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Pesticide effects on non-target terrestrial plants at individual, population and eco-system level (PENTA)

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Preface and acknowledgement

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Dansk sammendrag

Baggrund

Når markafgrøder sprøjtes med ukrudtsmidler kan lave doser af sprøjtemidlet via bl.a. vindafdrift spredes til omgivelserne og derved påvirke vilde planter og de fødekæder planterne er en del af negativt. En række undersøgelser har peget på afdrift af sprøjtemidler, som en væsentlig årsag for tilbagegangen af planter og tilknyttede dyrearter i nabohabitater til sprøjtede marker (fx Marrs et al. 1989; Bhatti et al. 1995; de Jong et al. 1995; Boutin and Jobin 1998; de Snoo 1999; Aude et al. 2003; Petersen et al. 2006; Gove et al. 2007; de Jong et al. 2008; Schmitz et al. 2013; Boutin et al. 2014). Ingen af disse undersøgelser præsenterer imidlertid samtidige målinger af afsat koncentration af sprøjtemiddel og påvirkninger på planterne.

Forud for godkendelsen af et sprøjtemiddel til markbrug skal der gennemføres en risikovurdering. I EU fremgår datakrav til brug for risikovurderingen af to forordninger: Kommissionens Forordning (EU) Nr. 283/2013 og Nr. 284/2013. Ifølge forordningerne skal der oplyses om mulige kort- og langtidsrisici for ikke-målarter, -populationer, -samfund og –processer. De standardtest, der gennemføres for at undersøge mulige påvirkninger på planter, omfatter sædvanligvis kun korttids potteforsøg af en varighed på 2 til 3 uger, hvor påvirkningen af plantebiomasse undersøges for en række afgrøde-arter, der vokser enkeltvis eller sammen med få individer sammen. Der stilles derfor hyppigt spørgsmål ved hvorvidt data fra standard test er velegnede og tilstrækkelige til at vurdere mulige påvirkninger af vilde planter og de fødenet og økosystemer planterne indgår i.

I risikovurderingen kan man, hvis der er behov for det, fastlægge restriktioner i anvendelsen af bekæmpelsesmidlet eller krav om at afdriftsreducerende tiltag som fx randzoner eller anvendelse af særlige dyssetyper ved udbringningen. Også i EUs landbrugspolitik (CAP) og nationalt findes en række virkemidler, der har til hensigt direkte eller indirekte at fremme biodiversitet, og dermed at bremse og reducere eventuelt negative påvirkning på biodiversitet i agerlandet bl.a. som følge af sprøjtemiddelanvendelsen. For at vurdere i hvilken udstrækning forskellige virkemidler yder den formodede reduktion i pesticidbelastningen og dermed beskyttelse af ikke-målarter er der behov for udvikling af pesticidindikatorer (EU 2009a). En væsentlig udfordring i forbindelse med udviklingen af indikatorer for reduceret herbicidbelastning er at finde en indikator, der direkte responderer på herbicideksponeringen og ændringer i denne. Samtidig skal det også være let at måle den pågældende indikator. Flere studier har vist at antallet af plantearter i nabohabitater til sprøjtede marker som fx hegn indeholder flere plantearter hvis herbicidpåvirkningen ophører eller reduceres væsentligt ved omlægning til økologisk jordbrug (fx Aude et al. 2003, Boutin et al. 2008, 2014, Strandberg et al. 2013). Inde i marken øges antallet af plantearter hurtigt efter omlægningen (Petersen et al. 2006, Jonason et al. 2011) hvorimod forøgelsen i artsantallet foregår meget langsommere i nabohabitater (Strandberg et al. 2013). På grund af denne forsinkelse i responset er artsantal ikke en velegnet indikator for reduceret herbicidbelastning. Blomstring af urterne i nabohabitater ser imidlertid lovende ud som indikator. Flere studier har påvist at herbicideksponering reducerer blomstringen signifikant (fx Schmiz et al. 2013, Boutin et al. 2014) og at ændringen i blomstring sker umiddelbart efter ændringen af belastningen, som det blev påvist for blomstring i hegn ved anlægges af en herbicid-fri randzone langs hegnet (Strandberg et al. 2013).

Formål

Formålet med projektet er at bidrage med yderligere viden omkring herbicideksponering som følge af vindafdrift og de påvirkninger af ikke-mål planter (non-target terrestrial plants, NTTPs), som lave herbiciddoser kan medføre. Det overordnede forskningsspørgsmål er: ”Hvordan og i

hvilket omfang påvirker herbicider ikke-mål planter (NTTPs) på individ- populations- og økosystemniveau?" og mere specifikt undersøge følgende hypoteser:

1. Planters reproduktion, målt som blomstring, frøproduktion eller spiringsdygtighed af F1-generation frø, er særlig følsom overfor påvitkning af lave herbicidkoncentrationer,

2. Plantepopulationer påvirkes af såvel konkurrence mellem arter og herbicideksponering og disse faktorer interagerer. Det er derfor nødvendigt at kende betydningen af begge faktorer hvis man skal vurdere herbicidpåvirkningen på populationsniveau,

3. Via påvirkninger på planters reproduktion kan herbicider have en uønsket effekt på plantesamfund og biodiversitet,

4. Urter i nabohabitater til sprøjtede marker udsættes ved vindafdrift for herbicidkoncentrationer, der påvirker blomstringen negativt,

5. Herbicideksponering af ikke-mål planter (vilde planter) reducerer og forsinker planternes blomstring og kan derved medføre tidsmæssig afkobling mellem blomstrende planter og besøgende insekter inklusive bier, og

6. Blomstring af urter i naturlige og semi-naturlige habitater kan benyttes som indikator for herbicideksponering.

Undersøgelser

Projektet består af følgende individuelle undersøgelser (i parentes fremgår hvilket kapitel undersøgelsen er beskrevet i):

- Væksthusforsøg hvor effekten af lave herbiciddoser på ikke-mål planter undersøges
  - Potteforsøg, der på individniveau undersøger effekten på biomasse, frøproduktion og spiring af F1-generation frø (Kapitel 2),
  - Potteforsøg, der på individniveau undersøger effekten på blomstring (Kapitel 3),
  - To-arts konkurrenceforsøg, der på populationsniveau undersøger samspil mellem herbicideffekt og plantekonkurrence (Kapitel 4),
- Afdriftforsøg hvor sammenhæng mellem herbicideksponering og påvirkningen af ikke-mål planter undersøges (Kapitel 5),
- Monitering af blomstring i randzonen langs vandløb (Kapitel 6), og
- Undersøgelse af blomstring som mulig indikator for herbicideksponering.

Undersøgelserne af korttidseffekter på individniveau følger standard test guidelines (OECD 2006a,b). For undersøgelser af langtidseffekter på individniveau og for konkurrenceforsøget har vi i projektet udviklet testprotokoller da sådanne ikke findes. For at vurdere reproducerbarheden af de udviklede protokoller mellem laboratorier har vi gennemført alle væksthusforsøg både i Canada og Danmark.

Konklusioner

Projektets overordnede konklusioner er:

1. Væksthusforsøg med eksponering af ikke-mål planter overfor lave herbiciddoser medførte signifikante påvirkninger i såvel kort- som langtids test målt som negativ påvirkning af biomasse, blomstring, frøproduktion og spiringsdygtighed af F1-generation frø og for flere plantearter resulterede eksponeringen overfor 5% af makdosis af glyfosat (= 72 g a.i./ha^{-1}) i at planterne døde før blomstring.

2. Den negative påvirkning af blomstring ved lave herbiciddoser omfattede både reduktion i det samlede antal blomster og forsinkelse af blomstringen. I potteforsøg var den samlede blomsterproduktion signifikant reduceret eller reduceret mere end 10%, men ikke signifikant, i 57% af de testede kombinationer (planteart*ukrudtsmiddel) og tidspunktet for maksimal blomstring forsinket i 40% af testene. Samtidig påvirkning af den samlede blomsterproduktion og tidspunktet for blomstring blev fundet i 25% af testene. Den største forsinkelse blev set for ioxynil+bromoxynil (27 dage) efterfulgt af glyfosat (24 dage), metsulfuron-methyl (22 dage), clopyralid (15 dage) og bromoxynil (9 dage).

3. Meta-analysen, der blev gennemført på data fra de individ-baserede test i væksthus, viste at planter generelt var mest følsomme i tidlige vækststadier. Denne overordnede konklusion dækker imidlertid over betydelig variation mellem test (planteart*ukrudtsmiddel) og for nogle arter blev der udelukkende fundet påvirkning ved eksponering på knopstadiet. En påvirkning, der ikke vil blive fundet i standard plantetest, hvor eksponeringen sker på 6-8 blad stadiet. Desuden skal tilføjes at data for påvirkning af blomstring ikke indgik i meta-analysen hvorfor vi ikke kan konkludere vedrørende følsomheden af blomstring i forhold øvrige mål (biomasse, frøproduktion, spiring) men som anført oven for var blomstring påvirket i betydeligt omfang.

4. Meta-analysen viste desuden at der for de undersøgte arter var en generel forskel i følsomhed mellem test udført i Danmark og Canada, hvor følsomheden var større i danske test sammenlignet med canadiske. Variationen i følsomhed var dog af en størrelse, der ikke er usædvanlig i plantetest og kan forklares ved en række mindre forskelle mellem landene uanset at samme protokoller blev anvendt. Det gælder fx væksthusbetingelser, som antal timer sollys, temperatur og fugtighed, der ikke kan kontrolleres fuldstændig. Desuden var der forskelle i sprøjteteknikken, der bevirkede at sprøjteudstyret ikke leverede samme mængde af sprøjtemiddel og planterne blev derfor ikke eksponeret fuldstændig ens selvom, der blev anvendt samme koncentration af sprøjtemidlerne. Endelige bidrager de lave koncentrationer, der blev anvendt, generelt til relativt større variation i testresultaterne end traditionelle dosis-repons forsøg, hvor en større andel af planterne dør.

5. Konkurrenceforsøget viste at konkurrence mellem planter påvirkede pesticidfølsomheden og at såvel konkurrence mellem individer tilhørende samme art (intraspecifik konkurrence) som konkurrence mellem forskellige arter (interspecifik konkurrence) var af betydning. Det er derfor ikke muligt på baggrund af standard plantetest at forudsige hvorledes herbicider vil påvirke plantepopulationer bestående af en eller flere arter ligesom det ikke er muligt på forudsige hvilken art, der vil blive dominerende under påvirkning af herbicider.

6. Felteksperimentet med vindafdrift fra marksprøjte med afdriftsreducerende dysser viste at de lave glyfosatkoncentrationer, der blev afsat på planter i nabohabitaten (højeste dosis var 2,8% af markdosis på 1440 g a.i. ha^{-1}), resulterede i signifikant reduktion i det samlede antal blomster for to af fire plantearter. Desuden var der en tendens til forsinket blomstring hos tre af de fire plantearter.

7. Ændringer i planters blomstring, som påvist i projektet, kan have negative konsekvenser for blomstersøgende insekter som fx bier og sommerfugle.

8. Selvom blomstring hos den enkelte planteart, der findes i naturlige eller semi-naturlige habitater, ikke et entydigt respons ved eksponering overfor lave herbiciddoser fandt vi at blomstringen hos samtlige arter i habitaten gav et entydigt og følsom indikation på de relativt små ændringer i herbicidpåvirkningen, der ses fx ved etablering af herbicid-fri randzoner eller ved omlægning til økologisk produktion. En af styrkerne ved at benytte blomstring som indikator for herbicidpåvirkningen er at blomstring er et funktionelt respons, der ikke er afhængig af tilstedeværelsen af specifikke plantearter. Desuden er blomstring relevant

som mål ikke bare for påvirkningen af plantesamfundet men også for de øvrige organismer, der er tilknyttet blomstrende planter fx bestøvende insekter.

Summary

Background

When crops in agricultural fields are sprayed by herbicides mostly sub-lethal doses of herbicides may be deposited on plants in habitats adjacent to the field and may potentially affect these non-target plant species and their corresponding food webs. Several studies have highlighted pesticide spray drift as a major factor affecting the flora and fauna of natural and semi-natural habitats adjacent to agricultural fields (e.g. Marrs et al. 1989; Bhatti et al. 1995; de Jong et al. 1995; Boutin and Jobin 1998; de Snoo 1999; Aude et al. 2003; Petersen et al. 2006; Gove et al. 2007; de Jong et al. 2008; Schmitz et al. 2013; Boutin et al. 2014). However, none of these studies include coherent measurements of herbicide deposition on the plants and plant responses.

Within the European Union, Commission Regulation (EU) No 283/2013 and No 284/2013 specify the information required before placing any pesticide on the market. According to the regulations, effects on NTTPs should be included. However, tests are mostly conducted with crop species as surrogates for the NTTPs and the core data required for authorisation of pesticides and other crop protection products only includes short-term effects on the biomass of plants grown individually in pots or in monoculture and harvested two to three weeks after spray. As a general requirement for substance approval, the Commission Regulation (EU) No 283/2013 also states, “the potential impact of the active substance on biodiversity including indirect effects via alteration of the food web, shall be considered”. The deviation between this overall protection goal and data underlying the risk assessment continuously results in the question of whether standard plant tests do provide sufficient data for the protection of NTTPs and biodiversity (e.g. EFSA PPR Panel 2014).

In order to follow-up on current policies (EU’s common agricultural policy (CAP), national initiatives) and mitigation measures that aim to reduce potential negative effects of spray drift on biodiversity in semi-natural habitats, there is a need to find common, harmonized pesticide indicators (EU 2009a). The challenge of identifying indicators that are valid as proxies for herbicide exposure comes both from the demand of a comprehensive data set and from a need of a close causality between the herbicide deposition and the response described by the indicator. Reductions in herbicide exposure may increase plant species richness in habitats adjacent to formerly sprayed fields and herbicide-free buffer zones (e.g. Aude et al. 2003, Boutin et al. 2008, 2014, Strandberg et al. 2013). Such an increase, however, contrary to the fast rise in plant diversity documented in some agricultural fields (Petersen et al. 2006, Jonason et al. 2011), has been shown to generally be a slow process outside the field (Strandberg et al. 2013). Therefore, species richness or the occurrence of sentinel species might not be fully suitable as indicators. In contrast, flowering of herbaceous plant species seems to be a strong candidate for use as a proxy for assessing herbicide effects on NTTPs. Several studies have documented that herbicide exposure reduces plant flowering (e.g. Schmitz et al. 2013, Boutin et al. 2014) and reduction of herbicide exposure due to establishment of herbicide-free buffer zones resulted in significant increases in plant flowering the year of establishment (Strandberg et al. 2013).

Objectives

This project aims at contributing to both the knowledge of deposition following spray drift and the effects of these mostly sub-lethal herbicide doses on NTTPs by answering the overarching research question: “How do herbicides affect non-target terrestrial plants (NTTPs) at individual, population and ecosystem levels?” Specifically, we test the following hypotheses:

1. Reproductive endpoints are particularly sensitive to sub-lethal doses of herbicides
2. Plant populations are affected by both herbicides and competition, and responses to both factors need to be measured in order to generate predictions on the effects on plant populations
3. Through effects on reproductive endpoints, herbicides have an important adverse effect on non-target plant populations and on biodiversity
4. Flowering of herbaceous species in natural and semi-natural habitats adjacent to fields treated with herbicides is affected by sub-lethal dosages of herbicides drifting into the habitats
5. Herbicide exposure of NTTPs reduces and delays flowering of herbaceous plants in semi-natural habitats leading to lack of resources and temporal mismatches between plants and pollinators
6. Flowering of herbaceous species in natural and semi-natural habitats are applicable as an indicator of herbicide exposure

Studies

The project comprises a number of individual studies including:

- Greenhouse experiments assessing the effects of low herbicide doses on NTTPs
  - Individual level effects of herbicides on NTTPs measured as effects on biomass, seed production and germinability of F1 generation seeds (Chapter 2)
  - Individual level effects of herbicides on NTTPs measured as effects on plant flowering (Chapter 3)
  - Population level effects of herbicide exposure and plant competition (Chapter 4)
- Spray-drift experiment measuring herbicide deposition and effects on NTTPs (Chapter 5)
- Monitoring of plant flowering in stream buffers (Chapter 6)
- Evaluation of plant flowering as a sensitive indicator for herbicide exposure (Chapter 7).

Studies of short-term effects at the individual level follow standard test guidelines (OECD 2006a,b) while we have formulated protocols for studies of long-term effects and for the competition experiment. In order to evaluate the reproducibility between laboratories of these protocols, the greenhouse experiments were carried out in Canada as well as Denmark.

Conclusions

The main conclusions from the project are:

1. The greenhouse study covering a broad spectrum of NTTPs (three annuals and six perennials belonging to six plant families) and herbicides (four herbicides with different modes of action, applied at rates of 1 and 5% of label rate, showed that low doses of herbicides found in spray drift may have significant, negative effects on NTTPs measured as effects on biomass, plant flowering, seed production and germinability of F1 seeds. For some plant species exposure to 5% of label rate of glyphosate (= 72 g a.i./ha^{-1}) resulted in mortality before the plants came to flower.

2. The low herbicide doses tested in the greenhouse experiments resulted in significant negative effects on total flower production and non-significant negative trends (>10% reduction) in 57% of all tested cases with reduction spanning from 4% to 100%. Delays to peak flowering were present either significantly or as trends towards a delay in 40% of all cases. Simultaneous effects on both flower production and time of peak flowering occurred in approximately 25% of all cases. The longest delays were observed for ioxynil+bromoxynil (27 days) followed by glyphosate (24 days), metsulfuron-methyl (22 days), clopyralid (15 days) and bromoxynil (9 days).

3. The meta-analysis performed on data from the present greenhouse studies confirmed that plants are generally most sensitive to herbicides at early life stages. This over-all conclusion, however, covers many different response patterns for individual cases (plant*herbicide) and it is evident that for some species effects were only observed when the plant was exposed at the bud stage. Such effects will not be covered by standard species tests where plants are only exposed at the 6-8 leaf stage. Furthermore, it has to be noted that plant flowering as an endpoint was not included in the meta-analysis, and therefore, it is not possible to conclude about the sensitivity of this endpoint relative to other endpoints, although we demonstrate the it is a sensitive endpoint (Chapter 3).

4. Furthermore, the meta-analysis showed that the tested species, generally, were more sensitive to the tested herbicides in Denmark than in Canada whatever the endpoint. However, the variability in sensitivity was in the range normally observed among laboratories and could be explained by minor differences between the study locations such as hours of sunshine, temperature and humidity, factors that can only be controlled to some extent. Moreover, variation in the spraying technique between countries resulting in differences in amount of herbicide delivered may also have contributed to the differences found. Finally, the low doses used in the present study may have contributed to a relatively larger variation in the responses that are commonly seen in dosis-response test in which a larger fraction of the plants dye.

5. The competition study conducted under greenhouse conditions showed that herbicide effects are influenced by intra- and interspecific competition. Based on individual species data as obtained in standard plant tests it could not be ascertained or predicted which species will be most sensitive when growing together and that the most sensitive species would likely be outcompeted when herbicide spray-drift is present as an additional stressor.

6. In the field experiment, it was shown that despite the low glyphosate concentrations (highest dose 2.8% of label rate (1440 g a.i.ha-1)) deposited on the vegetation in the spray drift experiment in which the sprayer was equipped with drift reducing nozzles, the cumulative number of flowers was significantly reduced for two of the four species found in the vegetation. Additionally, a trend towards delayed flowering was observed for three of the species although it was not significant.

7. The altered flowering of plants exposed to sub-lethal herbicide doses demonstrated in the present project may ultimately have negative consequences for flower-visiting insects including pollinators.

8. Although flowering of individual NTTPs does not respond uniformly, flowering of all NTTPs within a habitat was found to be a sensitive measure for herbicide exposure. One of the strengths of utilizing flowering of all species as indicator is that the indicator is not dependent on the occurrence of specific species but on the functional response of the species present. Furthermore, using flowering could be a potential indicator of the health of the plant community as a whole.

1. Introduction

1.1 Rationale

Before placing any pesticide on the market, the risk to human health and the environment is assessed in an environmental risk assessment (ERA). Within EU, the Commission Regulation (EU) No 283/2013 and No 284/2013 lay down data requirements for the authorization of active substances and plant protection products, respectively. Although the regulations require information on effects on non-target terrestrial plants (NTTPs), tests are mostly conducted with crop species as surrogates for the NTTPs. However, several traits distinguish crops from NTTPs, e.g. most crops are annuals while most NTTPs are perennials, and it is debatable whether or not ERA based on crop data represents an adequate safeguard for protection of NTTPs. The results of studies comparing sensitivity of crops and NTTPs to herbicides are inconclusive and some have documented no significant differences (Boutin and Rogers 2000; McKelvey et al. 2002; Olszyk et al. 2006; White and Boutin 2007; Carpenter and Boutin 2010; Strandberg et al. 2012), while others have found NTTPs to be significantly more sensitive than crops (Boutin and Rogers 2000; Schmitz et al. 2012; Strandberg et al. 2012; Boutin et al. 2014).

1.1.1 Short-term and long-term test – selection of end-point

While effects on reproductive output are routinely tested for some animals, these effects have received little attention when looking for effects of herbicides on plants; and reproductive endpoints are not an integrated part of the current ERA. Here only data on effects on seedling emergence and vegetative vigour are requested. As a consequence, no common protocol for plants using reproductive endpoints has been adopted. Recently, however, a number of studies have documented that herbicides significantly affect plant reproduction, i.e. flowering and seed production, even at low dosages and irrespective of time of application relative to plant life stage. Most of these studies found reproductive endpoints more sensitive than vegetative (Olszyk et al. 2006; Riemens et al. 2009; Carpenter and Boutin 2010; Strandberg et al. 2012; Boutin et al. 2012; Carpenter et al. 2013; Boutin et al. 2014). Based on the very limited number of studies available at the moment, seed production seems to be about ten times as sensitive as biomass when testing effects of herbicides on plants (EFSA PPR Panel, 2014). In addition, recent studies show that herbicides affect plant flowering (Schmitz et al. 2013, Boutin et al. 2014). Schmitz et al. (2013) found that flower density of *Ranunculus acris* was greatly reduced with recurrent application of sub-lethal doses of glyphosate and Boutin et al. (2014) found reductions in flowering intensity and delay of flowering of *Taraxacum vulgare* and *Trifoium pratense* as consequences of exposure to fluroxypur. The combined effects of fertilizer and herbicide (glyphosate) on flowering of individual plant species has been studied in a recent project (ECOMARG). In this study, flowering phenology of two composite species *Tanacetum vulgare* and *Leucanthemum vulgare* was investigated in a long-term experimental plot http://bios.au.dk/forskning/faciliteter/long-term-experimental-plot/. These results showed that glyphosate application reduced floral abundance (measured as the number of flowering stems) and delayed flowering of *Tanacetum vulgare*, whereas application of nitrogen moderately increased floral abundance, but had no significant effect on timing of flowering (Damgaard et al. 2016; Dupont et al., submitted).

Within the present project, we have tested and compared vegetative and reproductive endpoints as measures of sensitivity of selected NTTPs exposed to herbicides. We have formulated and used common test protocols that might subsequently form the basis for interlaboratory testing (round robin testing) with reproductive endpoints. In addition, effects size (Hedges'

g) have been calculated using the meta-analysis statistics to determine the most sensitive endpoint.

1.1.2 Effect at individual, population and ecosystem level

Whereas current ERA is carried out at the individual species level and only include short term effects, the protection goals are defined at the population and ecosystem level, encompassing short-term (acute) as well as long-term (chronic) effects (EU 2009b; Nienstedt et al. 2012). Today, very little is known about long-term herbicide effects on plant populations, and no tools are available to bridge the gap between data generated at the individual level and predictions of long-term chronic effects on non-target plant populations. At the moment, an urgent need to fulfil this gap is being formulated (EFSA PPR Panel 2014). A few microcosm studies (Riemens *et al.*, 2008; Riemens *et al.*, 2009; Dalton and Boutin, 2010), fields experiments (Perry *et al.*, 1996; Strandberg *et al.*, 2007; Strandberg *et al.*, 2012) and competition experiments (Strandberg *et al.*, 2007; Damgaard *et al.*, 2008; Strandberg *et al.*, 2012), form useful starting points; and within the present project, we have further developed methods for estimating and predicting the effect of herbicides on plant populations by carrying out dose response experiments in the greenhouse on plants growing in conspecific and heterospecific populations and by empirical modelling. Another approach to bridging the gap between standard plant tests and the protection goals formulated for population and ecosystem might by mechanistic models such as the one recently published by Reeg et al. (2017). This model, however, does only include plant community responses to grazing and not responses to herbicides. The authors intend to include herbicide effects as plant sensitivity in standard plant tests. Following such updating of the model, it still will need validation under field condition before it might be useful for assessment of community and landscape effects.

1.1.3 Measuring effects of herbicide spray drift on plants

Several studies have pointed out pesticide spray drift as a major factor affecting both the flora and fauna of natural and semi-natural habitats adjacent to agricultural fields, and applications of fertilizers and pesticides are regarded as playing important roles for the declines in plant species richness within agricultural areas (e.g. Marrs et al. 1989; Bhatti et al. 1995; de Jong et al. 1995; Boutin and Jobin 1998; de Snoo 1999; Aude et al. 2003; Petersen et al. 2006; Gove et al. 2007; de Jong et al. 2008; Strandberg et al. 2012; Schmitz et al. 2013; Boutin et al. 2014) with parallel declines being documented for pollinators and insect-pollinated plants (Biesmeijer et al. 2006; Carvell et al. 2006; Kleijn and Raemakers 2008).

Whereas both fertilizers and herbicides reduce the plant species richness, recent studies show that herbicides also affect plant flowering (Schmitz et al. 2013, Boutin et al. 2014) by reducing the number of flowers on the plants and delaying the flowering period of herbaceous species in field margins, meadows as well as in hedgerow ground vegetation. Thereby, herbicides have the potential to adversely affect both floral diversity and through effects on resources higher trophic levels including flowering visiting insects such as pollinators.

Within the present project, we investigated the effects on diversity and flowering by exposing experimentally established vegetation to drifting herbicide in order to establish causality between measured herbicide deposition and flowering under field conditions. In addition, we examined whether effects on diversity and flowering similar to those found in hedgerows, field margins and meadows can be found within stream buffers, which form another common type of habitat adjacent to agricultural fields and hence subjected to drifting herbicides.

1.1.4 Development of indicators for herbicide exposure

Facing the biodiversity crisis and the failing success of halting biodiversity decline in agricultural areas (EEA 2006; Butchart et al. 2010; EEA 2010), there is a growing need to protect semi-natural habitats from the potential effects of pesticide drift. In Europe, both EU and na-

tional agricultural policies have been implemented to reduce or revert the negative environmental impacts of modern agricultural practices (Kleijn and Sutherland 2003; Uthers et al 2011). However, the efficiency of these schemes to protect biodiversity has been questioned (Kleijn and Sutherland 2003; Hole et al. 2005; Brittain et al. 2010). The EU Directive for Sustainable Use of Pesticides, Directive 2009/128/EC (EU 2009a) specifies the need for common, harmonized pesticide indicators to follow up on mitigation measures. Reduction in pesticide exposure may increase plant species richness (e.g. Aude et al. 2003; Boutin et al. 2008); this increase, however, contrary to the fast rise in plant diversity in agricultural fields (Petersen et al. 2006; Jonason et al. 2011), has been shown to be a slow process in semi-natural habitats (Strandberg et al. 2013). Plant flowering of herbaceous species at the hedgerow ground, however, seems to be a sensitive and responsive indicator for both conversion to organic farming and establishment of herbicide-free buffer zones that responds the same year buffer zones are established (Strandberg et al. 2013; Boutin et al. 2014). Flowers are often conspicuous and easy to identify, and assuming the responses of plant flowering are robust and specific, plant flowering may fulfil the requirements of a good indicator (Heink and Kowarik 2010). Within the present project, we evaluated plant flowering as indicator for herbicide exposure, and developed a protocol for sampling flowering on a larger scale for application as a nationwide indicator and define how flowering may translate into a quantitative pesticide impact footprint.

1.2 Project hypotheses

In order to answer the overarching research question: “How do herbicides affect non-target terrestrial plants (NTTPs) at individual, population and ecosystem levels?” we have formulated the following hypotheses:

1. Reproductive endpoints are particularly sensitive to sub-lethal doses of herbicides
2. Plant populations are affected by both herbicides and competition, and responses to both factors need to be measured in order to generate predictions on the effects on plant populations
3. Through effects on reproductive endpoints, herbicides have an important adverse effect on non-target plant populations and on biodiversity
4. Flowering of herbaceous species in natural and semi-natural habitats adjacent to fields treated with herbicides is affected by sub-lethal dosages of herbicides drifting into the habitats
5. Herbicide exposure of NTTPs reduces and delays flowering of herbaceous plants in semi-natural habitats leading to lack of resources and temporal mismatches between plants and pollinators
6. Flowering of herbaceous species in natural and semi-natural habitats are applicable as an indicator of herbicide exposure

The project includes a number of different studies encompassing pot, competition and field experiment, field oberservations and mathematical modelling. Table 1 gives an overview of the different studies and the end-points they include and which hypotheses they test.

TABLE 1. Overview of the relationsship between the different studies, tested endpoints, hypotheses and the chapter where the studies and results are presented and discussed.

Study	End-points	Hypothesis	Chapter
Pot experiments - individual level effects (short- and long-term)	Biomass Seed production Seed germinability (F1)	1	Chapter 2
Pot experiments - individual level effects (long-term)	Plant flowering	1	Chapter 3

Two-species competition: herbicide exposure and competition (population level effects)	Biomass Seed production	2	Chapter 4
Reproductive endpoints measured at individual, population and ecosystem level	Plant flowering Seed production Seed germinability	3	Chapter 3, 4, and 5
Effects of spray drift: experimental approach and monitoring in stream buffers (Ecosystem level effects)	Measure of deposition from spray drift (Chapter 5) Plant flowering	4	Chapter 5 and 6
Effects of herbicides of plant flowering	Timing of flowering Cumulative number of flowers	5	Chapter 3 and 5
Plant flowering as indicator	Number of flowers	6	Chapter 7

1.3 Background

1.3.1 Individual level effects of herbicides on non-target terrestrial plants

Standard plant tests, i.e. using plant species grown individually in pots or in monoculture, only considering biomass two or three weeks after spray in dose-response experiments and following strict guidelines (OECD 2006a, b; USEPA 2012) are usually performed on annual species, often crops. Previous studies reported contradicting conclusions as to whether crops may or may not be suitable surrogates for NTTPs (Strandberg et al, 2012, Boutin et al., 2004, McKelvey et al., 2002, Boutin & Rogers 2000). Furthermore, annual and perennial species do not consistently differ in their sensitivity to herbicides in the short-term (Boutin et al., 2004; White et al., 2007; Carpenter and Boutin, 2010; Boutin et al., 2012; Carpenter et al., 2013; Strandberg et al., 2012) and it is questionable whether differences exist in the long-term. Additionally, plant sensitivity to herbicides may vary depending on their phenological stage at the time of exposure and responses may differ depending on the endpoints measured, whether vegetative or reproductive.

The most common endpoints used in ERA are biomass and assessments of visible effects such as stunted growth and chlorosis (EFSA PPR Panel 2014). Although plants are known to be very sensitive to herbicides at early growth stages, it has been demonstrated that reproductive measures was more sensitive measures of herbicidal effects than biomass in more than 50% of the examined cases (Boutin et al., 2014, EFSA PPR Panel 2014). Following exposure at the seedling stage, effects may be observed on growth, flowering and/or seed set. In contrast, exposure at the reproductive stage has been shown to result in larger responses on reproductive endpoints such as flower and seed production compared to biomass as plant growth has nearly ceased at this stage and vegetative endpoints may no longer be relevant effect measures when plants are exposed at reproductive stages (Strandberg et al., 2012).

