reducing the metabolic processes in the plants.

Figure 4.11 shows the isobolograms of repeated treatments with glyphosate as the first treatment. Most treatments were synergistic, 3 treatments were antagonistic and one treatment followed ADM on ***S. noctiflora*** while all

treatments were synergistic on ***S. vulgaris***. It seems like low dosages of glyphosate may enhance plant sensitivity to later herbicide treatments. This is in contrast to the findings of Cedergren et al (2007) who evaluated 10 binary mixtures of nine herbicides representing the most commonly used molecular target sites for controlling broadleaved weeds. The joint effect of two mixtures of herbicides with the same mode of action was additive. Approximately 70% of the mixtures with different sites of action showed significant antagonism while no synergistic interactions were observed. In this study, however, the herbicides were applied at the same time and not in sequences.

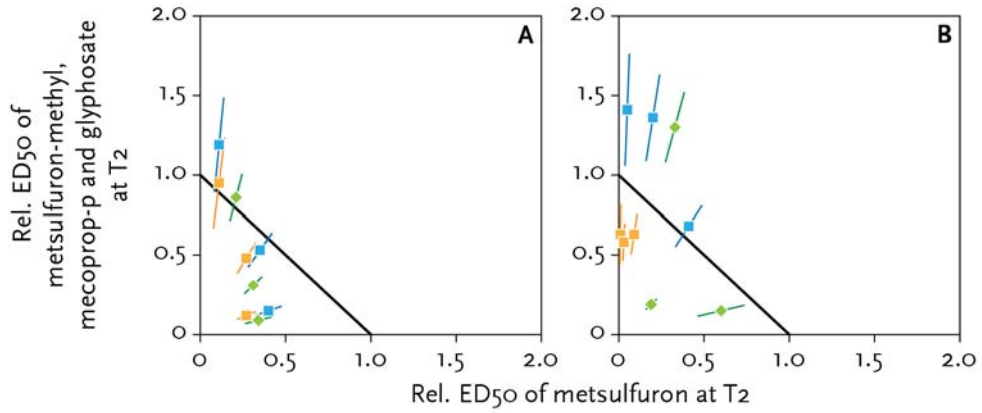


Figure 4.10. ADM isobolograms and estimated dosages for repeated treatments with metsulfuron-methyl at the first timing (T1) followed by metsulfuron-methyl (green), mecoprop-P (orange) and glyphosate (turkis) at the second timing (T2). Figure A shows results on S. noctiflora and figure B shows results on S. vulgaris. Bars indicate standard errors. The x- and y-axes are standardized so the ED50 dosages of the herbicides applied separately at T2 are fixed to 1.

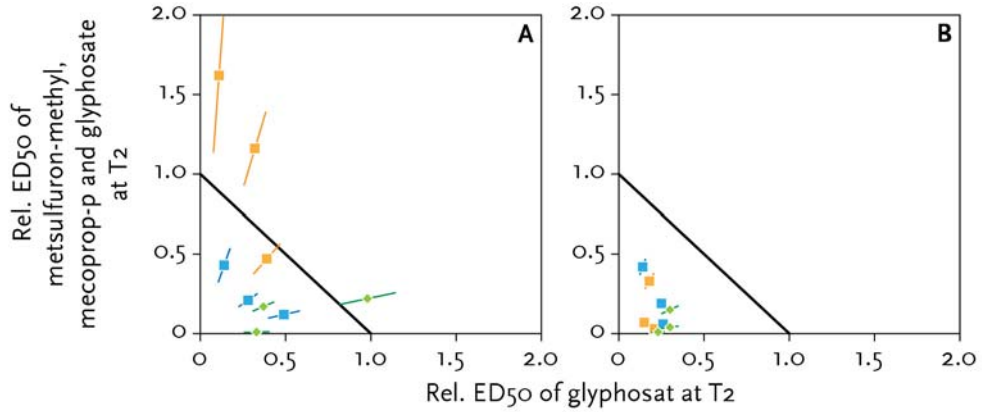


Figure 4.11. ADM isobolograms and estimated dosages for repeated treatments with glyphosate at the first timing (T1) followed by metsulfuron-methyl (green), mecoprop-P (orange) and glyphosate (turkis) at the second timing (T2). Figure A shows results on S. noctiflora and figure B shows results on S. vulgaris. Bars indicate standard errors. The x- and y-axes are standardized so the ED50 dosages of the herbicides applied separately at T2 are fixed to 1.

4.3 Exposure experiments in spraying chamber and agricultural field

4.3.1 Droplet size distribution

Visual expection of the water-sensitive papers clearly show that the droplets hitting plants, curlers and water-senstive paper in the spray chamber are much larger than the droplets drifting from the tractor in the field (Figure 4.12). The figure also shows that in the field exposure (total sprayed area) is higher close to the tractor.

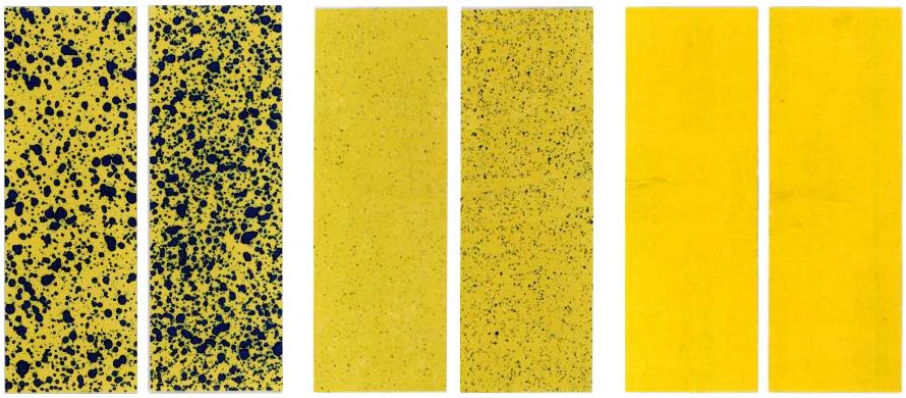


Figure 4.12. Water-sensitive paper exposed in spray chamber (left) and in the field, 0 m from the tractor (centre) and 36 m from the tractor (right) measured as distance from the neares end of the spraying boom.

A more detailed view of the distribution of droplet diameters is presented in Figure 4.13 for both field and spray chamber exposure.

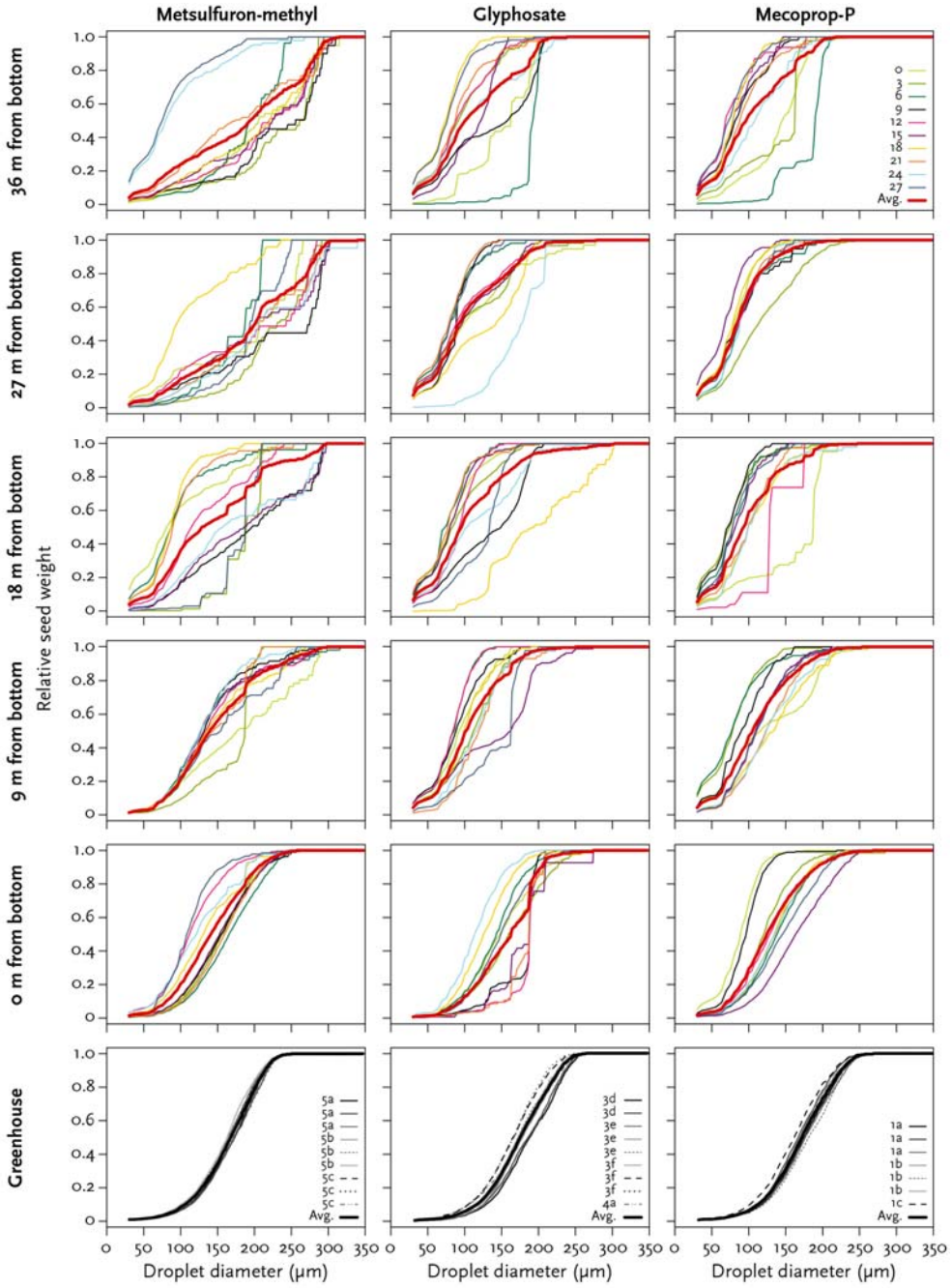


Figure 4.13. Cumulative droplet volume as function of droplet diameter for water-sensitive papers exposed in spray chamber (lower row) and in the field at different distances from the tractor (measured as distance to nearest end of the spraying boom). For the field samples, numbers 0 to 27 indicate the distance (m) going from the North edge of the field, parallel to the direction of the tractor (c.f. Figure 3.5), and mean values are shown as red lines. For the green house samples, the different lines show data for different replicates and pesticide concentrations, with averages shown as bold lines.

Comparison of e.g. the average droplet diameters representing a cumulative volume of 50 % confirms that droplets were generally larger in the spray chamber experiment. Furthermore, average droplet size decreases at increasing distance from the spraying boom, as expected. Droplet size also varies considerably at a given distance from the boom, probably as a consequence of variations in wind speed during the sparying events. It should, however, be noted that the digital analysis of the water-sensitive papers from

the greenhouse underestimates the proportion of large droplets, because very large droplets are identified as circles rather than spots.

4.3.2 Actual spray concentrations

In the field and spray chamber experiments actual herbicide and brilliant sulfaflavin tank concentrations varied as presented in Table 4.12. For all herbicide concentrations a brilliant sulfaflavin concentration of 200 mg/l was aimed at. Generally, there is a fairly good agreement between nominal and actual concentrations, but in the field experiments tank concentrations of mecoprop-P and metsulfuron-methyl were considerably lower than expected, and so were the concentrations of dye marker in the experiments with mecoprop-P and metsulfuron-methyl.

Table 4.12. Measured tank concentrations of herbicides and brilliant sulfaflavin in field and spray chamber experiments. Nominal concentrations are based on an output of 200 l/ha."l.r." = label rate

Herbicide	Exposure	Nominal herbicide concentration, mg/l	Measured dye concentration, mg/l	Measured herbicide concentration, mg/l
Mecoprop-P	Spray chamber, 0.5 % l.r.	90	210	88
	Spray chamber, 1 % l.r.	180	219	176
	Spray chamber, 2 % l.r.	360	185	324
	Spray chamber, 10 % l.r.	1,800	194	1780
	Spray chamber, 50 % l.r.	9,000	211	9940
	Field, 100 % l.r.	18,000	128	10,250
Glyphosate	Spray chamber, 0.5 % l.r.	0.5	208	19
	Spray chamber, 1 % l.r.	36	204	33
	Spray chamber, 2 % l.r.	72	210	64
	Spray chamber, 10 % l.r.	360	150	330
	Spray chamber, 50 % l.r.	1,800	112	1334
	Field, 100 % l.r.	3,600	193	3315
Metsulfuron methyl	Spray chamber, 0.5 % l.r.	0.094	222	0.17
	Spray chamber, 1 % l.r.	0.19	195	0.27
	Spray chamber, 2 % l.r.	0.38	206	0.48
	Spray chamber, 10 % l.r.	1.88	215	2.8
	Spray chamber, 50 % l.r.	9.38	205	12
	Field, 100 % l.r.	18.75	73	8.9

4.3.3 Exposure of curleres and leaves

Curler exposure to the three herbicides is shown in Figure 4.14. Field levels were lower than spray chamber levels. This is partly due to the lower tank concentrations in the field experiments (Table 4.12), but model calculations based on earlier experiements (Bruus et al. 2008) still predict a higher deposition because the high wind speed should increase the

spraydrift/deposition due to the spray being dispersed very soon after leaving the nozzles.

Irrespective of the low exposure levels in the field, there was a clear decrease in curler exposure with increasing distance to the tractor.