Additionally, reproductive measures can be expected to be more sensitive than vegetative measures to low doses of herbicides, as plants that are able to recover biomass likely had less resources to devote to flower or seed production (Carpenter and Boutin, 2010; Strandberg et al., 2012; Carpenter et al., 2013; Boutin et al., 2014). For instance, Egan et al. (2014b) also observed that for *Verbena utricifolia* and *V. hastata*, reproductive measures of inflorescence length were more sensitive than biomass for dicamba and glyphosate, but not for atrazine. In addition, the stage at which plants are exposed can further influence the magnitude of the observed effects (Gilreath et al., 2001; Riemens et al., 2009; Griffin et al., 2013; Aguilar-Dorantes et al., 2015).

Delays in flowering and/or reductions in flower (or fruit) production have been found for a variety of NTTP and crop species treated with low doses of a number of different herbicides including glufosinate (Boutin et al., 2014), chlorimuron (Carpenter et al., 2013; Boutin et al., 2014), dicamba (Bohnenblust et al., 2016; Colquhoun et al., 2014), 2,4-D (Hatterman-Valenti and Mayland, 2005), metsulfuron-methyl (Kjær et al. 2006), and fluroxypyr (Boutin et al., 2014).

1.3.2 Population level effects of pesticides on non-target terrestrial plants

The scheme for standard plant test is very remotely related to conditions in which NTTPs are found within agroecosystems whereby they grow within natural or semi-natural communities of several species interacting with each other and where conspecific and interspecific competition are very prominent. As phytotoxicity tests in ERA are performed using individually potted plants, these competitive interactions are not considered and make individual level and single species test results less realistic and trustworthy.

In greenhouse experimental trials for regulatory testing, plants do not experience predation, pathogens, adverse environmental conditions, fluctuation in resources or competition (Cairns 1984; Pfleeger et al. 2012). Under field conditions, for instance, neighbouring plants are an important limiting biotic factor for plant growth and reproduction, and the competition that arises is thought to be one of the most important factors influencing the composition of plant communities (Harper 1977; Weiher et al. 1998; Weiner 1993). Individual plants in a natural community will compete not only with conspecifics (intraspecific competition) but also with members of other plant species (interspecific competition) for limited resources (Harper 1977). As plant species have different sensitivities to herbicides, it can be expected that herbicide drift will affect competition by inhibiting some plant species more than others (Damgaard et al. 2014). If herbicide drift is affecting the competitive relationships between species, the result can be a community change in the long-term, as more sensitive species will be outcompeted and displaced by more tolerant ones (Boutin & Jobin 1998; Petersen et al. 2006; Gove et al. 2007). As such, plant-plant interactions may be important in two ways in terms of herbicide drift: herbicide deposition on individual plants can be affected by the structure of its neighbours, and any adverse effect on the performance of one species can promote the growth of others (Marrs et al 1991a). Therefore, the effect of an herbicide may depend strongly on plant competitors. For instance, in a greenhouse competition experiment by Damgaard et al. (2008) it was expected that *Geranium dissectum* L. would outcompete *Capsella bursa-pastoris* (L.) Medik. when exposed to the herbicide mecoprop-P. This was predicted based on the results of individual level tests, in which *G. dissectum* was less sensitive to mecoprop-P than *C. bursa-pastoris*. However, this expectation was not met in experiments with the two species growing together as the interspecific competitive abilities of both species increased significantly when exposed to the herbicide. It was concluded that in this case, the single species test was too conservative. In a field study by Damgaard et al. (2014), it was found that low doses of glyphosate altered the competitive interactions between two grass species, *Agrostis capillaris* L. and *Festuca ovina* L. With increasing levels of glyphosate, F. ovina became a better competitor than *A. capillaris*. This explained why *F. ovina* was found to be dominant in field plots treated with higher levels of glyphosate (Damgaard et al. 2014).

The most common endpoints used in ERA are biomass and assessments of visible effects such as stunted growth and chlorosis (EFSA PPR Panel 2014), even though several studies have shown that reproductive endpoints can be more sensitive than the vegetative ones, and adverse effects on reproduction can harm population and community dynamics (Fletcher et al. 1993; Riemens et al. 2009; Carpenter & Boutin 2010; Strandberg et al. 2012; Boutin et al. 2014). One of the reasons this may be is that while some plants have been noted to be able to recover from initial losses of biomass (Marrs et al. 1991b; Riemens et al. 2009; Carpenter et al. 2013), the energy required to do so may alter reproductive success (EFSA PPR Panel

2014). Recovery could be further hindered by competition, where again more sensitive species get outcompeted. As such, solely assessing the effects on vegetative structures may be underestimating the sensitivity of non-target terrestrial plants (NTTPs; EFSA PPR Panel 2014).

1.3.3 Ecosystem level effects of pesticides on non-target terrestrial plants

Biodiversity within agroecosystems is provided primarily by small non-crop habitats situated at the edge of crop-fields or interspersed within farmlands. These habitats comprise herbaceous field margins, woody hedgerows, riparian boundaries, and small wetlands and woodlots which harbour numerous plants, invertebrates and other animals. Non-crop habitats, however, are subjected to numerous perturbations and stressors, such as drift and runoff of pesticides that are applied in the neighbouring fields in conventional agriculture. Several studies have indicated that displacement of pesticides and fertilizers are major factors affecting the flora of natural and semi-natural habitats adjacent to agricultural fields, resulting in overall declines of plant species richness within agricultural areas (e.g. Marrs et al. 1989; Bhatti et al. 1995; de Jong et al. 1995; Boutin and Jobin 1998; de Snoo 1999; Aude et al. 2003; Petersen et al. 2006; Gove et al. 2007; de Jong et al. 2008; Strandberg et al. 2012; Schmitz et al. 2013; Boutin et al. 2014. Furthermore, from 1980 onwards, parallel declines have been documented for pollinators and insect-pollinated plants (Biesmeijer et al. 2006; carvel et al. 2006; Kleijn and Raemakers 2008; Potts 1980; Holzschuh et al 2007; Egan et al. 2014a). Whereas both fertilizers and pesticides (mostly herbicides) reduce the plant species richness, recent studies showed that sub-lethal doses of herbicides also affect plant flowering. Several studies have shown reductions in flowering caused by sub-lethal doses of herbicides (e.g. Marrs et al. 1991a; Gove et al. 2007; Boutin et al. 2014; Schmitz et al. 2014; Bohnenblust et al. 2016). Delays in flowering have also been noted in several NTTPs (Carpenter et al. 2013; Boutin et al. 2014; Bohnenblust et al. 2016) and crop varieties exposed to a wide range of herbicides (Wall et al. 1995; Pline et al. 2003a; Pline et al. 2003b; Bohnenblust et al. 2016).

Reductions in flowering due to herbicide exposure can have many repercussions in a plant community. Both delays and reductions in flowering can have subsequent effects on the population by affecting the number of seeds produced that year. Reductions in the amount of seeds produced by several species have been documented for many herbicides (Fawcett and Slife 1978; Isaacs et al. 1989; Marrs et al. 1989; Fletcher et al. 1996; Taylor and Oliver 1997; Riemens et al. 2008; Rokich et al 2009; Carpenter and Boutin 2010; carpenter 2013). Fewer seeds produced in a year also affect the seedbank – the natural store of seeds in the soil (Ball 1992; Rokich et al. 2009; Albrecht 2005; Crone et al. 2009). Herbicides can also be detrimental to seed viability. A number of studies have found that herbicides affect F1 seed germination (Nelemans et al. 2017; Blackburn and Boutin 2003 and references therein). Smaller plant populations, that would naturally have fewer flowers, have been shown to attract fewer pollinators overall, and generally have lower quality pollinators (Wilcock and Neiland, 2002; Kremen et al., 2007; Ison and Wagenius, 2014 and references therein). Klinkhamer et al. (1989) reported lower bumblebee visitation to *Cynoglossum officinale* plants containing fewer flowers. Timing of flowering in a plant population is more complex; plants need to ensure that they have the proper energy requirements to produce viable seed, but also need to ensure that they still have resources for growth and defense (Franks, 2015). Asynchronous flowering between individuals or between populations could potentially reduce both pollen transfer and gene flow within the community, especially for species that rely on outcrossing. Augspurger (1981) observed differences in *Hybanthus prunifolius* (Schult.) Schulz seed output when plants were experimentally stimulated to flower earlier in order to create asynchrony with the natural population. Temporally isolated plants had lower pollination success than the normal population, and were also more prone to seed predation. Further hindering this could be physiological changes in affected plants that further reduce reproductive success. For instance, anther deformations and reductions in pollen quality and production in affected individuals have been

documented for the herbicides glyphosate and glufosinate ammonium (Baucom et al., 2008; Londo et al., 2014; Gauvrit and Chauvel, 2010).

Delays in flowering could potentially lead to asynchrony with the emergence or presence of certain pollinators, likely reducing their available food supply. A plant could miss a key, highly effective pollinator species (Franks, 2015), or the absence of an attractive plant species (often termed a “cornucopian” species) may fail to bring in pollinators to a given patch (Jennersten, 1988; Kammerer et al. 2016), thus affecting all plant species within the plot. Conversely, delays could lead to an overlap in flowering periods between otherwise non-overlapping species, increasing pollinator competition between plants especially if one species offers significantly better floral rewards (Mosquin, 1971). Both floral resources and floral diversity have been correlated with pollinator abundance and richness, as both can increase the diversity of niches (i.e. nectar resources, floral morphology, etc.) within a habitat (Kremen et al., 2007). From a community perspective, having many plants of different species flowering together could increase the attractiveness of a patch to pollinators, thus leading to species facilitation and complementation; conversely, too many flowers could lead to oversaturation and thus lead to competition (Elzinga et al., 2007). Pollinators may avoid patches containing fewer or poorer quality flowers, thus further limiting pollen flow and transfer between individual plants (Kearns et al., 1998). Lack of flowers in a particular corridor may also act as a barrier to pollinator movement within the landscape, potentially creating isolated patches of plants that pollinators may selectively avoid. If there is a continuous population of flowers (i.e. there are no patches), the movement of pollinators is shorter and will likely result in more successful pollination events (Wilcock and Neiland, 2002). For instance, Jennersten (1988) observed fewer pollinators in isolated patches of *Dianthus deltoides* even though overall plant densities were similar to non-isolated patches. In addition, flowers were visited less, and fewer seeds produced in these isolated patches.

1.3.4 Using plant flowering as indicator for pesticide exposure

The EU Directive for Sustainable Use of Pesticides, Directive 2009/128/EC, (EU, 2009a) specifies the need for common, harmonized pesticide indicators to follow up on risk assessment including mitigation measures.

Over the last two decades, indicators for ecosystem impact due to pesticide application have received attention (e.g. Kruijne et al., 2010; Kjær et al., 2008; Gutsche and Strassenmeyer, 2007; Reus and Leendertse, 2000; Møhlenberg et al., 2001; Clausen, 1998; Gustavson et al., 2008; Claeys et al., 2005; Vercruysse and Steurbaut, 2002; Lewis et al., 2003; Juraske et al., 2007; van der Werf and Zimmer, 1998; Alister og Kogan, 2006). The indicators developed in these studies are load indicators or mathematical models that estimates the toxicological load without predicting effect full-scale levels. In these indicators, records of used quantity of the active ingredient are combined with toxicological and physio-chemical data from standardized laboratory testing to calculate indexes for one or a number of endpoints and fate properties that are considered significant. Such indicators will only be relativistic by predicting one ecosystem as being more or less impacted by pesticides than another ecosystem (Sørensen et al., 2009). The reasoning is that it is impossible to predict the real impact on ecosystems by using indicators that are only based on laboratory testing data, assumed pesticide application and fate. This type of indicator can instead predict the impact due to one pesticide application as more or less strong compared to the impact due to another pesticide application.

In ecology, however, the quality of an indicator is mainly determined by a close relationship between indicator and indicandum, i.e. the indicated phenomenon, while the relevance of an indicator for a given issue is of paramount importance for conservation policy (Heink and Kowarik, 2010). The development of stream invertebrate community as an indicator for pesticide exposure (Liess and von der Ohe 2005) is an example of an ecological-type indicator.

The challenge of making indicators that are valid as proxy for the indicandum comes both from the demand of a comprehensive data set and from a need of a close causality between the impact due to the pesticide application, and the response described by the indicator. However, this investigation will face those challenges by analysing how the identified reduction of flowering due to pesticide application may be useful in developing a pesticide indicator.

2. Individual level effects of herbicides on non-target terrestrial plants - biomass, seed production, germinability

2.1 Introduction

Using parallel studies conducted in greenhouses in Denmark and Canada we seek to further address the effects of low, sub-lethal herbicide exposure on non-target terrestrial plants (NTTPs) using different endpoints including vegetative (biomass) as well as reproductive (seed production, germinability of F1 seeds) measures.

The present study focuses on providing more evidence on some of the issues raised in the EFSA opinion (EFSA PPR Panel, 2014) and addresses the following questions of which the first and second relate to the projects hypothesis that reproductive endpoints are particularly sensitive to sub-lethal doses of herbicides:

1. Are NTTPs more sensitive to herbicide spray drift at the reproductive growth stage (bud stage) compared to the vegetative stage (seedlings)?
2. Are reproductive endpoints more sensitive parameters than the vegetative endpoints?
3. Are annual and perennial NTTPs equivalent in their sensitivity to spray drift? and
4. Are effects of low doses of herbicides on NTTPs reproducible between laboratories when following a common protocol?

2.2 Material and methods

The study was carried out as pot experiments in greenhouses at two locations – Aarhus University, Research Centre Flakkebjerg in Denmark (DK, GPS: 55.324382, 11.390041) and Environment and Climate Change Canada in Ottawa Canada (CA, GPS: 45.3876, 75.6960).

2.2.1 Test plants

Nine non-target terrestrial plant species from six plant families were tested. They consisted of three annual species: *Centaurea cyanus* L., *Silene noctiflora* L., and *Viola arvensis* Murray, and six perennial species: *Cerastium arvense* L., *Cirsium arvense* (L.) Scop., *Epilobium montanum* L., *Knautia arvensis* (L.) J.M. Coult., *Taraxacum officinale* F.H. Wigg. and *Trifolium pratense* L. The same seed stocks were used in both the Danish and Canadian experiments, with the following exceptions. Due to unknown reasons, seeds of *K. arvensis* failed to germinate sufficiently in the Canadian trial, and thus this species was replaced with another perennial, *T. pratense* in the Canadian trial only. The nine species have been selected as they respresent a broad spectrum of species including annuals as well as perennials which are all insect pollinated and important as food resources for flower-visiting insects including pollinators.

Seeds of each species were sown separately into 2 L pots containing formulated potting soil. Soil composition varied between the experiments conducted in Denmark and those in Canada due to the availability of specific base materials. The Danish soil was composed, by weight, of 38.7% dry field collected soil (a sandy loam comprised of 12% clay, 6.5% silt, 80% sand, and

1.5 % organic matter), 25.8% coarse sand/gravel, and 35.5% peat medium (Fin-Peat B2). The Canadian soil was designed to best replicate the composition of the Danish soil, and was composed of 60.5% all-purpose horticultural sand, 34.9% peat-based potting soil (Pro-Mix® BX General Purpose Growing Medium, Premier Horticulture Ltd., Rivière-du-Loup, QC, CAN), and 4.6% kaolin clay (Edgar Minerals Inc., Edgar, FL, USA) by dry weight. All soils were added the same micro- and macro-nutrients (0.11% nitrogen, 0.04% phosphorus and 0.07% potassium).

After emergence the number of plants per pot was reduced to three for pots used for biomass measurement and one for pots used for seed harvest.

2.2.2 Herbicides

Four widely used herbicides with different modes of action were included in the study (Table 2).

TABLE 2. Herbicides and doses used in the study

Active ingredient	Commercial product DK	Commercial product CA	Mode of action	Recommended dose in g a.i./ha (1% and 5% of recommended dose)
Glyphosate	Glyphogan, 360 g a.e./L, Adama Northern Europe B.V.	Glyphos® 360 g a.e./L Cheminova Canada Inc.	Inhibition of EPSPS synthase	1440* (14.4, 72)
Metsulfuron-methyl	Ally SX, 200 g a.i./kg, Du Pont Denmark ApS	Not available in Eastern Canada	ALS-inhibitor	6 (0.06, 0.3)
Ioxynil+bromoxynil in DK Bromoxynil in CA	Briotril 400 SC, 160 + 240 g a.i./L, Adama Northern Europe B.V.	Pardner® 280 g a.i./L bromoxynil Bayer CropScience Inc.	Inhibition of photosynthesis II	240 DK; 280 CA (2.4, 12) DK (2.8, 14) CA
Clopyralid	Matrigon SG, 720 g a.e./kg, DOW AgroSciences Denmark A/S	LontrelTM 360 360 g a.e./L DOW AgroSciences Canada Inc.	Synthetic auxin	80 (0.8, 4)

*Recommended dose for control of *Elytrigia repens*

Glyphosate (N-(phosphonomethyl)glycine) is the world's most commonly used herbicide in agriculture (Benbrook 2015). It is a post-emergent, systemic and non-selective herbicide used for the control of annual and perennial grasses and broad-leaved weeds (Franz et al. 1997). It is taken up through the leaves, after which it is translocated primarily via the phloem throughout the entire plant, concentrating in the actively growing tissues such as meristems (Franz et al. 1997; Tomlin 2000). With the introduction of glyphosate tolerant crops in 1996, its use increased, with glyphosate replacing many other herbicides (Young 2006). Glyphosate mode of action is by inhibition of the activity of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase involved in the shikimic acid pathway (Liu et al. 1997), which is used for biosynthesis of several aromatic plant metabolites, including the amino acids tyrosine, tryptophan and phenylalanine (Franz et al. 1997; Tomlin 2000). The result is the disruption of protein synthesis and growth, leading to plant death (Monsanto 2014). In the experiments, Glyphos® (Cheminova Canada Inc.) was used in Canada and Glyphogan (Adama Northern Europe) was used in

Denmark. Both products contain 360 g a.e. L^{-1} of glyphosate as an isopropyl amine salt and the formulations include tallow amine ethoxylat. We expect that glyphosate has a relatively large effect on all test species.

Metsulfuron-methyl (methyl 2-[[[[(4methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate) is a sulfonylurea herbicide used to control dicot weeds in cereals. The herbicide is taken up mainly by the foliage and translocated via xylem and phloem. Sulfonylureas are selective herbicides that inhibit the enzyme acetolactate synthase (ALS), which catalyzes the synthesis of three branched amino acids, valine, leucine and isoleucine (Ray 1986; Bacmaga et al. 2015; Yuan et al.; Brown 1990). The inhibition of ALS enzyme results in a rapid cessation of growth and eventual plant death occurs within one to three weeks. Visual symptoms such as chlorosis and discolouration appear a few days later. Selectivity in some species is due to metabolic inactivation to non-phytotoxic compounds (Brown 1990, Beyer et al 1988 in Boutin 2000). In the experiments, Ally SX® formulated as a water-soluble granule and containing 200 g kg-1 metsulfuron-methyl was used (Du Pont Denmark ApS). Ally is expected to have a large effect on *S. noctiflora* and *V. arvensis*, a medium effect on *C. arvensis* and a low effect on all other test species.

Ioxynil and bromoxynil belong to the nitriles, which are a group of photosystem II inhibitor herbicides. Both active ingredients are primarily contact herbicides as they are readily absorbed through the cuticle with little or no basipetal movement to other plant parts (Davies et al. 1968). They inhibit the photosynthesis by blocking electron transport in the photosystem II complex. Ioxynil and bromoxynil are used in cereals, onion, poppies, rice flax and pastures. In Denmark, ioxynil and bromoxynil were marketed as a co-formulation until February 2015 when the approval of ioxynil expired. In Canada, bromoxynil is used as a solo product. Both ioxynil + bromoxynil and bromoxynil are expected to have a relatively little effect on all test species.

Clopyralid is a synthetic auxin causing epinastic bending and twisting of stems and petioles, stem swelling and leaf curling. This is followed by chlorosis at the growing points, growth inhibition, wilting and necrosis. Clopyralid is absorbed mainly by foliage and is readily transported in plant tissues, primarily via the symplasm and it readily accumulates in the actively growing plant parts. Clopyralid is used in a wide range of crops e.g. sugar beets, Christmas trees, maize, grasses and oilseed rape to control many annual and perennial broadleaf weeds. Clopyralid is expected to have large effect on *C. cyanus, Cirsium arvense, E. montanum, T. officinale* and *T. pratense*.

2.2.3 Herbicide application

To assess effect of spray drift exposure on plants the herbicides were applied at 1% and 5% of recommended doses (Table 2). Glyphosate is used for several purposes and the recommended dose used in this study refer to the one for control of *Elytrigia repens* (1440 g a.i./ha). For consistency and replicability, application rates of glyphosate and clopyralid in the Canadian part of the experiment were similar to those used in Denmark.

In Denmark the herbicides were applied using a cabinet pot sprayer (JK Design, Denmark) equipped with two Hardi ISO-02-110 nozzles. The nozzles were operating at a pressure of 300 kPa and a velocity of 5.8 km/h delivering a spray volume of 160 L/ha. In Canada plants were sprayed with a track spray booth (DeVries Manufacturing, MN) equipped with Teejet 8002E flat fan nozzle delivering 77 L/ha at a pressure of 207 kPa.

Groups of pots were sprayed when the plants had either the 6-8 leaves or at the flower bud stage. In each case, a series of plants were harvested three weeks after spray to assess short-term vegetative effects (pots containing three plants), and a second set of plants was allow to flower and produce seed in order to assess effects on reproductive endpoints during the full life cycle (pots containing a single plant).

The experiment included 1360 pots in Denmark and 1024 pots in Canada (8 plant species x 2 growth stages at spray x 4 herbicides in Denmark/3 herbicides in Canada x 2 doses x 2 harvesting times (vegetative and reproductive) with 3 replicates for biomass (plus 6 controls for each growth stage) and 5 replicates for reproduction and germinability (plus 10 control for each growth stage).

2.2.4 Measurements

The effects of herbicide exposure were recorded for aboveground biomass (dry weight) in both short- and long-term studies, and in the long-term study also for number of seeds and seed germinability of the F1 generation.

2.2.4.1 Short term study (biomass)

One group of pots was harvested three weeks after herbicide application for recording the vegetative endpoint (biomass). Plants were cut at the soil surface and dried at 70-80 ºC in an oven for at least 48 hours prior to biomass measurement. Average dry weight per plant was calculated for each pot and was used for statistical analyses.

2.2.4.2 Long-term study (seed production)

Another group of plants was used for assessing the effects on reproductive endpoints. When plants began flowering bees were released in the greenhouses to facilitate pollination of the plants. In Canada, bumblebees (*Bombus impatience* CR.) were used and in Denmark, honeybees (*Apis mellifera* L.). A high number of flower visitations were observed at both locations indicating that plants were well-fertilized.

When seed set was initiated, mature seed heads were counted and cut off twice a week and thereafter kept in a paper bags. When flowering ceased, plants were cut at the soil surface and placed in air permeable bags to dry.

Seeds were harvested by cleaning the plant material through sieves and using an air blower. The final seed cleaning was performed by hand. The total number of seeds and the seed weight (100 seeds) were measured using an automatic seed counter (in Denmark: JK Design, Denmark, in Canada: Elmor C1, Switzerland).

Seed germination was tested in Petri dishes (100 seeds per plant) with two filter papers. If fewer than 100 seeds were present, all seeds were tested and percentages were adjusted accordingly. To break seed dormancy, four ml of a 0.2% KNO3 solution was applied to each Petri dish. The Petri dishes were placed in climate cabinets running at 22ºC (16 hrs.) and 15ºC (8 hrs.) at a light intensity of 175 µmol m^{-2} s^{-1}. Germinated seeds were counted and removed twice a week, and water was added as required to maintain moisture. The number of germinable seeds (NGS) was calculated as the proportion of seeds that germinated in the germination test times the total seed production for a given plant.

2.3 Statistics and meta-analysis

The responses to herbicide treatments for each combination of plant species and growth stage were examined separately using one-way analysis of variance. The assumption of normally distributed residual variation was checked by inspecting residual plots. When required, data were transformed to meet the assumptions. Each treatment group was compared directly to the control group using Dunnett's one way post hoc comparison to determine if a significant effect was found. All analyses were performed in SAS (SAS; 2010).

In addition, the overall responses of all combinations of herbicides and plant species were analyzed in a meta-analysis using a linear mixed model. The effect size for each treatment was measured by Hedges' g.

$$Hedges'g = \frac{\mu_t - \mu_c}{\sigma} \quad (1),$$

where μ_t is the treatment mean, μ_c is the control mean, and σ is the pooled standard deviation (Hedges, 1981) estimated by:

$$\sqrt{\frac{(n_t-1)s_t^2+(n_c-1)s_c^2}{n_t+n_c-2}} \quad (2).$$

Thus, the absolute value of Hedges' g increases with the difference between the treatment mean and control mean and decreases with the sampling variance of the study. A positive value of Hedges g indicates that the treatment had a higher mean value than the control. The calculated Hedges' g's were analyzed in a mixed model with Lab (DK or CA), life span (annual or perennial), growth stage at spray (6-8 leaves stage or bud stages), herbicide dose, and the herbicide*dose interaction as fixed effects and plant species as a random effect assuming normally distributed residual variation. The assumption of normally distributed residual variation was checked by inspecting residual plots. All analyses were made in R (CRAN.r-project.org/).

2.4 Results

The trials produced a large amount of data and to get an overview of the effects of all combinations of herbicide and doses on the different plant species following exposure at the two growth stages the data are presented in response tables (Table 3 to 5). Effects were rated in five different classes. One class represents results showing growth stimulation. The other classes represent reductions in biomass/ number of seeds/ number of germinable seeds < 25%, 25 – 50%, 50 – 75%, and > 75%. Each herbicide treatment was tested for significant difference from the control using Dunnett's one-way post hoc comparison.

2.4.1 Short-term effects on biomass

In most cases, the 1% herbicide doses had low effect (<25% reduction) on biomass at both growth stages (Table 3). In the Canadian study, effects were most frequently observed with the 5% doses, with reductions not exceeding the 50% level, while in the Danish, biomass of several plant species (e.g. *V. arvensis* exposed to glyphosate) was reduced by more than 50% specifically following exposure at the 6-8 leaf stage of plants. Growth stimulation was recorded for many combinations of herbicides and plant species exposed at the bud stage, but primarily in the Danish studies (Table 3).

Overall, exposure to glyphosate resulted in the highest number of significant reductions in biomass (Table 3). The Danish study, showed statistically significant biomass reductions for the 1% and 5% doses of glyphosate when applied at the 6-8 leaf stage for *Cirsium arvense*, *T. officinale* and *K. arvensis*; and at the 5% dose for additionally three species: *S. noctiflora*, *V. arvensis*,and *Cerastium arvense*. The Canadian study only showed significant biomass reductions for *Cirsium arvense* and *T. pratense* at the 5% dose.

Metsulfuron-methyl at 1% significantly reduced biomass of *S. noctiflora* and *T. officinale* when applied at the 6-8 leaf stage and *C. cyanus* when applied at the bud stage (Table 3). The 5% dose caused significant reductions in biomass of *S. noctiflora* and *T. officinale* at both growth stages.

In Denmark, the 1% dose of ioxynil + bromoxynil applied at the 6-8 leaf stage caused a significant reduction in biomass of *T. officinale* (Table 3). The 5% dose reduced biomass of *S. noctiflora*, *T. officinale* and *Cirsium arvense* when applied at the 6-8 leaf stage, and *C. cyanus* and *S. noctiflora* when applied at the bud stage. In Canada, the 1% dose of bromoxynil applied at

the 6-8 leaf stage caused a significant reduction in biomass of *T. officinale*, and the 5% dose reduced biomass of *C. cyanus*, *Cerastium arvense* and *T. officinale*.

Clopyralid had low effect on biomass and only few plant species showed significant responses to the treatments (Table 4). In Denmark, *S. noctiflora* (1% dose, bud stage); in Canada, *T. officinale* (5% dose, 6-8 leaf stage) and *T. pratense* (5% dose, bud stage).

2.4.2 Long-term effects on reproduction (seed production)

In most cases, herbicides applied at the 1% doses had less than 25% effect on the number of seeds produced (Table 4). In contrast, the 5% herbicide doses reduced seed production by more than 75% for some plant species in the Danish study, with most pronounced effects when the plants were sprayed at the 6-8 leaf stage.

Glyphosate applied at the 1% dose significantly reduced seed production of *C. cyanus* when applied at the 6-8 leaf stage in Canada and of *V. arvensis* when applied at the bud stage in Denmark (Table 4). For *C. cyanus* the negative effect on number of seeds persisted at the 5% dose, although not significant. In Denmark, the 5% dose significantly reduced seed production of *C. cyanus*, *V. arvensis* and *T. officinale* following application at both growth stages and of *E. montanum* and *K. arvensis* when applied at the 6-8 leaf stage. In Canada, a significant reduction in seed production of *T. pratense* was obtained with the 5% dose of glyphosate applied at the bud stage but not at the 6-8 leaf stage.

When metsulfuron-methyl was applied at the 6-8 leaf stage, significant reductions in the number of seeds produced was observed at the 1% dose for only two species: *T. officinale* and *K. arvensis*, and at the bud stage *V. arvensis* was the only species being affected at this dose (Table 4). No negative effect was found for *K. arvensis* at the 5% dose indicating that the response at the 1% dose may be due to plant variability. For other plant species effects became more pronounced at the 5% dose, with reduced seed production being detected for *S. noctiflora* and *T. officinale* when applied at the 6-8 leaf stage and for *V. arvensis* and *T. officinale* when applied at the bud stage.

In the Danish study, ioxynil + bromoxynil caused significant reductions in seed production of *S. noctiflora* following application at the bud stage (1% and 5%) (Table 4). In Canada, the 5% dose of bromoxynil reduced seed production of *C. cyanus* and *Cerastium arvense* when applied at the 6-8 leaf stage.

Clopyralid had no adverse effects on seed production of the studied plant species in Denmark, while in Canada, significant reductions in seed production were found for *T. pratense* (5% dose applied at both growth stages). The significant reduction obtained for *C. cyanus* following the 1% dose applied at the 6-8 leaf stage was not persistent at the 5% dose (Table 4).

TABLE 3. Herbicide effects on aboveground biomass (DW) (treated compared to controls) for plants exposed to herbicides at either the 6-8 leaf or bud stage. Herbicide effects were determined using ANOVA; significant effects for each herbicide*dose combinations compared to controls were determined using Dunnett's one-way post hoc comparison. Effects are grouped into five classes: <25% effect, 25-50% effect, 50-75% effect, >75% effect, growth stimulation. Statistically significant effects are shown by * p≤ 0.05, **, p≤ 0.01 and *** p≤0.001

Plant species	Herbicide	Denmark				Canada			
		6-8 leaf		Bud stage		6-8 leaf		Bud stage	
		1%	5%	1%	5%	1%	5%	1%	5%
C. cyanus	Glyphosate			***	***		*		
	Metsulfuron			**					
	Ioxynil + Bromoxynil [a]				*		*		
	Clopyralid								
S. noctiflora	Glyphosate		***						
	Metsulfuron	*	***		***				
	Ioxynil + Bromoxynil [a]		*		**				
	Clopyralid			*					
V. arvensis	Glyphosate		*						
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
E. montanum	Glyphosate				**				
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
Cirsium arvense	Glyphosate	***	*						
	Metsulfuron		*						
	Ioxynil + Bromoxynil [a]		**				*		
	Clopyralid								
T. officinale	Glyphosate	*	***		**				
	Metsulfuron	**	**		*				
	Ioxynil + Bromoxynil [a]	*	*			**	*		
	Clopyralid						*		
Cerastium arvense	Glyphosate		*						***
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
K. arvensis	Glyphosate	*	***						
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
T. pratense	Glyphosate						**		***
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								*

[a] Bromoxynil only in Canada

TABLE 4. Herbicide effects on total number of seeds produced (treated compared to controls) for plants exposed to herbicides at either the 6-8 leaf or bud stage. Herbicide effects were determined using ANOVA; significant effects for each herbicide*dose combinations compared to controls were determined using Dunnett's one-way post hoc comparison. Effects are grouped into five classes: <25% effect, 25-50% effect, 50-75% effect, >75% effect, increased number of seeds. Statistically significant effects are shown by * p≤ 0.05, **, p≤ 0.01 and *** p≤0.001

Plant species	Herbicide	Denmark				Canada			
		6-8 leaf		Bud stage		6-8 leaf		Bud stage	
		1%	5%	1%	5%	1%	5%	1%	5%
C. cyanus	Glyphosate		*		***	*			
	Metsulfuron								
	Ioxynil + Bromoxynil [a]						*		
	Clopyralid					*			
S. noctiflora	Glyphosate								
	Metsulfuron		**						
	Ioxynil + Bromoxynil [a]			*	*				
	Clopyralid								
V. arvensis	Glyphosate		*	***	***				
	Metsulfuron			*	**				
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
E. montanum	Glyphosate		*						
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
Cirsium arvense	Glyphosate								
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
T. officinale	Glyphosate		***		**				
	Metsulfuron	***	***		**				
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
Cerastium arvense	Glyphosate								
	Metsulfuron								
	Ioxynil + Bromoxynil [a]						*		
	Clopyralid								
K. arvensis	Glyphosate		**						
	Metsulfuron	*							
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
T. pratense	Glyphosate								***
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid						**		*

[a] Bromoxynil only in Canada

TABLE 5. Herbicide effects on total number of germinable seeds (treated compared to controls) for plants exposed to herbicides at either the 6-8 leaf or bud stage. Herbicide effects were determined using ANOVA; significant effects for each herbicide*dose combinations compared to controls were determined using Dunnett's one-way post hoc comparison. Effects are grouped into five classes: <25% effect, 25-50% effect, 50-75% effect, >75% effect, increased number of germinable seeds. Statistically significant effects are shown by * p≤ 0.05, **, p≤ 0.01 and *** p≤0.001

Plant species	Herbicide	Denmark				Canada			
		6-8 leaf		Bud stage		6-8 leaf		Bud stage	
		1%	5%	1%	5%	1%	5%	1%	5%
C. cyanus	Glyphosate		*		**	**			
	Metsulfuron								
	Ioxynil + Bromoxynil [a]					**	**		
	Clopyralid					*			
S. noctiflora	Glyphosate								
	Metsulfuron		**		*				
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
V. arvensis	Glyphosate		*		*				
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
E. montanum	Glyphosate		*						
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
Cirsium arvense	Glyphosate								
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
T. officinale	Glyphosate		***						
	Metsulfuron	***	***						
	Ioxynil + Bromoxynil [a]						*		
	Clopyralid								
Cerastium arvense	Glyphosate								
	Metsulfuron								
	Ioxynil + Bromoxynil [a]						*		
	Clopyralid								
K. arvensis	Glyphosate		**						
	Metsulfuron	*							
	Ioxynil + Bromoxynil [a]								
	Clopyralid								
T. pratense	Glyphosate								*
	Metsulfuron								
	Ioxynil + Bromoxynil [a]								
	Clopyralid						*		

[a] Bromoxynil only in Canada

2.4.3 Long-term effects on reproduction (seed germinability F1)

For many plant species herbicide treatments had negative effects on the number of germinable seeds (Table 5). Significant responses on germinability naturally followed those for total seed production shown in Table 4. However, the number of significant responses was lower for the number of germinable seeds compared to the number of seeds reflecting that in some cases seed germinability was higher for sprayed plants compared to control (*S. noctiflora* treated with ioxynil + bromoxynil, *V. arvensis* treated with metsulfuron-methyl, *T. officinale* treated at the bud stage with glyphosate and metsulfuron-methyl). It should be noted that in

three cases seed germinability was significantly reduced at herbicide levels not affecting the total seed production: *C. cyanus* at 1% and *T. officinale* at 5% bromoxynil when sprayed at the 6-8 leaf stage; and *S. noctiflora* at 5% metsulfuron-methyl when sprayed at the bud stage.

2.4.4 Comparison of endpoints

In order to compare the different endpoints, mean values of biomass was plotted against the mean values of number of seeds per plant (Fig. 1A) and against the mean values of number of germinable seeds (Fig 1B) for all treatments (plant species*growth stages*herbicide*doses). All endpoints are shown as relative values compared to the controls. The straight line (x=y) inserted in the graphs illustrates the response window of equivalent effects on the measured endpoints. For data points above the straight line reproductive endpoints (relative number of seeds or relative number of germinable seeds) are less sensitive to the herbicide treatments than biomass and conversely for data points below the line the reproductive endpoints are more sensitive than biomass. The graphs show that some plant species were highly sensitive to low doses simulating herbicide drift, as used in the present study, while other plant species were tolerant or growth or seed production was stimulated. They also show that reproductive endpoints, i.e. number of seeds and number of germinable seeds, were more sensitive than biomass when treatments had more than 60% effect on biomass (relative DW<40 illustrated by the blue areas in the graphs). For treatments having less than 60% effect on biomass (relative DW>40 in the graphs) the number of data points above and under the straight line was more or less equal indicating no general trend in any of these endpoints being more sensitive than the other.

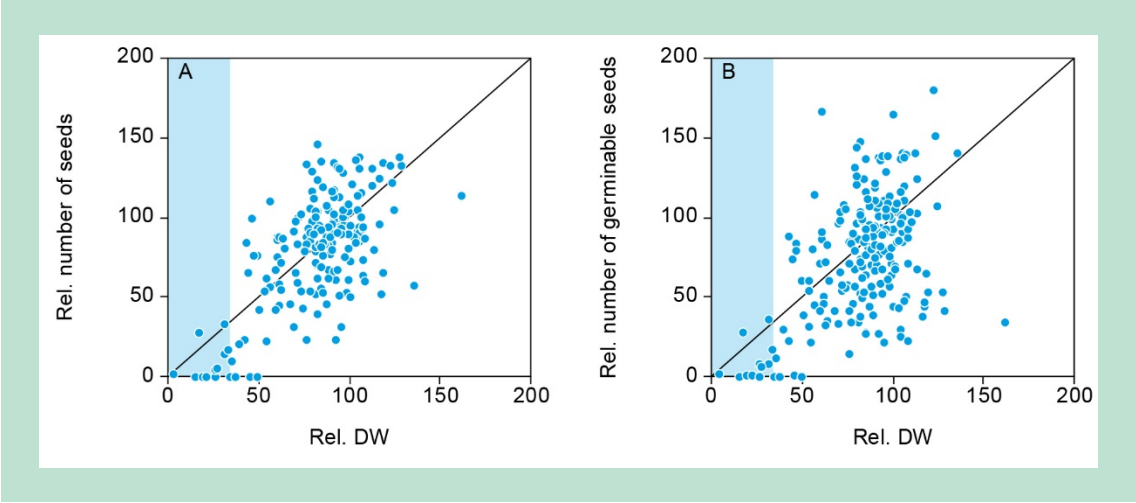

FIGURE 1. Correlation between mean values of plant biomass (dry weight) and number of seeds (A) and number of germinable seeds (B) for all treatments (plant species*growth stages*herbicide*doses. All values are expressed as relative figures compared to controls. The straight line (x=y) illustrates similar sensitivity of the variables

Analysis of the responses to herbicide treatments for each combination of plant species and treatment showed significant reduction in biomass following exposure at the 6-8 leaf stage for 28% of the cases in Denmark but only for 16% of the cases in Canada (Table 6). In both countries, the percentages of studies showing significant reduction in biomass following exposure at the bud stage were much lower (17% and 7%, respectively). These figures indicate that regarding biomass the sensitivity of plants sprayed at the bud stage was lower compared to plants sprayed at the 6-8 leaf stage.

For seed production, significant reductions were recorded in 6-15% of the cases included in the study. In Denmark, the percentage of herbicide treatments showing significant reductions

in number of seeds following exposure at the 6-8 leaf stage was much lower than the percentage of cases showing significant reductions in biomass (14% versus 28%). In contrast, similar percentages of significant responses for biomass and number of seeds were found following exposure at the bud stage in Denmark and both growth stages in Canada. In Denmark, the percentage of cases with significant reductions in number of seeds was similar on plants exposed at the 6-8 leaf stage (14 %) and at the bud stage (15%), while in Canada, a higher number of significant responses was recorded when plants were exposed at the 6-8 leaf stage (14%) compared to the bud stage (6%). In conclusion, sensitivity in number of seeds was either equal for exposure at the two growth stages (Denmark) or more sensitive when plants were exposed at the 6-8 leaf stage compared to the reproductive stage (Canada). For plants sprayed at the 6-8 leaf stage the percentage of treatments causing significant reductions in number of germinable seeds was similar to the percentage found for number of seeds but a lower number of significant effects were found when sprayed at the bud stage indicating that seed quality in terms of germination rate was less affected at the late growth stage.

TABLE 6. Number of plant species showing significant negative effects of given herbicides on biomass (BIO), number of seeds (NOS) and number of germinable seeds (NGS). Species were counted if they showed a significant effect at either the 1% or 5% doses

	Denmark						**Canada**					
	6-8 leaf stage			**Bud stage**			***6-8 leaf stage***			**Bud stage**		
	BIO	**NOS**	**NGS**	**BIO**	**NOS**	**NGS**	**BIO**	**NOS**	**NGS**	**BIO**	**NOS**	**NGS**
Glyphosate	9	5	5	4	4	2	2	1	1	2	1	1
Metsulfuron-methyl	5	4	4	3	3	0						
Ioxynil + bromoxynil[a]	4	0	0	2	2	0	4	2	4	0	0	0
Clopyralid	0	0	0	1	0	0	1	2	2	1	1	0
Number	18	9	9	11	9	3	7	5	7	3	2	1
%	28	14	14	17	15	5	16	14	19	7	6	3

[a] bromoxynil only in Canada

2.4.5 Sensitivity of annual and perennial NTTPs

Results of both the Danish and Canadian part of the study showed that the percentage of cases with significant reductions on biomass for annual plant species was slightly higher than for perennial plant species (25% versus 21% in DK, 17% versus 13% in Canada) (Table 7). Differences in sensitivity of annual and perennial plants were more pronounced when assessed on reproductive endpoints. For annual plants, the percentage of treatments showing significant reductions in number of seeds and number of germinable seeds was two fold higher than the percentage on perennial plants (Table 7).

TABLE 7. Number of annual and perennial NTTP species showing significant reductions to low doses of herbicides measured as biomass (BIO), number of seeds (NOS) and number of germinable seeds (NGS)

	Denmark						Canada					
	Annual			Perennial			Annual			Perennial		
	BIO	NOS	NGS	BIO	NOS	NGS	BIO	NOS	NGS	BIO	NOS	NGS
Glyphosate	4	5	4	10	4	2	1	1	1	3	1	1
Metsulfuron-methyl	4	3	2	4	4	1						
Ioxynil + bromoxynil	3	2	0	3	0	0	1	1	2	4	1	2
Clopyralid	1	0	0	0	0	0	0	1	1	1	2	1
Number	12	10	6	17	8	3	2	3	4	8	4	4
%	25	21	13	21	10	4	17	13	17	13	8	8

2.4.6 Effects in different labs

In Denmark, significant reductions in biomass of the herbicides were found in 21-25% of the treatments while in Canada, the percentage of cases with significant reductions was 13-17% (Table 7). For number of seeds the respective figures were 10-21% in Denmark and 8 to 13% in Canada (Table 7). These figures indicate that plants were more sensitive in the Danish experiment compared to the Canadian when assessed on biomass and number of seeds.

2.4.7 Meta-analysis

In order to provide a more general assessment of the importance of the growth stage at exposure, the lifespan of plants (phenology) and the use of different endpoints for plant sensitivity we carried out a meta-analysis using a linear mixed-effect model analysis of the calculated effect sizes, i.e. the Hedges' g values. Laboratories (Denmark compared to Canada), lifespan (perennial compared to annual) and growth stage at exposure (bud stage compared to 6-8 leaf stage) were defined as fixed effects and plant species as a random effect (Table 8). A negative value of Hedges' g shows that the mean effect of the first mentioned variable was larger than the mean effect on the second, and consequently a higher sensitivity was observed for the first variable. Conversely, a positive value of Hedges' g indicates that mean effect on the first mentioned variable was lower than the mean effect on the second. Furthermore, the absolute value of Hedges' g is a measure of the differences in effect sizes.

The values of Hedges' g's for laboratories (Table 8) (Denmark compared to Canada) were negative showing that the measured effects were larger, i.e. plant species were more sensitive, in Denmark compared to Canada for all measured endpoints, i.e. biomass, number of seeds and number of germinable seeds. The largest differences between laboratories were found for biomass (highest absolute figure) followed by number of seeds and the smallest difference was for number of germinable seeds. The differences between laboratories were significant for biomass and number of seeds but not for number of germinable seeds.

TABLE 8. Effects size (Hedges' g) of herbicides measured on three different endpoints (biomass, number of seeds and number of germinable seeds) at two laboratories (Denmark (DK) and Canada (CA)) for plants having different lifespan (annual, perennial) and exposed to herbicides at two life-stages (6-8 leaf stage and bud-stage)

	Hedges' g (P-value)		
	Biomass	**Number of seeds**	**Number of germinable seeds**
Laboratories (Denmark compared to Canada)	-1.09 (**0.0066**)	-0.61 (**0.0217**)	-0.15 (0.5339)
Life span (perennial compared to annual)	0.81 (0.2550)	0.15 (0.6708)	0.13 (0.6820)
Growth stage at exposure (bud stage compared to 6-8 leaf stage)	0.84 (**0.0065**)	0.19 (0.2903)	0.40 (**0.0099**)

Effect size or Hedges' g values, were positive for importance of plant life span for all endpoints but none of the results was significant. The positive values indicate that herbicide effects tended to be lower on perennial plant species compared to annual. As for laboratories, the differences were largest for biomass.

Finally, the positive values comparing effects of exposure at the bud stage to effects at the 6-8 leaf stage show that effects were lower when plants were exposed at the bud stage compared to the 6-8 leaf stage with significant effects for biomass and number of germinable seeds. Similar to the other comparisons of fixed effects the largest value was found for biomass but in this case, biomass was followed by number of germinable seeds with number of seeds showing the lowest and non-significant value.

2.5 Discussion

In contrast to previous studies, focusing on a few plant species and one or a few herbicides the present study covers a wide spectrum of plant species and herbicides, which can allow a more general assessment on the influence of different factors on plant sensitivity to herbicide drift.

In the present study, nine NTTP species with different lifespan (three annuals, six perennials) and belonging to six plant families were exposed to herbicides with different modes of action. The herbicides were applied at two low doses, i.e. 1% and 5% of label rate, simulating drift, and plants were exposed either at the 6-8 leaf stage or at the bud stage. Effects on biomass were recorded in the short- and the long-term study whereas effects on reproductive endpoints, i.e. seed production and germinability of the F1 seeds, were recorded in the long-term study. Overall, the results of the study allow comparison of the sensitivity of annual and perennial plants exposed at different phenological stages using different endpoints representing the whole life cycle.

In order to test whether effects of low doses of herbicides were reproducible between laboratories, the plant tests were conducted under controlled conditions in two greenhouses, one located in Denmark and one in Ottawa, Canada. The meta-analysis showed that the tested species, generally, were more sensitive to the tested herbicides in Denmark than in Canada what-

ever the endpoint. This finding must be assessed in view of the generally wide variation between species sensitivity (ER50s) found in different studies for crops as well as non-target plants (e.g. Strandberg et al. 2012, Boutin et al. 2010, Fletcher et al. 1985, 1990). Although aiming at following the same protocols, minor differences between the study locations were unavoidable. In most greenhouses, factors such as hours of sunshine, temperature and humidity can only be controlled to some extent and these are factors known to affect herbicide performance in a way that can easily result in the observed differences (Kudsk, 2017). The same batches of seeds were used at both locations, however, *K. arvensis* did not germinate well in Canada and was replaced by *T. pratense*, and some plant species did not flower properly in Canada (*S. noctiflora* and *Cirsium arvense*). Consequently, reproductive endpoints could not be assessed for these latter two species. Furthermore, the low doses used in the present study may also have contributed to the differences found, as performance of many of the herbicide-treated plants did not differ significantly from the control. Moreover, the spraying technique varied between countries resulting in 77 L/ha and 160 L/ha delivered in the Canadian and Danish trials, respectively, another difference that can easily explain the observed discrepancies in sensitivity. Finally, only one of the four herbicides tested were was the same in the two locations. Glyphosate was used in the same formulation (isopropylamin salt formulated with tallow amide) in both countries. For clopyralid a granular formulation was used in Denmark while a soluble concentrate formulation was used in Canada. Metsulfuron-methyl was not available in Eastern Canada and therefore only used in Denmark while ioxynil and bromoxynil was used as a co-formulation in Denmark and bromoxynil as a solo product in Canada.

Previous studies have concluded that responses to herbicides may depend on the growth stage at exposure and on the specific endpoint measured. Plants exposed at early growth stages will have their vegetative parts affected, and although they may recover in terms of biomass, negative impacts on reproductive endpoints such as seed production may appear at later life stages (Boutin et al. 2014). Plants exposed at the reproductive stage may have their seed production and/ or the seeds of the F1 generation impacted (Boutin et al, 2014). Impact on biomass is less likely as vegetative growth has more or less ceased at late growth stages and previous reports have shown that effects on biomass results in unrealistically high ER10 and ER50 values (Strandberg et al., 2012). Therefore, plants exposed to low doses of herbicides at early growth stages usually show greater responses on biomass than plants exposed at later growth stages. Reproductive structures (flowers, seeds, fruit) are suggested as being more appropriate endpoints than biomass to assess effects, following exposure at late growth stages (Strandberg et al. 2012, Boutin et al. 2014).

One of the research questions raised in the EFSA opinion (EFSA PPR Panel 2014) was whether NTTPs are more sensitive to spray drift at the reproductive growth stage (bud stage) compared to the 6-8 leaf stage. This question cannot be fully answered with addressing the second research question of the present study concerning sensitivity of vegetative and reproductive endpoints. Based on the present study, we cannot confirm that plants are more sensitive at the reproductive stage. Generally, we observed that the tested species were more sensitive to herbicides when exposed at the 6-8 leaf stage compared to the bud stage (Table 6). Sensitivity measured as effects on number of seeds was either equal at the two growth stages or plants were more sensitive at the 6-8 leaf stage compared to the bud stage, and, sensitivity measured as biomass and the number of germinable seeds were higher at the 6-8 leaf stage compared to bud stage. In the ANOVA analyses individual responses among cases (species * herbicides) were observed and for some species effects were only observed when the plant was exposed at the bud stage. Such species will not be covered by standard species test where plants are only exposed at the 6-8 leaf stage. We were surprised to find several cases of reductions in biomass for plants exposed at the bud stage showing no significant effects at the 6-8 leaf stage (i.e. *E. montanum* exposed to 5% glyphosate, *C. cyanus* exposed to 1% glyphosate, 1% metsulfuron and 5% ioxynil+bromoxynil).

The meta-analysis confirmed that herbicide effects, generally, were lower at the bud stage compared to the 6-8 leaf stage or equally sensitive regardless of endpoint (biomass, number of seeds or number of germinable seeds) (Table 8). Significant effects were obtained only for biomass and number of germinable seeds, and the responses were most pronounced for biomass. However, plant flowering has not been included in the meta-analysis. Consequently, when looking at biomass, number of seeds and number of germinable seeds, as endpoints, we cannot confirm that reproductive endpoints are more sensitive parameters than vegetative endpoints as reported in several studies (Olszyk et al., 2009, Riemens et al., 2009, Carpenter and Boutin, 2010, Strandberg et al, 2012, Carpenter et al., 2013). Boutin et al. (2014) summarized 59 cases and found reproductive endpoints were more sensitive than biomass in 58% of all cases, biomass was more sensitive than reproduction endpoints in 32% of the cases, and the two endpoints were equally sensitive in the remaining 10%. For plants exposed to herbicide drift at early growth stages, reproductive endpoints were three times more sensitive than biomass.

The overall conclusion taking all data from the present study into account is that biomass is the most sensitive endpoint. This is based on the value of Hedges' g for biomass being larger than for germinable seeds. The ANOVA analyses also showed that in some cases effects on biomass were more pronounced than effects on seed production, in other cases the sensitivity of biomass and number of seeds or number of germinable seeds was equal (Table 5). However, this overall conclusion covers many different response patterns and it is evident that some effects may not be found in the standard test used. For example, significant effects were obtained on number of seeds but not on biomass for glyphosate and metsulfuron-methyl applied to *V. arvensis* at the bud stage and on the number of seeds following application of clopyralid to *T. pratense* at the 6-8 leaf stage. These effects would not have been discovered in standard tests sprayed at the 6-8 leaf stage and harvested 3 weeks later.

Blackburn and Boutin (2003) demonstrated a negative effect on the germination rate of seeds produced by plants exposed to glyphosate depending on the degree of seed maturity at exposure. Similarly, Nelemans et al. (2017) reported significant negative effects on seed germination rates of several species following exposure to metsulfuron-methyl. In our study we only observed a few cases of significant reductions in number of germinable seeds for which no significant effect was found on number of seeds (*C. cyanus* sprayed with 1% bromoxynil at the 6-8 leaf stage, *T. officinale* sprayed with 5% bromoxynil at the 6-8 leaf stage, *S. noctiflora* at bud stage sprayed with 5%metsulfuron-methyl, Table 5 and 6). The differences in responses in different studies indicate that sensitivity depends on plant stage at exposure as well as plant species.

The standard guidelines for risk assessment of NTTPs (OECD 2006a, 2006b, US EPA 2012a) only include exposure of test plants at young growth stages and effects are measured as vegetative vigour or biomass. It has been suggested that reproductive endpoints should be added as part of the risk assessment and it has been argued that NTTPs are exposed to pesticides at different growth stages and that reproductive endpoints might be more affected than biomass at certain phenological stages. The present study shows that for an overall assessment on several plant species and herbicides biomass was the most sensitive endpoint. However, the results also confirm that the most sensitive endpoint varies among cases as concluded by Boutin et al. (2014). Reproductive endpoints, i.e. number of seeds and number of germinable seeds in F1 generation, were generally more sensitive than biomass in cases where biomass was reduced by more than 60%. If assessment of species sensitivity to herbicides is only based on data on biomass, the effects of herbicide drift may be underestimated as cases with reproductive endpoints being more affected would not be found and consequently a number of NTTPs will not be protected by the assessment.

Another issue raised in the EFSA opinion for effects of plant protection products on NTTPs was whether responses on annual crop plants sufficiently mimic responses on NTTPs (EFSA PPR Panel, 2014). Results of previous studies comparing sensitivity of crops and NTTPs to herbicides are inconclusive. Some have reported no differences in sensitivity between crop species and NTTPs (Boutin and Rogers, 2000, White and Boutin, 2007, Carpenter and Boutin, 2010, Strandberg et al. 2012) while others have found NTTPs to be significantly more sensitive than crops (Boutin and Rogers, 2000, Boutin 2004; Schmitz et al. 2012, Boutin et al. 2014). The present study addresses the fact that a high proportion of NTTPs in the field boundaries are perennial plants. Previous studies have shown no systematic differences in sensitivity of annual and perennial plants on the short term (Strandberg et al., 2012). However, perennial plants exposed to herbicide drift at young growth stages have a longer period to recover than annual plant species and at later growth stages they may allocate resources from their root system to compensate for growth inhibition although Kjær et al. (2006) did not find full recovery from herbicide exposure of hawtorn the following season.

The meta-analysis on life span shows that annual NTTPs tended to be more affected by herbicides than perennial plant species for all endpoints but the differences were not significant (Table 8). In conclusion, our results did not confirm any significant difference in sensitivity of annual and perennial plants. However, it should be noted that under field conditions perennial plants might be exposed to herbicides several times during their life cycle.

Based on the results of our studies we can conclude that NTTP's are not more sensitive to spray drift at the flowering stage compared to the vegetative growth stage (question 1) and that reproductive endpoints (seed production) are not more sensitive endpoints than vegetative endpoints (biomass) (question 2). Annual plant species tended to be more sensitive than perennial (question 3) and effects were not reproducible between laboratories (question 4).

3. Individual level effects of herbicides on non-target terrestrial plants - effects on plant flowering

3.1 Introduction

Using parallel studies conducted in greenhouses in Denmark and Canada we seek to further address the effects of low, sub-lethal herbicide exposure on the flowering of wild, non-target terrestrial plants. Specifically, we want to evaluate if these exposures lead to decreases in total flower output as well as to delays in peak flowering and/or to reductions in peak flower production when plants are exposed at either the seedling (6-8 leaf) or the bud stages and addresses the following hypotheses:

1. Reproductive endpoints, such as plant flowering, are particularly sensitive to sub-lethal doses of herbicides
3. Through effects on reproductive endpoints, herbicides have an important adverse effect on non-target plant populations and on biodiversity

3.2 Material and methods

The study was carried out as pot experiments in greenhouses at two locations – Aarhus University, Research Centre Flakkebjerg in Denmark (DK, GPS: 55.324382, 11.390041) and Environment and Climate Change Canada, in Ottawa Canada (CA, GPS: 45.3876, 75.6960).

3.2.1 Test plants, herbicides and herbicide application

Nine non-target terrestrial plant species from six plant families were exposed to low herbicide doses (1% and 5% of label rate). They consisted of three annual species: *Centaurea cyanus*, *Silene noctiflora*, and *Viola arvensis*, and six perennial species: *Cerastium arvense*, *Cirsium arvense*, *Epilobium montanum*, *Knautia arvensis*, *Taraxacum officinale* and *Trifolium pratense*. The study was carried out as described in Chapter 2 when it comes to test plants, herbicides and herbicide application.

For assessment of effects on plant flowering five plant replicates were used for all herbicide doses (1 and 5% treatments). Either five (for *Cerastium arvense*, *Knautia arvensis*, *Silene noctiflora*, *Taraxacum officinale* and *Viola arvensis* – DK experiments) or ten plant replicates (all remaining treatments) were used for the controls. Prior to treatment, plants were sorted by size across all doses and the controls to minimize starting size effects.

3.2.2 Counting of flowering

After spraying, plants were monitored regularly (daily in Canada or on count days in Denmark) to obtain the date of first flowering for each individual plant. A flower (including the compound inflorescences of *C. cyanus*, *Cirsium arvense*, *K. arvensis* and *T. officinale*) was considered in bloom when the petals had emerged from the bud. For the compound flowers of *T. pratense*, this was when the first fully developed floret in the inflorescence was observed. Once flowering had commenced, flower counts (or flower head counts) were performed, on average, two to three times a week to obtain measures of flowering over time for each individual of each species. At each count, the number of flowers currently on each plant as well as the number of fruit/seed heads was recorded. Total flower production for a given date was calculated as the

total number of flowers on the plant at that time plus the total cumulative number of fruit/seed heads produced by that time point. Measurements were recorded for the duration of flowering up until either the natural senescence of the plants or the point of seed release (to accommodate measurements for other experiments).

In order to control plant pests within the greenhouses, biological controls were applied in Canada. These consisted of ladybugs (*Hippodamia convergens* Guerin-Meneville)) and predatory midges (*Aphidoletes aphidimyza* Rondani) for aphid control, predatory mites (*Neoseiulus cucumeris* Oudemans and *Stratiolaelaps scimitus* Womersley) for fungus gnat and thrip control, and parasitic wasps (*Encarsia formosa* Gahan) for white fly control. No biological controls were applied in Denmark. In addition, all plants of a given species were supplemented with additional fertilizer (540 mg per pot of Plant-Prod® 20-20-20 All Purpose Fertilizer in Canada; 50 ml per pot of a 5:1:4 liquid fertilizer diluted 1:200 in Denmark) during the course of the experiments when the controls started showing signs of nutrient stress (discolored leaves).

Though testing began and proceeded with both *S. noctiflora* and *Cirsium arvense* in the Canadian trial, overall lack of flowering uniformity for both *S. noctiflora* (many plants did not bolt across all tested herbicides x doses) and *Cirsium arvense* (plants bolted, but did not produce many flowers) across the controls and herbicide doses led to the decision to exclude the Canadian flowering data for these species from all analyses.

3.3 Statistics

3.3.1 Model

The flowering data for different combinations of species and herbicides was modelled using a Bayesian hierarchical model where cumulative number of flowers was assumed to follow a modified Gompertz growth curve (Seber and Wild, 1989; Damgaard et al., 2016):

$$f(h_i, t) = (\alpha_0 + \alpha_1 h_i)\,\mathrm{Exp}[-\mathrm{Exp}[-\kappa(t - (\gamma_0 + \gamma_1 h_i))]] \quad (1),$$

where h_i is the level of herbicide in pot i, and t is time in days. The effect of herbicide on flowering was modelled by allowing two of the parameters in the Gompertz growth curve, i.e., α that models the asymptotic cumulative number of flowers and γ that models the inflection point of the curve, to be simple linear functions of the herbicide level (Fig. 2).

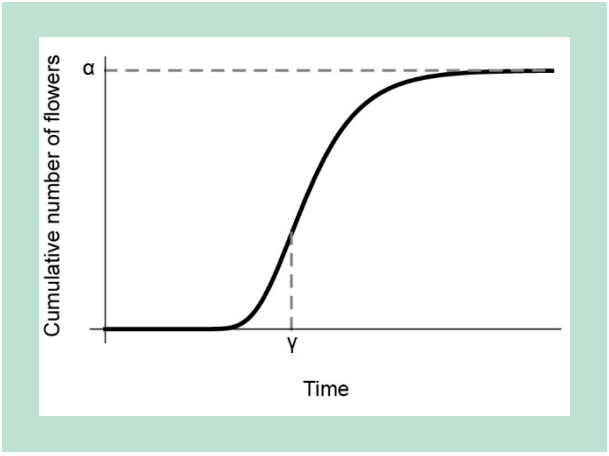

FIGURE 2. Graphical illustration of the interpretation of two of the parameters in the Gompertz growth model. The parameter ***α*** measures the asymptotic cumulative number of flowers and ***γ*** measures the inflection point of the curve, i.e. the time of peak flowering

The observed cumulative number of flowers at time *t* in pot *i*, $y_{i,t}$, was assumed to be Poisson distributed with the mean determined by the Gompertz growth curve:

$$y_{i,t} \sim Poisson\big(f(h_i, t)\big) \quad (2).$$

The models were parameterized with numerical maximum likelihood methods using the NMaximize procedure in *Mathematica* (Wolfram, 2016). The model fit was checked by inspecting plots of the observed and predicted cumulative number of flowers. Statistical inferences ware made using asymptotic likelihood ratio tests.

The results of the different cases were summarized by the estimated quantities α_1/α_0 and γ_1/γ_0, which are the relative change in the asymptotic cumulative number of flowers by a unit dose of the herbicide (i.e. per 1 g a.i. ha^{-1}), and the relative change in the inflection point (time to peak flowering) by a unit dose of the herbicide, respectively, in relation to the controls.