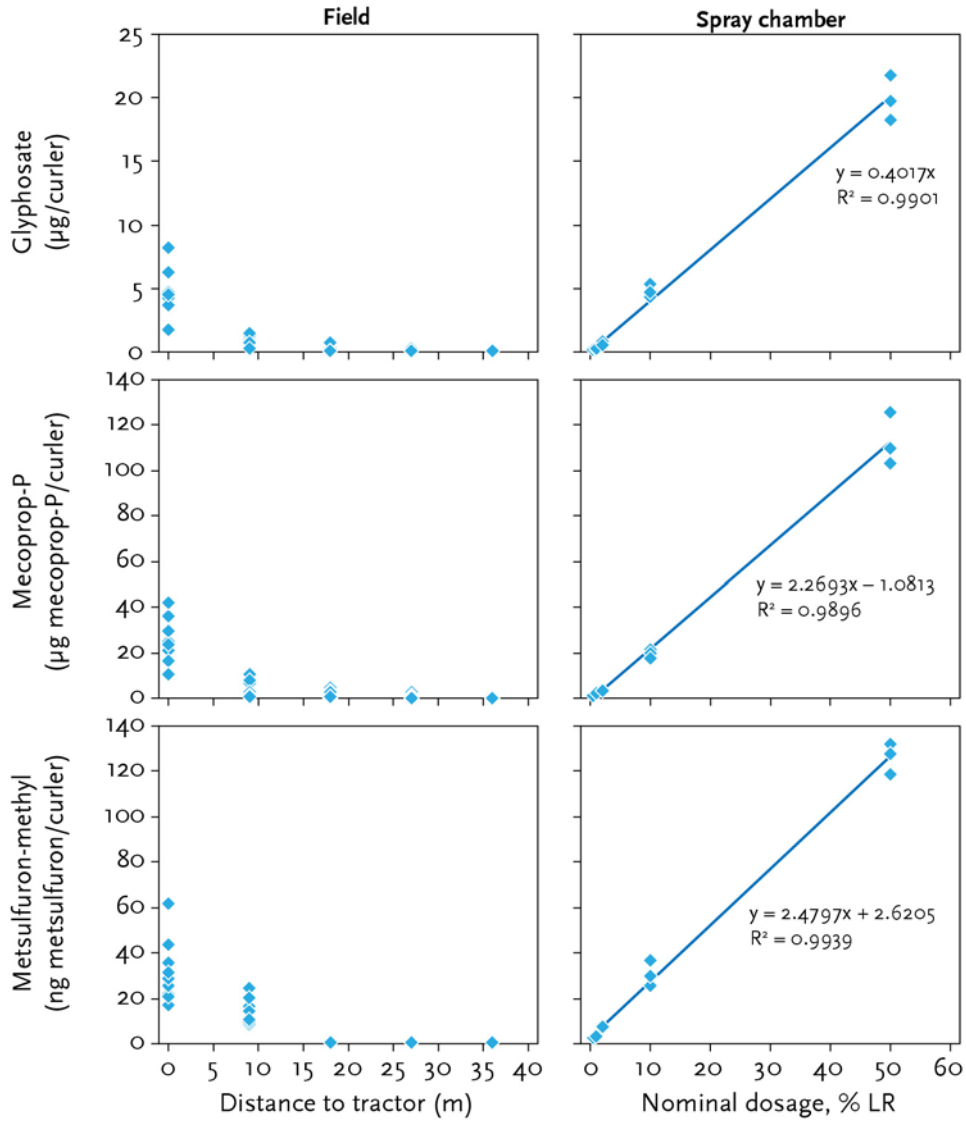


Figure 4.14. Exposure of curlers to glyphosate, mecoprop-P and metsulfuron-methyl methyl in field (left) and spray chamber (right).

In contrast, plant exposure hardly varied with distance to tractor, as shown in Figure 4.15.

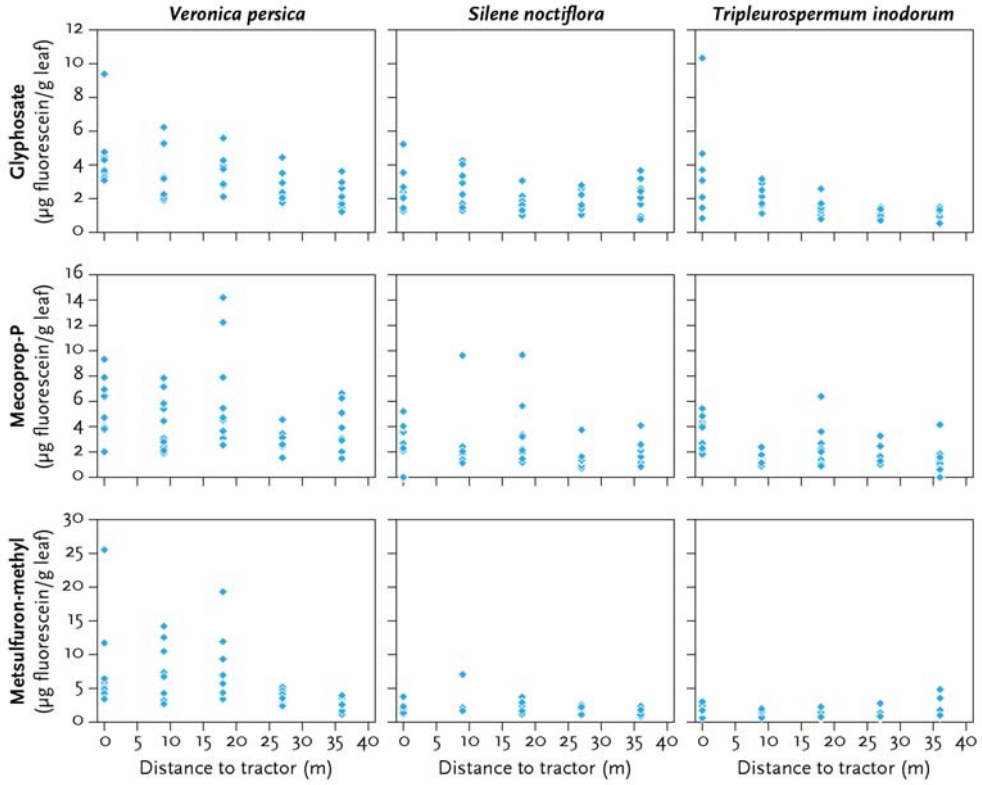


Figure 4.15. Plant exposure to dye marker (fluorescein) in the field as function of the distance to tractor. Three experiments were run, i.e. dye marker in combination with glyphosate , mecoprop-P and metsulfuron-methyl.

As expected from earlier experiments (Bruus et al. 2008), the relation between dye marker and herbicide deposition on curlers was fairly good, although not too convincing for glyphosate (Figure 4.16).

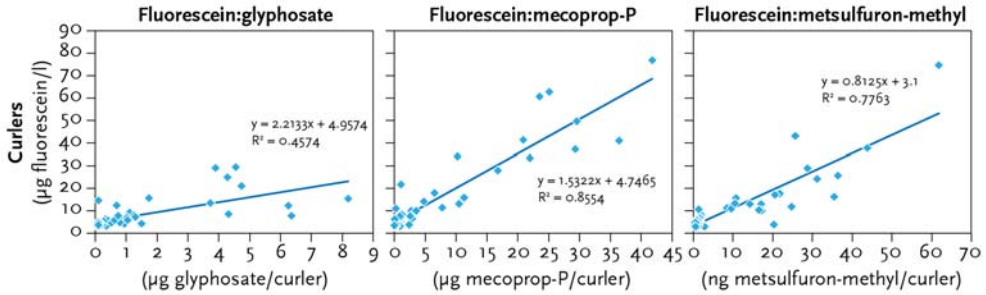


Figure 4.16. Relation between dyemarker (fluorescein) and glyphosate, mecoprop-P and metsulfuron-methyl) deposition on curlers exposed in field experiment.

However, the relations between dye deposition on leaves and curlers were very poor (Figure 4.17). This was rather unexpected, since earlier studies found good relations (Bruus et al. 2008). Possibly, the reason for the differences in exposure is the combination of fairly high wind speed and the physical layout of the experiment. The dye may have been blown over the pots, almost not hitting the plants, while the curlers, which extended more from the pots, were more exposed to the dye, as sketched in Figure 4.18.

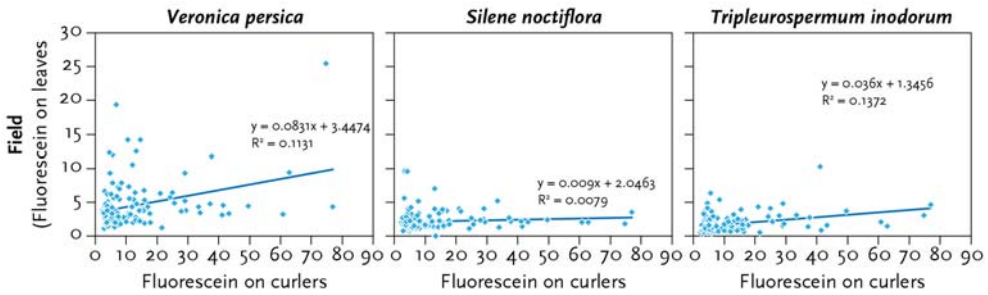


Figure 4.17. Relation between dyemarker (fluorescein) deposition on leaves (Veronica persica, Silene noctiflora and Tripleurospermum inodorum) and curlers exposed in field experiment.

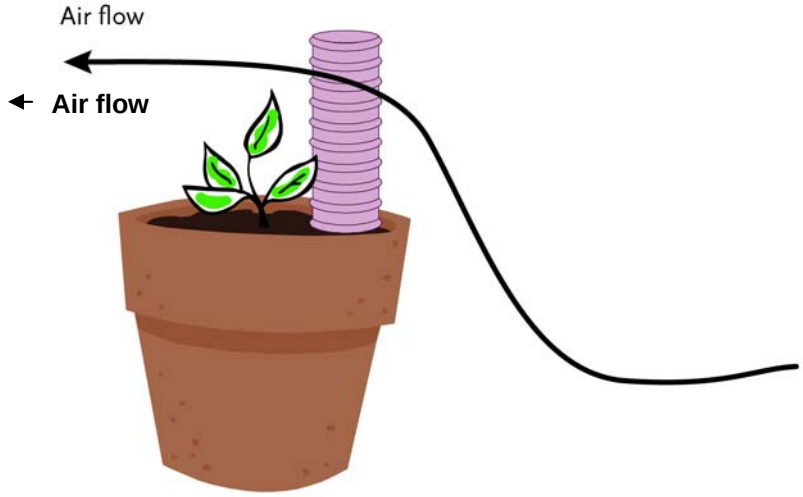


Figure 4.18. Outline of expected air flow over pot with plant and curler at high wind speed.

4.3.4 Effects on plants exposed in the spraying chamber and agricultural field

Plants exposed to the three herbicides in the spray chamber were clearly affected, as shown in Figure 4.19. Effects are expressed as function of curler exposure, because leaf exposure was not measured.

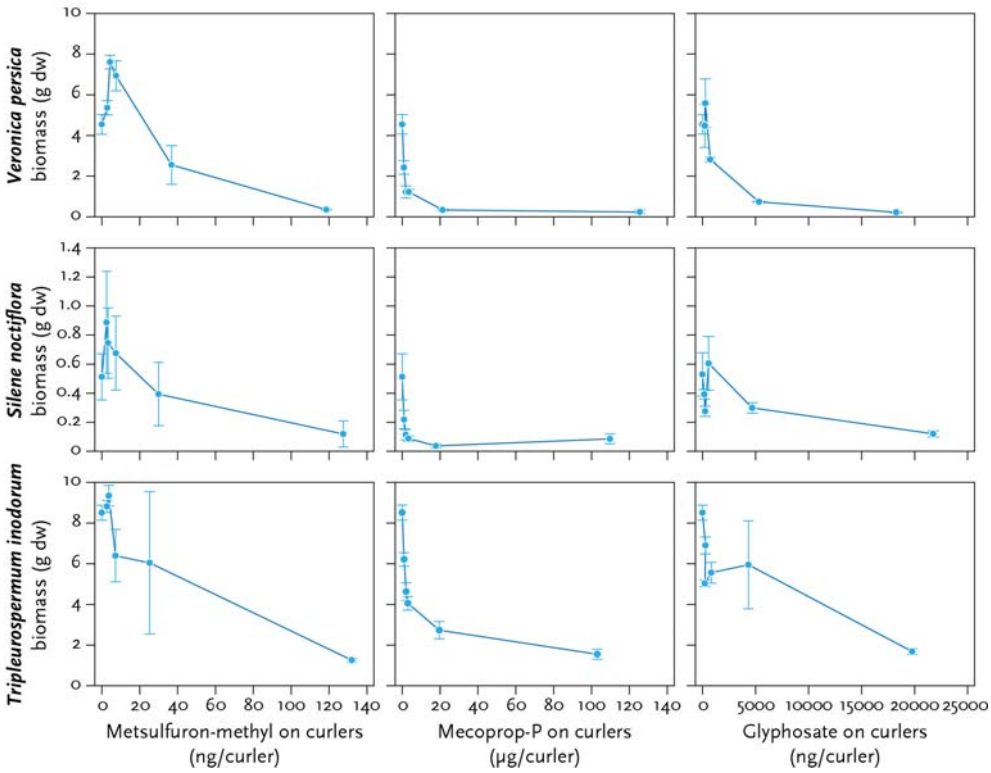


Figure 4.19. Mean aboveground biomass ± SEM of Silene noctiflora, Veronica persica and Tripleurospermum inodorum exposed to metsulfuron-methyl methyl, mecoprop-P and glyphosate in spray chamber. Curler exposure levels correspond to 0, 0.5, 1, 2 and 50 % label rate.