3.3.2 Statistical analyses

The results of the model for cumulative flowering and time of peak flowering were assessed based on the probabilities of α_1/α_0 and γ_1/γ_0producing a significant result. Negative values for α_1/α_0indicate that the herbicide treatment resulted in reduction in the cumulative number of flowers produced; whereas positive values for γ_1/γ_0indicate a delays to peak flowering for the herbicide treated plants.

To assess differences in effects between the two life stage treatments (6-8 leaf and bud stages) for α_1/α_0 or γ_1/γ_0, paired t-tests were performed separately for each herbicide, with the Canadian and Danish data combined. Each pair represented a given species (for clopyralid and glyphosate that were tested both in Canada and Denmark using the same species, each test was considered a separate pair) with the α_1/α_0 or γ_1/γ_0 results of the 6-8 leaf stage compared directly to its corresponding bud stage value.

3.4 Results

3.4.1 Cumulative number of flowers as an endpoint measure

3.4.1.1 Effects following exposure at 6-8 leaf stage

Significant negative effects of 1% herbicide application rates on total flowering were only observed in three cases for plants sprayed at the 6-8 leaf stage: *T. officinale* and *K. arvensis* with metsulfuron-methyl in the Danish experiments, and *C. cyanus*-DK with clopyralid in the Canadian experiments (Table 9). However, for both *K. arvensis* and *C. cyanus*-DK, though negative trends were present at the 5% dose, the effect was no longer significant. Effects were much more pronounced for 5% herbicide application rates, with 13 identified cases of significantly reduced flowering in response to herbicide exposure across all herbicides and locations (Denmark and Canada). The majority of these cases were for glyphosate in the Danish experiments. Plant death was the driver of many of these trends: all (or mostly all) *C. cyanus*-DK, V. *arvensis*, *Cirsium arvense*, *K. arvensis*, *T. officinale*, and *Cerastium arvense* replicates died at the 5% glyphosate dose, hence producing either no flowers or few flowers prior to death. *T. officinale* (Denmark) was the only species to experience significantly reduced flowering at the 5% doses of all four herbicides tested (similar to the results for glyphosate, all metsulfuron-methyl treated plants died). Metsulfuron-methyl (1 and 5%) was also observed to have a negative effect on *S. noctiflora* (1% metsulfuron-methyl two plants did not flower, and at 5% four plants did not flower); however, the trend was not significant due to the variability in the control counts (some controls also did not flower).

TABLE 9. Percent changes in total flower production for plant species exposed to 1 and 5% field application rates of five herbicides as compared to control plants. Plants were either sprayed at the 6-8 leaf stage or the bud stage, and experiments were conducted in either Denmark (DK) and/or Canada (CA). ANOVA was used to determine statistically significant effects (blue cells); exposure stage and labs were assessed separately. Dunnett's one-way post hoc comparison was used to determine levels of significance. * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001. Plant name abbreviations: CENCY *Centaurea cyanus*, SILNO *Silene noctiflora,* VIOAR *Viola arvensis*, CERAR *Cerastium arvense,* CIRAR *Cirsium arvense,* EPIMO *Epilobium montanum,* KNAAR *Knautia arvensis,* TAROF *Taraxacum officinale,* TRFPR *Trifolium pratense*

Plant species	Herbicide	Denmark				Canada			
		6-8 leaf		Bud stage		6-8 leaf		Bud stage	
		1%	5%	1%	5%	1%	5%	1%	5%
C. cyanus - CA	loxynil + Bromoxynil [a]					-17,2	**-65.6*** **	-6,1	**-53.2***
	Metsulfuron								
	Clopyralid					9,5	-14,6	11,0	4,5
	Glyphosate					12,9	**-73.9*** **	37,6	-16,7
C. cyanus - DK	loxynil + Bromoxynil [a]	56,3	-34,2	-12,1	**-37.4***	-27,2	**-38.2***	19,4	-13,9
	Metsulfuron	-4,9	-68,6	-15,8	-19,8				
	Clopyralid	-7,9	12,8	-6,6	-26,0	**-43.9****	-33,5	31,3	56,7
	Glyphosate	-1,2	**-100.0*** **	-34,2	**-98.8*** **	-35,8	-25,9	30,8	27,4
S. noctiflora	loxynil + Bromoxynil [a]	12,9	-14,6	**-24.8***	**-28.9****				
	Metsulfuron	-53,2	-88,4	**-25.5***	2,8				
	Clopyralid	69,5	39,9	-17,6	-13,6				
	Glyphosate	26,2	97,0	-7,9	-16,6				
V. arvensis	loxynil + Bromoxynil [a]	3,1	-14,1	17,0	14,9	-9,0	-19,0	2,7	-0,1
	Metsulfuron	24,5	-31,3	-36,7	-25,5				
	Clopyralid	11,7	-44,8	-2,3	-18,6	-14,3	-8,3	16,2	15,8
	Glyphosate	-3,7	**-100.0*** **	**-63.3***	**-94.7*** **	-23,2	-20,3	5,1	7,5
E. montanum	loxynil + Bromoxynil [a]	38,4	-1,0	32,7	4,5	-1,2	-7,0	-0,1	0,5
	Metsulfuron	15,1	3,4	45,0	27,4				
	Clopyralid	-6,7	-1,9	44,2	38,6	-40,6	-17,8	0,1	10,2
	Glyphosate	28,8	**-94.9****	40,1	**-93.3*** **	-18,1	-37,1	-0,2	2,2
Cirsium arvense	loxynil + Bromoxynil [a]	-9,9	19,9	60,4	55,2				
	Metsulfuron	3,3	45,4	-2,6	-4,4				
	Clopyralid	-15,5	-10,2	13,3	26,8				
	Glyphosate	-27,8	**-100.0*** **	39,4	-18,9				
T. officinale	loxynil + Bromoxynil [a]	1,2	**-37.7***	-6,3	-3,8	4,6	-9,8	-8,1	13,5
	Metsulfuron	**-79.0*** **	**-100.0*** **	12,0	**-94.9*** **				
	Clopyralid	-9,7	**-31.1***	1,3	-12,0	2,0	-4,6	-2,7	-16,2
	Glyphosate	-26,1	**-98.4*** **	-1,9	**-93.7*** **	-15,0	-22,9	0,0	-16,2
Cerastium arvense	loxynil + Bromoxynil [a]	-64,4	-79,0	15,7	23,5	-1,3	-27,9	-1,2	-7,0
	Metsulfuron	-26,7	-5,7	32,8	40,0				
	Clopyralid	-40,0	-50,7	0,0	6,1	-7,0	-15,5	-40,6	-17,8
	Glyphosate	-57,8	**-100.0*** **	4,6	-47,6	-32,7	12,7	-18,1	-37,1
K. arvensis	loxynil + Bromoxynil [a]	46,9	-16,7	-24,4	-3,5				
	Metsulfuron	**-70.7***	-37,8	-28,3	-47,3				
	Clopyralid	17,6	-22,9	13,8	-26,0				
	Glyphosate	-29,0	**-96.8****	-26,4	-27,7				
T. pratense	loxynil + Bromoxynil [a]					-29,7	-38,0	-23,3	*-18,2*
	Metsulfuron								
	Clopyralid					-18,3	-51,9	6,2	42,6
	Glyphosate					45,2	-32,3	-17,0	**-48.3***

[a] Bromoxynil only in Canada

3.4.1.2 Effects following exposure at the bud stage

There were 10 cases where exposure at the bud stage (at either 1 and/or 5% application rates) resulted in significant reductions in the cumulative number of flowers produced at the end of the experiment (Table 9). Similar to the results for the 6-8 leaf stage, the majority of effects were observed for glyphosate (five cases); however, unlike the 6-8 stage, significant effects were driven by plants that did not flower (or produced fewer flowers) as opposed to dead plants. There were four cases where an effect on the bud stage was observed, but no effects on the equivalent 6-8 leaf stage were present. These were *C. cyanus*-DK treated with 5% ioxynil + bromoxynil (DK), *S. noctiflora* treated with 1% metsulfuron or 1 and 5% ioxynil + bromoxynil (DK), and *T. pratense* treated with glyphosate (5%). In one case (*S. noctiflora* with ioxynil + bromoxynil), an effect was observed at 1% but not at 5%.

3.4.2 Model results

3.4.2.1 Effects of bromoxynil on plant flowering

More subtle effects of bromoxynil on flowering were revealed in the model, with negative effects on *V. arvensis*, *Cerastium arvense*, and *E. montanum* that had not been identified when solely addressing final flower counts (Figure 3a, Table 10a). Significant reductions in both the numbers of flowers produced as well as delays to peak flowering were observed for *C. cyanus*-CA (both life stages) as well as for *Cerastium arvense* (6-8 leaf stage) (Figure 4a,b). Significant reductions in flower number without a corresponding delay to peak flowering were observed for *C. cyanus* -DK and *V. arvensis* (6-8 leaf stages), while significant delays to peak flowering without a reduction in maximum flowering was observed for *E. montanum* (both stages). Based on the parameters of the model, significant reductions in total flower number spanned from 4% (*Cerastium arvense* – 6-8 leaf stage; however a positive effect on flowering was recorded for *E. montanum* at the bud stage) to 38% (*C. cyanus* -DK – 6-8 leaf stage) and delays in flowering from 4% (*E. montanum* – bud stage; approximately a 1 d delay) to 37% (*C. cyanus*-CA – 6-8 leaf stage; approximately a 9 d delay) at the 5% dose (Table 10a). Though not significant according to the model ($p > 0.05$), a further two species (*T. officinale* and *T. pratense*) did trend towards reduced flowering (>10% reductions) in at least one life stage following bromoxynil exposure (Table 10a). Overall, there were eleven cases (out of 14) where either a significant reduction ($p < 0.05$; $n = 6$) or a negative trend (>10% reduction, $n = 5$) in total flower number were present; while there were five cases where significant delays in flowering were detected.

TABLE 10. Percent effect values of herbicide exposure on the max number of flowers produced and the time to peak flowering at 5% herbicide exposure calculated from the predicted values of α1/α0 and γ1/γ0. A. Bromoxynil, B. Ioxynil + Bromoxynil, C. Metsulfuron-methyl. Values for predicted percent effect are calculated as the value of α1/α0 or γ1/γ0 times the g a.i. ha^{-1} present at the 5% dose for each herbicide separately. Negative values for α1/α0 indicate reductions in the number of flowers, whereas positive values for γ1/γ0 indicate flowering delays. Delay (days) reflects the time required to reach peak flowering at 5% exposure; positive values indicate a delay, while negative values indicate earlier flowering. Significant effects (p-values) for α and γ are highlighted in bold. Plant name abbreviations: CENCY *Centaurea cyanus*, SILNO *Silene noctiflora,* VIOAR *Viola arvensis*, CERAR *Cerastium arvense,* CIRAR *Cirsium arvense,* EPIMO *Epilobium montanum,* KNAAR *Knautia arvensis,* TAROF *Taraxacum officinale,* TRFPR *Trifolium pratense*

A. Bromoxynil								
		Maximum Number of Flowers			Time to Peak Flowering			
Species	Stage	α1/α0	Percent Effect at 5%	pα1	γ1/γ0	Percent Effect at 5%	Delay (Days)	pγ1
CENCY CA	6-8 leaf	-0,02390	-33,46	**0,000**	0,02645	37,03	9	**0,000**
	Bud	-0,02632	-36,85	**0,000**	0,02311	32,36	8	**0,002**
CENCY DK	6-8 leaf	-0,02703	-37,84	**0,006**	-0,00240	-3,36	-1	0,779
	Bud	-0,01342	-18,79	0,282	0,00229	3,20	1	0,801
VIOAR	6-8 leaf	-0,00993	-13,90	**0,003**	0,00344	4,81	1	0,266
	Bud	-0,00223	-3,12	0,572	0,00145	2,03	0	0,678
CERAR	6-8 leaf	-0,00287	-4,02	**0,014**	0,00530	7,42	3	**0,000**
	Bud	-0,00733	-10,26	0,074	-0,00086	-1,20	0	0,662
EPIMO	6-8 leaf	0,00073	1,02	0,862	0,00585	8,18	2	**0,000**
	Bud	0,00209	2,92	**0,000**	0,00290	4,06	1	**0,021**
TAROF	6-8 leaf	-0,01909	-26,73	1,000	-0,00147	-2,06	-3	0,729
	Bud	0,00226	3,16	0,887	-0,00307	-4,30	-3	0,913
TRFPR	6-8 leaf	-0,01353	-18,94	0,199	-0,00858	-12,01	-3	0,374
	Bud	-0,01571	-22,00	0,129	-0,00231	-3,23	-1	0,861

B. Ioxynil + Bromoxynil								
		No. Flowers at Peak Flowering			Time to Peak Flowering			
Species	Stage	α1/α0	Percent Effect at 5%	pα1	γ1/γ0	Percent Effect at 5%	Delay (Days)	pγ1
CENCY DK	6-8 leaf	-0,01889	-22,66	**0,019**	-0,00092	-1,11	0	0,823
	Bud	-0,00993	-11,91	**0,035**	0,00410	4,92	1	0,136
SILNO	6-8 leaf	-0,01950	-23,40	0,433	0,01146	13,75	3	0,296
	Bud	-0,00254	-3,05	0,897	0,01928	23,13	5	**0,000**
VIOAR	6-8 leaf	-0,01469	-17,63	0,597	0,00780	9,36	3	0,539
	Bud	0,00704	8,45	0,776	0,00437	5,24	2	0,640
CERAR	6-8 leaf	-0,00001	-0,01	1,000	0,00881	10,57	27	**0,000**
	Bud	-0,00030	-0,36	0,882	-0,00173	-2,08	-2	**0,005**
CIRAR	6-8 leaf	0,01531	18,37	0,302	0,01451	17,41	4	0,057
	Bud	0,02691	32,29	0,202	0,00321	3,85	1	0,597
EPIMO	6-8 leaf	-0,01183	-14,19	0,383	-0,00302	-3,62	-1	0,536
	Bud	-0,00393	-4,72	0,734	-0,00173	-2,08	-1	0,531
KNAAR	6-8 leaf	-0,01317	-15,80	0,675	0,00757	9,08	5	**0,040**
	Bud	0,00292	3,50	0,869	-0,00459	-5,51	-3	0,413
TAROF	6-8 leaf	-0,01620	-19,44	0,619	0,00944	11,32	9	0,085
	Bud	0,00556	6,67	0,837	-0,00195	-2,34	-2	0,836

C. Metsulfuron								
		No. Flowers at Peak Flowering			Time to Peak Flowering			
Species	Stage	α1/α0	Percent Effect at 5%	pα1	γ1/γ0	Percent Effect at 5%	Delay (Days)	pγ1
CENCY DK	6-8 leaf	-0,86667	-26,00	**0,028**	0,89676	26,90	7	**0,000**
	Bud	-0,37674	-11,30	0,196	0,16229	4,87	1	0,116
SILNO	6-8 leaf	-1,69100	-50,73	0,260	4,11235	123,37	22	**0,000**
	Bud	0,01424	0,43	0,965	-0,04757	-1,43	0	0,740
VIOAR	6-8 leaf	-1,21832	-36,55	0,180	-0,64618	-19,39	-7	0,160
	Bud	-0,69428	-20,83	0,531	0,30318	9,10	3	0,546
CERAR	6-8 leaf	0,00001	0,00	1,000	0,05724	1,72	5	**0,001**
	Bud	0,00029	0,01	0,976	-0,10181	-3,05	-3	**0,000**
CIRAR	6-8 leaf	0,02317	0,70	0,592	0,03168	0,95	0	0,869
	Bud	-0,89549	-26,86	0,216	-0,59689	-17,91	-6	0,081
EPIMO	6-8 leaf	0,01414	0,42	0,875	-0,01689	-0,51	0	0,884
	Bud	0,00233	0,07	0,934	-0,04834	-1,45	-1	0,434
KNAAR	6-8 leaf	-0,54837	-16,45	0,427	-0,16151	-4,85	-2	0,508
	Bud	-1,09731	-32,92	0,053	0,08347	2,50	1	0,725
TAROF	6-8 leaf	-3,54982	-106,49	0,622	5,54678	166,40	154	**0,000**
	Bud	-3,06681	-92,00	**0,021**	-0,42717	-12,82	-10	0,601

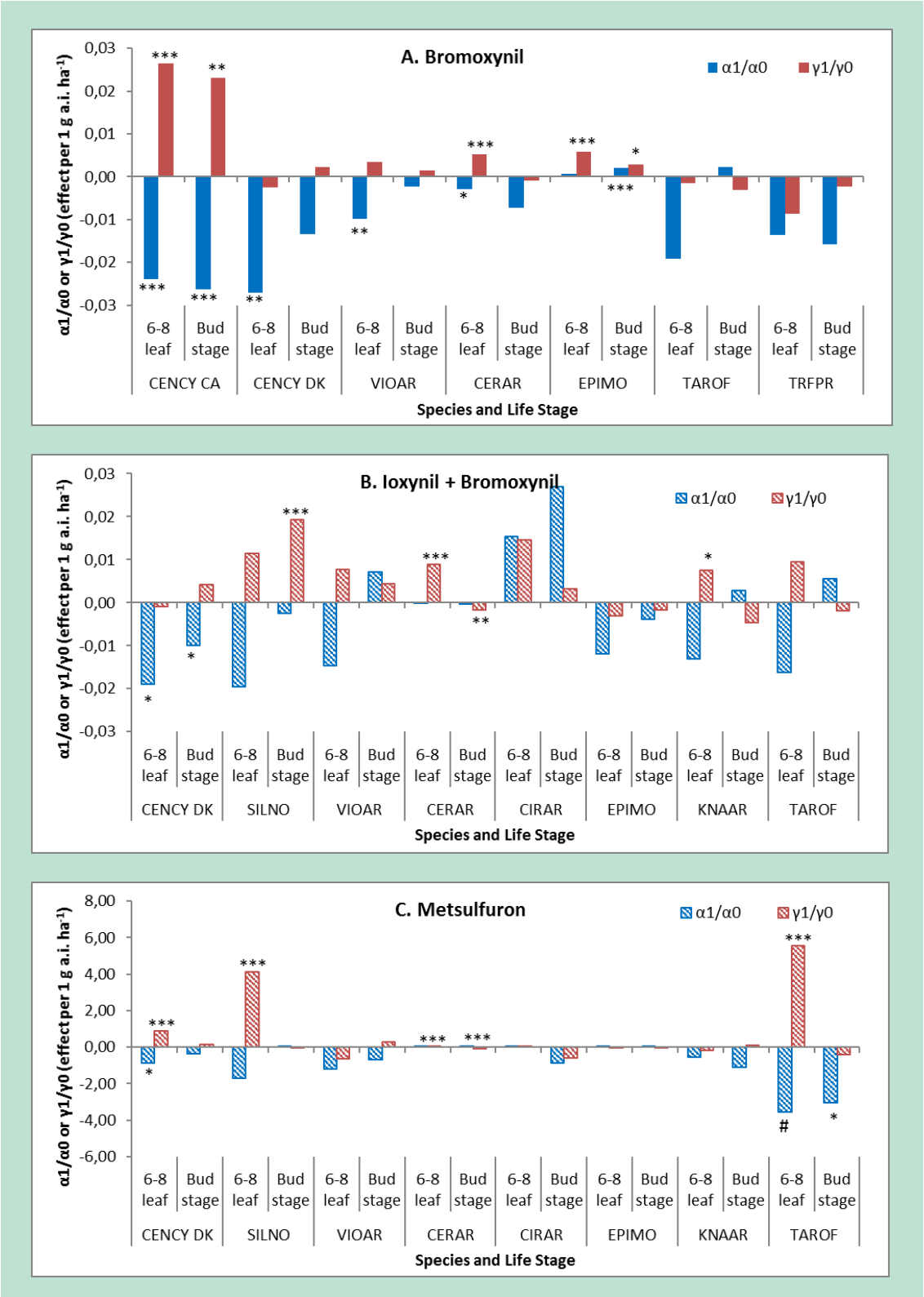

FIGURE 3. Predicted values of α1/α0 and γ1/γ0 for plant species exposed to the herbicides A. bromoxynil (Canada), B. ioxynil + bromoxynil (Denmark), or C. metsulfuron-methyl (Denmark). Values are calculated through models of cumulative flowering over time, taking into consideration the effects of 1 and 5% herbicide exposure levels on the parameters. Negative values for α1/α0 indicate reductions in the maximum number of flowers produced, while positive values for γ1/γ0 indicate delays in time to peak flowering. Values correspond to the predicted level of

effect at 1 g a.i. ha^{-1}; i.e. a value of ± 0.10 would thus correspond to a ± 10% increase/decrease in the parameter at 1 g a.i. ha^{-1}. Significant effects (as indicated by asterisks) are shown below the x-axis for α1/α0 and above the x-axis for γ1/γ0. Effect levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. # indicates a non-significant result; however, plants died at the 5% dose. Plant name abbreviations: CENCY *Centaurea cyanus*, SILNO *Silene noctiflora,* VIOAR *Viola arvensis*, CERAR *Cerastium arvense,* CIRAR *Cirsium arvense,* EPIMO *Epilobium montanum,* KNAAR *Knautia arvensis,* TAROF *Taraxacum officinale,* TRFPR *Trifolium pratense*

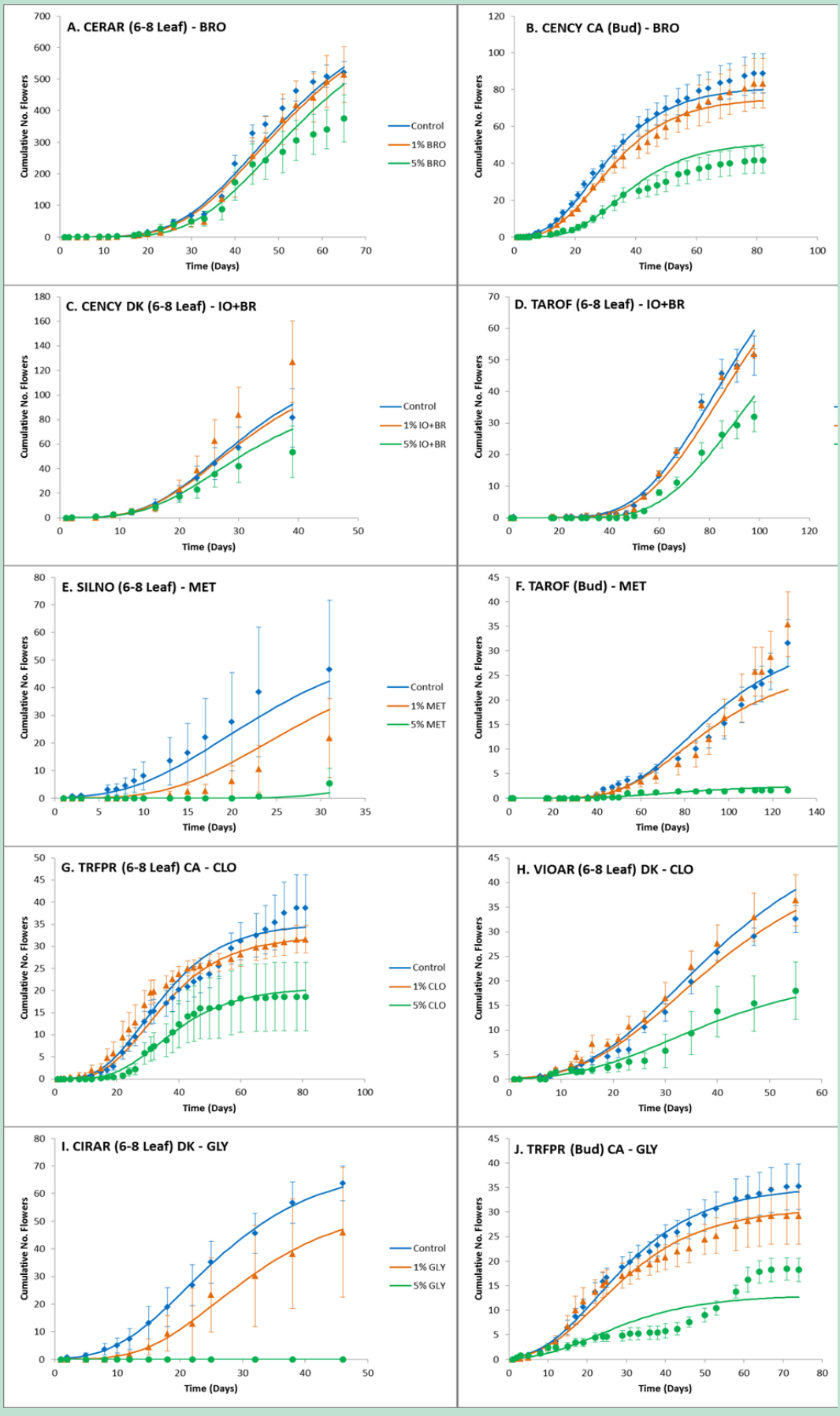
A. CERAR (6-8 Leaf) - BRO
Cumulative No. Flowers
Time (Days)
Control
1% BRO
5% BRO
B. CENCY CA (Bud) - BRO
Cumulative No. Flowers
Time (Days)
C. CENCY DK (6-8 Leaf) - IO+BR
Cumulative No. Flowers
Time (Days)
Control
1% IO+BR
5% IO+BR
D. TAROF (6-8 Leaf) - IO+BR
Cumulative No. Flowers
Time (Days)
E. SILNO (6-8 Leaf) - MET
Cumulative No. Flowers
Time (Days)
Control
1% MET
5% MET
F. TAROF (Bud) - MET
Cumulative No. Flowers
Time (Days)
G. TRFPR (6-8 Leaf) CA - CLO
Cumulative No. Flowers
Time (Days)
Control
1% CLO
5% CLO
H. VIOAR (6-8 Leaf) DK - CLO
Cumulative No. Flowers
Time (Days)
I. CIRAR (6-8 Leaf) DK - GLY
Cumulative No. Flowers
Time (Days)
Control
1% GLY
5% GLY
J. TRFPR (Bud) CA - GLY
Cumulative No. Flowers
Time (Days)

FIGURE 4. Selected graphs showing the cumulative flower production over time of various plant species following exposure to 1% (Δ orange lines) and 5% (○ – green lines) herbicide application rates, as well as non-exposed control plants (◊ - blue lines). A. *Cerastium arvense* (CERAR) treated with bromoxynil at the 6-8 leaf stage; B. *Centaurea cyanus* (CENCY-CA) treated with bromoxynil at the bud stage; C. *C. cyanus* (CENCY-DK) treated with ioxynil + bromoxynil at the 6-8 leaf stage; D. *Taraxacum officinale* (TAROF) treated with ioxynil + bromoxynil at the 6-8 leaf stage; E. *Silene noctiflora* (SILNO) treated with metsulfuron-methyl at the 6-8 leaf stage; F. *T. officinale* treated with metsulfuron-methyl at the bud stage; G. *Trifolium pratense* (TRFPR) (Canada) treated with clopyralid at the 6-8 leaf stage; H. *Viola arvensis* (VIOAR) (Denmark) treated with clopyralid at the 6-8 leaf stage; I. *Cirsium arvense* (CIRAR) (Denmark) treated with glyphosate at the 6-8 leaf stage; J. *T. pratense* (Canada) treated with glyphosate at the bud stage. Fitted lines were determined through the modified Gompertz growth curves. Error bars represent the standard error on the mean number of flowers for each dose

3.4.2.2 Effects of ioxynil + bromoxynil on plant flowering

Few significant effects were detected for ioxynil + bromoxynil by the models (Figure 3b, Table 10b). The only species for which the model predicted significant decreases in the number of flowers was *C. cyanus* –DK (Figures 3b, 4c), with effects present at both the 6-8 and bud stage sprays (predicted reductions at 5% were 23 and 12% respectively); however, there were no corresponding delays in flowering for this species (Table 10b). Significant delays in flowering were noted for *Cerastium arvense* and *K. arvensis* at the 6-8 stage as well as for *S. noctiflora* at the bud stage (flowering took 9 to 23% longer at the 5% dose, corresponding to delays ranging from 5 to 27 d), though no effects on flower number were observed (Figure 3b, Table 10b). Conversely, a slight, significant positive effect (earlier flowering) was observed for *Cerastium arvense* at the bud stage (Figure 3b, Table 10b). Though not significant, five other species (*S. noctiflora*, *V. arvensis*, *E. montanum*, *T. officinale* (Figure 4d), and *K. arvensis*) did trend towards reduced flowering (predicted decreases >10% at the 5% dose) when exposed at the 6-8 stage, while one species trended towards increased flowering (*Cirsium arvense*; both stages) (Table 10b). Likewise, non-significant trends towards delays to peak flowering (>10% delays) were also noted for the 6-8 stages of *S. noctiflora*, *Cerastium arvense*, and *K. arvensis*. When examining all of the results, there are seven cases where flower number was either significantly reduced (n = 2) or trended negatively (> 10% reductions, n = 5) and six cases where delays to peak flowering were either significant (n = 3) or followed a negative trend (>10% delay, n = 3).

3.4.2.3 Effects of metsulfuron-methyl on plant flowering

The only species to experience a significant reduction in flowering as well as a delay in time to peak flowering was *C. cyanus*-DK treated at the 6-8 leaf stage (26% decrease in max flowering, and a 27%, or 7 d, delay to peak flowering at the 5% dose; Figure 3c, Table 10c). Significant delays in flowering were noted for both *S. noctiflora* (123%, or 22 d, delay at the 5% dose; Figure 4e) and *T. officinale* (166% at the 5% dose due to plant death) at the 6-8 leaf stage; however, the models did not predict significant reductions in number of flowers for these species (Figure 3c, Table 10c). For *S. noctiflora*, the model predicted a non-significant reduction in flowering of 51% at the 5% dose (driven by the fact that only one of the five replicates flowered); however, overall low flower production by some controls probably limited the model's predictive ability. Likewise, though no flowers were produced by *T. officinale* at the 5% dose (due to plant death), and the model predicted a 106% reduction in flowering at this dose, the result was considered non-significant (Figure 3c, Table 10c). In contrast, the model did predict a significant reduction in flowering for *T. officinale* at the bud stage (92% reduction at the 5% dose), but no corresponding delay (Figures 3c, 4f, Table 10c). The remaining significant results were for *Cerastium arvense* (Figure 3c, Table 10c), which showed a significant earlier flowering at the 6-8 leaf stage (by approximately 3 d) and a significant delayed flowering at the

bud stage (by approximately 5 d). Non-significant trends for reduced flowering (>10% reductions as predicted by the model) were also noted for *C. cyanus* -DK (bud stage), *V. arvensis* (both stages), *Cirsium arvense* (bud stage), and *K. arvensis* (both stages); while non-significant earlier flowering (>10% earlier) was observed for *V. arvensis* R (6-8 leaf), *Cirsium arvense* R (bud), and *T. officinale* (bud) (Table 10c). In terms of overall effects on flowering, there were ten cases (out of 14) where either a significant reduction ($p < 0.05$; n = 2) or a negative trend (>10% reduction, n = 8) in maximum flower number were observed. Similarly, there were four cases where significant delays to peak flowering were detected, but interestingly, there were four cases where species attained peak flowering earlier (one significant, three trending).