Assuming a good relation between dye marker and herbicide exposure of plants, Figure 4.20 shows that plants exposed to herbicides in the field were hardly affected. As shown above, plant exposure hardly varied with distance to tractor, and curler exposure in the field corresponded with spray chamber exposure levels that caused no or very small effects (cf. Figure 4.14, herbicide exposure of curlers in field and spray chamber).

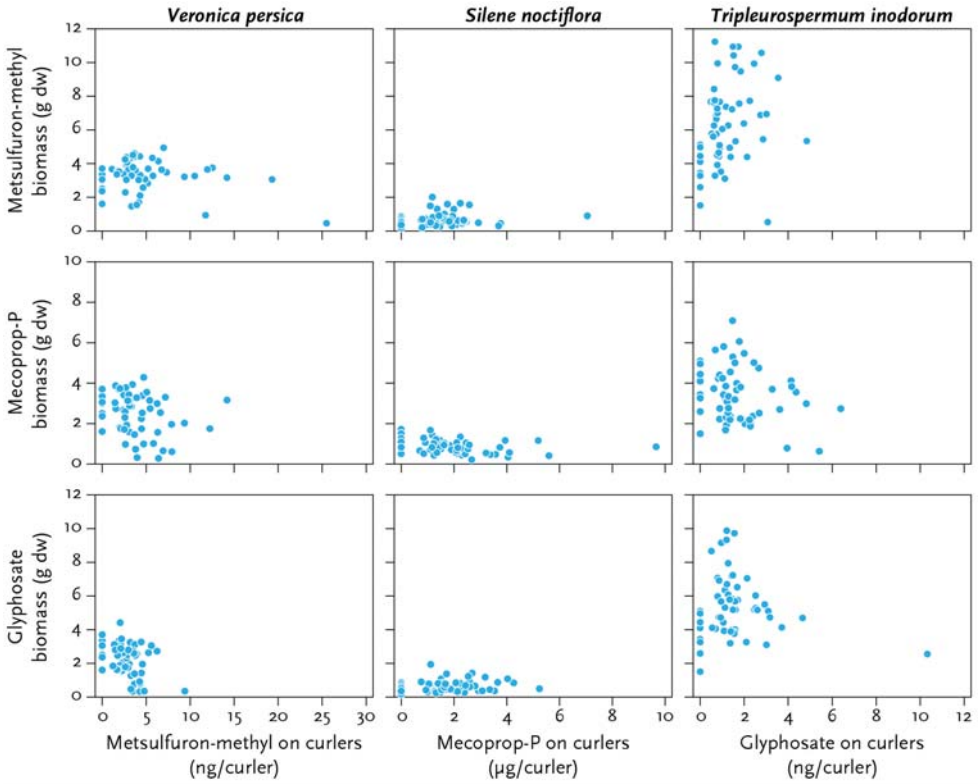


Figure 4.20. Mean aboveground biomass of Veronica persica, Silene noctiflora and Tripleurospermum inodorum exposed to metsulfuron-methyl, mecoprop-P and glyphosate in the field as function of dye marker (fluorescein) exposure of leaves.

4.4 Assessment of community effects of herbicide and nitrogen on experimentally established vegetation

4.4.1 Effects of glyphosate and nitrogen on species richness and species composition

Spraying experimentally established vegetation with glyphosate at concentrations relevant for estimation of effects of spray drift, i.e. respectively 0, 14.4, 72 and 360 g a.i./ha, equivalent to 0, 1, 5 and 25 % of recommended field dosage, affected the vegetation at the experimental plot visibly (Fig. 4.21). Although the effects of glyphosate concentrations of 14.4 and 72 g a.i./ha mainly were sublethal, glyphosate at these concentrations resulted in some visual effects e.g. curly, yellow-coloured or dead leaf lips. At 360 g a.i./ha, corresponding to 25 % of full rate, glyphosate resulted in mortality and dead plant material and uncovered soil was seen in all years (Fig. 4.22). Dead plant material and bare soil constituted a greater part of the coverage in 2008 than in previous years both before and after plot treatments, especially within plots receiving both glyphosate (360 g a.i./ha) and nitrogen (100 kg/ha) at the highest concentrations. The regrowth over the summer was good.

Figure 4.21. Glyphosate effects on the vegetation at the Kalø experimental plot. Un-treated plot (left) and plot that has received 360 g/ha glyphosate (right) two weeks after application. Both plots have received 100 kg N/ha. Photo: B. Strandberg.

Over the years, the vegetation has gradually changed both with respect to species richness (Figs. 4.23 and 4.24) and species composition (Fig. 4.22). Generally, the number of species decreased over the years and both application of nitrogen and glyphosate affected the species number negatively (Figure 4.23 and 4.24). However, at highest nitrogen level (100 kg N/ha) the application of glyphosate to some extent counteracts the negative effect of nitrogen (Figure 4.23).

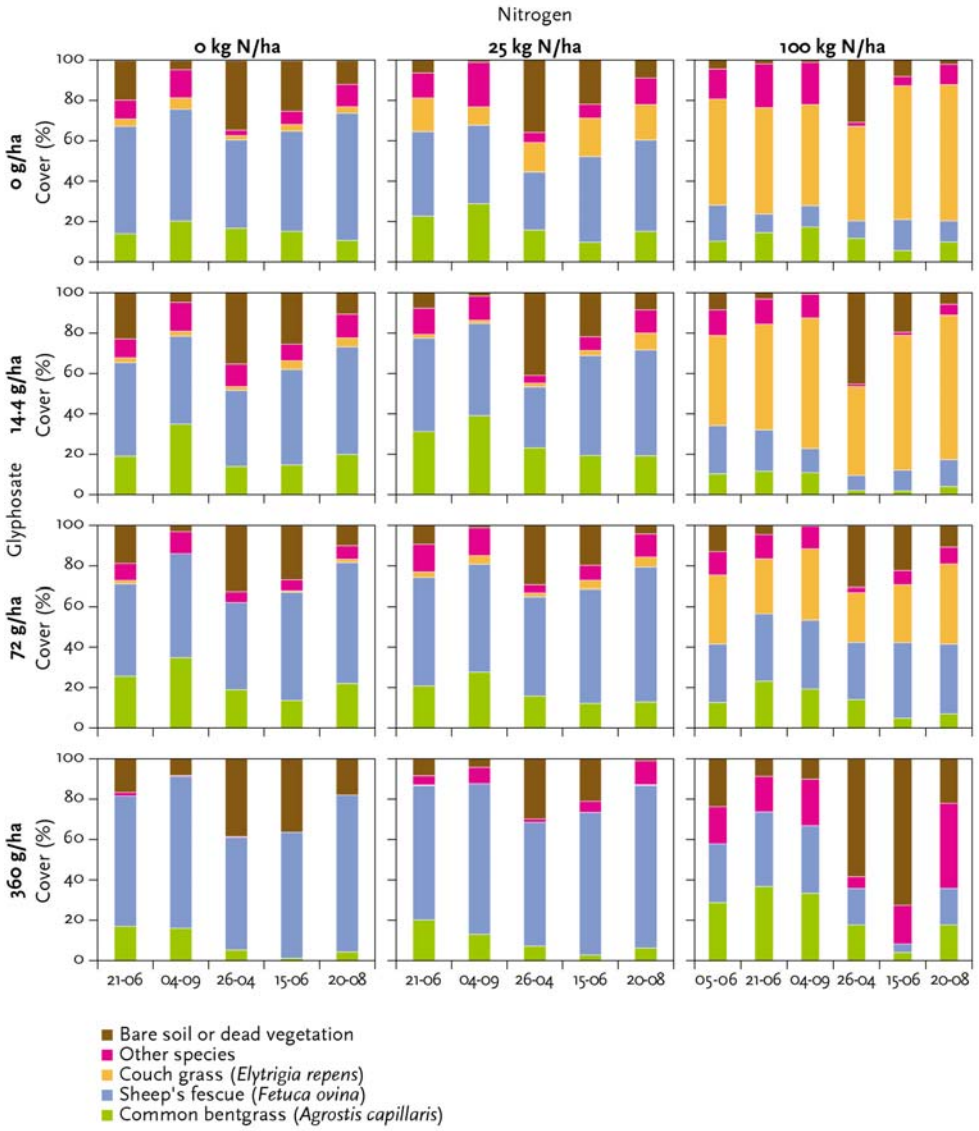


Figure 4.22. Species composition, during the season 2007 and 2008, shown as mean cover of five species: *Agrostis capillaris*, *Festuca ovina*, *Elytrigia repens*, a group comprising other species and finally soil and dead vegetation at experimental plots receiving a combination of the following treatments of Glyphosate (0, 14.4, 72 AND 360 g a.i./ha) and nitrogen (0, 25 and 100 kg N/ha)

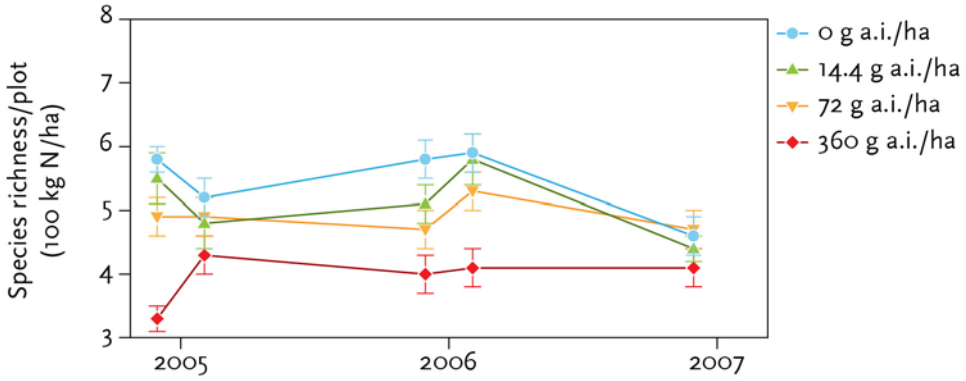


Figure 4.23. Species richness, i.e. number of species per 0.56m2 (mean ± SE), within the experimental plot Kalø in the period 2005-2007 based on sampling within randomly selected plots. Only data for plots receiving 100 kg N is shown.

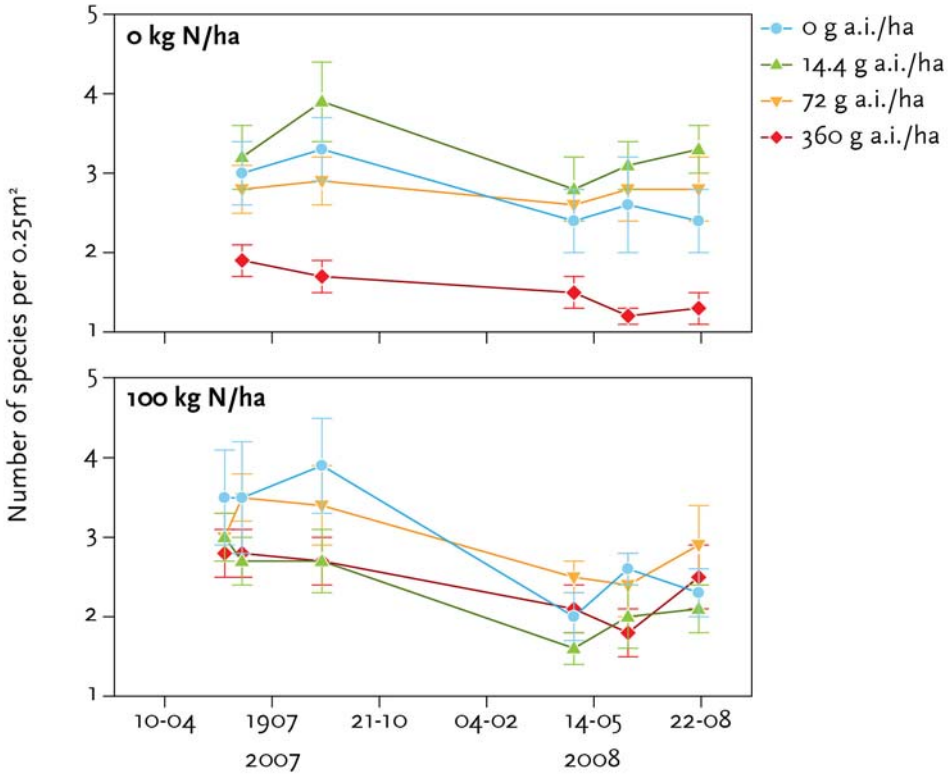


Figure 4.24 Species richness, i.e. number of species per. 0.25m² within the Permanent plots at the experimental plot Kalø in the period 2007-2009.

Two major changes in species composition have occurred over the experimental period (Fig 4.22, Table 4.13 and App. 2). First, ***Elytrigia repens*** increased significantly in cover and biomass over the period, especially in plots receiving 100 kg N/ha and no or low to intermediate concentrations of glyphosate (Fig. 4.22 and 4.25). Also, the number of plots where the species is present has increased (Table 4.13). Secondly, the number of biennials and the cover these species have decreased over the period, e.g. spectacular species such as ***Verbascum thapsus***, ***V. nigrum***, and ***Oenothera biennis*** that were commonly found in 2005 (Fig. 4.26, Tabel 4.12) have nearly disappeared despite viable seeds of ***V. thapsus*** were among the most common in 2004 (Holst et al. 2008). In addition, perennial herbs such as ***Tanacetum vulgare***, ***Linaria vulgaris*** and ***Hieracium pilosella*** that were found in many plots in 2005 have also become less frequent (Tabel 4.13). ***Convolvulus arvensis*** is an example of a species that became more frequent and apparently is relatively tolerant to glyphosate (Tabel 4.13).