3.4.2.4 Effects of clopyralid on plant flowering

Significant reductions in the number of flowers were observed for only three species: *Cerastium arvense* (Canada), *T. pratense* (Canada; Figure 4g), and *V. arvensis* (Denmark; Figure 4h), all at the 6-8 leaf stage, with predicted reductions ranging from 4% to 58% at the 5% dose (Figure 5a; Table 11a). Conversely, significantly increased flower production was predicted for both *E. montanum* (Canada; increase of 1% at the 5% dose) at the 6-8 leaf stage and *T. pratense* (Canada; increase of 22% at the 5% dose) at the bud stage (Figure 5a, Table 11a). Significant delays in flowering were noted in six instances: *C. cyanus*-CA (Canada; both stages), *C. cyanus*-DK (Denmark; 6-8 leaf stage); *Cerastium arvense* (Canada; bud stage), *S. noctiflora* (Denmark; bud stage), and *Cerastium arvense* (Denmark; 6-8 leaf stage), with predicted delays spanning 6% to 21% (3 to 15 d depending on the species) at the 5% dose (Figure 5a, Table 11a). In contrast, significantly earlier flowering was predicted in five cases: *Cerastium arvense* (Canada; 6-8 leaf stage), *Cerastium arvense* (Denmark; bud stage), *Cirsium arvense* (Denmark; both stages) and *E. montanum* (Denmark; bud stage), with plants predicted to flower 1% to 37% (1 to 8 d, depending on the species) faster than the controls at the 5% dose (Figure 5a, Table 11a). In only one case, *Cerastium arvense* (Canada; 6-8 leaf stage), were both the effects on flower production and flowering time significant (Figure 5a, Table 11a). In this case, sprayed plants flowered slightly earlier (by approximately 1 d) but produced fewer flowers. In the remaining cases, non-significant trends towards reduced flowering (>10% reductions in flowering at the 5% dose as predicted by the model) were observed in nine cases: *C. cyanus*-CA (Canada), *C. cyanus*-DK (Canada), *Cirsium arvense* (Denmark), and *T. officinale* (Denmark) at the 6-8 leaf stage; *T. officinale* F (Canada), *C. cyanus* -DK (Denmark) and *T. officinale* (Denmark) at the bud stage; and *K. arvensis* (Denmark) at both stages, while a potential increase in flowering (>10% increase) was observed in one case (*C. cyanus*-DK (Canada); Table 11a). Additionally, there were five cases where species non-significantly trended towards delays in peak flowering: *T. pratense* (CA; both stages), *V. arvensis* (DK; bud stage), and *K. arvensis* (both stages) (Table 11a). In total, there were 12 cases (out of 30) where reductions in number of flowers was either significant (n = 9) or followed a negative trend (>10% reduction; n = 3), and 11 cases (out of 30) where flowering was significantly delayed (n =6) or followed a negative trend (>10% delay; n = 5).

3.4.2.5 Effects of glyphosate on plant flowering

Significant effects of glyphosate on the number of flowers at peak flowering and/or time to peak flowering were present for all species (Figure 5b, Table 11b). Reductions in the number of flowers and delays in flowering were most evident for the DK results, as either plant death or severely reduced flowering was common at the 5% dose for plants (*C. cyanus*-DK, *V. arvensis*, *Cerastium arvense*, *Cirsium arvense* (Figure 4j), *K. arvensis*, and *T. officinale*) sprayed at the 6-8 leaf stage as indicated earlier; however, the model did not always identify significant effects on α_1/α_0 and γ_1/γ_0 for these scenarios (Figure 5b).

Taking this into consideration, for plants exposed at the 6-8 leaf stage where plants survived at the 5% dose (nine cases), the models predicted significantly reduced number of flowers for *C. cyanus*-CA, *E. montanum*, and *V. arvensis* (6% to 29% predicted reductions at the 5% glyphosate dose) tested in Canada, and significantly increased flowering for *Cerastium arvense*

(Canada; 18% increase at 5% glyphosate) and *S. noctiflora* (Denmark; 206% increase at 5%), though the increased flowering for SILNO was likely skewed due to unproductive control plants (Figure 5b, Table 11b). Though *E. montanum* (DK) plants exposed to 5% glyphosate produced significantly fewer flowers than the controls (Table 9), there was no significance of α_1/α_0 ($p\alpha$ = 1). This finding was likely the result of the increased flowering that was observed at the 1% dose. Examination of non-significant trends found that in three other cases (*C. cyanus*-DK, *T. officinale*, and *T. pratense*; all in Canada), the model predicted reductions in flowering (>10% reduction) at the 5% glyphosate dose (Table 11b). For plants sprayed at the bud stage, significant reductions in the number of flowers were observed in four cases: *T. pratense* (Canada; Figure 3.3j) and *Cirsium arvense*, *V. arvensis*, and *T. officinale* (Denmark), with predicted reductions ranging from 44% to 93% at the 5% dose (Figure 5b, Table 11b). Non-significant trends (>10% reductions) were present in four cases: *T. officinale* and *V. arvensis* (Canada), and *S. noctiflora* and *K. arvensis* (Denmark) while a potential positive trend of increased flowering was noted for *C. cyanus*-DK (Canada) (Table 11b). Similar to the results for the 6-8 leaf stage, the bud stage of *E. montanum* (Denmark) trended towards increased flowering at 1%; however, flowering was severely reduced at 5% (Table 9). This result this was not accurately captured by the model. Reductions in the number of flowers was found to be significant in 16 cases (including the cases where plants died, but where the models did not detect a decrease), with negative trends (>10% reductions) present in a further seven (n = 30 for all cases).

Significant delays in time to peak flowering for plants sprayed at the 6-8 leaf stage were observed for *C. cyanus*-CA, *E. montanum*, and *T. pratense* in the Canadian trials, and for *C. cyanus*-DK, *S. noctiflora*, *Cerastium arvense*, *Cirsium arvense*, *E. montanum*, and *T. officinale* in the Danish trials, with predicted delays spanning from 7% to 120% (2 to 65 days) at 5% glyphosate doses (Figure 4b, Table 11b). If delays within the Danish trials are calculated for the 1% dose only, omitting data for the 5% dose due to the high mortality, they spanned approximately 1-7 days. Surprisingly, no significant delays were noted for either *V. arvensis* (model indicates no trend) or *K. arvensis* (model indicates a significant earlier flowering at 5%) in the Danish trials even though plants had died at the 5% dose. For plants treated at the bud stage, five cases: *C. cyanus*-CA and *Cerastium arvense* (Canada), and *C. cyanus*-DK, Cerastium arvense, and *E. montanum* (Denmark), indicated significant delays in time to peak flowering, with predicted delays spanning 11% to 89% (10 to 24 d depending on the species) at the 5% dose (Table 11b). Significant earlier flowering (18 d) was also predicted for *Cirsium arvense* (Denmark). *C. cyanus*-DK (Canada) trended towards delayed peak flowering, while *T. officinale* (Canada) and *V. arvensis* and *T. officinale* (Denmark) trended towards earlier peak flowering (Table 11b). Overall, significant delays to peak flowering were observed in 16 cases (including cases where plants died at the 5% dose), with one additional case showing a negative trend (>10% delay). Omitting the six cases where there was death or zero flowering at the 5% dose (hence obvious effects on flowering parameters), there were only four other cases where a simultaneous effect on both the number of flowers produced and time to peak flowering were observed (Table 11b); however, the trends were different for each scenario. *C. cyanus*-CA and *E. montanum* (both in Canada) sprayed at the 6-8 leaf stage experienced both a significant reduction in the number of flowers produced as well as a delay in time to peak flowering. *S. noctiflora* (Denmark) at the 6-8 leaf stage had an increase in the number of flowers following glyphosate exposure, but time to peak flowering was delayed. In contrast, *Cirsium arvense* (Denmark) at the bud stage attained peak flowering earlier after exposure, but had reductions in the number of flowers present at the time of peak flowering.

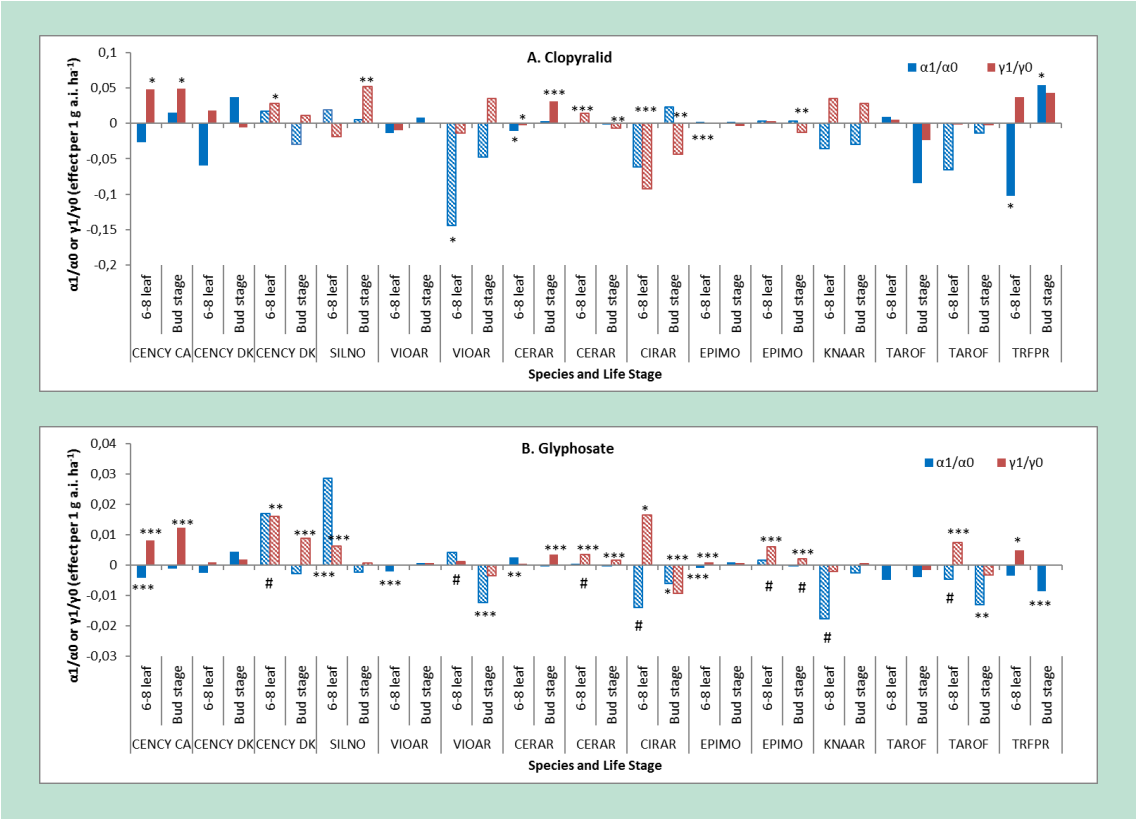

FIGURE 5. Predicted values of α1/α0 and γ1/γ0 for plant species exposed to the herbicides A. clopyralid or B. glyphosate in both Canada and Denmark. Canadian data are depicted as solid bars; Danish data as patterned bars. Values are calculated through models of cumulative flowering over time, taking into consideration the effects of 1 and 5% herbicide exposure levels on the parameters. Negative values for α1/α0 indicate reductions in the maximum number of flowers produced, while positive values for γ1/γ0 indicate delays in time to peak flowering. Values correspond to the predicted level of effect at 1 g a.i. ha^{-1}; i.e. a value of ± 0.10 would thus correspond to a ± 10% increase/decrease in the parameter at 1 g a.i. ha^{-1}. Significant effects (as indicated by asterisks) are shown below the x-axis for α1/α0 and above the x-axis for γ1/γ0. Effect levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. # indicates a non-significant result; however, plants died at the 5% dose. Plant name abbreviations: CENCY *Centaurea cyanus*, SILNO *Silene noctiflora,* VIOAR *Viola arvensis*, CERAR *Cerastium arvense,* CIRAR *Cirsium arvense,* EPIMO *Epilobium montanum,* KNAAR *Knautia arvensis,* TAROF *Taraxacum officinale,* TRFPR *Trifolium pratense*

TABLE 11. Percent effect values of herbicide exposure on the max number of flowers produced and the time to peak flowering at 5% herbicide exposure calculated from the predicted values of α1/α0 and γ1/γ0. A. Clopyralid, B. Glyphosate. Both herbicides were evaluated in Canada and Denmark. Values for predicted percent effect are calculated as the value of α1/α0 or γ1/γ0 times the g a.i. ha^{-1} present at the 5% dose for each herbicide separately. Negative values for α1/α0 indicate reductions in the number of flowers, whereas positive values for γ1/γ0 indicate flowering delays. Delay (days) reflects the time required to reach peak flowering at 5% exposure; positive values indicate a delay, while negative values indicate earlier flowering. Significant effects (p-values) for α and γ are highlighted in bold. Plant name abbreviations: ENCY *Centaurea cyanus*, SILNO *Silene noctiflora,* VIOAR *Viola arvensis*, CERAR *Cerastium arvense,* CIRAR *Cirsium arvense,* EPIMO *Epilobium montanum,* KNAAR *Knautia arvensis,* TAROF *Taraxacum officinale,* TRFPR *Trifolium pratense*

A. Clopyralid

	Species	Stage	No. Flowers at Peak Flowering α1/α0	Percent Effect at 5%	pα1	Time to Peak Flowering γ1/γ0	Percent Effect at 5%	Delay (Days)	pγ1
CANADA	CENCY CA	6-8 leaf	-0.02648	-10.59	0.260	0.04846	19.38	5	**0.027**
		Bud	0.01558	6.23	0.538	0.04917	19.67	5	**0.029**
	CENCY DK	6-8 leaf	-0.05997	-23.99	0.092	0.01808	7.23	2	0.533
		Bud	0.03762	15.05	0.062	-0.00595	-2.38	-1	0.758
	VIOAR	6-8 leaf	-0.01338	-5.35	0.338	-0.00995	-3.98	-1	0.377
		Bud	0.00872	3.49	0.094	0.00071	0.29	0	0.945
	CERAR	6-8 leaf	-0.01092	-4.37	**0.030**	-0.00306	-1.22	-1	**0.013**
		Bud	0.00321	1.28	1.000	0.03118	12.47	5	**0.000**
	EPIMO	6-8 leaf	0.00206	0.82	**0.000**	0.00052	0.21	0	0.915
		Bud	0.00195	0.78	0.523	-0.00320	-1.28	0	0.370
	TAROF	6-8 leaf	0.00885	3.54	0.969	0.00500	2.00	6	1.000
		Bud	-0.08447	-33.79	1.000	-0.02413	-9.65	-7	0.856
	TRFPR	6-8 leaf	-0.10289	-41.16	**0.011**	0.03671	14.68	4	0.418
		Bud	0.05379	21.52	**0.036**	0.04362	17.45	4	0.137
DENMARK	CENCY DK	6-8 leaf	0.016903	6.76	0.340	0.02861	11.45	3	**0.041**
		Bud	-0.02955	-11.82	0.173	0.01162	4.65	1	0.149
	SILNO	6-8 leaf	0.01929	7.72	0.761	-0.01907	-7.63	-1	0.229
		Bud	0.00568	2.27	0.924	0.05174	20.69	4	**0.001**
	VIOAR	6-8 leaf	-0.14466	-57.86	**0.050**	-0.01351	-5.40	-2	0.772
		Bud	-0.04746	-18.98	0.516	0.03540	14.16	4	0.296
	CERAR	6-8 leaf	0.00001	0.00	0.983	0.01431	5.72	15	**0.000**
		Bud	-0.00175	-0.70	0.873	-0.00647	-2.59	-2	**0.006**
	CIRAR	6-8 leaf	-0.06169	-24.68	0.059	-0.09298	-37.19	-8	**0.000**
		Bud	0.02358	9.43	0.762	-0.04396	-17.58	-6	**0.001**
	EPIMO	6-8 leaf	0.00347	1.39	0.931	0.00179	0.72	0	0.895
		Bud	0.00363	1.45	0.744	-0.01242	-4.97	-3	**0.007**
	KNAAR	6-8 leaf	-0.03592	-14.37	0.644	0.03486	13.94	6	0.058
		Bud	-0.02991	-11.96	0.668	0.02796	11.18	5	0.114
	TAROF	6-8 leaf	-0.06544	-26.18	0.249	-0.00045	-0.18	0	0.974
		Bud	-0.01399	-5.60	0.865	-0.00135	-0.54	0	0.221

B. Glyphosate

	Species	Stage	Maximum Number of Flowers α1/α0	Percent Effect at 5%	pα1	Time to Peak Flowering γ1/γ0	Percent Effect at 5%	Delay (Days)	pγ1
CANADA	CENCY CA	6-8 leaf	-0.00409	-29.42	**0.000**	0.00821	59.08	15	**0.000**
		Bud	-0.00123	-8.85	0.613	0.01233	88.79	24	**0.000**
	CENCY DK	6-8 leaf	-0.00250	-18.02	0.230	0.00091	6.54	2	0.561
		Bud	0.00434	31.28	0.113	0.00195	14.03	5	0.197
	VIOAR	6-8 leaf	-0.00202	-14.54	**0.001**	0.00003	0.24	0	0.956
		Bud	0.00060	4.30	0.459	0.00069	4.95	1	0.325
	CERAR	6-8 leaf	0.00245	17.66	**0.009**	0.00043	3.10	1	0.201
		Bud	-0.00056	-4.05	1.000	0.00342	24.64	10	**0.000**
	EPIMO	6-8 leaf	-0.00090	-6.49	**0.000**	0.00093	6.67	2	**0.000**
		Bud	0.00100	7.23	0.260	0.00060	4.29	1	0.071
	TAROF	6-8 leaf	-0.00481	-34.61	0.248	-0.00036	-2.56	-4	0.687
		Bud	-0.00390	-28.09	1.000	-0.00155	-11.16	-8	0.961
	TRFPR	6-8 leaf	-0.00358	-25.76	0.215	0.00478	34.41	11	**0.030**
		Bud	-0.00873	-62.82	**0.000**	-0.00028	-2.03	0	0.935
DENMARK	CENCY DK	6-8 leaf[a]	0.01687	121.47	0.320	0.01603	115.42	29	**0.004**
		Bud	-0.00284	-20.41	0.162	0.00876	63.08	23	**0.000**
	SILNO	6-8 leaf	0.02868	206.46	**0.000**	0.00621	44.69	8	**0.000**
		Bud	-0.00234	-16.87	0.398	0.00075	5.40	1	0.436
	VIOAR	6-8 leaf[a]	0.00425	30.58	0.850	0.00113	8.16	2	0.905
		Bud	-0.01242	-89.39	**0.001**	-0.00357	-25.69	-6	0.316
	CERAR	6-8 leaf[a]	0.00002	0.11	0.985	0.00358	25.79	65	**0.000**
		Bud	-0.00007	-0.51	0.923	0.00155	11.14	10	**0.000**
	CIRAR	6-8 leaf[a]	-0.01388	-99.91	0.241	0.01660	119.53	24	**0.035**
		Bud	-0.00607	-43.72	**0.027**	-0.00923	-66.42	-18	**0.000**
	EPIMO	6-8 leaf[b]	0.00166	11.93	1.000	0.00614	44.23	20	**0.000**
		Bud	-0.00007	-0.53	0.924	0.00218	15.67	16	**0.000**
	KNAAR	6-8 leaf[a]	-0.01771	-127.48	0.147	-0.00210	-15.11	-7	0.646
		Bud	-0.00258	-18.56	0.416	0.00034	2.47	1	0.744
	TAROF	6-8 leaf[a]	-0.00457	-32.93	0.776	0.00746	53.74	41	**0.001**
		Bud	-0.01298	-93.45	**0.007**	-0.00331	-23.83	-18	0.475

a - Death of all, or majority of, replicates at the 5% glyphosate application rate

b - Very low production of flowers at the 5% glyphosate dose, but increased production at 1% as compared to controls

3.4.3 Comparison of flowering for plants exposed at 6-8 Leaf and Bud Stages

Paired t-tests were performed comparing the 6-8 leaf and bud stage results for a given species in terms of either the α1/α0 or the γ1/γ0 (Table 12). Herbicides were evaluated separately. In terms of the effects on the number of flowers (α1/α0), only the results for ioxynil + bromoxynil tended to indicate an effect of stage at spray, with plants being sprayed at the 6-8 leaf stage tending towards higher reductions in flowering as compared to corresponding plants sprayed at the bud stage (t = -4.901, df = 7, p =0.002). No other herbicides showed a significant difference between the 6-8 leaf and bud stages (p = 0.109 to 0.469). Likewise, results for paired t-tests comparing γ1/γ0 values for the different stages found no trends for increased sensitivity of either the 6-8 stage or the bud stage as compared to each other (p = 0.073 to 0.685). Overall, this indicates that, with the exception of perhaps ioxynil + bromoxynil, the effect of herbicides on the flowering of plants is highly variable, and cannot be easily predicted from the life stage at which plants are sprayed.

When the results for all herbicide models were combined (Table 12), there was no overall trend for either the 6-8 leaf stage or the bud stage being the most sensitive in terms of the predicted values for α1/α0 or γ1/γ0 (p > 0.05).

TABLE 12. Paired t-tests for comparison of results of the 6-8 leaf stage to the bud stage to determine if stage of spray can predict sensititivy to herbicide as predicted by α1/α0 or γ1/γ0. Each pair represented one species that was sprayed in either Canada or Denmark. Herbicides were evaluated separately and as a combined group

	Peak Number of Flowers					Time to Peak Flowering				
	Mean α1/α0		t	df	p	Mean γ1/γ0		t	df	p
Herbicide	6-8	Bud				6-8	Bud			
Bromoxynil	-0,014	-0,009	-1,370	6	0,220	0,004	0,003	0,425	6	0,685
Ioxynil + Bromoxynil	-0,010	0,003	-4,901	7	**0,002**	0,007	0,003	1,504	7	0,176
Metsulfuron	-0,980	-0,764	-0,766	7	0,469	1,228	-0,084	1,530	7	0,170
Clopyralid	-0,031	-0,004	-1,714	14	0,109	0,003	0,010	-0,892	14	0,387
Glyphosate	0,000	-0,003	1,046	14	0,313	0,005	0,001	1,935	14	0,073
All	-0,160	-0,118	-1,012	52	0,316	0,189	-0,009	1,428	52	0,159

3.4.4 Laboratory comparison

To evaluate replicability, individual results for α1/α0 and γ1/γ0 for a given species, life stage, and herbicide combination were qualitatively compared between labs. This was done for the two herbicides (clopyralid and glyphosate) and the five plant species (C. cyanus-DK, *V. arvensis*, *Cerastium arvense*, *E. montanum*, and *T. officinale*) that were used in common, giving a total of 20 cases (two life stages x two parameters (α1/ α0 and γ1/ γ0) x five species) for comparison per herbicide.

For clopyralid, results were similar in 13 cases where no significant effects were predicted in either Canada or Denmark (though in some cases the Denmark results did trend more negatively, for instance the number of flowers for bud stage *V. arvensis*). In the other seven cases, a significant effect was detected in one lab but not the other (five cases: two in Canada and three in Denmark; for instance, flowering was delayed in *E. montanum* at the bud stage in Denmark but not in Canada) or significant effects were detected in both labs, but the direction of the effects were different (two cases; for instance, *Cerastium arvense* at the 6-8 leaf stage had earlier peak flowering in Canada but delayed flowering in Denmark).

Differences between labs were more noticeable for glyphosate, where exposure at the 5% level resulted in either the death of most plant species or in significantly reduced flowering

when sprayed at the 6-8 leaf stage in the Danish studies. This was not observed in Canadian studies. The only consistencies between Denmark and Canada for the 6-8 bud stage were for *E. montanum* and *V. arvensis*, where a significant decrease (though of lower magnitude) in the max number of flowers was also noted in Canada (plants died or produced few flowers in Denmark at the 5% dose). A significant effect was detected on *Cerastium arvense* max flowering in Canada; however, the effect was positive (increased flowering). The only common delay in flowering at the 6-8 leaf stage was for *E. montanum*. Negative effects at the bud stage were also more common for the Danish data. For four species, statistically significant negative effects were only recorded in Denmark for this life stage (reductions in peak flowering for *V. arvensis* and *T. officinale*; delays in flowering for *C. cyanus*-DK and *E. montanum*). Similar results between labs were only observed for *Cerastium montanum* (bud stage), where delays to peak flowering were observed by both labs.

3.5 Discussion

The effects of sub-lethal herbicide exposure on NTTP species growing near agricultural areas is of increasing concern due to the benefits that these species provide to ecosystem services. Edge habitats and high quality edge floral communities are important resources for bees and other pollinators (Baude et al., 2016; Kammerer et al., 2016), supporting abundant and species-rich pollinator communities for agricultural areas and providing different floral resources over the course of a year (Mandelik et al., 2012). The presence of small, narrow field margins, though they may only contain a few plant species, is still vital to creating corridors in intensively cultivated areas (Kremen et al., 2007; Hahn et al. 2014). Plant communities, however, within these margins and adjacent natural areas are at risk of low dose herbicide exposure due to drift (Kleijn and Snoeijing, 1997; de Snoo and van der Poll, 1999; de Jong et al., 2008). Given that field margins and buffer strips are often very small, or narrow, in some agricultural landscapes, it is possible that all plants within the patch could experience some degree of exposure, and thus the plant community composition may change based on individual species sensitivities to any given herbicide (Rodriguez and Jacobo, 2010; Pfleeger et al., 2012; Olszyk et al., 2013; Schmitz et al. 2014; Reeg et al., 2017). Many studies have documented direct effects on plant biomass following exposures simulating drift; however, few studies have addressed the longer-term effects on overall flower production, and fewer still have addressed the concepts on delayed flowering. In this study, cumulative flowering over time was used to model the effects of sub-lethal herbicide exposure on maximum flower production as well as on the time to peak flowering. Overall, the models were able to predict subtle negative effects on these ecologically relevant parameters.

One of our primary hypotheses was that exposure of herbicides would result in reduced flowering in non-target species. Total flower production as an end-point provides information on the potential reproductive output of an individual, which is important for maintaining seed recruitment and thus population size. In this experiment, we observed that all tested herbicides were found to negatively affect total flower production in at least one species, and all species were affected by at least one herbicide when sprayed at either the 6-8 leaf or bud stages.

Glyphosate was found to have the most adverse effect on the total number of flowers produced by plants, however, high mortality when plants were exposed at the 6-8 leaf stage to 5% glyphosate in experiments conducted in Denmark was a major driver. In total, significant negative effects on total flower production or non-significant trends towards reduced flowering (>10% reductions according to model parameters) were observed in 57% of all cases (61/106), with reductions spanning from 4% to 100%. This finding supports our hypothesis that reproductive endpoints are particularly sensitive to low level herbicide exposure.

Overall, trends indicate that the 6-8 leaf stage may be the more sensitive than the bud stage (62% of the 61 significant/trending cases were for plants exposed at the 6-8 leaf stage), and this was found to be true for ioxynil + bromoxynil; however, there were instances where either

significant reductions were only observed for plants sprayed at the bud stage (i.e. *T. pratense* tested with glyphosate) or the negative effects on the bud stage were larger (i.e. *Cirsium arvense* tested with metsulfuron-methyl). This indicates that though the classical protocol of evaluating plants at the seedling stage (6-8 leaves) may still identify the majority of potential risks to wild plants, it is also likely to miss effects for some species that are more sensitive at later life stages, with possible repercussions at the community level.

One limitation of this study was that all flowering was treated equally, that is, both healthy and unhealthy/malformed flowers were counted as part of the total floral count. Though a sprayed plant may have produced as many flowers as an unsprayed control, the viability of these flowers was not assessed. Flower deformations resulting from herbicide exposure has been documented. Petal, anther, and pollen deformations have been reported for *Brassica* spp. flowers exposed to low doses of glyphosate (Londo et al., 2014) and anther malformations and pollen quality reductions have been reported for imazethapyr (Qian et al., 2015). Floral traits are important to pollinators (Bosche et al., 1997; Sargent and Ackerly, 2008); small (malformed) flowers/petals would reduce visitation rates, as would the absence of rewards (Wilcock and Neiland, 2002). In an area where flowering is sparse (for instance, a plant species affected by herbicide), or where malformed flowers with poor rewards are present, a pollinator may be less consistent to a given species, and may explore more options including using other species, or moving to a healthier patch (Chittka et al., 1999). Gezon et al. (2006), in an experiment designed to examine the effects of climate-related phenological shifts on flowering in *Claytonia lanceolata*, observed fewer pollinator visits to smaller flowers than larger flowers of this species.

Many plant species, especially long-lived species, naturally exhibit forms of asynchronous flowering within a given population; however, short-lived species would require a higher degree of synchronization (Rathcke and Lacey, 1985; Rafferty et al., 2015; Valverde et al., 2016). Delays in, or asynchronous flowering, within a population has the potential to reduce outcrossing potential between individual plants due to minimized pollen transfer (Elzinga et al., 2007). Additionally, long delays may put affected plants at a disadvantage if it means they are now competing with new plant species for pollinators (Mitchell et al., 2009), or if environmental conditions are now unfavorable for flower development. Small shifts in phenology may also negatively affect plant reproduction. Gezon et al. (2016) identified differences in pollinator occurrence on *Claytonia lanceolata*, a spring ephemeral with a short flowering period, after experimentally altering flowering time to either approximately one week earlier or one week later than controls. Though there was overlap between pollinator species, overall visitation rates in the later flowering plants were five times lower than the controls, and foraging times 3.6 times lower, with plants in the late treatment being significantly pollen limited.

In this experiment we hypothesized that exposure to low levels of herbicides would lead to delays in flowering. Similar to our results for total flower production, time to peak flowering was also negatively affected in at least one species for all herbicides thus supporting the above hypothesis. Though slightly more difficult to assess than total flower production (i.e. an affected plant may only produce one flower, but may produce it before the controls, hence there would not be a delay), delays to peak flowering were present as either a statistically significant delay, or as trending towards a delay (>10% delay) in a minimum of 40% of all cases (42/106), providing further support for the hypothesis that reproductive endpoints are sensitive measures for assessing sub-lethal herbicide exposures. Again, the majority of these cases were for the 6-8 life stage (64%; approx. 27/42); however, variability in the magnitude of the effects between the 6-8 and bud stage results for some species and herbicide combinations did indicate that predicting delays based on one life stage may be inappropriate (Table 12). Across all life stages, the predicted longest delays at 5% herbicide exposure were 9, 27, 22, and 15 days for bromoxynil, ioxynil + bromoxynil, metsulfuron, and clopyralid, respectively, and 24 days for glyphosate (for plants that survived the 5% exposure).

Simultaneous effects on both the total number of flowers produced and the time to peak flowering occurred in approximately 25% of all of our study cases. For instance, both *T. pratense* and *C. cyanus*-CA (Canadian study) exposed to 5% glyphosate at the 6-8 leaf stage experienced significant reductions in flower production (26% and 29%, respectively) and delays to peak flowering (34% and 59%, or 11 and 15 days, respectively). Not only would this equate to reduced seed output for both species, but also the approximate two-week delays would likely temporally restrict pollen transfer and out-crossing in the affected patches and lead to assortative mating for the unhealthy individuals. In addition, both *C. cyanus* and *T. pratense* have been identified as highly important pollen species (García and Miñarro 2014; Ricou et al., 2014). If both were present in the same patch, and if both served as attractants to pollinators, not only would lower flower numbers reduce their own visitation rates, but it may also reduce visitation rates to other plant species in the patch regardless if those plants had been negatively affected by herbicide exposure. Bohnenblust et al. (2016) recorded reduced flowering and/or delays to first flowering for *Medicago sativa* and *Eupatorium perfoliatum* at drift level rates of dicamba exposure (1%), but did note that there was recovery over time. Reductions in pollinator visits were noted for both species, with significant reductions observed for *E. perfoliatum*; however, pollen analysis found no reduction in pollen quality for *E. perfoliatum*.