Table 4.13 Occurrence (number of plots out of ten in which the species occurs) of eight selected species (*Agrostis capillaris*, *Festuca ovina*, *Elytrigia repens*, *Linaria vulgaris*, *Tanacetum vulgare*, *Hieracium pilosella*, *Verbascum thapsus*, and *Convolvulus arvensis*) relative to nitrogen and glyphosate treatments in the period. Note, plots receiving 0 kg N/ha was only sampled in early spring 2005 during the period 2005-2007. (continued overleaf)

Number of plots out of ten in which the species occured, sampling was performed in 6 randomly located quadrates within each treatment

Indicates the occurence of the species within permanent plots, 2007-2009

Agrostis capillaris

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
2005 before/May	10	10	10	10	10	10	10	10	10	10	10	10
2006 before/May					10	10	10	10	10	9	9	9
2006 after/June					10	10	10	10	10	9	9	7
2006 Aug.					10	10	9	9	10	9	9	7
2007 before/May					10	10	9	9	10	9	7	7

Elytrigia repens

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
2005 before/May	2	1			3	3	3		8	8	8	1
2006 before/May					10	9	8		10	9	9	4
2006 after/June					10	9	8	1	10	9	9	4
2006 Aug.					5	6	3	1	10	9	10	2
2007 before/May					6	9	3	2	10	10	8	3

Festuca ovina

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
2005 before/May	10	10	10	10	10	10	9	9	8	9	9	10
2006 before/May					9	10	9	10	8	10	10	10
2006 after/June					8	9	10	10	8	8	9	10
2006 Aug.					10	10	9	10	7	8	10	10
2007 before/May					10	10	9	10	8	8	10	10

Linaria vulgaris

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
2005 before/May	7	5	5	2	9	6	7	3	10	9	10	6
2006 before/May					7	4	5	1	8	8	6	1
2006 after/June					7	4	5	1	9	8	7	1
2006 Aug.					9	8	7	4	10	9	6	1
2007 before/May					3	4	3	1	9	5	4	1

Table 4.13, continued.

Tanacetum vulgare

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
2005 before/May	4	8	4	3	8	9	8	7	10	10	6	6
2006 before/May					7	9	8	4	9	8	8	10
2006 after/June					8	9	9	4	9	8	8	10
2006 Aug.					10	9	7	6	9	10	10	7
2007 before/May					7	3	3	1	10	9	7	5

Hieracium pilosella

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
2005 before/May	5	2	3	4	2	5	4	7	1	3	2	1
2006 before/May					3	4	3	2		1	1	
2006 after/June					3	4	3	2		1	1	
2006 Aug.					2	4	3	2		1	1	1
2007 before/May					2	4	3	2		1		

Verbascum thapsus

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
seed bank*	5,1	9,6	8,5	6,8	26,2	15,8	16	5	55	27	15	9,7
2005 before/May	3	2	4	3	2			1	5	4	3	6
2006 before/May					1	1	1			3		5
2006 after/June					1	1	1			3		5
2006 Aug.								2	1	2		3
2007 before/May					1				1	2		2

* mean (number of seeds pr. 0.6 x 10^{-3} m^3), sampled Sep. 2004 (Holst et al. 2008)

Convolvulus arvensis

	0 kg N/ha				25 kg N/ha				100 kg N/ha			
	0	14,4	72	360	0	14,4	72	360	0	14,4	72	360
2005 before/May			1	3	1	1	1	2	2	3	1	2
2006 before/May						2	2	2	3	1		2
2006 after/June						2	2	2	3	1		2
2006 Aug.					2	1	2	5	3	2	1	3
2007 before/May					2	1	3	5	2	2	2	4

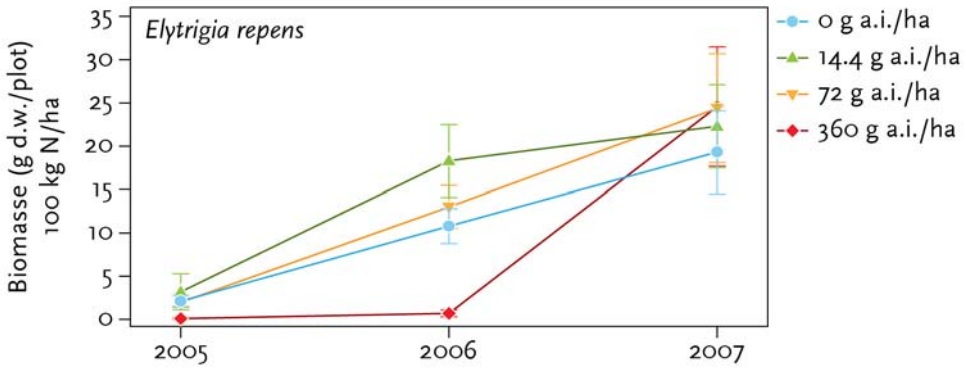
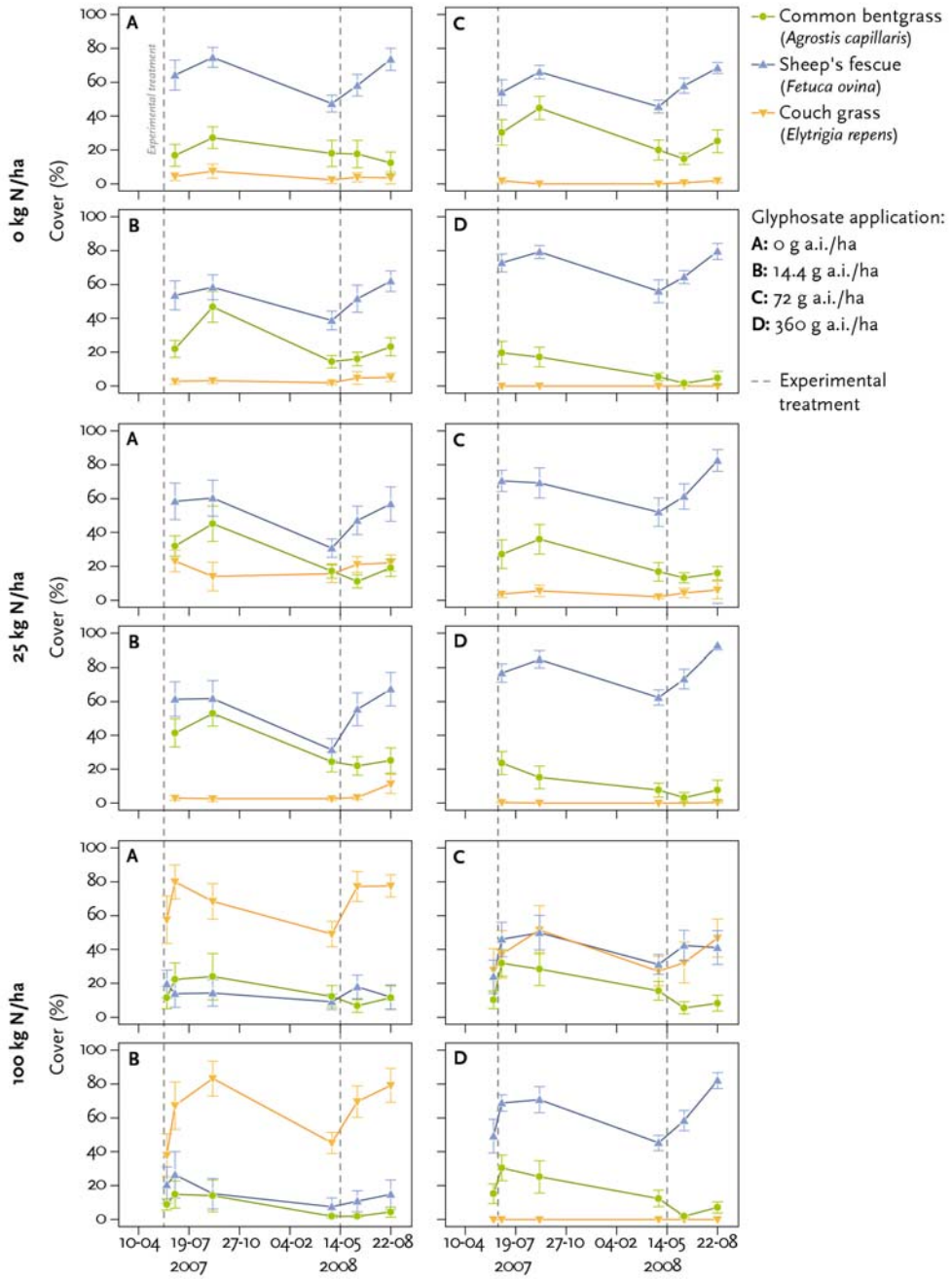


Figure 4.25 Biomass of *Elytrigia repens* within experimental plots at the kalø experimental plot receiving a combination of nitrogen (100 kg n /ha) and glyphosate (0, 14.4, 72 and 360 g a.i/ha) over the period 2005-2007.

Figure 4.26. View of the experimental plot at Kalø, late August 2005. *Verbascum thapsus* is seen at right of the photo and *Tanacetum vulgare* mainly in backgrounds. Photo: B. Strandberg.

Species composition is affected both by application of nitrogen and glyphosate (Fig 4.22 and Tabel 4.13). Grasses made up the main part in all plots and four grass species including ***Agrostis gigantea*** in addition to the three dominating grasses, ***Agrostis capillaris***, ***Festuca ovina*** and ***Elytrigia repens***, were the only species that were found regardless of the treatment (Tabel 4.14). These grasses dominated the vegetation independently of treatment and year and covered at least 50-60% of the ground (Figure 4.22). These grasses, however, responded differently to both glyphosate and nitrogen, and the treatment determined which of the grasses that had the largest cover. ***Festuca ovina*** thrived at low and intermediate nitrogen level (0 and 25 kg N/ha, respectively). At highest nitrogen level (100 kg N/ha), the cover was much lower except in plots treated with both nitrogen and the highest levels of glyphosate (Fig. 4.27). This is in accordance with its normal occurrence at nutrient poor locations and, generally, the species is described as a hardy plant. In contrast, ***Elytrigia repens*** did badly at low and intermediate nitrogen levels, but in plots receiving 100 kg N/ha annually it became dominant except for plots that in addition to nitrogen received high concentrations of glyphosate. ***Agrostis capillaris*** did best at intermediate levels of both nitrogen and glyphosate.

Most species responded negatively to glyphosate and their cover decreased significantly with increasing glyphosate concentrations. ***Festuca ovina*** was the only plant that showed increased cover (Fig. 4.27) and biomass (Fig. 4.28) with increasing glyphosate concentrations ($p<0,001$). Nitrogen addition had a significant and positive effect on cover of ***Elytrigia repens*** ($p<0.0001$) and ***Agrostis gigantea*** ($p<0.0001$), but had no or little effect on cover of species such as ***Tanacetum vulgare*** or even a negative effect on other species. A few third order interactions (species x year x plot) were found, and if found the species behaved differently in no more than one of the ten replicates.



Figur 4.27 Cover (mean ± SE) for the three dominant grasses *Agrostis capillaris*, *Festuca ovina* and *Elytrigia repens* in 2007 and 2008. Time of the experimental treatment is indicated by red arrows.

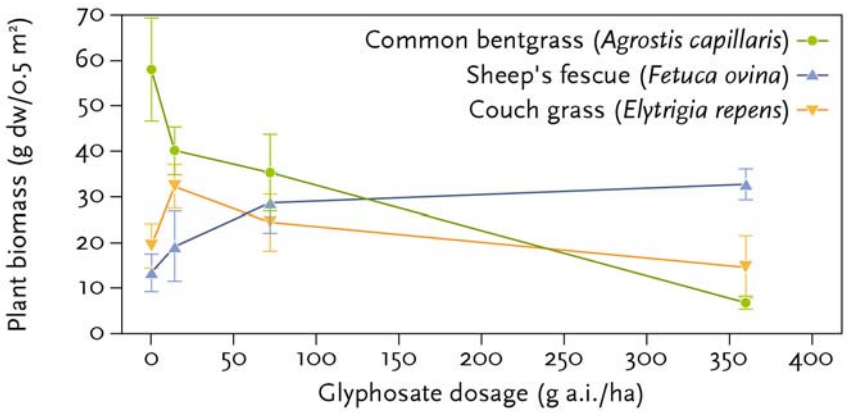


Figure 4.28. Biomass (mean ± SE) of *Agrostis capillaris*, *Festuca ovina* and *Elytrigia repens* in 2006 estimated non-destructively as a function of glyphosate concentrations (g a.i./ha) in plots receiving 100 kg N/ha.

Equal numbers of plants belonging to the C-, S-, and R-strategy were sown within all plots in April 2001. The species that were sown in 2001 and the affiliation regarding to the CSR-strategy (sensu Grime 2001) is indicated in Table 4.13.

Table 4.14. Occurrences of plant species within permanent plots at the Kalø experimental field in 2008 shown in relation to nitrogen (0, 25 og 100 kg N/ha) and glyphosate (0, 14.4, 72 og 360 g a.i./ha) treatments. The colours indicate the plant strategy (CSR-strategy, sensu Grime 2001) for the plants sown in 2001. xxx C-strategy, xxx S-strategy, xxx R-strategy, xxx mixed strategy (following Bruus et al. 2004).