When considering the effects of herbicides on plant communities, both the number of flowers within the patch at a given time as well as the presence of specific high quality pollen or nectar species is important for attracting pollinators to a patch. For plants, this means that the chances for a successful pollination event are increased, while for pollinators it ensures that a healthy, diverse array of food resources is available. Grundel and Pavlovic (2000) found that the presence of the short-lived adults of the endangered butterfly *Lycaeides melissa samuelis* was often determined by the presence and quality of nectar plants. Adults tended to only fly short distances (approximately 70 m) and that they generally choose the nectar species with the most flowers in a patch, though preference for certain plant species is sometimes given regardless of floral number.

Disruption to plant-pollinator systems as a result of sub-lethal herbicide exposure is of significant concern. Overall declines in nectar diversity have been reported in the UK (Baude et al., 2006), and declining bee species richness has been reported in both the UK and the Netherlands, with simultaneous declines in obligately outcrossing plant species also occurring in the Netherlands (Beismeijer et al., 2016). Generally, decline of wildflowers and, consequently, food limitation of flower-visiting insects is a major concern and is thought to be an important driver of historical pollinator decline (Biesmeijer et al. 2006; Potts et al. 2010). However, significant knowledge gaps still exist in plant-pollinator systems; specifically how pollinator diversity fluctuates through space and time (Allen-Wardell et al., 1998), and thus it is difficult to predict how pollinators and plants will be affected with any shifts in plant phenology, including following herbicide exposure. The loss of flowers in a habitat may limit pollinator movement. For instance, bees have been shown to have site constancy with a preference for moving along continuous corridors (Osbourne and Williams, 2001). Damaged flowers may discourage bees from foraging in herbicide-exposed patches, thus causing them to look elsewhere for better rewards. Forrest and Thomas (2011; and references within) state that what can potentially be more detrimental to early flowering plants and their pollinators is floral damage (in their case frost damage; in our case herbicide damage), that may lead to future gaps in the food supply for pollinators. Memmott et al. (2007) simulated the effects of earlier flowering (one to three weeks) on food availability for pollinators. They found that these subtle time shifts could result in 17-51% of all pollinators suffering a food supply disruption at some point in their lifecycle. Heglund et al. (2009) also indicate that shifts may be more detrimental to pollinators than to plants, as pollinator populations could potentially crash if food is scarce or lacking during a critical period, while many plant species are still capable of self-reproduction. If a pollinator normally has multiple generations in a year, this in turn could have more repercussions on future

flowering plant species. However, Memmott et al. (2004), also indicate that pollination networks are robust, with few specialist-specialist interactions, and that the removal of one species (either a plant or pollinator) is unlikely to result in major changes to the community (i.e. local extinctions) unless it is a highly connected species (i.e. highly efficient, generalist pollinator or a high quality pollen/nectar producing plant). When assessing the risks of herbicides on non-target plants it is therefore important to consider the effects on total flower production of not only individual species, but the entire plant community as a whole, as well as the availability and quality of floral resources (both for pollen transfer between individual plants and for pollinators). Decline in floral availability could trigger pollinator decline, which can in turn lead to decline in pollination of wild flowers. Moreover, decline of pollinators may ultimately result in reduced seed set of insect-pollinated plants and reduced yield in crops (Garibaldi et al. 2013).

4. Population level effects of herbicides on non-target terrestrial plants - Plant competition and herbicide exposure

4.1 Introduction

The main objective of this study was to evaluate the effect of herbicides on the competitive interactions between two plant species in greenhouse phytotoxicity tests, by measuring vegetative and reproductive endpoints. Specifically, the hypothesis tested was that herbicides would influence the competitive interactions between the model species *Centaurea cyanus* and *Silene noctiflora* by altering their competitive abilities. It was predicted that increasing intra- and inter specific densities would have a negative effects on plant performance (vegetative and reproductive parameters) but there is no way to predict how the herbicide will affect a plant's ability to compete with its neighbours, as its response to herbicide may also depend on the response of the other species. In three separate experiments, the two plant species were grown individually and in varying densities and proportions of conspecifics and the competing species, and were sprayed with doses of glyphosate (Experiment 1) or metsulfuron-methyl (Experiment 2 – short-term, Experiment 3 – long-term). By using a Bayesian modelling approach, the intra- or interspecific competitive abilities of the two plant species as affected by low doses of the herbicides were assessed on the vegetative and reproductive parameters. Effectively, the intent was to determine if plants grown individually or in monoculture provide sufficient information for the protection of non-target terrestrial plants (NTTPs) populations and communities and test the following project hypothesis:

2. Plant populations are affected by both herbicides and competition, and responses to both factors need to be measured in order to generate predictions on the effects on plant populations

4.2 Material and methods

The study was carried out as pot experiments in greenhouses at two locations – Aarhus University, Research Centre Flakkebjerg in Denmark (DK, GPS: 55.324382, 11.390041) and Environment and Climate Change Canada, in Ottawa, Canada (CA, GPS: 45.3876, 75.6960).

4.2.1 Test species

Two annual plant species, *Centaurea cyanus* L. (Cornflower) and *Silene noctiflora* L. (Night-flowering catchfly) were chosen as the NTTPs. Both species live in arable fields, and are commonly found growing together along agricultural field margins in Europe and to a lesser extent in North America. Both serve as important sources of food for pollinating insects. The bright blue flowers of *C. cyanus* emerge between May and August, and rely on insect pollination as they are self-incompatible (Bellanger et al 2014). *S. noctiflora* flowers between June and September; its white flowers are nocturnal, opening at night to release a fragrance to attract nocturnal moths. However, the species is self-compatible (McNeill 1980). Both are considered weed species in agricultural areas, and are in decline in their native habitats (Sutcliffe & Kay 2000).

C. cyanus seeds were obtained from commercial seed suppliers (OSC seeds, jubilee gem variety of bachelor's button) for the experiment in Canada. *S. noctiflora* seeds were shipped to Canada from Denmark. *C. cyanus* (used in Danish experiments) and *S. noctiflora* seeds were collected from wild populations in 1995 at Flakkebjerg, Denmark, and propagated in the greenhouses in 2007.

4.2.2 Herbicides

Two different herbicides, i.e. glyphosate and metsulfuron-methyl, were used for the experiments. For more details on the herbicides, see Chapter 2.2.2.

4.2.3 General experimental set-up

Three experiments were conducted with C. cyanus and S. noctiflora, 1) Experiment 1 in Canada, using glyphosate with plants harvested and appraised at mature stage, 2) Experiment 2 in Denmark, with metsulfuron-methyl including plants harvested three weeks after spray, and 3) Experiment 3 with metsulfuron-methyl in Denmark with plants assessed throughout mature stage.

Experiments in the greenhouses at Carleton University, in Ottawa, Canada, were conducted between January and August 2015. Average temperature in the greenhouses ranged from 17.3 ±3.5 to 38.0 ±7.3°C, average photosynthetically active radiation (PAR) (with no artificial lighting) ranged from 49 (cloudy day) to 1505 µmol/ms2 (sunny day) and average humidity ranged from 35.1 ±16.4 to 74.1 ±14.1%. Biological control agents (Predatory mites *Neoseiulus cucumeris*, Ladybugs *Hippodamia convergens*, and mealybug destroyers *Cryptolaemus montrouzieri*) were used when necessary to control for greenhouse pests (fungus gnats, thrips, aphids, and mealybugs), although there were never any large infestations. Fertilizer was added when nutrient stress was detected (through discolorations) to ensure they were not limiting (Plant-Prod, 20-20-20 mix of nitrogen, phosphoric acid and soluble potash at a concentration of 2.5mL/L, Plant Products Co. Ltd., Brampton, ON, Canada). All pots were treated equally with respect to biological control and nutrient addition.

In Denmark, the experiment with biomass as endpoint was conducted from June to August 2016. During this period the average temperature in the greenhouse ranged from 19.3 to 20.3 ºC, average photosynthetic active radiation ranged from 65.5 to 630 µmol/ms2 and average humidity from 64.3% ±14.7 to 67.1 ± 13.6. The experiment with reproductive endpoint was conducted between July and November 2016 with mean temperatures from 7.9ºC ±2.5 to 20.3ºC ±3.9, photosynthetic light from 56 to 630 µmol/ms2 and humidity from 67.1% ±27.1 to 82.7% ±10.4. Fertilizer was added as required. There was no need for controlling pests and diseases.

The experimental set up consisted of 26 plant combinations x 3 doses (including controls) x 3 replicates. In each of Experiments 1 and 3, 234 experimental boxes/units were used, containing 7,416 plants – 3,672 *C. cyanus* and 3,744 *S. noctiflora*. The 26 combinations were chosen based on a response-surface design (Figure 6). In manipulated plant competition experiments, this type of design is recommended, whereby both density and proportions are varied to cover a wide array of realistic conditions in natural populations (Inouye 2001). Plants of each species were grown individually, in six densities of monoculture, and in 14 different densities and proportions relative to one another (Figure 6). Due to a lack of seedlings, the number of combinations was reduced in the short-term Experiment 2 with metsulfuron-methyl (marked in red in Figure 6), and only two replicates were used.

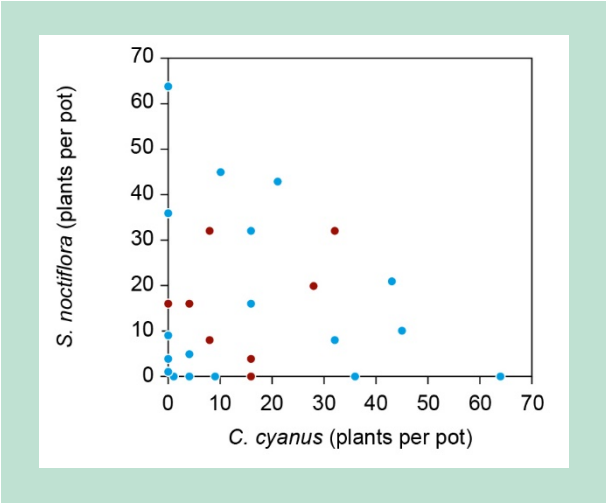

FIGURE 6. Compositions of the densities and proportions in pots used to study herbicide drift and competition between *Centaurea cyanus*. and *Silene noctiflora*. Dots in red are those that were not used in Experiment 2 whereby plants were harvested three weeks after spray

All seeds were surface sown in small trays containing enriched soil (88% Pro-mix BX and 12% sand, plus clay and calcium carbonate) and placed in the greenhouses. After emergence, seedlings of both species were transplanted to boxes/units using templates to standardize arrangements for each combination into containers (in Canada: opaque plastic containers 39L x 28W x 12H cm; in Denmark: Styrofoam container 40L x 40W x 11H cm), containing similar enriched soil mixture. The potting mixture in Denmark was a sandy loam soil (12% clay, 6.5% silt, 80% sand, 1.5% organic matter), with the addition of peat (Kekkila, Finland), and sand (0.5-1 mm, Dansand, Denmark) (2:1:1 w/w) containing all necessary micro and macro nutrients (0.11% nitrogen, 0.04% phosphorus and 0.07% potassium). In Canada, the soil used was made to best replicate that of the Danish soil. Based on dry weights, the soil consisted of: 60.5% all-purpose horticultural sand; 34.9% peat-based potting soil (Pro-Mix® BX General Purpose Growing Medium, Premier Horticulture Ltd., Rivière-du-Loup, QC, CAN), and 4.6% kaolin clay (Edgar Minerals Inc., Edgar, FL, USA), and was supplied with the same nutrient composition as the Danish. Pots were randomly placed in the greenhouse and watered daily.

Plants were sprayed at the juvenile 6-8 leaf stage with Glyphos® (active ingredient glyphosate) or Ally SX® (active ingredient metsulfuron-methyl). Three doses were used for each combination: for glyphosate doses included control (no herbicide spray) and 14.4 and 72.0 g a.i./ha. For metsulfuron-methyl, in addition to control, doses included 0.06 and 0.3 g a.i./ha. Doses are equivalent to 1% and 5% of the recommended label rates of glyphosate (1440 g a.i./ha) and of metsulfuron-methyl (6 g a.i./ha) in Denmark, and simulates herbicide drift.

In Canada, plants were sprayed using a track spray booth (DeVries Manufacturing, MN) equipped with a Teejet 8002E flat-fan spray nozzle, which delivers 7.75 mL/m2 (77.5 L/ha) at a pressure of 206.84 kPa. In Denmark, herbicide application was made using a cabinet pot sprayer equipped with two ISO-02-110 flat fan nozzles. The nozzles were operating at a pressure of 300 kPa and a velocity of 5.8 km/h delivering a spray volume of 160 L/ha. In all cases, each box/unit was assigned a numerical ID tag with treatment randomly assigned so that the observer would be unaware of the dose during assessments in order to prevent bias. For all combinations except the individual plants, a sample of four plants of each species was used to represent the box/unit for assessments throughout the experiment. These four plants were chosen randomly before assessments began, but were from positions in the center of the boxes/units as to avoid edge effects.

4.2.4 Assessment of vegetative endpoints

In the Canadian experiment, most *S. noctiflora* failed to flower, therefore plants were harvested for above ground biomass after six months, when the *C. cyanus* plants began dying after flowering (Experiment 1). In the first Danish experiment, plants of the two species were harvested three weeks after spray, and above ground biomass was measured (Experiment 2). In all cases, healthy plants were cut at the soil surface and bagged individually. They were then dried in a drying oven at approximately 70-80˚C for 48-72 hours prior to weighing.

4.2.5 Assessment of reproductive endpoints

Reproductive endpoints used included number of flowers and seeds of *C. cyanus* (Experiment 1) and of both species (Experiment 3). When plants began flowering, bumblebees (*Bombus impatience* Cr.) in Canada or honeybees (*Apis mellifera* L.) in Denmark were released in greenhouses to pollinate the plants. Plants were examined each day before the beginning of flowering, and after flowering began the number of flowers (flower heads) and seed heads was counted twice a week. To prevent seed losses from shedding, ripe fruit/dried seed heads were cut and counted throughout the experiment and put in labeled envelopes. The seeds from each plant were counted using a seed counter (in Canada Elmor C1, Switzerland, in Denmark JK Design, Denmark) to give a total seed count for each plant.

4.3 Statistical model for the effect of herbicide on plant competition

Variables measured for each experiment and used for modelling effects of herbicide on competitive interactions were as follow: Experiment 1 (Canadian), biomass of *S. noctiflora*, flower and seed production of *C. cyanus*; Experiment 2 (Danish), biomass three weeks after spray for *S. noctiflora* and *C. cyanus*; Experiment 3 (Danish), seed production of the two species.
The effect of herbicide treatment on the measured variables (biomass, flower or seed counts) of the two competing species was modelled by a generalization of a discrete hyperbolic competition model (Damgaard, 2003, Damgaard et al., 2008). We assume that within the limited domain of the present study there is a linear effect of the herbicide on the competitive interactions and on the measured variables of the susceptible plant species, i.e. the expected measured variables of species i when grown in competition with species j is:

$$\mu_i = \left\{ Exp(a_i + \alpha_i\, h) + Exp(b_i + \beta_i\, h)\left[x_i + Exp\left(c_j + \gamma_j\, h\right)x_j\right]^{Exp(d_i)} \right\}^{-Exp(-f_i)} \quad (1),$$

where h is the level of the herbicide treatment, x_i is the density of plant species i, d_i and f_i are shape parameters of the response function of plant species i that are assumed to be independent of the herbicide treatment (Damgaard, 2003, Damgaard et al., 2008). The measured variables of species i at low density in a monoculture were measured by $Exp(a_i + \alpha_i\, h)^{-Exp(-f_i)}\ and$ (- $Exp(b_i + \beta_i\, h)$ measured the competitive effect of conspecific neighbouring plants. The relative competitive effect of heterospecific neighbouring plants j compared to conspecific plants (competition coefficient) was measured by $Exp\left(c_j + \gamma_j\, h\right)$.

The expected measured variable (1) was fitted to the average measured variables of four plants of each plot (k:1,…,N) assuming data were gamma distributed with scale parameters v_i, i. e. the likelihood function is:

$$\prod_k^N \frac{Exp\left(-\frac{y_{i,k}\, v_i}{\mu_{i,k}}\right)\left(\frac{y_{i,k}\, v_i}{\mu_{i,k}}\right)^{v_i}}{y_{i,k}\, \Gamma(v_i)} \quad (2).$$

The joint Bayesian posterior distribution of the parameters in the competition model was sampled using the Metropolis-Hastings algorithm with a multinomial candidate distribution

(300,000 iterations with a burn-in period of 50.000) assuming uniform prior distributions of the parameters (Carlin & Louis, 1996).

The sampling procedure was checked by visual inspections of the evolution of the deviance and the mixing properties of the sampling chains. The statistical inferences on the parameters were assessed using the sampling properties of the parameters and the 95% credible interval. The *ED*10, i.e. the herbicide dose required to reduce the measured endpoint (biomass or seed production) by 10% for *C. cyanus*, was expected to depend on the density of the two competing plant species, and $ED10_i$ was calculated by solving eq. (3) for h, after inserting the mean of the estimated parameters.

$$\frac{\mu_i(x_i,x_j,h)}{\mu_i(x_i,x_j,0)} = 0.9 \qquad (3).$$

4.4 Results

In all three experiments, it was evident that density effects occurred, although to different extents. As density increased, plants were generally negatively affected, in all three variables measured: biomass, number of flowers and the number of seeds. The added effect of sub-lethal doses of herbicide was more subtle.

4.4.1 Experiment 1 – long-term experiment with exposure to glyphosate

The hyperbolic curve was fitted to biomass measurements and counts of flowers and seeds in order to understand the effects of glyphosate on competition. Density effects were evident in monoculture with no herbicide (parameter a and b in Tables 13 and 14). Both α and β, which measure herbicide treatments in monoculture, were significant for the seed production but not for flower production of *C. cyanus*, nor for the biomass of *S. noctiflora* (Tables 13 and 14). This demonstrates that glyphosate affected *C. cyanus*'s ability to compete with conspecifics at both low and high densities while producing seeds. However, *S. noctiflora*'s ability to compete with conspecifics was unaltered.

When examining the two-species mixtures for interspecific competition with no herbicide (the competition coefficient, c), it is shown that *C. cyanus* was a stronger competitor than *S. noctiflora* (Tables 13 and 14, $c_{sn} < c_{cc}$, both greater than respective parameters a and b). With herbicide exposure, the competitive interactions between the two species were altered. The competitive effect of *C. cyanus* on *S. noctiflora* (γ_{cc}) biomass was significantly weakened by glyphosate (Tables 13 and 14), as was *S. noctiflora*'s competitive effect on *C. cyanus*'s (γ_{sn}) seed production (Table 14). This means that while *C. cyanus* could not suppress the growth of *S. noctiflora* as well when exposed to the herbicide, *S. noctiflora* was not able to suppress the seed production of *C. cyanus* as efficiently either. These negative impacts on competitive effects were, however, more pronounced for *S. noctiflora*, as shown by the more negative parameter values.

Nevertheless, the density of *S. noctiflora* (competitor) was an influent element in determining the ED10 of C. cyanus (Figures 7 and 8), especially at low *C. cyanus* density. The ED10 of *C. cyanus* was at its highest (less affected by glyphosate) when *S. noctiflora* increased to high density. In fact, *C. cyanus* density (conspecific) did not greatly affect its own response to the herbicide, particularly when *S. noctiflora* was not present.

TABLE 13. Calculated percentiles of the marginal posterior distribution of parameters of *Silene noctiflora* L. (Sn) biomass (mg dry weight) and *Centaurea cyanus* L. (Cc) flower count.

Plants growing at different densities were exposed to 1% and 5% glyphosate. Bolded numbers indicate parameter significance, with probability values of <0.05 or >0.95 being significant

Parameter percentiles			*Silene noctiflora*					*Centaurea cyanus*			
			2.5	50	97.5	P (X>0)		2.5	50	97.5	P (X>0)
Parameter and definition											
a	low density effects in monoculture	a_{sn}	**-50.040**	**-24.190**	**-7.399**	**0.000**	a_{cc}	**-77.160**	**-53.170**	**-15.930**	**0.000**
b	high density effects in monoculture	b_{sn}	**-19.560**	**-7.842**	**-5.357**	**0.000**	b_{cc}	**-48.570**	**-30.740**	**-10.260**	**0.000**
c	competitive effect on other species	c_{sn}	**-2.554**	**-1.622**	**-0.969**	**0.000**	c_{cc}	**0.104**	**0.442**	**0.808**	**0.996**
α	herbicide influence on competitive effect in low density monoculture	α_{sn}	-6.339	-1.388	1.939	0.285	α_{cc}	-1.019	4.396	7.158	0.819
β	herbicide influence on competitive effect in high density monoculture	β_{sn}	-0.043	0.002	0.053	0.549	β_{cc}	-0.056	0.094	0.294	0.903
Υ	herbicide influence on competitive effect on other species	Υ_{sn}	-0.923	-0.093	0.207	0.299	Υ_{cc}	**-0.265**	**-0.128**	**-0.009**	**0.017**

TABLE 14. Calculated percentiles of the marginal posterior distribution of parameters of *Silene noctiflora* L. (Sn) biomass (mg dry weight) and *Centaurea cyanus* L. (Cc) seed number. Plants growing at different densities were exposed to 1% and 5% glyphosate. Bolded numbers indicate parameter significance, with probability values of <0.05 or >0.95 being significant

Parameter percentiles			*Silene noctiflora*					*Centaurea cyanus*			
			2.5	50	97.5	P (X>0)		2.5	50	97.5	P (X>0)
Parameter and definition											
a	low density effects in monoculture	a_{sn}	**-64.800**	**-45.790**	**-14.400**	**0.000**	a_{cc}	**-111.70**	**-82.310**	**-19.740**	**0.000**
b	high density effects in monoculture	b_{sn}	**-63.970**	**-48.420**	**-15.920**	**0.000**	b_{cc}	**-31.020**	**-17.790**	**-7.518**	**0.000**
c	competitive effect on other species	c_{sn}	**-4.738**	**-2.119**	**-0.990**	**0.000**	c_{cc}	**0.095**	**0.454**	**0.831**	**0.996**
α	herbicide influence on competitive effect in low density monoculture	α_{sn}	-5.366	-1.205	0.785	0.186	α_{cc}	**-0.098**	**2.710**	**5.880**	**0.972**
β	herbicide influence on competitive effect in high density monoculture	β_{sn}	-0.157	0.018	0.213	0.605	β_{cc}	**0.007**	**0.080**	**0.199**	**0.986**
Υ	herbicide influence on competitive effect on other species	Υ_{sn}	**-7.799**	**-5.326**	**-1.590**	**0.000**	Υ_{cc}	**-0.273**	**-0.133**	**-0.007**	**0.019**

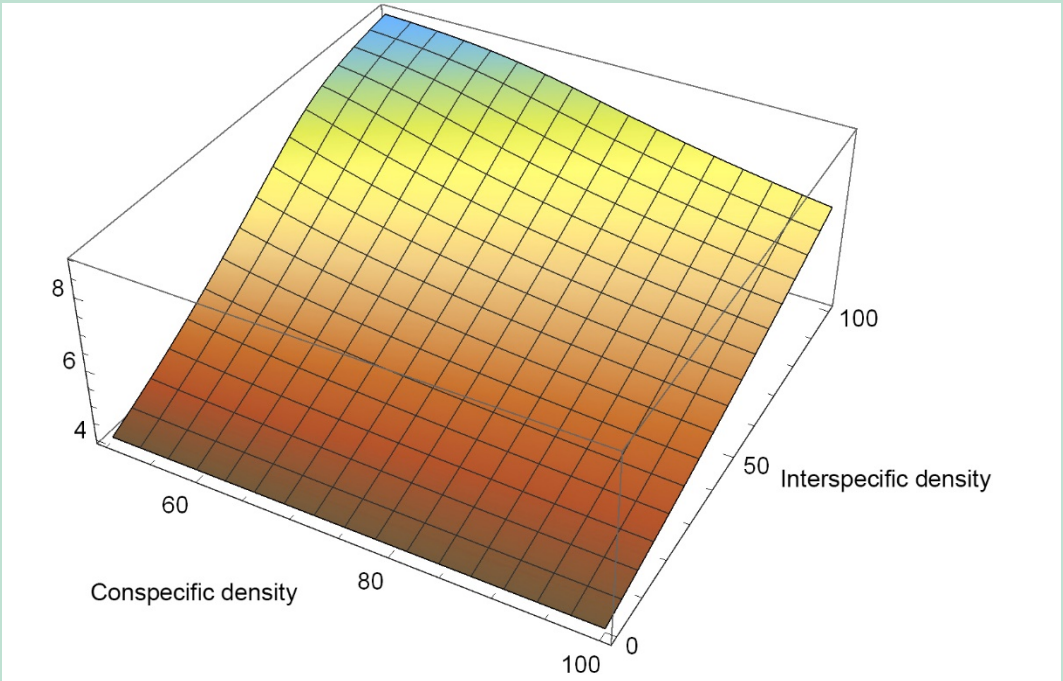

FIGURE 7. The ED10, i.e. the herbicide dose required to reduce the measured number of flowers of *Centaurea cyanus* L. by 10% (Z-axis), presented as a function of the intraspecific density (conspecific) and the interspecific density (competition with *Silene noctiflora* L.). This is the result of Experiment 1 with glyphosate conducted in Canada.

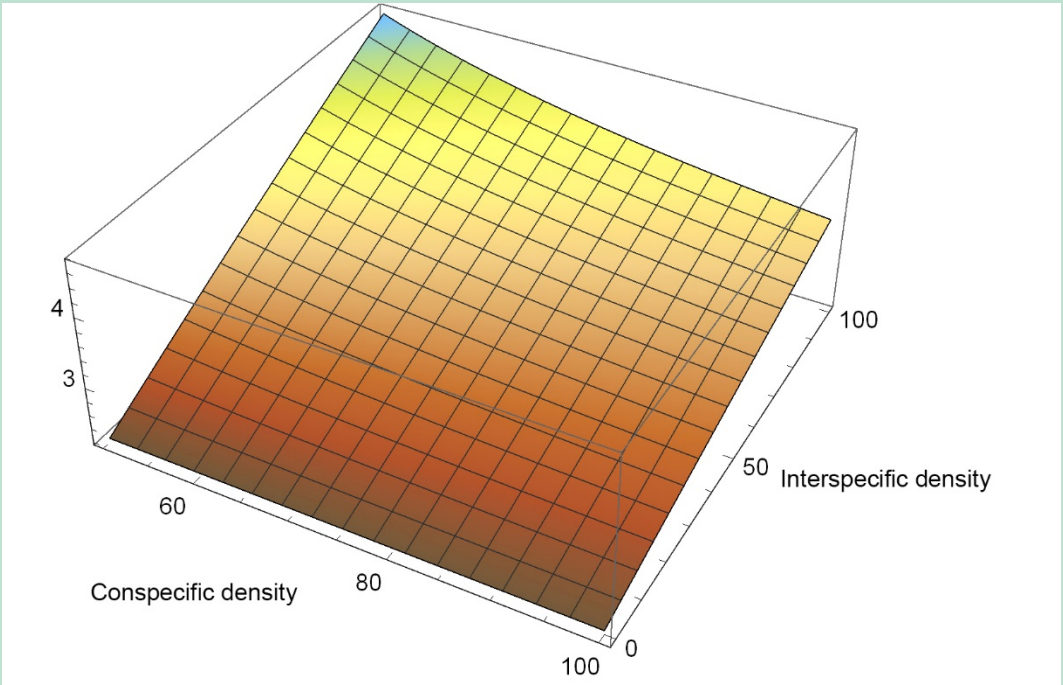

FIGURE 8. The ED10, i.e. the herbicide dose required to reduce by 10% the measured number of seeds of *Centaurea cyanus* L. (Z-axis), presented as a function of the intraspecific density (conspecific) and the interspecific density (competition with *Silene noctiflora* L.). This is the result of Experiment 1 with glyphosate conducted in Canada

4.4.2 Experiment 2 – short-term experiment with exposure to metsulfuron-methyl

As in experiment 1, the hyperbolic curve was fitted to biomass measurements (three weeks after spray) of the two species in order to understand the effects of metsulfuron-methyl on competition. As expected, there was a significant density effect in monoculture when no herbicide was present, both at low and high density (coefficients *a* and *b*). There was a mixed effect of the herbicide metsulfuron-methyl on plants in monoculture (α and β). While there was a significant change induced by the herbicide on *S. noctiflora* at low density (α_{sn}), no effect was observed at high density (β_{sn}). Conversely, an effect was solely observed at high density in the case of *C. cyanus* (compare coefficients α_{cc} and β_{cc}).

In mixtures, interspecific competitive effects were apparent: in both species, the coefficient *c* was greater than coefficients *a* and *b*. In addition, the competitive ability of *S. noctiflora* was significantly altered by *C. cyanus* (C_{sn}) while the competitive ability of *C. cyanus* was not significantly altered by the density of *S. noctiflora* (C_{cc}). The addition of the herbicide metsulfuron-methyl modified the interaction of *C. cyanus* with *S. noctiflora* (γ_{cc}). Figure 9 shows that at the young stage (three weeks after spray) conspecific density was very important in shaping the sensitivity of *C. cyanus* to metsulfuron-methyl (lower ED10 when *C. cyanus* increased). In contrast, the density of the competitor *S. noctiflora* had limited influence on the sensitivity of *C. cyanus* to metsulfuron-methyl (little change in ED10).