Nitrogen (kg N/ha)		0				25				100			
glyphosate (g a.i./ha)		0	14	72	360	0	14	72	360	0	14	72	360
Agrostis capillaris	alm. hvene												
Festuca ovina	fåresvingel												
Elytrigia repens	alm. kvik												
Agrostis gigantea	stortoppet hvene												
Leucanthemum vulgare	hvid okseøje												
Linaria vulgaris	torskemund												
Tanacetum vulgare	rejnfan												
Euphorbia esula	langbladet vortemælk												
Poa pratensis ssp. angustifolia	smalbladet rapgræs												
Hieracium pilosella	håret høgeurt												
Hypericum perforatum	prikbladet perikon												
Hypochoeris radicata	alm kongepen												
Poa pratensis	eng rapgræs												
Artemisia vulgaris	gråbynke												
Rumex acetosella	rødknæ												
Myosotis arvensis	mark forglemmigej												
Holcus lanatus	fløjlsgræs												
Campanula rotundifolia	liden klokke/blåklokke												
Lepidium campestre	Salomons lysestage												
Cirsium arvense	ager tidsel												
Fallopia convolvulus	snerle pileurt												
Galium mullogo	hvid snerre												
Achillea millefolium	alm. røllike												
Urtica dioca	stor nælde												
Verbascum thapsus	filtbladet kongelys												
Oenothera biennis	toårig natlys												
Chenopodium album	hvidmelet gåsefod												
Viola tricolor	alm. stedmoder												
Convolvulus arvensis	agersnerle												

The CSR-strategy seems not to play an important role for the species response to the herbicide and nitrogen treatments. The only general pattern in occurrence in relation to the CRS-strategy is that R-strategs have become relatively rare.

The statistical analyses of cover of the three most commonly occurring species within the experimental plots, i.e. ***Festuca ovina***, ***Agrostis capillaris*** and ***Elytrigia repens***, showed that both glyphosate and nitrogen significantly affected the species cover and they also showed interaction of glyphosate and nitrogen. For the less commonly occurring species, some general pattern in species occurrence in relation to the treatments may also be appearent (Tables 4.13, 4.14 and Appendix 2) and based on this we were able to separate plants at the Kalø experimental plot into 7 major groups:

1: Species that occurred within all treatments
2: Species that occurred independently of glyphosate concentrations but avoided low nitrogen levels

3: Species that occurred within most treatments except for the combination of low nitrogen and high glyphosate where they were rare or absent
4: Species that occurred at all nitrogen levels but avoid high concentrations of glyphosate
5: Species that only occurred at low concentrations of glyphosate
    5a: and low nitrogen level
    5b: and intermediate and high nitrogen levels
6: Species that only occurred at high nitrogen levels
7: Species with a ruderal occurrence (not permanently established within the plots).

The analyses of effects of the treatment on the individual species give some indications of the overall effects on the plant community, and in addition to these we made multivariate statistical analyses to evaluate the overall effect of the treatments on the plant community. These showed that plots that received the highest concentrations of glyphosate (360 g a.i./ha) encircled by a blue line on Fig. 4.29 may be distinguised from plots receiving lower concentrations (0, 14.4 and 72 g a.i./ha) based on the cover of the species occurring within the plots and that glyphosate played the most important role for this pattern

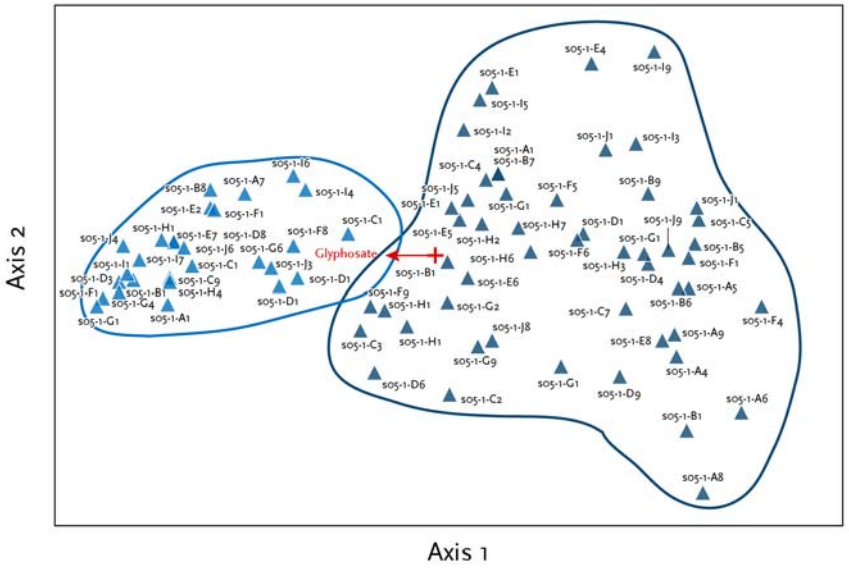


Figure 4.29. DCA ordination based on data on plant cover in June 2006.

Grasses dominated the vegetation regardless of the treatment at the Kalø experimental plot. This corresponds with monitoring data from natural and semi-natural habitats such as hedgerows (e.g. Aude et al. 2003, Holst et al. 2008). However, the composition of the grass community depended on the treatment. Three grasses, ***Agrostis capillaris***, ***Festuca ovina*** and ***Elytrigia repens***, made up the main part of the vegetation and covered at least 50-60% of the ground in all experimental plots. ***Festuca ovina*** was the species least sensitive to glyphosate among the plants at the Kalø experimental field, and plots receiving high concentrations of glyphosate (360 g a.i./ha) had a high cover and biomass of ***Festuca ovina***. This grass, however, is rarely found in semi-natural habitats in agricultural areas presumably due to the high nitrogen levels found within these habitats both at organic and at conventional farms (Aude et al. 2003). We will not expect ***Festuca ovina*** to be a good competitor compared to a number of tall-growing grasses and herbs such as ***Elytrigia***

repens, ***Dactylis glomerata***, ***Arrhenatherum elatius***, ***Urtica dioca*** and ***Cirsium arvense*** that dominate these habitats. The performance of ***Elytrigia repens*** (Figures 4.22 and 4.27) within the experimental plots also corresponds well to the species performance in natural and semi-natural habitats. It had the highest cover in plots receiving high levels of nitrogen (100 kg N/ha) and with low or intermediate glyphosate concentrations. ***Agrostis capillaris*** was sensitive to competition from other species and did best at low and intermediate glyphosate and nitrogen concentrations. This is in accordance with the findings of Holst et al. (2008). They showed that the sensitivity of ***Agrostis capillaris*** to glyphosate was affected by the presence of the less sensitive species ***Festuca ovina***. At glyphosate concentrations equal to those found in spray drift ***F. ovina*** did well and lowered the ED_x dosages of ***A. capillaris***, an effect that might not be predicted in a standard plant test.

4.5 Modelling of plant competition

The sampled pin-point data of cover and compactness was analysed using the state-space competition model that is outlined in Fig. 3.11 p. 41 and explained in equations (1) – (5) in section 3.9.3. The results are reported in Table 4.15 by the calculated percentiles of the marginal posterior distribution of the parameters. From the reported percentiles, it is possible to read the median estimate of the parameter as well as the 95% credibility interval of the parameter. Furthermore, using the 95% credibility intervals it is possible to read if the marginal posterior distributions of the more interesting parameters, i.e. the parameters that quantify the effects of nitrogen, glyphosate, or competition, are significantly different from zero.

Table 4.15. The calculated percentiles of the marginal posterior distribution of the parameters. The parameters are explained in the text connected to the equations (1) – (5) in section 3.9.3. The indices j and k has the following interpretation: for Festuca oviana, j: Agrostis capillaris, k: other species, for A. capillaris, j: F. oviana, k: other species, for other species, j: A. capillaris, k: F. oviana. The parameter of interest has been colour coded: competition coefficients have orange background, parameters that measure effects of nitrogen have green background, parameters that measure effects of glyphosate have blue background, and parameters that measure interaction effects of nitrogen and glyphosate have cyan background. Bold numbers indicate that a parameter of interest deviated significantly from zero. Note that the parameters in process equations (2) and (3) have the same notation; this does not mean that the parameters of the two processes are identical, but only that the parameters with the same notation have an analogous interpretation.

parameter	Festuca ovina			Agrostis capillaris			Other species		
percentiles	2.5%	50%	97.5%	2.5%	50%	97.5%	2.5%	50%	97.5%
Measurement equation 2:									
σ	0.96	1.03	1.11	0.31	0.33	0.36	1.32	1.41	1.52
Process equation 1:									
α	-0.0171	-0.0120	-0.0067	-0.00918	-0.00308	0.00311	-0.0412	-0.0317	-0.0236
β	0.585	0.653	0.721	-0.237	-0.145	-0.057	0.593	0.693	0.788
σ_{12}	0.368	0.404	0.445	0.428	0.477	0.528	0.566	0.620	0.684
Process equation 2:									
a	59.8	64.9	70.5	251.1	253.6	255.9	48.7	62.0	69.3
b	0.756	0.868	0.974	1.97	1.99	2.00	1.00	1.10	1.21
c_j	0.062	0.578	0.897	0.225	0.236	0.257	0.236	0.627	0.985
c_k	0.807	1.185	1.610	1.181	1.206	1.238	-0.128	0.116	0.346
d_j	0.85	1.75	1.99	1.65	1.81	1.98	0.51	0.96	1.82
d_k	1.31	1.87	1.99	1.65	1.68	1.73	0.52	1.05	1.94
e	-0.0015	-0.00034	0.00149	-0.00246	-0.00238	-0.00225	0.00281	0.00381	0.00578
f	-0.00491	0.000219	0.00492	-0.0246	-0.0233	-0.0224	-0.0183	-0.0056	0.00486
ef	-2.4E-05	7.58E-05	0.000151	6.18E-05	7.12E-05	8.28E-05	6.03E-05	0.000198	0.000339
σ_{23}	2.17	2.32	2.53	0.017	0.023	0.032	1.88	2.02	2.19
Process equation 3:									
a	0.120	0.279	0.361	0.204	0.275	0.333	0.211	0.311	0.396
b	0.502	0.534	0.704	0.500	0.513	0.570	0.501	0.522	0.598
c_j	-0.0156	-0.0106	-0.00552	-0.00633	-0.00104	0.00426	-0.0170	-0.0101	-0.0028
c_k	-0.0237	-0.0183	-0.0128	-0.00532	0.000794	0.00716	-0.0184	-0.0121	-0.0059
e	-0.00777	-0.00417	-0.00088	-0.01009	-0.00675	-0.00324	0.000853	0.00549	0.00989
f	-0.0064	0.00450	0.01572	-0.0382	-0.0249	-0.0117	-0.0379	-0.0195	-0.00254
ef	-6.1E-05	0.000137	0.000342	7.34E-05	0.000298	0.000517	-0.00032	-3.7E-05	0.00025
g	-1.95	-1.53	-1.07	-2.89	-2.53	-2.17	-2.72	-2.26	-1.83

4.5.1 Effect of glyphosate on cover (P1)

The change in cover in the relative short period from the first recording immediately before the glyphosate and nitrogen treatments and the second recording approximately two weeks later was found to be significantly negatively affected by the glyphosate treatment for ***F. ovina*** and the aggregated class of other species, whereas the cover of ***A. capillaris*** was not significantly affected by the glyphosate treatment. The negative effect of glyphosate on the cover of ***F. ovina*** and other species were surprising results and contradicted the results of a simple standard statistical analysis of the effect of glyphosate on the change in the cover of ***F. ovina*** and other species from $\boldsymbol{t_1}$ to $\boldsymbol{t_2}$ (see Fig. 4.27), as well as earlier dose-response experiments of ***F. ovina*** and ***A. capillaris*** where ***A. capillaris*** were found to be more sensitive to glyphosate than ***F. ovina*** (Holst et al. 2008). A possible explanation of the surprising results of the model may be that the model assumption that the period between the two recordings was insufficient for the variable level of

nitrogen in the different plots to have any effect on the change in cover (but see also P3).

4.5.2 Competitive growth (P2)

Inter-specific competitive interactions among ***F. ovina***, ***A. capillaris*** and the class of other species were demonstrated during growth from t_2 to t_3. This is based on the estimated competition coefficients during competitive growth that were significantly higher than zero for all species combinations, except the effects of ***F. ovina*** on other species. This means that increasing cover of one of the species at ***t***2 had a negative effect on the compactness of the other species at t_3.

There were significant and positive interaction effects of glyphosate and nitrogen treatments on the growth of ***A. capillaris*** and other species. No significant effects of either glyphosate or nitrogen treatments on the growth of ***F. ovina***. The estimated effects of the glyphosate and nitrogen treatments on the growth of ***A. capillaris*** and ***F. ovina*** may be compared to the observed relative growth of the two species in the randomly placed plots (Fig. 4.30).

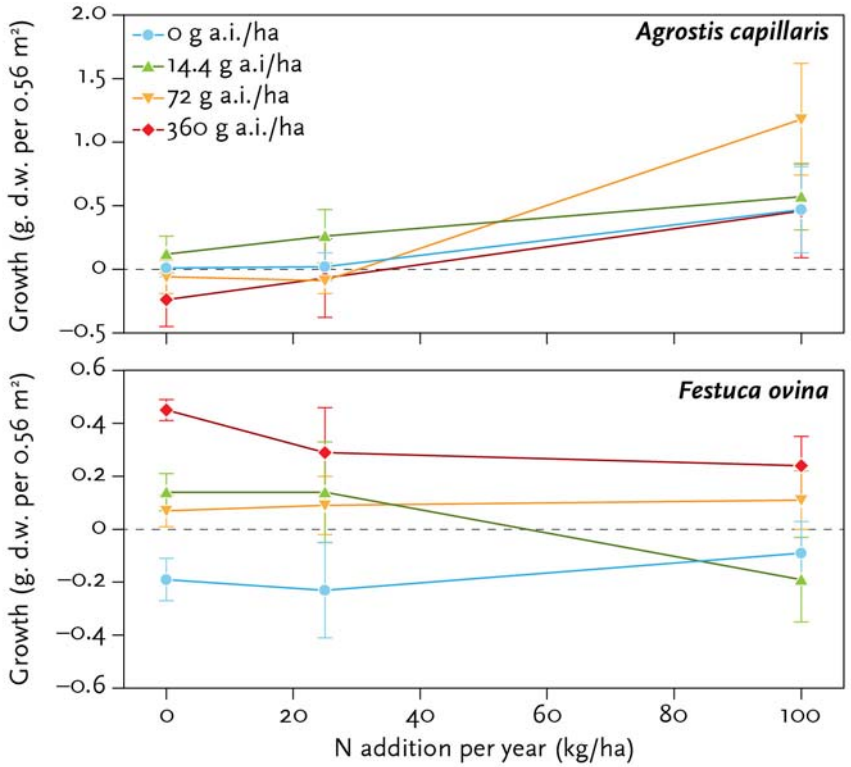


Figure 4.30 Mean growth (g d.w. per 0.56m^2) ± s.e. of *Agrostis capillaris* and *Festuca ovina*

In the used model (P2), the effects of glyphosate, nitrogen, the interaction of glyphosate and nitrogen, and interspecific competition are partitioned, which hinders a direct comparison of the effects, but the observed relative growth of ***A. capillaris*** (Fig. 4.30) is congruent with the estimated positive interaction effect using the model. A possible positive interaction between the effects of glyphosate and nitrogen may be important for the ecological success of ***A. capillaris*** in field margins, supported by monitoring results showing ***A. capillaris*** to be one of the most common species in hedgerow ground

vegetation (Aude et al. 2003, Strandberg et al. on-going projects). In the case of ***F. ovina***, the observed relative growth in the randomly placed plots (Fig. 4.30) displayed a positive effect of glyphosate on growth, which may seem to be contradicted by the insignificant response to glyphosate found using the competition model. However, the positive effect on growth is almost surely due to the competitive advantage of ***F. ovina*** at the high glyphosate treatment (Holst et al. 2008) which in the competition model is modelled by the competition coefficients.

Generally, it would have been an advantage if the competition coefficients in the competition model were assumed to be functions of the levels of glyphosate and nitrogen. This would allow for testing of the effect of glyphosate, nitrogen, and the interaction of glyphosate and nitrogen on the competitive interactions. However, such a formulation of the model with up to twelve additional parameters of interest would require a significant increase in the dimension of the experimental design, which unfortunately was impossible due to resource constraints.

4.5.3 Survival and establishment (P3)

There were significant negative inter-specific competitive effects of the compactness at $\boldsymbol{t}_3$ on the cover at $\boldsymbol{t}_1$ the following year for all species combinations.

There was a significantly negative effect of nitrogen on survival and establishment from $\boldsymbol{t}_3$ to $\boldsymbol{t}_1$ the following year. This negative effect of nitrogen is most likely due to an increased competitive ability of ***Elytrigia repens*** af higher nitrogen availabity.

There was a significant negative effect of glyphosate on survival and establishment from $\boldsymbol{t}_3$ to $\boldsymbol{t}_1$ the following year for ***A. capillaris*** and other species. Note that the survival and establishment of ***F. ovina*** was not negatively affected by glyphosate. This result is in agreement with the observed trends in the glyphosate treated plots and may partly explain the surprising result in P1.

As was the case for competitive growth, there was a significant positive interaction effect between nitrogen and glyphosate on survival and establishment from $\boldsymbol{t}_3$ to $\boldsymbol{t}_1$ the following year for ***A. capillaris***.

4.5.4 All processes (P1- P3)

From the above-reported results of the three studied processes, it may be concluded that the observed ecological success of ***F. ovina*** on the behalf of ***A. capillaris*** in glyphosate treated plots primarily are due to altered plant growth responses and, consequently, altered competitive interactions during the growing season and unaffected survival or establishment outside the growing season, rather than an immediate effect of die back due to poising. Additionally, the results suggest that positive interactions between the effects of glyphosate and nitrogen may be important for the ecological success of ***A. capillaris*** in field margins.

However, in retrospect, it must be concluded that the increasing and variable abundance of couch grass (***Elytrigia repens***) and the relative small dimension of the experiment made it unfeasible to model the effect of nitrogen, glyphosate, and the interaction of the two on the competitive interactions in

such a way that predictions on the effect of nitrogen and glyphosate on the population dynamics of ***F. ovina*** and ***A. capillaris*** can be made.

5 General discussion

Testing the following null hypotheses:

1. The sensitivity of non-target plants to herbicides measured as survival and biomass does not vary significantly from the sensitivity of crop species
2. Selection of end-point does not influence the outcome of the risk assessment of herbicides
3. The effects of repeated herbicide exposure to sublethal dosages of herbicides with different modes of action do not differ from the Additive Dose Model (ADM)
4. Differences in droplet size and in herbicide concentration within droplets between spray drift and direct exposure in spraying chambers do not result in any differences in the effects observed on plants at a given herbicide dosage
5. The interactions of natural habitats and the inherent complexity do not have any effects on the responses to herbicide spray drift quantified as the ecological success, i.e. biomass and reproductive allocation of the plants growing in these habitats
6. Effects of nitrogen fertilizers do not interact with effects of herbicide spray drift in natural and semi-natural habitats

we were able to confirm hypothesis 1, while we have to reject the hypotheses 2, 3, 5 and 6. The project did not make it possible to fully assess the fourth hypothesis. Below you will find a detailed discussion of the project results and findings.

5.1 Species sensitivity to herbicides

Except for the test by Boutin and collaborating Danish scientists (Boutin et al. 2004), few dose-response experiments have been performed with non-target terrestrial plants (Holst et al. 2008, Gove et al. 2007, Strandberg et al. 2006b, McKelvey et al. 2002, Asman et al. 2001, and Marrs et al. 1993). So far, the expectations (e.g. Boutin et 2004, Marrs et al. 1993) have been that wild plants or non-target plants are more sensitive to herbicides than crop plants normally used in standard tests for risk assessment of herbicides. On the other hand, an analysis on species sensitivity based on response data from US regulatory testing (McKelvey et al. 2002) suggested that crop species sensitivity to test substances is likely to be representative for non-crop species (32 non-crop species). This study, however, had very little in common with the "European" studies. Metsulfuron-methyl was the only herbicide occurring in both studies, only two species, black bindweed (***Polygonum convolvulus*** syn. ***Fallopia convolvulus***) and wild mustard (***Sinapis arvensis***), were common to the analyses, and the comparison of relative sensitivity was based on different end-points.

We therefore made a re-analysis of existing toxicity data found in the databases EUROTOX and PHYTOTOX. In accordance with the finding by Boutin et al. (2004), our calculations suggest that crop plants are less sensitive than non-crop species to a number of common herbicides. The present comparison of species sensitivity (ED_{25}) included the five herbicides

glyphosate, bromoxynil, dicamba, metolachlor and pendimethalin. This analysis also showed that the crop plants we tested were significantly less sensitive to glyphosate than non-crop plants (Chap. 4, Figure 4.1) while there was a non-significant trend of crop plants being less sensitive than non-crop plants to the other four herbicides. However, test data for the two groups of plants originated from different experiments. While data on crop plant sensitivity originated from the ECOTOX database, data on non-crop plants came from the study by Boutin and co-workers. The ECOTOX database contains test data generated according to the OECD test guideline, but it does not specify how exposure levels were calculated, under which abiotic conditions the test were conducted and the experimental conditions in general (for example how many specimens were present in the pot etc.).

In order to overcome the weaknesses of the previous comparisons of sensitivity of crop species and non-target species to herbicides that have all been based on analyses of data belonging to different studies, we conducted a dose-response experiment with 10 crop species and two of the non-crop species, ***Centaurea cyanus*** and ***Papaver rhoeas***, tested by Boutin et al. (2004). Test conditions of this study were in accordance with Boutin et al. in order to make the results comparable. This experiment showed ED_{50} dosages for ***C. cyanus*** and ***P. rhoeas*** comparable to Boutin et al. (2004) for glyphosate and bromoxynil, while the sensitivity to metsulfuron-methyl was higher in the present experiment. Consequently, it is possible to compare the sensitivity of crop and non-target species between the two studies. Our results indicate that the sensitivity of non-target species to at least glyphosate and bromoxynil was within the range found for crop species, i.e. the study did not show that non-target species were more sensitive than crop species when tested under the same conditions. The differences in test conditions may be the main reason for the differences in sensitivity calculated both by Boutin et al (2004) and in our analyses based on data found within the databases. The present investigation, therefore, supports our first null hypothesis assuming that the sensitivity of non-target plants does not differ significantly from the sensitivity of crop species when measured as effect on survival and biomass. This finding both has implications for the selection of relevant test species for standard tests and calls for further investigations regarding the importance of varying test conditions for the species sensitivity. Furthermore, it highlights the importance of documentation of test conditions in the databases.

One may argue that ***C. cyanus*** and ***P. rhoeas*** are poor representatives for non-target plants, as they are mainly found as weeds (i.e. target species) in agricultural fields. However, they are also found in natural and semi-natural habitats, but not as frequent as in agricultural fields. As representatives of wild/non-target plants, ***C. cyanus*** and ***P. rhoeas*** differ from the plant species dominating the flora in natural and semi natural habitats, because they are both annuals. Lifespan is one of the main factors that differ between the recommended test species, mainly annual crop species, and plants in natural and semi-natural habitats. Our studies of crops, weeds and perennials showed in accordance with most other studies that sensitivity measured as effect on biomass was larger when young plants were exposed compared to older plants, independently of the longevity of the test species. Only one species, ***Agrostis capillaris***, a commonly occurring grass within Danish natural and semi-natural habitats, did not show a decline in sensitivity with age (Holst et al. 2008). The biomass end-point, however, is only relevant and ecologically meaningful when dealing with exposure of young plants as is the case in standard plant tests. For older plants with little vegetative growth and

especially plants in the reproductive stage, biomass is not a good effect end-point (see below, for discussion of seed production as end-point).

Another important finding of the present project based on dose-response experiments with pairs of taxonomically closely related annual and perennial species is that taxonomically relationship was not the determining factor for species sensitivity to herbicides in our study. Unlike Fletcher et al. (1990) who found that the ED_{50} values of species belonging to the same genus or taxonomically closely related species had a higher degree of similarity than taxonomically more distant species, we found that other characteristics may be of higher importance for species' sensitivity to herbicides than taxonomic relationship. This could be morphological characters or plant trait such as leaf shape, leaf thickness, hairs or wax layer or other plant traits. The higher sensitivity of ***Silene noctiflora*** than of ***S. vulgaris,*** for example, may be caused by the differences in leaf size and surface characteristics. ***S. vulgaris*** has smooth, relatively thick and waxy leaves that may exclude at least some herbicides from being absorbed while ***S. noctiflora*** has broad, thin leaves with sticky hairs.

5.2 Relationship between way of herbicide exposure and effects on plants

5.2.1 Dose-response: longevity, growth stage and end- point

One of our major findings is that seed production is a more sensible end-point for risk assessment of herbicides than biomass independently of time of exposure (early vegetative stage and during flowering), see Fig. 4.7 p 60. For both annual and perennial species, the ED_{50} based on seed production was lower than the ED_{50} dosages based on biomass data. We, therefore, have to reject the second null-hypothesis assuming that selection of end-point does not influence the outcome of the risk assessment of herbicides. The result is in contrast to the findings of Asman et al. (2001), while the few other studies dealing with exposure during reproduction concurrently with the present study have shown that the reproductive structures such as flowers, pollen, and fruits or seeds seem to be particularly sensitive to herbicide exposure (Blackburn & Boutin 2003, Felsot et al. 1996, Bhatti et al. 1995, Marrs et al 1993, Christensen 2008). As indicated by the application scheme for the three herbicides glyphosate, metsulfuron-methyl and MCPA, Fig. 3.2 p. 27, there are only short periods during the growing season without any herbicide exposure. The flowering period of plants occurring in natural and semi-natural habitats varies a lot. Herbicides, therefore, may affect the plants quite differently depending on the synchronization of the herbicides used in the neighbouring fields and the flowering period of the plants. Some species are ephemerals, i.e. they complete their live cycle including flowering during a short period in spring, summer or autumn, while others flower for a longer period. Generally, plants that need to produce viable seeds and go through the sensitive early growth stages from time to time in order to persist as part of the flora are more vulnerable to herbicides than clonal plants that have the potential to propagate through buds, roots or rhizomes. Particularly, this is important for annuals and biennials but also perennials need to reproduce by seeds time by time. Over a five year period (2004-2008) biennial species such as ***Verbascum thapsus***, ***V. nigrum***, and ***Oenothera biennis*** that were commonly found at the Kalø experimental plot in 2005 (Fig. 4.26, Tabel 4.12) have nearly disappeared despite viable seeds of ***V. thapsus*** were among the most common in 2004 (Holst et al. 2008). In addition, perennial herbs such as

Tanacetum vulgare, ***Linaria vulgaris*** and ***Hieracium pilosella*** that were found in many plots in 2005 have also become less frequent.