TABLE 15. Calculated percentiles of the marginal posterior distribution of parameters of *Silene noctiflora* L. (Sn) and *Centaurea cyanus* L. (Cc) biomass (g dry weight). Plants growing at different densities were exposed to 1% and 5% metsulfuron-methyl. Plants were harvested three weeks after spray. Bolded numbers indicate parameter significance, with probability values of <0.05 or >0.95 being significant

Parameter percentiles			*Silene noctiflora*					*Centaurea cyanus*			
			2.5	50	97.5	P (X>0)		2.5	50	97.5	P (X>0)
Parameter and definition											
a	low density effects in monoculture	a_{sn}	**-144.200**	**-100.880**	**-61.229**	**0.000**	a_{cc}	**-76.730**	**-18.773**	**-8.142**	**0.000**
b	high density effects in monoculture	b_{sn}	**-330.180**	**-240.470**	**-154.190**	**0.000**	b_{cc}	**161.758**	**-44.510**	**-21.875**	**0.000**
c	competitive effect on other species	c_{sn}	**-2.072**	**-1.204**	**-0.482**	**0.000**	c_{cc}	-1.091	-0.224	0.506	0.301
α	herbicide influence on competitive effect in low density monoculture	α_{sn}	**5.571**	**8.764**	**12.241**	**1.000**	α_{cc}	-6.291	-1.167	4.511	0.378
β	herbicide influence on competitive effect in high density monoculture	β_{sn}	-3.718	-1.232	1.071	0.247	β_{cc}	**3.327**	**5.832**	**8.219**	**1.000**
Υ	herbicide influence on competitive effect on other species	Υ_{sn}	-4.807	-1.802	0.662	0.096	Υ_{cc}	**-0.216**	**2.937**	**6.784**	**0.959**

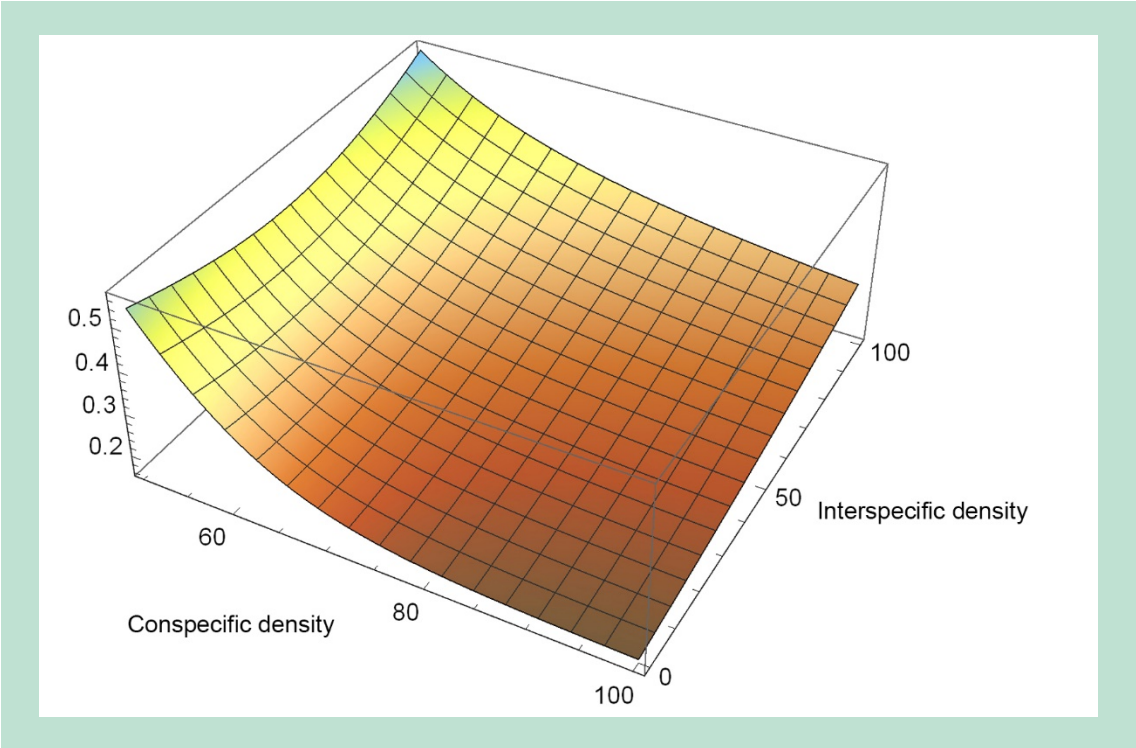

FIGURE 9. The ED10, i.e. the herbicide dose required to reduce by 10% the measured biomass of *Centaurea cyanus* L. (Z-axis), presented as a function of the intraspecific density (conspecific) and the interspecific density (competition with *Silene noctiflora* L. g dry weight). This is the result of Experiment 2 with metsulfuron-methyl conducted in Denmark

4.4.3 Experiment 3 – long-term experiment with exposure to metsulfuron-methyl

The hyperbolic curve fitted to seed production of the two species revealed that the competitive effect was important in all situations. These results also revealed that *C. cyanus* was a better

competitor than *S. noctiflora* when no herbicide was used since coefficients were always lower for the latter (comparing a, b and c between the two species). The competitive effect, however, was increased when the herbicide metsulfuron-methyl was applied, with *C. cyanus* remaining a better competitor than *S. noctiflora*. As with glyphosate, the density of *S. noctiflor*a (competitor) had a marked effect on the ED10 of *C. cyanus* (Figure 10). *C. cyanus* was less affected by metsulfuron-methyl (higher ED10) when *S. noctiflora* increased to high density. Likewise, *C. cyanus* density (conspecific) was not a defining element in shaping the sensitivity of the herbicide metsulfuron-methyl, particularly at low density of *S. noctiflora*.

TABLE 16. Calculated percentiles of the marginal posterior distribution of parameters of *Silene noctiflora* L. (Sn) and *Centaurea cyanus* L. (Cc) seed production. Plants growing at different densities were exposed to 1% and 5% metsulfuron methyl. Bolded numbers in all situations indicate parameter significance, with probability values being between <0.05 or >0.95

Parameter percentiles			*Silene noctiflora*				*Centaurea cyanus*			
			2.5	50	97.5	P (X>0)	2.5	50	97.5	P (X>0)
Parameter and definition										
a	low density effects in monoculture	a_{sn}	**-228.523**	**-131.098**	**-39.254**	**0.000**	**-180.944**	**-85.881**	**-40.746**	**0.000**
b	high density effects in monoculture	b_{sn}	**-328.184**	**-156.339**	**-62.387**	**0.000**	**-153.807**	**-66.118**	**-22.179**	**0.000**
c	competitive effect on other species	c_{sn}	**-5.929**	**-4.152**	**-1.303**	**0.000**	**0.925**	**1.467**	**2.020**	**1.000**
α	herbicide influence on competitive effect in low density monoculture	α_{sn}	**4.068**	**6.048**	**7.759**	**1.000**	**0.852**	**6.093**	**10.372**	**1.000**
β	herbicide influence on competitive effect in high density monoculture	β_{sn}	**-5.940**	**-3.959**	**-1.370**	**0.000**	**3.259**	**4.973**	**8.569**	**1.000**
Υ	herbicide influence on competitive effect on other species	Υ_{sn}	**-4.438**	**-1.819**	**0.201**	**0.043**	**3.023**	**4.308**	**5.326**	**1.000**

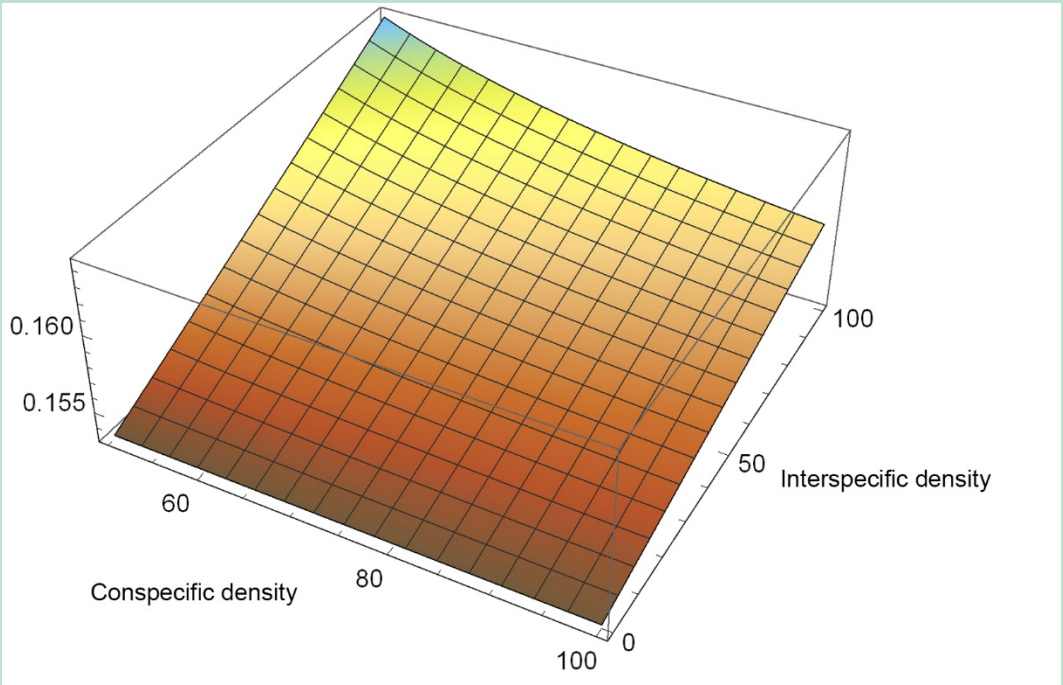

FIGURE 10. The ED10, i.e. the herbicide dose required to reduce by 10% the number of seeds of *Centaurea cyanus* L. (Z-axis), presented as a function of the intraspecific density (conspecific) and the interspecific density (competition with *Silene noctiflora* L.). This is the result of Experiment 3 with metsulfuron-methyl conducted in Denmark

4.5 Discussion

Environmental Risk Assessments (ERAs) for herbicides are performed to ensure that there are no unacceptable effects on the environment and on non-target organisms such as NTTPs, which includes both short and long-term effects. This goal for NTTPs is considered accomplished not only when populations of non-target species are unaffected, but also when communities are not negatively impacted (EFSA PPR Panel 2014). These goals are considered predictable based on the results of individual level or single species tests. Plant species, however, do not grow in isolation and are part of populations and communities of plants. The hypothesis tested here is that plant populations are affected by both herbicides and competition, and that responses to both factors need to be measured in order to generate predictions on the effects on plant populations. The hypothesis was tested using a simplified two-species experiment whereby it was predicted that sub-lethal doses of herbicides would alter the competitive interactions between *C. cyanus* and *S. noctiflora,* assessing both vegetative and reproductive parameters.

4.5.1 Changes in competitive interactions

The competition model demonstrated that the competitive interactions were altered between *C. cyanus* and *S. noctiflora* when exposed at the 6-8 leaf stage to low doses of herbicides representing herbicide drift, affecting both vegetative and reproductive endpoints. In the current study, *S. noctiflora* appear to be more affected by herbicides than *C. cyanus*, thus placing *S. noctiflora* at disadvantage. As interspecific competition influences plant communities (e.g. Weiher et al. 1998), changes in interspecific interactions can affect the population dynamics between the two species over time (Damgaard et al. 2008), possibly leading to effects at the community level as the susceptible species are displaced by the more tolerant ones (Boutin & Jobin 1998; Gove et al. 2007; Petersen et al. 2006). The composition of field margins can be hundreds of species (EFSA PPR Panel 2014), making it likely that there is a range of responses. Even slight effects can put more sensitive species at greater disadvantages than the

more tolerant ones (EFSA PPR Panel 2014), and these sensitive species can experience increased competition from less-affected neighbours (Riemens et al. 2008).

Using the same competition model as this study, Damgaard et al. (2008) showed that the interspecific competitive abilities of two weed species were increased at low doses of the herbicide mecoprop-P. With a different competition model examining plant coverage of field quadrats, Damgaard et al. (2014) found that the competitive effect of one grass increased while the other grass decreased with increasing doses of glyphosate. A similar conclusion was reached in the greenhouse with the same grass species (Damgaard and Fayolle 2010). Other studies have also documented differences in sensitivities between terrestrial plants grown individually at different densities or in monocultures and those grown in mixtures (Humphry et al. 2001; Riemens et al. 2008; Riemens et al. 2009; Dalton & Boutin 2010), suggesting competition plays a role in responses to herbicides. Furthermore, changes in community composition caused by herbicide drift have been seen in previous studies (e.g. Marrs et al. 1991a; Kleijn and Snoeijing 1997; Gove et al. 2007; Strandberg et al. 2012). Marrs & Frost (1997) used microcosms to show that the response of a species to herbicide drift depends on the herbicide used and on which species are present in the mixture – some species showed a response to some herbicides but not others, and responses varied depending on the presence of grasses in the mixture.

In the experiments with metsulfuron-methyl (Experiments 2 – short-term and 3 – long-term), where effects were measured at two phenological stages, it was found that in both cases competition was an important component in determining the sensitivity of species to metsulfuron-methyl. Interestingly though, in *C. cyanus*, intraspecific competition dominated when effects on biomass (young vegetative stage) was considered (Figure 9) while interspecific competition was more pronounced for seed production (mature stage, Figs. 7, 8, 10) as seen with changes in the ED10 of this species. It is unclear why this occurred since both *S. noctiflora* and *C. cyanus* were of comparable sizes at the time of spray and grew to similar heights and spans during the growing season. More subtle changes in morphological traits may explain this situation (Chacalis et al. 2001; González-Torralva et al. 2010). For instance, belowground traits (Mokany and Ash 2008) and physiological parameters (González-Torralva et al. 2010) may play a critical role in species susceptibility and were not assessed here.

Furthermore, in our experiment on effects measured at the young vegetative stage (Fig. 9), the high density of *C. cyanus* induced a higher sensitivity to the herbicide metsulfuron-methyl (lower ED10 – biomass endpoint at young stage) in *C. cyanus* while *S. noctiflora* had a limited effect. In contrast, Humphry et al. (2001) tested two densities of the grass *Agrostemma githago* L. with the herbicide 2, 4-D on plants sprayed at a young stage and harvested two weeks after spray and they found that at high density (64 plants per pot), the ED50 was nearly 15 times higher than at low density (two plants per pot). Likewise, Winkle et al. (1981) found that as density of plants per pot increased, more of the herbicide atrazine was required to cause fresh weight reduction of the weed to be suppressed (measured at the young vegetative stage). They attributed the difference in sensitivity between the increasing densities to the smaller leaf area and less horizontal positioning of the leaves at high density. Conversely, in our experiment conducted until the mature stage and assessing flower or seed outputs, the situation was reversed: an increased density of *C. cyanus* did not alter its sensitivity to herbicides but the influence of *S. noctiflora* on *C. cyanus* was more pronounced causing a reduced herbicide effect in *C. cyanus* (Figs. 7, 8, 10).

4.5.2 Effects of endpoints

This study also demonstrated that plants assessed at a young stage (three weeks after spray measuring biomass) can show a response to herbicides that differs from plants assessed at a mature stage (using seed production as the endpoint). Effects of metsulfuron-methyl on both *C. cyanus* and *S. noctiflora* were more pronounced when quantifying seed production during

the course of the experiment than when biomass was measured at one point in time at an early growth stage, as in regulatory testing. A few studies have demonstrated that plants sprayed at the young vegetative leaf-stage can later suffer impact on their reproduction, and this with numerous herbicide types, e.g. glufosinate ammonium (Riemens, et al. 2008, 2009; Carpenter and Boutin 2010), 2,4-D, dicamba and picloram (Rinella et al 2010) or sulfonylureas (Gealy et al. 1995; Carpenter et al. 2013). Other studies where plants were sprayed with herbicides at the reproductive stage demonstrated considerable effects on reproductive measures (Guo et al. 2009; Clay and Griffin 2000; Blackburn and Boutin 2003). Sulfonylurea herbicides are known to affect plant reproduction at sometimes doses that are below 1% of the label rate (Fletcher et al. 1993). In the field experiment, see Chapter 5.4.1, exposure to glyphosate corresponding to 40.3 g a.i. equivalent ha^{-1} (or 2.8% label rate) caused significant effects on plant flowering. Likewise, in Denmark, hawthorns (*Crataegus monogyna* L.) growing in hedgerows and sprayed with metsulfuron-methyl at reproductive stage showed effects soon after the spray event, which continued to be significant one year after the herbicide application (Kjær et al., 2006a, b). Nevertheless, other studies have shown that sensitivity was higher when juvenile stages were assessed (Boutin et al. 2014; EFSA PPR Panel 2014 and references therein). These studies altogether demonstrate that impacts on reproductive output are common but often uncorrelated to vegetative effects at the young stage.

5. Effect of glyphosate spray drift on plant flowering

5.1 Introduction

Whereas both fertilizers and pesticides (mostly herbicides) reduce the plant species richness, recent studies showed that sub-lethal doses of herbicides also affect plant flowering (Schmitz et al. 2013, 2014; Boutin et al. 2014). The number of flowering plants and flowering intensity, i.e. the number of flowers per plant, were reduced (Boutin et al. 2014, Schmitz et al. 2013, 2014) and the flowering period of herbaceous species was delayed and shortened (Boutin et al. 2014, Dupont et al. submitted) for plants exposed to sub-lethal concentrations of herbicides. Thus, herbicide spray drift may adversely affect flower diversity and availability of floral resources, which in turn may affect higher trophic levels, including flowervisiting insects that act as pollinators.

In non-target areas adjacent to sprayed crop fields, spray drift deposits are in the order of 5-25% of field application rate, depending on the spraying equipment, weather conditions, distance from the field edge and height of the vegetation (Weisser et al. 2002; Nordbo et al. 1993).

Herbicide exposure in the above-mentioned studies of effects of herbicide exposure on flowering has either involved direct overspray with low doses of herbicide (Schmitz et al. 2013, 2014) or spray drift in studies that monitor plant flowering of hedgerow ground vegetation adjacent to either organic (no exposure, controls) or conventional fields (Boutin et al. 2014). In the latter study, hedgerows adjacent to conventional fields may have been exposed to spray drift from a number of different spraying events including different herbicides. Exact levels of herbicide exposure was not measured in any of the studies, and the documented differences in plant flowering were assumed to be caused by differences in exposure.

The overall aim of the current study was to establish the relationship between herbicide spray drift exposure of non-target plants and plant flowering and, thereby, test the following project hypotheses:

4. Flowering of herbaceous species in natural and semi-natural habitats adjacent to fields treated with herbicides is affected by sub-lethal dosages of herbicides drifting into the habitats
5. Herbicide exposure of NTTPs reduces and delays flowering of herbaceous plants in semi-natural habitats leading to lack of resources and temporal mismatches between plants and pollinators

More specifically, we addressed the following questions:

1. What is the spatial exposure pattern of spray drift under light wind conditions in distances up to 25 meters from the field margin?
2. Is floral abundance of herbaceous plant species affected by herbicide exposure at spray drift concentrations? and
3. Do all flowering species respond the same way?
4. To answer the above question a field experiment was set up where effects of wind drift exposure of glyphosate on planted NTTPs were assessed.

5.2 Material and methods

The study was conducted as an experiment in an agricultural field located at Skovgaard, Gjessø in Mid-Jutland (GPS: 56.109376, 9.536714).

5.2.1 Experimental set up

The experimental area (25 x 100 m) was placed in the middle of the field and had a north-south orientation. The fields surrounding the experimental area was used for grazing animals and no pesticides were used in a distance of 1 km from the area, hence contamination with other herbicides than the experimentally applied herbicide may be excluded. In Mid-Jutland, the prevailing winds are from west. The spray drift exposure of the area was established using a conventional spaying equipment mounted on a tractor driving northwards on the wind side of the field (Figure 11). Glyphosate in the Roundup Bio formulation was selected for the experiment as it is the most widely used herbicide in the world, including Europe, and it is used during most parts of the growing season for different purposes. Glyphosate is a non-selective herbicide affecting all plant species to some degrees. The dye marker, sodium fluorescein, was sprayed together with glyphosate for the measurement of the exposure at each sampling plot as well as of the drift gradient from the sprayer across the experimental area. Sampling of the herbicide, dye marker and plant response parameters (see below), were carried out on ten trajectories across the experimental area (parallel to the wind direction, i.e. covering the exposure gradient), and each trajectory had eleven sampling plots each 0.5 by 0.5 m (110 sampling plots total). On each trajectory, the first eight plots were placed close together, covering the first four meters nearest the sprayer, thereafter the distance between sampling plots increased and the three remaining plots were centered at 6.25, 13.25 and 20 m (Figure 11). This stratified sampling was planned to ensure the best description of the expected exposure gradient and subsequent changes in the vegetation.

The area was sown with a mixture of eight perennial NTTPs, see below. After sowing, plots were left untouched until June the following year. The experimental exposure to herbicide spray drift was performed 26 June 2015.

Before spraying, passive samplers (hair curlers, previously used in e.g. Løfstrøm et al. (2013)) were set up to collect deposits from spray drift. Two hair curlers were placed in the centre of each 0.5 by 0.5 m sampling plot, just above the vegetation. For every fixed distance from the sprayer, three passive samplers were randomly selected for glyphosate analysis (Figure 11). All other passive samplers (187 samplers) were analysed for the content of dye marker. Thus, there were 33 passive samplers for glyphosate analysis, and for each of these sampling plots, the second passive sampler was analysed for dye marker to get corresponding measurements of dye marker and herbicide.

After sampler placement and immediately before spraying, a 8 m wide strip along the edge opposite the side of spraying was covered by plastic foil to secure a close to zero exposure condition (control). The plastic foil covered the sampling plots furthest away from the tractor in each trajectory and was gently removed 10 minutes after spraying.

Immediately upon exposure, the curlers were collected, placed in plastic cylinders and stored in darkness to prevent photo degradation of glyphosate and the dye. The curlers for dye marker analysis were placed in cylinders containing extraction fluid (0.1 M Na_2HPO_4 buffer). The period of time between spraying until all samples were placed in darkness did not exceed 20 minutes, therefore, the risk of photo degradation in the field was considered negligible.

5.2.2 Test species

The experimental area was sown late August 2014 with a mixture of eight perennial herbs, *Trifolium pratense* L., *T. repens* L., *Lotus corniculatus* L., *Cichorium intybus* L., *Taraxacum vulgare* L., *Knautia arvensis* (L.) Coulter, *Carum carvi* L. and *Sanguisorba minor* Scop., belonging

to five families (Fabáceae, Asteráceae, Dipsacáceae, Apiáceae, Rosaceae). Seeds of the four first species were added at 2 kg/ha and the rest at 1 kg/ha. The plants selected for the experiment were insect-pollinated herbaceous species, which are common in field-margins in Northern Europe.

5.2.3 Herbicide application

Glyphosate in the Roundup® Bio formulation (360 g/L) at label rate of 1440 g a.i./ha was used for the experiment together with the dye marker sodium fluorescein at 2 g/l. A sample from the spray tank was taken before spraying to check the concentration of glyphosate and dye marker. The sprayer was mounted with drift reducing nozzles. The weather on the day of spraying was dry, partly clouded, temperatures around 15°C and average wind speed of 4.5 m/s but slight changes in both speed and direction of the wind was seen during the spaying.

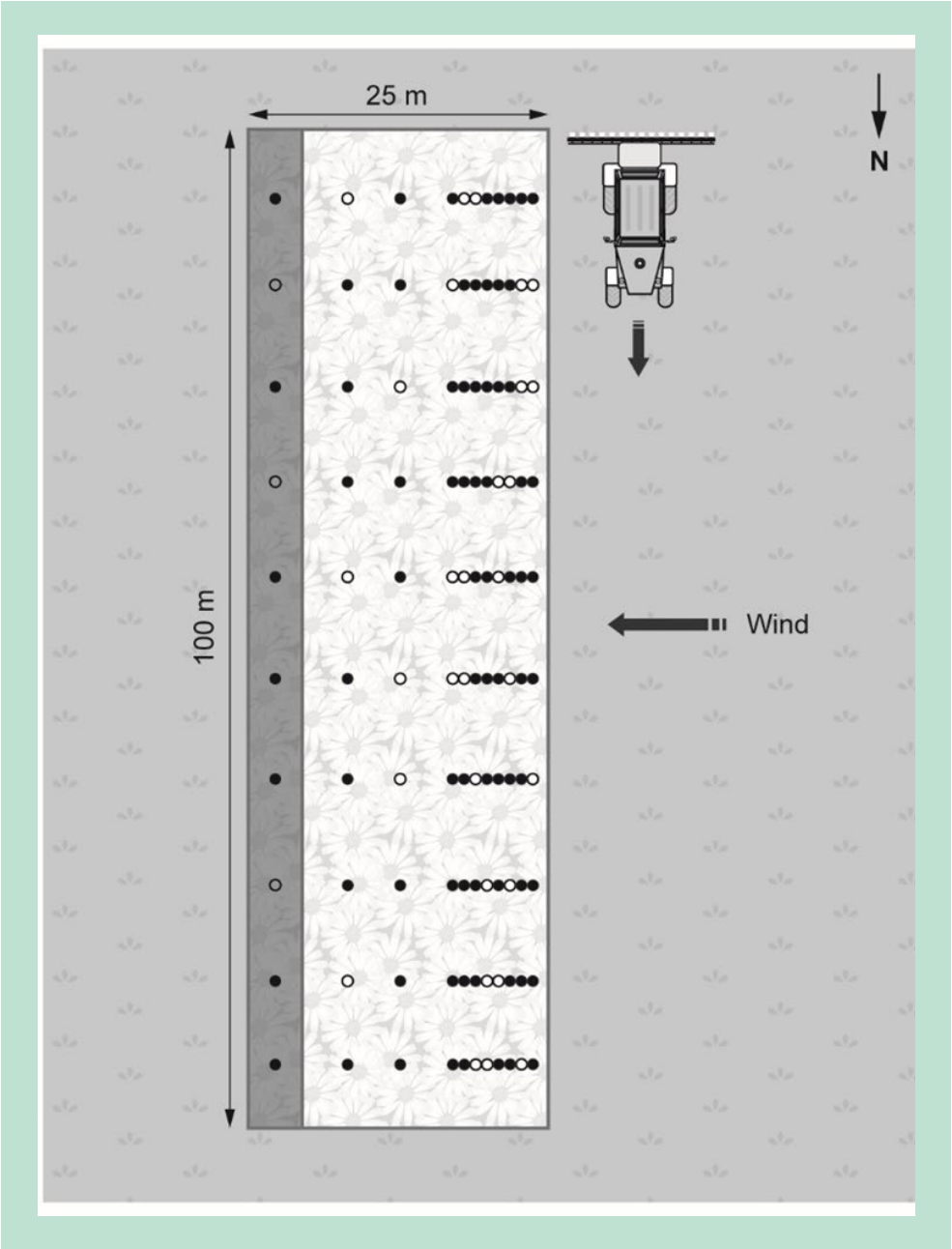

FIGURE 11. Sketch showing the set-up of the experiment at Skovgaard. The shaded area in the left part of the experimental plot was covered by plastic folio during the spraying to secure a close to zero exposure condition (control). Dots show the position of the sampling sites.

White dots represent samples analysed for both glyphosate and dye marker, black dots samples only analysed for dye marker. Plant cover and flowering were analysed at all sample sites.

5.2.4 Sampling of plant cover and flowering

Plant cover and flowering were recorded within each sampling plot before herbicide treatment and at regular intervals over the rest of the flowering period. Plant cover was sampled three times (24 June, 31 July, and 21 August 2015). Plant flowering was recorded the same three dates and at four additional dates (13 July, 7 August, 9 and 25 September 2015). At the end of September, flowering had ceased. The period between samplings was sufficiently long so that a flower was not sampled twice.

Plant cover was estimated by the point intercept method (Levy and Madden 1933, Kent and Coker 1992) using a pin-point frame with the dimension of the sampling plot, i.e. 05 x 0.5 m. The frame had 25 pin positions placed regularly with a distance of 10 cm. At each pin position, a thin pin was passed vertically through the vegetation and the species passed through or touched by the pin is noted. The cover of a specific plant species was defined as the relative number of pins where the species was hit.

All sown plant species produced flowers in dense inflorescences, flower heads. Plant flowering was estimated for each species as the number of open, pollen producing inflorescences (hereafter named flowers) per sampling plot.

5.2.5 Analyses of glyphosate deposited on curlers

5.2.5.1 Chemicals

Glyphosate 99.7%, a certified standard, was obtained from Fluka (Buchs, Switzerland). Isotope labeled glyphosate 13C, 15N, 96% (100 µg ml^{-1}) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). FMOC chloride (FMOC-Cl), derivatization grade (≥99.0%) was purchased from Sigma Aldrich (Copenhagen, Denmark). Analytical grade disodium tetraborate decahydrate and sodium hydroxide (NaOH) were obtained from Merck (Darmstad, Germany). For the HPLC mobile phase preparation, ammonium formate (puriss., Fluka) and acetonitrile (Lichrosolv, Merck) were used. Ultrapure water was prepared with a Super-Q water purification system (Millipore, Darmstad, Germany).

A primary stock solution of the glyphosate was prepared in water at a concentration of 1 mg ml-1. Borate buffer was prepared by dissolving 4.75 g of disodium tetraborate decahydrate in 250 ml Milli-Q water and the pH was adjusted to 9 with 1M NaOH. FMOC-Cl was dissolved in acetonitrile at a concentration of 1.5 mg ml-1 and used the same day.

5.2.5.2 Sample preparation

Samples were transferred to 50 ml polypropylene tubes and spiked with isotope labelled glyphosate (2 µg). Milli-Q water (20 ml), 2.5 ml sodium tetraborate buffer and 2.5 ml FMOC-Cl solution were added to the tubes. After vortexing, the samples were left 4 hours for derivatization. Calibration standards (range: 25-500 ng ml^{-1}) were freshly prepared following the same derivatization procedure used for the samples. 1 ml aliquots of samples and standards were transferred to HPLC vials and analyzed by LC-MS-MS.

5.2.5.3 Instrumental analysis

The LC-MS-MS system consisted of an Agilent 1200 series (Agilent Technologies, Wilmington, DE, USA) and a QTrap 5500 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with an electrospray ionization source (ESI). Chromatographic separation was carried out on a PRP-1 column, 250 x 2.1 mm, particle size 7µm (Hamilton, Reno, NV, USA).

The mobile phase consisted of 20 mM ammonium formate, pH adjusted to 8.5 (solvent A) and acetonitrile (solvent B). Analyte separation was obtained by running a gradient from 90% A/10% B to 10% A/90% B in 25 minutes at a flow rate of 0.2 mL/min. The injection volume was 10 µL.

The ESI source was operated in positive mode at a temperature of 600° C and an ion spray voltage of +4500 V. Nitrogen was used as collision gas. The analyses were performed with a multiple reaction monitoring (MRM) method that monitored two mass transitions (parent ion/product ion) for each analyte (m/z 392/179 and 392/88 for glyphosate and m/z 394/216 and 394/172 for isotope labelled glyphosate). The values of the voltages applied to the sampling cone, focusing lenses, collision cell and quadrupoles were optimized in MRM mode by direct infusion of a solution containing the analytes. Detection was based on retention time and the most abundant mass transition corresponding to an authentic standard. Confirmation of analyte identity was based on the relative response of the secondary mass transition to the primary mass transition. Analyst software 1.5.1 (AB Sciex) was used for system control and data processing.

5.2.5.4 QC procedures

Blank samples consisting of Milli-Q water and recovery samples (Milli-Q water spiked with glyphosate at a concentration of 10 ng/ml) were extracted with the samples. The recovery of Glyphosate was 101 ± 1%. The detection limit was 2.5 ng/ml, corresponding to 62.5 ng/sample.

5.2.6 Analysis of dye marker deposited on curlers

Fluorescein was excited at 492 nm and was detected at 513 nm by a fluorescence HPLC monitor within 3.0 ml extraction fluid the day after spraying (Shimadzu RF-551, Shimadzu, Kyoto, Japan; detection limit 0.01µg l^{-1}). Prior to the experiment, the potential decay of sodium fluorescein was estimated and no decay could be detected during two days of storage at room temperature, which indicated that no measurable decay took place between sampling and analysis.

5.3 Statistics

5.3.1 Estimation of glyphosate deposition

The relationship between dye marker and glyphosate deposited on curlers was established based on the thirty sampling plots where one curler was used for measurement of fluorescein and one for glyphosate. The correlation was established as a linear regression and analysed for significance. The correlation was, thereafter, used to estimate deposition of glyphosate on curlers for all sampling plots.

In order to get an estimate of glyphosate deposition on plants within the experimental plot we used the relationship between fluorescein and metsulfuron methyl deposition on curlers and on hedgerow vegetation reported by Kjær et al. (2014) and assumed that this relationship could also be used to estimate deposition of glyphosate on herbs.

5.3.2 Model

The flowering data of the different species was modelled using a Bayesian hierarchical model similar to that used for individual plants in pots (Chapter 3.3.1). However, the model was parameterized a little different as flowering data for the different NTTPs was sampled in a plant community with a number of different species, i.e. the experimentally established vegetation. Within the model, the cumulative number of flowers was assumed to follow a modified Gompertz growth curve (Seber & Wild, 1989, Damgaard et al. 2016):

$$f(q_i, h_i, t) = ((\alpha_0 + \alpha_1 \mathrm{Log}[h_i + 1]) * {q_i}^b) \, \mathrm{Exp}[-\mathrm{Exp}[-\kappa(t - (\gamma_0 + \gamma_1 \mathrm{Log}[h_i + 1]))]] \quad (1),$$

where q_i was the cover of the species in plot i, b was a species-specific non-linear term, h_i was the measured amount of a die deposit in plot i, and t was time in days. For the modelling amount of deposited die marker was used as proxy. The effect of herbicide on flowering was modelled by allowing two of the parameters in the Gompertz growth curve, i.e., α that estimate the asymptotic cumulative number of flowers and γ that estimate the inflection point of the curve, to be simple linear functions of the logarithm to the herbicide concentration (Figure 2, p. 39).