5.2.2 Exposure to repeated sublethal dosages

The study of staggered herbicide treatments, i.e. several treatments given with a period of time between the exposures to the different herbicides, has shown that sequences of drift of different herbicides have a higher effect than expected from the individual effect profiles of the herbicides. Thus, the third null hypothesis, assuming additive effects of repeated dosages of herbicides, is not sustained. This contrasts with most findings in previous investigations studying the joint action of herbicide mixtures applied simultaneously. Mathiassen & Kudsk (1993) found antagonism in mixtures of MCPA and sulfonylurea herbicides and reported later mixtures of tribenuron and ioxynil + bromoxynil to be additive while the joint action of tribenuron and mecoprop-P was additive or antagonistic depending on the mecoprop-P formulation (Kudsk & Mathiassen, 1995). Streibig et al. (1999) found mixtures of photosystem II inhibitors to be additive. Cedergren et al (2007) evaluated 10 binary mixtures of nine herbicides representing the most commonly used target side for controlling broadleaved weeds. They found that the joint effect of two mixtures of herbicides with the same mode of action and same target site was additive. Approximately 70% of the mixtures with different sites of action showed significant antagonism, while no synergistic interactions were observed. However, Kudsk & Mathiassen (2004) reported mixtures of metsulfuron-methyl and glyphosate/ glyfosinate to be synergistic and suggested that the formulation constituents in the spray solution led to a higher activity than expected. As mentioned the herbicides were applied at the same time and not in sequences over a period of time in these studies. Presumably, the discrepancy between the previous studies and the present is due to this difference in timing of the applications. The first application may render the test plant more sensitive to later applications because it is already weakened by herbicide exposure. On the other hand, the plant may be expected to be less sensitive to later exposure due to reduced translocation of the herbicide in the plant as a response to the previous (first) exposure. Plants living in natural and semi-natural habitats may experience both the exposure to several herbicides at the same time and staggered exposures. To represent an adequate safeguard for environmental protection of these species and habitats, both ways of exposure need to be considered during the risk assessment of herbicides. The method used in the present study for investigating whether the effect of the total herbicide load is additive, antagonistic or synergistic may serve as an example.

5.2.3 Droplet size and concentration of pesticide

In accordance with previous findings (e.g. Elliot & Wilson 1983, Hewitt et al., 2002, Bruus et al. 2008), spray droplets were larger in the spray chamber experiment than in the field experiment, and droplet size also decreased with increasing distance to the tractor. Similarly, total exposure measured on curlers decreased with increasing distance to the tractor. Unfortunately, this was not the case for the plants exposed in the field; these plants all received very low dosages, irrespective of the distance to the tractor, and hardly any effects on growth were observed. We believe that the reason for this lack of plant exposure may be the rather high wind speed in combination with the physical properties of the pots, in which the plants were placed. Because of the low exposure of plants in the field experiment and consequent lack of effects, we are not able to assess null hypothesis four assuming that

differences in droplet size and in herbicide concentrations within droplets between spray drift and direct exposure in spraying chamber do not result in any differences in the effects observed at a given herbicide dosage.

5.3 Herbicide and fertilizer interactions on species and habitats

We found that application of glyphosate at drift relevant concentrations of 0, 14.4, 72 and 360 g a.i./ha equivalent to 0, 1, 5 and 25 % of recommended field dosage, respectively, and nitrogen at concentrations of 0, 25 and 100 kg N/ha affected the vegetation at the Kalø experimental field significantly. The experiment was started in 2001 in order to evaluate the combined effects of drifting herbicides and fertilizers on vegetation in grassland habitats neighbouring agricultural fields. Over the years, the vegetation has gradually changed both with respect to species richness and species composition. Generally, application of nitrogen as well as glyphosate affected the species number negatively. However, at highest nitrogen level (100 kg N/ha) the application of low dosages of glyphosate to some extent counteracts the negative effect of nitrogen. The negative effect of nitrogen on species number is well-documented in the literature (e.g. Clark and Tilman 2008, Gough et al. 2000, Bobbink et al. 1998) and Stevens et al (2004) found a 23 % species reduction in grasslands in UK at a mean chronic deposition rate of 17 kg N/ha annually compared to grasslands receiving the lowest levels of nitrogen deposition. The negative effect of drift relevant concentrations of glyphosate on species number is not surprising. Pesticide applications have been hypothezised to be one of the main reasons for the declining biodiversity in agricultural areas in Europe (e.g. Green, 1990; Fuller et al., 1995; Andreasen et al., 1996; Rich and Woodruff, 1996; Chamberlain et al., 2000; Donald et al., 2000; Atkinson et al., 2002; Benton et al., 2002). The way glyphosate appears to compensate the negative effect of nitrogen on species richness at the highest nitrogen level may be explained by glyphosate reducing the fast growth of the dominant species. Glyphosate, as well as other herbicides, acts as “a disturbance” that removes biomass and thereby gives room for other species. The notion that disturbance can increase biodiversity opposes the idea that diversity is highest in undisturbed ecosytems, first proposed by Grime (1973), but today the “intermediate disturbance hypothesis” proposed by Connell (1979) is generally accepted. It states that some intermediate level of disturbance not being too strong, occurring too frequent or at a too large spatial scale results in highest species numbers. However, monitoring of species diversity of hedgerow ground flora shows significantly higher species richness in hedgerows at organic farms compared to hedgerows at conventional farms with comparable soil nitrogen levels (Aude et al. 2003; Strandberg et al. in prep.). This leads to the hypothesis that disturbances in conventional hedgerows are too strong or too frequent for high species diversity and that organic hedgerows offer less disturbed conditions. This is meaningfull if herbicides and other pestcides are regarded as disturbances.

Grasses dominated the vegetation regardless of the treatment at the Kalø experimental field, and the three grasses, common bentgrass (***Agrostis capillaris***), sheep’s fescue (***Festuca ovina***) and couch grass (***Elytrigia repens***) made up the main part of the vegetation. This corresponds well with monitoring data from natural and semi-natural habitats such as hedgerows (e.g. Aude et al. 2003, Holst et al. 2008). The treatment, however, determined the composition of the grass community. ***Festuca ovina*** was the species least sensitive to glyphosate among the plants at the Kalø experimental field, and plots receiving high concentrations of glyphosate (360 g a.i./ha) had a high

cover and biomass of this grass. The performance of ***Elytrigia repen***s within the experimental plots also corresponds well to the species performance in natural and semi-natural habitats. It had the highest cover in plots receiving high levels of nitrogen (100 kg N/ha) and with low or intermediate glyphosate concentrations. ***Agrostis capillaris*** did best at low and intermediate glyphosate and nitrogen concentrations and it seemed to be sensitive to competition from ***F. ovina*** or ***E. repens***. This is in accordance with the findings of Holst et al. (2008). They showed that the sensitivity of ***A. capillaris*** to glyphosate was affected by the presence of the less sensitive ***F. ovina***. At glyphosate concentrations equal to those found in spray drift, ***F. ovina*** did well and lowered the EDx dosages of ***A. capillaris***, an effect that might not be predicted in a standard plant test. The extrapolation from single species standard test in which the growth conditions should be optimal except for the addition of the herbicide to field conditions where plants of varying sensitivity to the herbicide and of varying response to the actual nitrogen level grow together are difficult. The analyses of community data support the conclusion that the treatments influence the community composition differently. The species composition is determined by the combined treatment of herbicide and nitrogen but the results suggest that glyphosate had the stongest impact on the species composition at the Kalø experimental plot.

When observing changes in the abundance of different species in natural plant communities, it is common to refer to competition for a limiting resource as the cause for the observed changes (Grime 2001), or to assume a more general effect of neighboring plants on growth and reproduction, which has been demonstrated in a number of empirical studies (Goldberg and Barton, 1992). However, only a few studies have attempted to quantify the competitive interactions in natural plant communities in a design that enable quantitative preditions to be made (Pacala and Silander, 1990, Rees et al., 1996, Turnbull et al., 2004, Damgaard et al., 2009).

The experimental design in the present study has been especially developed for measuring and quantifying competitive interactions in plant communities that are dominated by perennial species and where it is difficult to distinguish individual plants. Significant effects of nitrogen, glyphosate, and the interaction of ***Agrostis capillaris*** and ***Festuca ovina*** on plant growth were demonstrated using the model. The model findings strengthen the conclusions in Holst et al. (2008) and in this report, that the effect of nitrogen and glyphosate on the abundance of ***F. ovina*** and ***A. capillaris*** is mediated by how nitrogen and glyphosate affects the competitive interactions between ***F. ovina*** and ***A. capillaris***. Furthermore, the availability of nitrogen has previously been shown to affect the competitive interactions between two ***Eriophorum*** species (McGraw and Chapin, 1989) and have important effects on plant communities (Clark and Tilman, 2008, Stevens et al., 2004).

Summarizing the results on the combined effects of glyphosate and nitrogen on individual species, species interactions and community composition we have to reject the two last null-hypotheses (# 5 and 6) assuming that nitrogen fertilizers do not interact with effects of herbicide spray drift in natural and semi-natural habitats and that the interactions within natural habitats and the inherent complexity do not have any effects on the response to herbicide spray drift quantified as the ecological success, i.e. biomass and reproductive allocation of the plants growing in these habitats.

5.4 Evaluation of current risk assessment as safeguard for environmental protection of non-target species and their habitats

The present project improves the general knowledge on sensitivity of non-target species to herbicides and the importance of herbicide exposure for vegetation in natural and semi-natural habitats, and the results have implications for the way plant testing should be conducted in the future. Below, we will discuss the results of this project in relation to the current risk assessment of herbicides. Specifically, we will address a number of issues relevant for the regulatory plant tests including selection of test species, end-points, time of exposure, and documentation of test conditions.

The question "How representative are crop species for the sensitivity of non-target terrestrial plants to herbicides?" has been debated during the last decade, and test guideline requirements have been changed towards including more non-target species (See App. 1). The revised OECD guidelines (e.g. OECD 208) have added a list of 52 non-crop species to the list of possible test plants. The arguments for using crop species for testing are that seeds of crop species are easy to produce, they have a high and uniform germination and they usually have a fast and uniform growth. As investigated by Pallett et al. (2007), there are some problems of the 52 non-crop species to meet the OECD guideline validity criterion for seedling emergence of 70%. Furthermore, they found that the variability of the biomass end-point was larger than normal biomass variability of crop species in plant testing. One way to overcome the problem of low emergence is to separate the emergence testing from the growth testing and run the growth experiment on uniform pre-cultivated plants that have been transplanted before the herbicide exposure as it was done by Boutin et al. (2004). The present study, however, showed that crop species, in general, were not less sensitive to herbicides (glyphosate, metsulfuron-methyl and mecoprop-P) than non-target species when the dose-response experiments were run under the same conditions. Sensitivity was more dependent on the efficacy spectrum of the herbicide and whether the test species was a monocot or a dicot. Furthermore, the study indicated that variation in test conditions and end-points may be more important for the previously observed differences in sensitivity of crops and non-target species (e.g. Boutin et al., 2004). The argument for including non-target species in the test battery when testing effects of herbicides according to the guidelines, i.e. using biomass as end-point for effects of herbicides to early growth stages, may therefore not be strong. On the other hand, it may be argued that test plants should cover different traits (characteristics).

OECD guidelines require a visual evaluation of the effects of the chemical on the test plants as well as determination of the effects on the biomass. However, Boutin et al (1995) and Obrigawitch et al. (1998), who both reviewed databases and field studies on effects of herbicides on non-target plants, found that the most commonly used end-point was visual evaluation converted into percentage effect. Some studies include effect on biomass (e.g. Holst et al. 2008, Gove et al. 2007, Strandberg et al. 2006b, Boutin et al. 2004, Asman et al. 2001, Marrs et al. 1993), but effects on seed production are rarely reported although some studies of effects of glyphosate on seed production exist (see Blackburn and Boutin 2003 for review). The present study, however, indicated that the most sensitive end-point was seed production, irrespective of plant species, lifespan (annual, biennial or perennial) and the life stage at the time of exposure (vegetative and reproductive). Today effects on seed production are not covered by the risk

assessment procedure and presumably risk assessment based on biomass and visual effects underestimates the sensitivity of non-target plants.

Selection of end-point needs to be made with respect to time of exposure. In natural and semi-natural habitats, the plants may vary both in age and functional stage at the time of herbicide exposure. Standard plant tests only cover exposure of young plants normally in an early vegetative stage having 4-10 leaves. Many studies, including the present, confirm that young plants are more sensitive than older plants when effect on biomass is used as end-point and effects of drift relevant herbicide dosages normally are sublethal to older plants. However, for older plants, and especially for plants in the reproductive stage, effect on biomass may be directly misleading for effects on plant fitness, i.e. the ability of the plant to run through a full life cycle, produce viable off-spring and establish a new generation. We found that when using seed production as end-point, two year old plants in the bud stage were at least as sensitive as plants in the young stages (Table 4.11, Fig. 4.7 p. 60). The conclusion that older plants are less sensitive than younger, therefore, is misleading. Previously, a few other studies have indicated that reproductive structures such as flowers, pollen, and fruits or seeds are particularly sensitive to herbicide exposure (Blackburn & Boutin 2003, Felsot et al. 1996, Bhatti et al. 1995, Marrs et al 1993, Christensen 2008). However, herbicide effects on plants in the reproductive stage are not covered by current risk assessment.

6 Conclusions

Non-target plants, i.e. plants in natural and semi-natural habitats, may unintentionally be exposed to pesticides drifting from the agricultural fields. Monitoring has pointed at pesticide spray drift as a major factor affecting both flora and fauna within these habitats (e.g. Aude et al. 2003, Bruus Pedersen et al. 2004, Petersen et al. 2006, Bhatti et al. 1995) and pesticides are regarded to play an important role for the decline of species richness in agricultural areas (Fuller et al., 1995; Andreasen et al., 1996; Rich and Woodruff, 1996; Chamberlain et al., 2000; Donald et al., 2000; Atkinson et al., 2002; Benton et al., 2002; Strandberg & Krogh 2011).

Before a new herbicide is approved for placement on the market, it needs to be evaluated in accordance with The Plant Protection Products Directive (Council Directive 91/414/EEC 15 July 1991). According to this Directive and the Annexes II and III, there are no specific data requirements for effects on non-target plants although the effects of herbicides, in particular, are considered to be critical for such plants. However, the data requirements and testing of effects on non-target terrestrial plants are under revision. Therefore, there is an urgent need to know to what extent the vegetation of natural and semi-natural habitats is protected by the current risk assessment and if and how the assessment should be improved for an adequate protection of these habitats.

Our results suggest that the current risk assessment provides insufficient protection of non-target species and their habitats in several areas.

We found that conclusions regarding the sensitivity of a species to herbicides based on biomass measurements not always are valid for effects on seed production which may be regarded as more ecological relevant for non-target species. Seed production was found to be a more sensible end-point for risk assessment of herbicides than biomass independently of plant species, life-span of the plant (annual, biennial, perennial) and functional stage at the time of exposure (early vegetative stage and during flowering). Today effects on seed production are not a commonly used end-point for risk assessment and risk assessment based on biomass and visual effects presumably underestimates the sensitivity of non-target plants.

We showed that the crop species we tested, in general, were not less sensitive to herbicides (glyphosate, metsulfuron-methyl and mecoprop-P) than non-target species when dose-response experiments were run under the same conditions. Sensitivity was more dependent on the efficacy spectrum of the herbicide and whether the test species was a monocot or a dicot species. Furthermore, our results indicate that variation in test conditions may be more important for the previously observed differences in sensitivity of crops and non-target species found based on data from the PHYTOTOX and EUROTOX databases (Boutin et al. 2004) than whether it is a crop or a non-target-species. Today documentation of test conditions and end-points are normally lacking in the databases. Consequently, wrong or misleading conclusions on species sensitivity may be drawn if information on test

conditions and end-point are not available. Therefore, we recommend that information concerning these factors become included in the databases.

Finally, we found that interactions between species with different sensitivity to glyphosate and different responses to nitrogen are important for species composition on experimental plots that resemble natural and semi-natural habitats which are exposed to agrochemicals. Glyphosate dosages representative for spray drift resulted in decreased biodiversity within the experimental plots and changed species composition. At present interactions between species and between herbicides and fertilizers are not part of the risk assessment even though it may render some species more sensitive to common agricultural practice than expected based on data from standard plant tests.

7 Perspectives

Below, we will discuss the implication of the project results for future research on herbicides and effects on non-target terrestrial plants and their habitats and the administative improvements that are needed to ensure that risk assessment represent an adequate safeguard for environmental protection of these species and their habitats.

We found several areas where risk assessments today are insufficient for protection of non-target species and their habitats. These include the selection of the end-points for risk assessment, timing of exposure of different growth stages, selection of end-point relative to time of exposure, and effect of species interactions for the outcome of the risk assessment.

7.1 Test conditions

This study highlights the importance of documentation of test conditions and end-points in the databases. The lack of such information may lead to wrong conclusions on for example species sensitivity. Test guidelines need to allow for some variability of the test conditions otherwise plant testing will be highly monopolized. One way to make results from different tests comparable could be to include common reference species in every test. The reference species needs to include both a monocot and a dicot species in order to cover differences in response relative to the herbicide efficacy spectrum.

7.2 Crops as test plants

Our results suggest that crop species are good representatives for effects of herbicides on early plant stages of non-target plants. Crop species are often easier to purchase, seed germination rate is high and the uniformity of plants is better than for non-target plants. However, more studies on importance of species selection and test conditions for outcome of the risk assessment are needed before any changes in guidelines are performed. Specifically, there needs to be focus on the representativity of short-term tests on annual species for assessment of long-term effects on perennial species.

7.3 Selection of end-point

We found that seed production needs to be included as end-point for effect assessment as it is the most sensitive end-point despite growth stage at the time of exposure and lifespan of the test plant (annual, biennial, perennial). Certainly, there needs to be much more focus on herbicide effects on the reproductive output including effects on both numbers and size of the seeds/fruits produced. This, however, calls for much more studies of the representativity of tests of seed production of annual species for assessment of effects on perennial species. Furthermore, future studies also should comprise effects of herbicides on production of pollen and nectar, as these plant products are highly important for pollinating insects and, therefore, also for a successful fertilization of the flowers.

7.4 Sensitivity of related plants

We found that the taxonomic relationship did not tell much about species sensitivity to herbicides. Research on plant functional traits, such as plant growth form, above/belowground biomass ratio, leaf shape and surface structure, and their importance for species sensitivity, is needed.

7.5 Competitive interactions

We found that competitive interactions between species having different sensitivity to herbicides are important for the species responses in natural and semi-natural habitats. More studies on the importance of these interactions for responses to herbicides are needed to suggest how this can be included in the risk assessment of pesticides.

7.6 Interaction of herbicide and fertilizers

We found that both herbicides and nitrogen affected the individual species and the plant community. More studies on community responses are needed. The Kalø experimental plot that is run by Aarhus University, DMU, (contact Beate Starndberg) is very useful for this.

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Appendix 1. Guidelines and procedures for testing effects of herbicides on terrestrial plants

The effects of chemical substances are being examined through tests developed by the ASTM, ISO and OECD. Pesticides are approved by the EU in accordance with Directive no. 91/414/EEC. Commission directive 93/71/EEC amending Council Directive 91/414/EEC identifies the areas that are required to be addressed to justify pesticide effectivenss and lack of unacceptable adverse effects. This directive does not specifically demand testing of effects on non-target plants, however, it does require that all unwanted effects be reported and that further studies be carried out when effects are indicated.

The Guidance Document on Terrestrial Eco-toxicology (EPPO guideline 226/1), which serves as a background document for Council Directive 91/414/EEC, includes 3 Tiers, where Tier 1 is a preliminary screening of at least 6 species belonging to various taxa. For herbicides, dose-response data is required from acute-toxicity tests on at least 6 species of crops, i.e. 3 monocotyledons and 3 dicotyledons using full field dose. Out of these 6 species, at least one species must be a nitrogen-fixing leguminous plant, and of the remaining species, one must belong to the Brassica and Avena families, respectively. For non-herbicides, 6 species of crops are also required (3 monocotyledons and 3 dicotyledons), however, for these pesticides 3 sets of normal-dose data are sufficient. In case these tests indicate a risk of damage, dose-response tests must be conducted as is the case for herbicides. Tier 2 requires dose-response testing of 6-10 species. It is recommended to test as many species as possible and to include both highly sensitive and less sensitive species. It is further recommended that exposure be as realistic as possible. In Tier 3, semi-field tests using realistic exposures are employed. For Tier 1 and 2 tests, it is recommended to follow the test guidelines developed by OECD (OECD 208 and 227, prior to the revision in 2005, only OECD 208) or OPPTS test guidelines developed by the US EPA. There are no guidelines for conducting Tier 3 tests.

Most EU countries refer to the guidelines in EU directive 91/414/EEC, however, in connection with all applications for approvals of pesticides Germany requires specific data on effects on non-target terrestrial plants if at all there is a risk of exposure of non-target plants. In reality, this is the case for all pesticides, except for seed mordants.

Based on the calculated PEC (Predicted Environmental Concentration) values and the estimated EC_{25}, the TER (Toxicity Exposure-Ratio) is calculated. In case the TER>10, the pesticide has a half-life of less than 1 year, and, if the pesticide is used twice a year at the most, no further testing is required and the pesticide can be approved. In case the TER<10, it must be determined whether the risk of effects on non-target plants can be reduced so that the TER is greater than 10, e.g. by labelling of spraying distance requirements or setting restrictions on spraying equipment as to driftage. The

maximum spraying distance requirement in Germany is 5 metres, as larger distance requirements are considered to be unrealistic.

In case the abovementioned precautions fail to increase the TER to above 10, or if the half-life is more than 1 year or the pesticide is used more than twice a year, supplementary documents are required, e.g. a “Plant Life Cycle Test”. As a rule, this test must be conducted on the most sensitive species from the acute-toxicity test. If this test shows a TER>5, the pesticide can be approved. If the test shows a TER<5 and risk precautions are unable to increase this value, supplemental tests must be conducted under realistic circumstances, e.g. in terrestrial eco-system models or in the field. Based on the results of these tests, the decision can be made as to whether the pesticide should be approved or not.

Previously, the effects of herbicides on plants have mainly been tested on crops and weeds. The revised OECD guidelines (OECD 208 and 227) have added a list of 52 non-crop species to the list of possible test plants. Thus, the complete list of test species contains 82 species, of which 62 are dicotyledons (belonging to 20 families) and 20 are monocotyledons, of which 19 belong to Poaceae.

Appendix 2. Species occurrence at the Kalø experimental plot

C-strategy
S-strategy
R-strategy

	Common names (Danish)	Sown April 2001	Germinated June, 2001	2003	Seed bank 2004	2005	2006-1	2006-2	2007
Artemisia vulgaris	gråbynke	x	x	x	x	x	x	x	x
Cirsium arvense	ager-tidsel	x			x	x	x	x	x
Elytrigia repens	kvik	x	x	x		x	x	x	x
Euphorbia esula	langbladet vortemælk	x		x		x	x	x	x
Leucanthemum vulgare	hvid okseøje	x	x	x	x	x	x	x	x
Tanacetum vulgare	rejnfan	x	x	x	x	x	x	x	x
Urtica dioca	stor nælde	x	x	x	x	x	x	x	x
Campanula rotundifolia	liden klokke	x	x	x	x	x	x	x	x
Festuca ovina	fåresvingel	x	x	x	x	x	x	x	x
Filipendula vulgaris	knoldet mjødurt	x							
Hieracium pilosella	håret høgeurt	x	x	x	x	x	x	x	x
Lotus corniculatus	alm. kællingetand	x	x	x					
Pimpinella saxifraga	alm. pimpinelle	x		x					
Solidago virgaurea	gyldenris	x	x	x		x	x	x	x
Aphanes arvensis	alm. dværgløvefod	x	x						
Lapsana communis	haremad	x	x	x	x				
Lepidium campestre	Salomons lysestage	x	x	x	x	x	x	x	x
Myosotis arvensis	mark-forglemmigej	x	x	x	x	x	x	x	x
Oenothera biennis	toårig natlys	x	x	x	x	x	x	x	x
Poa annua	enårig rapgræs	x	x						
Verbascum thapsus	filtbladet kongelys	x	x	x	x	x	x	x	x
Agrimonia eupatoria	alm. agermåne	x	x	x		x			
Agrostis capillaris	alm_ hvene	x	x	x	x	x	x	x	x
Agrostis gigantea	stortoppet hvene	x		x	x	x	x	x	x
Centaurea cyanus	kornblomst	x	x						
Convolvulus arvensis	ager-snerle	x	x	x		x	x	x	x
Galium verum	gul snerre	x	x	x		x	x	x	
Hypericum perforatum	prikbladet perikon	x	x	x	x	x	x	x	x
Hypochoeris radicata	alm. kongepen	x	x	x	x	x	x	x	x
Linaria vulgaris	torskemund	x	x	x	x	x	x	x	x
Lychnis viscaria	tjærenellike	x		x		x		x	
Achilea millefolium	alm_ røllike					x	x	x	x
Agrostis hybrid	hvene hybrid				x				
Anthriscus sylvestris	vild kørvel						x		
Arabidopsis thaliana	alm. gåsemad				x				
Arenaria serpullifolia	alm markarve				x				
Centaurea jacea	alm. knopurt							x	
Cerastium arvense	alm. hønsetarm					x			
Chenopodium album	hvidmelet gåsefod				x	x	x	x	
Chrysanthemum sgetum	gul okseøje						x		

Epilobium anagustifolium	alm. gederams		x	x	x		x
Epilobium sp.	dueurt		x	x	x		
Fallopia convolvulus	snerle-pileurt		x	x	x	x	
Galeopsis tetrahit	alm. hanekro			x			
Galium aparine	burresnerre			x	x		x
Galium boreale	trenervet snerre			x			
Galium mollugo	hvid snerre			x	x	x	x
Gnaphalium sylvaticum	rank evighedsblomst		x				
Gnaphalium uliginosum	sumpevighedsblomst	x				x	
Holcus lanatus	fløjlsgræs			x	x		
Juncus bufonius	tudse siv		x				
Luzula pilosa	håret frytle		x				
Papaver argemone	kølle valmue		x				
Plantago lanceolatum	lancet vejbred			x			
Poa pratenis ssp. angustifolia	smalbladet rapgræs			x	x	x	x
Poa pratense	eng rapgræs		x				
Poa trivialis	alm. rapgræs				x		x
Polygonum aviculare	vej pileurt			x			
Reseda lutea	gul reseda		x				
Rumex acetosella	rødknæ		x	x	x	x	
Senecio vernalis	vår brandbæger			x			
Silene alba	aften-pragtstjerne					x	
Silene dioica	dag pragtstjerne			x			
Silene vulgaris	blæresmælde			x	x	x	
Spergula arvensis	alm. spergel		x	x		x	
Spergularia rubra	mark hindeknæ		x				
Stellaria holostea	stor fladstjerne			x			
Taraxacum sp.	mælkebøtte		x	x		x	
Teesdalia nudicaulis	flipkrave		x				
Verbascum nigrum	mørk kongelys		x	x		x	
Veronica arvensis	mark ærenpris		x				
Viola tricolor	alm. stedmoderblomst	x	x	x	x	x	x

Summary
The report presents the results on effects of herbicides on plants found in natural habitats within the agricultural land. Furthermore, it evaluates whether the current risk assessment of herbicides represents an adequate safeguard for protection of these species and habitats. We found several areas where risk assessment seems to be insufficient. The most extensive conclusion is that seed production is a more sensible end-point for risk assessment of herbicides than the currently used end-point biomass. Crop species, in general, were not less sensitive to herbicides than non-target species. Finally, we found that interactions between species are important for their responses to herbicides.

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