The rationale underlying the model was to describe the increase in the cumulative number of flowers with a general and relatively flexible growth model where the asymptote and inflection point were parameterized. Furthermore, it was assumed that increasing plant cover will have an increasing non-linear effect on the cumulative number of flowers in the sampling plot, and that the effect of the herbicide on the number and flowers and the inflexion point of the curve were best modelled using a log-transformed herbicide concentration.

The observed cumulative number of flowers at time t in plot i, $y_{(i,t)}$, was assumed to be Poisson distributed with the mean determined by the Gompertz growth curve,

$$y_{i,t} \sim Poisson\big(f(q_i, h_i, t)\big) \quad (2).$$

The plot specific variables q_i and h_i were modeled as latent variables and constrained by the observed number of pin-point hits, x_i, out of 25 possible hits assuming a binomial distribution, and the measured levels of die deposit on the curlers (proxy for herbicide concentration), $z_{(i,k)}$ assuming a reparametrized gamma distribution, respectively.

$$x_i \sim Binomial(25, q_i) \quad (3),$$

$$z_{i,k} \sim Gamma(h_i, v)) \quad (4),$$

where h_i was the mean die deposit at plot i and v was a scale parameter that model the observed heterogeneous variance, i.e. the variance was observed to be an increasing function of the mean.

5.3.2.1 Estimation

The model was parameterized using numerical Bayesian methods, where the joint posterior distribution of the parameters and the latent variables were calculated using Markov Chain Monte Carlo (MCMC), Metropolis-Hastings algorithm, simulations with a multivariate normal candidate distribution and using a MCMC run of 100,000 iterations with a burn-in period of 10,000 iterations.

The prior distributions of all parameters and latent variables were assumed to be uniformly distributed either as improper priors or in their domain.

Plots of the sampling chains of all parameters and latent variables were inspected in order to check the mixing properties of the used sampling procedure. Additionally, the overall fitting properties of the model were checked by inspecting the regularity and shape of the marginal distribution of all parameters as well as the distribution of the deviance $(= -2 \log L(Y|\theta))$. The efficiency of the MCMC procedure was assessed by inspecting the evolution in the deviance.

Statistical inferences on the parameters were based on the marginal posterior distribution of the parameters.

5.4 Results

5.4.1 Glyphosate deposited as spray drift

The deposition of sodium fluorescein and glyphosate on curlers was found to be significantly ($p<0.001$) and positively correlated, when three outliers were omitted ($R^2 = 0.74$, Figure 12). The omitted data points were consistent with neighbouring data points with respect to fluorescein deposition, but not with respect to glyphosate measurements. Therefore, we assumed that an error must have occurred during the handling of samples or in the lab for these three samples. Thereafter, the deposition of glyphosate for all sampling points could be estimated from the measured deposition of sodium fluorescein.

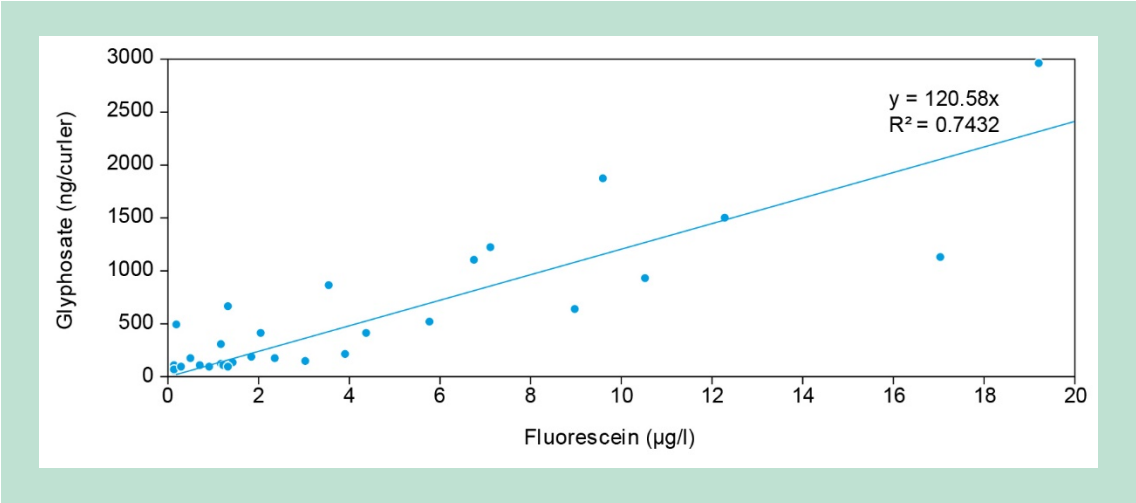

FIGURE 12. Correlation between fluorescein (µg/l) and glyphosate deposition on curlers (ng/curler)

In general, the estimated deposition of glyphosate within the experimental area decreased rapidly with increasing distance from the spraying track (Figure 13). In addition, the deposition close to the track also varied spatially along the track with the highest deposition rates at one end of the track indicating that turbulence and local changes in wind speed and/or direction occurred during the spraying event to an extent that affected the deposition rates.

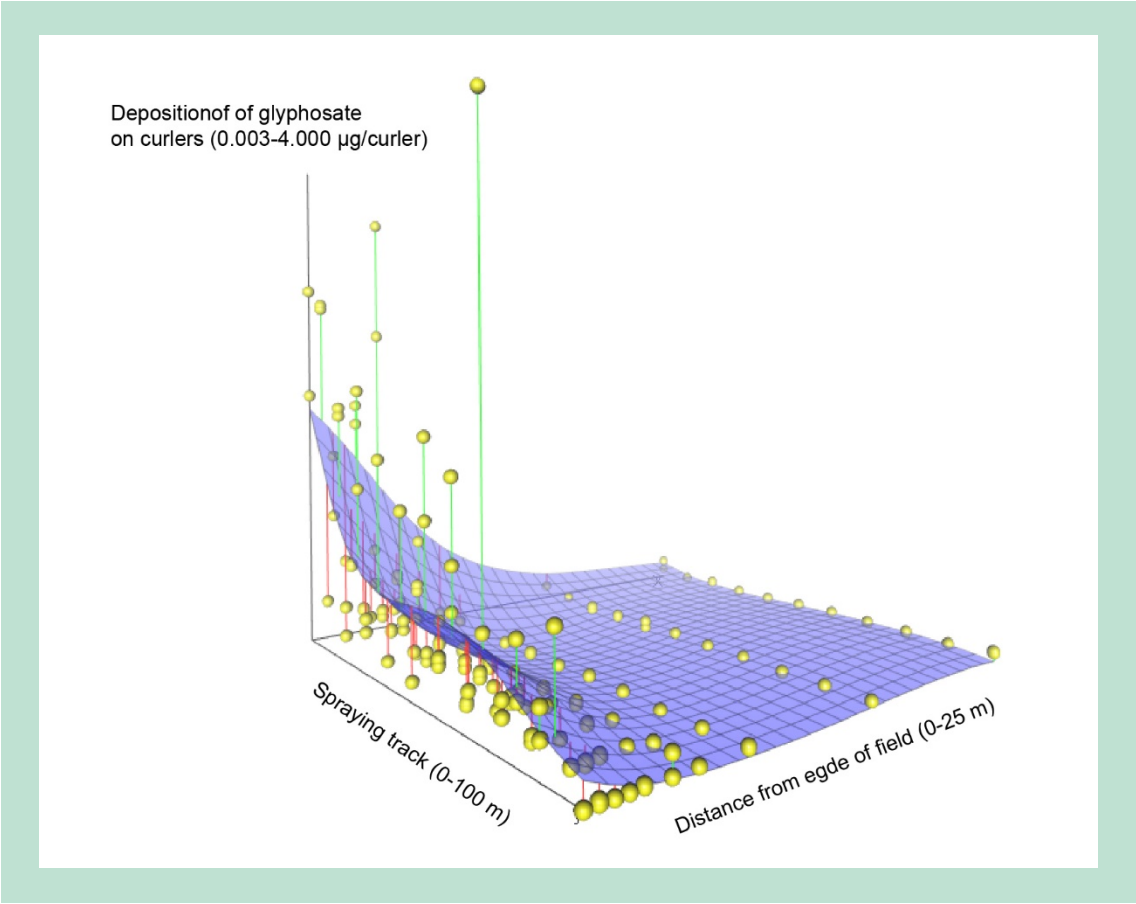

FIGURE 13. Estimated glyphosate spray drift deposition on curlers (mean of two replicates). The curlers were placed in the centre of each sampling plot at the Skovgaard experimental area. The estimates are based on the relation between measured fluorescein and glyphosate (Figure 12)

In order to estimate the fraction of the dose, i.e. 1440 g a.i./ha, that reached the plants in the experimental area we used the relationship between deposition of fluorescein and the herbicide metsulfuron-methyl on curlers on hedgerows in a previous study (Kjær et al. 2014) assuming that deposition on hedgerow plants was identical to deposition on herbs. Using this rough assumption we found that the maximum deposition was 2.8% of the label rate, i.e. 40.3 g glyphosate/ha and most sampling plots received 0.1% of label rate or less (Figure 14).

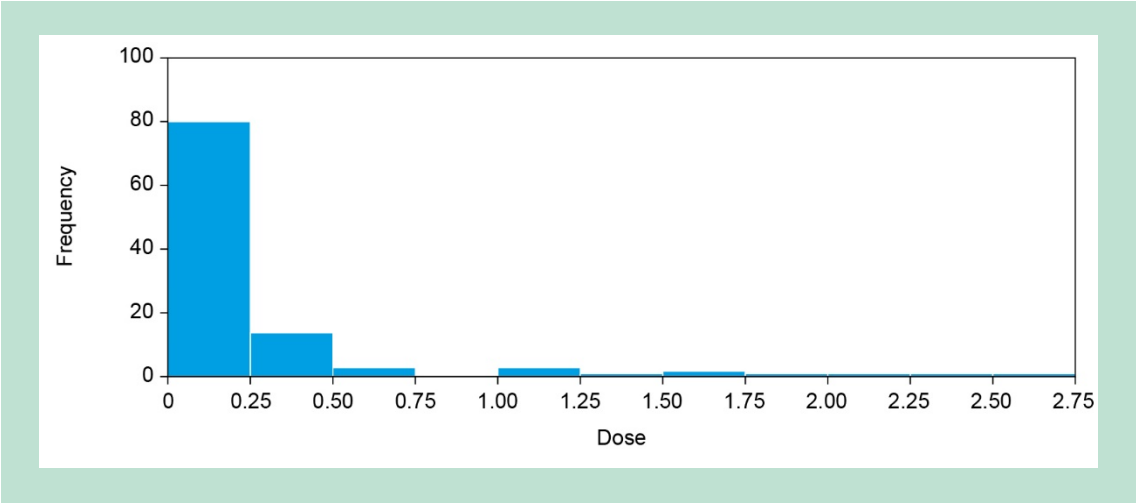

FIGURE 14. Number of samples that received a certain glyphosate deposition (% of recommended dose) at the Skovgaard experimental area. Recommended dose for Roundup Bio is 1440 g a.i./ha.

5.4.2 Species composition at the experimental plot – plant cover

Four of the sown species, *Trifolium pratense*, *T. repens*, *Lotus corniculatus*, and *Cichorium intybus*, all sown at 2 kg ha^{-1}, were well established and abundant in the vegetation the year after sowing when the spray drift experiment took place. The cover of the remaining four species, all sown at 1 kg ha^{-1}, was sparse and no further analyses are presented for these species. In addition to the sown species, a few plant species established within the experimental area. None of these reached a cover above five percent and they have not been included in the analyses.

T. pratense was the most abundant species having a cover of 70-100 percent in most plots (Table 17, Figure 16). This highly competitive species appeared to affect the growth of other species, as these species reached covers up to 80% in plots where *T. pratense* was less dominant. *T. repens*, in general, had low coverage in the plots (mostly <30%), while *L. corniculatus* varied highly in coverage among plots (1-93%). Seasonal change in cover was minor for all species, except for *C. intybus* (Table 16). In this species, cover increased from 30.4 ± 15.0 percent the day before spraying to 69.8 ± 23.2 percent in mid-August (Table 17).

TABLE 17. Percent cover (mean ± s.d.) of *Trifolium pratense*, *T. repens*, *Lotus corniculatus*, and *Cichorium intybus* estimated by the pin-point method 24 June (one day before glyphosate treatment), 31 July and 21 August, at the experimentally established vegetation at Skovgaard

	Trifolium pratense	Trifolium repens	Lotus corniculatus	Cichorium intybus
24 June	83.3 ± 16.1	7.5 ± 10.9	21.8 ± 17.6	30.4 ± 15.0
31 July	92.4 ± 15.7	7.9 ± 11.6	30.4 ±20.6	62.3 ± 22.3
21 August	95.4 ± 8.3	9.3 ± 14.1	33.0 ± 25.7	69.8 ± 23.2

Plant cover was not correlated to fluorescein deposition for any of the four species. As glyphosate correlates well to the deposition of fluorescein (Figure 12), we assume that the sub-lethal concentrations of glyphosate within the spray drift had no negative effect on plant cover. Plant height was, not measured, although visual inspection indicated decreasing plant heights with decreasing distance to the spraying track two weeks after exposure (Figure 15).

FIGURE 15. The experimental area seen from North facing South. The spaying track is seen in right side of the photo. The sample plots are marked with sticks that are 0.5 m tall. The part of the sticks above vegetation decreases to the left, i.e. away from the spraying track giving an indication of the herbicidal effect on plant height. Photo: Beate Strandberg, 13 July 2015

5.4.3 Plant flowering within the experimental plot

All four study species were mid to late season flowering plants, and only *T. repens* had initiated flowering at the time of exposure. *T. repens* had relatively few flower heads per plot (<12), but flowered continuously from late June until early August, flowering ceased in late August (Figure 16). The most dominant species, *T. pratense* started flowering in mid-July, peaking (with up to 56 flower heads per plot), and flowering until the end of September, when the experiment ended (Figure 16). *L. corniculatus* initiated flowering in mid-July, peaked in flowering in early August (with up to 13 flower heads per plot), and ended flowering in late September. *C. intybus* started flowering in late July, peaked (with up to 19 flower heads per plot) in late August to early September, and ended flowering at the end of September (Figure 16). Floral abundance varied highly between species and among sample plots over the sampling period. However, floral abundance was dependent on cover, and for plants with high cover, i.e. *T. pratense* and *L. corniculatus*, a negative correlation between flower abundance and glyphosate concentration was observed (Figure 16). On the other hand, *C. intybus* appeared to have a different response, floral abundances peaking at intermediate levels of glyphosate (Figure 16).

The cumulative number of flowers of the four species was modeled using the Gompertz growth model and the resulting marginal posterior distributions of the parameters are summarized in Table 18a-d. The effect of glyphosate spray drift on the cumulative number of flowers was measured by the parameter α_1. We found that glyphosate spray drift had a significant negative effect on the cumulative number of flowers of both *T. pratense* and *L. corniculatus* (Table 18, Figures 17a, 18), whereas it had no effect on the cumulative number of flowers of *T. repens* but positive but not significant effect on cumulative number of flowers of *C. intybus* (Figures 17b, 18).

There was no significant effect of glyphosate spray drift on the timing of flowering, i.e. the inflection point of the cumulative number of flowers curve, as measured by the parameter γ_1 (Figure 18, Table 18), in any of the four species. However, glyphosate spray drift tended to delay flowering of all species except *C. intybus* (Figure 18).

The effect of plant cover on the number of flowers deviated significantly from a linear relationship for three species (*T. pratense*, *T. repens*, *L. corniculatus*). For all three species, the number of flowers increased more than linearly with cover, as indicated by the parameter b (Table 18).

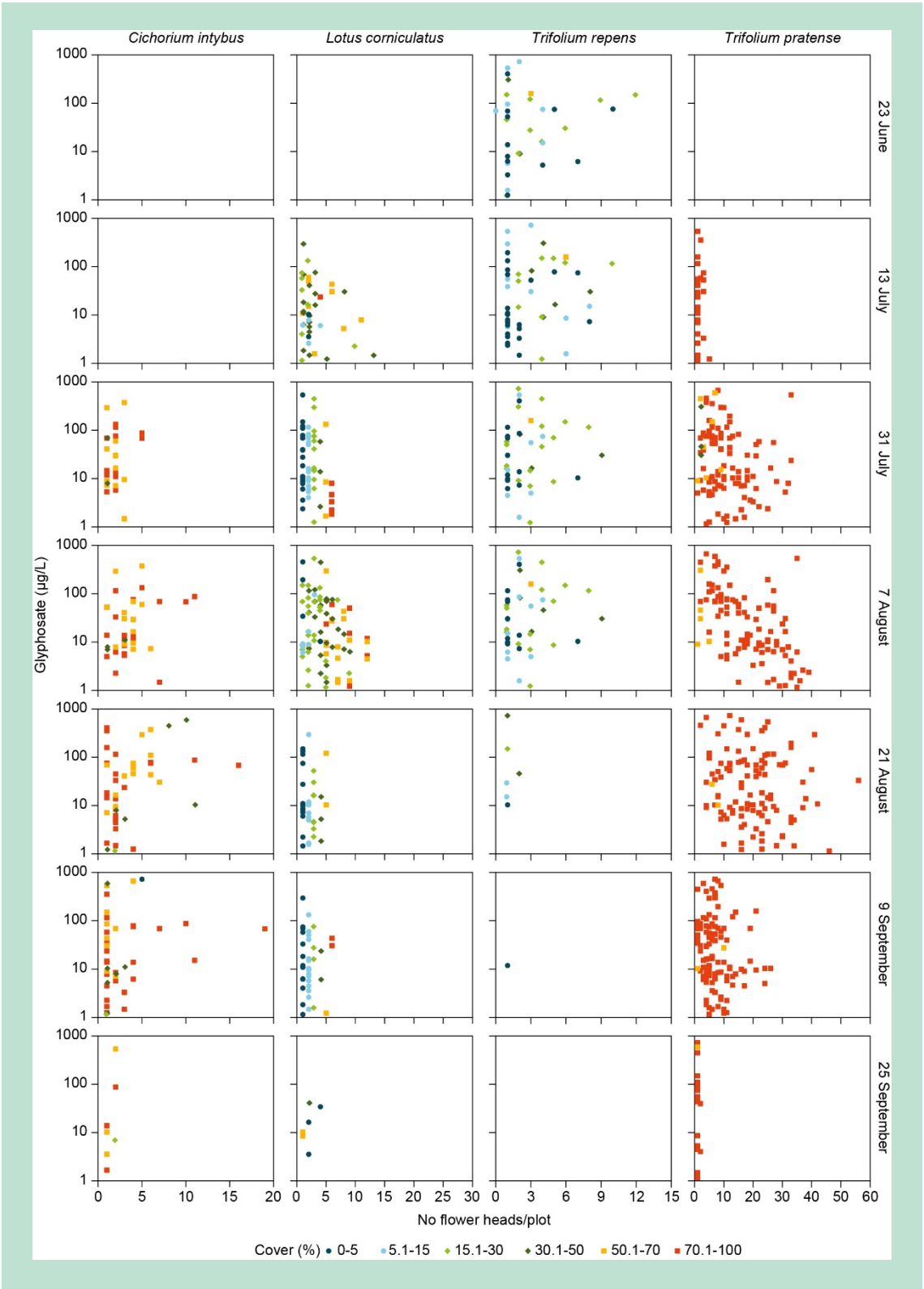

FIGURE 16. Flowering per sample plot (0.5 by 0.5 m) of *Trifolium pratense*, *T. repens*, *Lotus corniculatus*, and *Cichorium intybus* within the experimental plot as function of estimated glyphosate concentration on curlers

TABLE 18. The cumulative number of flowers on a) *Trifolium pratense*, b) *T. repens*, c) *Lotus cornicularius*, and d) *Cichorium intybus* was modeled using the Gompertz growth model (1). The resulting marginal posterior distributions of the parameters are summarized by the 2.50%,

50%, and 97.50% percentiles of the distribution, as well as the probability that the parameter is larger than zero (when relevant)

a)

Parameter	2.50%	50%	97.50%	P(> 0)
b	1.764	2.326	2.909	-
κ	0.072	0.080	0.089	-
α_0	84.89	92.1	99.7	-
α_1	-13.199	-10.526	-7.481	0
γ_0	48.20	49.90	51.62	-
γ_1	-0.345	0.569	1.464	0.88
ν	3.318	4.572	6.240	-

b)

Parameter	2.50%	50%	97.50%	P(> 0)
b	1.037	1.209	1.389	-
κ	0.018	0.041	0.072	-
α_0	58.475	106.423	162.679	-
α_1	-14.803	-2.573	10.320	0.39
γ_0	3.343	21.914	46.766	-
γ_1	-9.743	-0.744	7.716	0.40
ν	3.778	5.171	6.762	-

c)

Parameter	2.50%	50%	97.50%	P(> 0)
b	0.971	1.149	1.339	-
κ	0.059	0.077	0.102	-
α_0	43.089	52.313	64.011	-
α_1	-11.240	-7.870	-5.072	0
γ_0	31.506	35.985	40.514	-
γ_1	-1.150	2.000	4.813	0.9
ν	3.755	5.032	6.513	-

d)

Parameter	2.50%	50%	97.50%	P(> 0)
b	0.666	1.229	1.921	-
κ	0.053	0.096	0.178	-
α_0	2.732	4.604	7.837	-
α_1	1.012	2.336	4.131	-
γ_0	45.929	51.802	60.094	-
γ_1	-1.775	0.848	3.456	0.77
ν	3.713	4.979	6.554	-

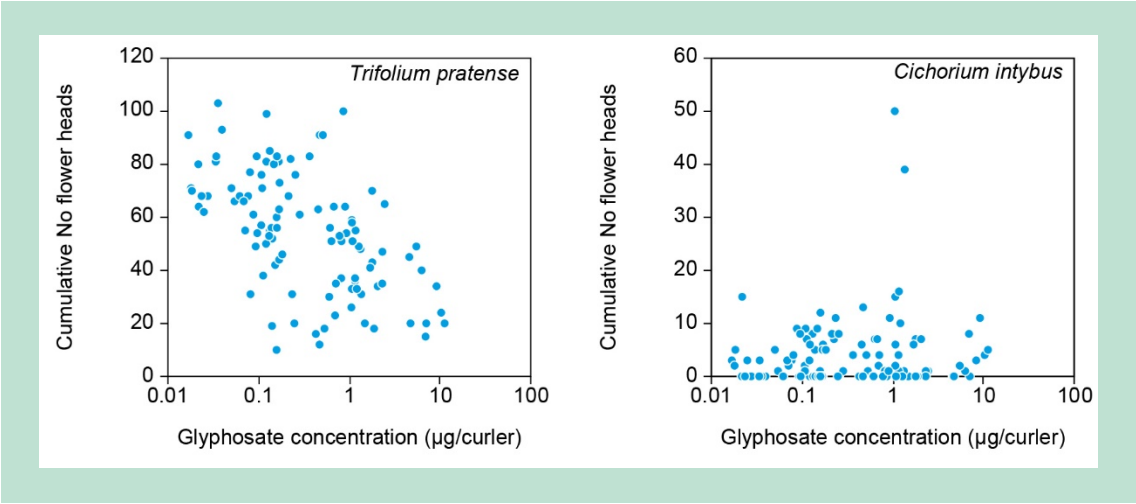

FIGURE 17. The cumulative number of flowers (flower heads) of a) (top) *Trifolium pratense* (red clover) and b) (bottom) *Cichorium intybus* (chicory) exposed to spray drift of glyphosate within the experimental area at Skovgaard, Mid-Jutland. Concentration of glyphosate was estimated based on the relationship between deposited fluorescein and glyphosate shown in Figure 12.

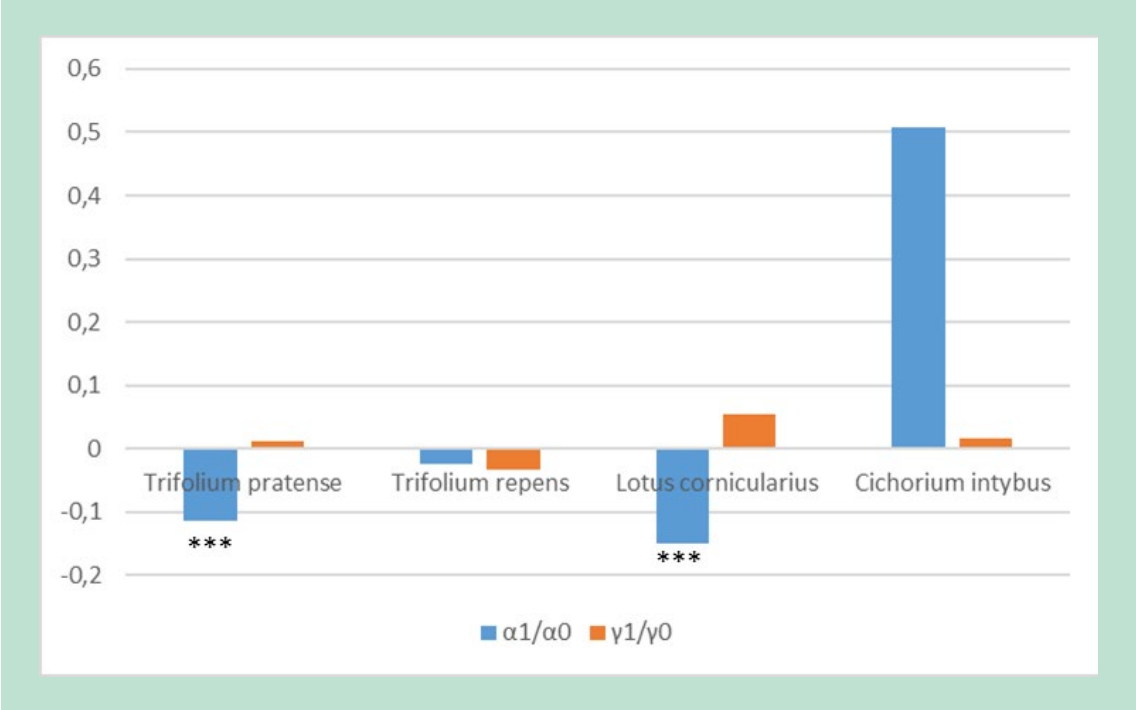

FIGURE 18. Predicted values of α1/α0 and γ1/γ0 for *Trifolium pratense*, *T. repens*, *Lotus corniculatus* and *Cichorium intybus* exposed to glyphosate spray drift at the experimental area at Skovgaard, Denmark Values are calculated through models of cumulative flowering over time. Negative values for α1/α0 indicate reductions in the maximum number of flowers produced, while positive values for γ1/γ0 indicate delays in time to peak flowering. Significant effects (as indicated by asterisks) are shown below the x-axis for α1/α0 and above the x-axis for γ1/γ0. Effect levels: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001

5.5 Discussion

To our knowledge this is the first field study on effects of spray drift deposition on vegetation where the vegetation was exposed to real spray drift and where spatially explicit deposition as

well as plant responses have been measured. Furthermore, the present study not only includes response parameters such as species composition and plant cover but also plant flowering which has been shown to be a sensitive measure of sub-lethal herbicide doses (Schmitz 2013, 2014, Boutin et al. 2014). Previous studies include (1) semi-field approaches in which non-target vegetation was exposed to overspray with drift realistic herbicide concentrations (e.g. Schmitz 2013, 2014, Kleijn and Snoeijing 1997, Dupont et al. submitted) or (2) field studies, which monitored effects of spray drift in habitats adjacent to herbicide treated fields and therefore potential exposed to spray drift (e.g. Marrs and Frost 1997, de Snoo and van der Poll 1999, Boutin et al. 2014). None of these studies, however, have included measurement of the deposited herbicides.

Previously, flowering of hedgerow ground vegetation at conventional farms have been shown to be reduced compared to hedgerows at organic farms, presumably due to differences in exposure to herbicide spray drift (Boutin et al. 2014). Conversely, plants at hedgerow grounds adjacent to fields with herbicide-free buffer zones had more flowers already the year of establishment of the buffer zone (Strandberg et al. 2103). The spray drift experiment support the assumption that differences in herbicide exposure were the main reason for these findings.

The sprayer in the present study was mounted with modern spraying equipment, i.e. drift reducing nozzles, an equipment used by most farmers. As a consequence only a small fraction, 0.1-2.8% of the dose (1440 g glyphosate/ha), reached the plot. Despite the low doses, we found significant effects on plant flowering. The fact that plant flowering is affected even at the low glyphosate concentrations seen in the present experiment makes it likely that the exposure by small droplets in the spray drift has to be taken serious in future investigations.

We found various responses on the cumulative number of flowers on the four test species, *T. pratense*, *T. repens*, *L. corniculatus*, and *C. intybus*, of glyphosate but no effect on timing of flowering. Flowering of *T. pratense* and *L. corniculatus*, was significantly and negatively affected, whereas glyphosate had no effect on flowering of the other two species, i.e. *T. repens* and *C. intybus*. Marrs and Frost (1997) also found various responses of the different species within microcosms with eight dicots placed 2 to 4 meter downwind of a sprayer and exposed to one of the herbicides glyphosate, mecoprop and MCPA. However, all species were negatively affected in at least one of the three years (Marrs & Frost 1997). In the present experiment, *T. pratense* made up a dense cover during the entire season whereas the other species and especially the leaves of *C. intybus* and *T. repens* were found under the red clover foliage. The cover and structure of the four species may result in differences in exposure that may explain the different responses of plant flowering. Although glyphosate is a non-selective herbicide, the four species might also have different sensitivity to the herbicide. Marrs and Frost (1997) also found that *L. corniculatus* was among the sensitive species in their microcosms study. Overall, the project results confirm the hypothesis that flowering of herbaceous species in habitats adjacent to fields treated with herbicides are affected by the sub-lethal dosages drifting into the habitat and that it reduces plant flowering. However, we did not find any significant delay of flowering although there was a tendency towards delayed flowering for the test species except for *T. repens*.

The variation in curler deposition in the direction of spraying disclosed in Figure 13 shows how true wind fields can affect the relation between deposition and distance to the spraying track. For mitigation measures such as buffer zones, distance to the edge of the field is the only governing factor for reduced herbicide deposition. However, as shown in Figure 13, this statement is highly problematic for exposure close to field edge and it is highly important in drift experiments to measure the actual deposition in every plot without any a priori assumption of a functional relation to the sprayed area. The finding of this study of effects on plant flowering at very

low exposure rates combined with the large variation in glyphosate deposition at the same distance to the spraying track, can challenge the conventional believe of existing buffer zones as sufficient protective.

Effects of herbicide spray drift exposure on floral abundance may secondarily affect pollination interactions. Floral scarcity negatively affects flower-visiting insect populations and, could hence result in cascading effects on natural ecosystems. Decline in floral availability could trigger pollinator decline, which can in turn lead to decline in pollination of wild flowers. Moreover, decline of pollinators may ultimately result in reduced seed set of insect-pollinated plants and reduced yield in crops (Garibaldi et al. 2013). Few studies have investigated herbicidal effects on trophic interactions (but see Dupont et al, submitted). Results of the current study indicate that flower availability if affected negatively in at least two insect pollinated wild flowers when exposed to field-realistic spray drift levels of glyphosate. In intensively farmed agricultural landscapes, areas with natural vegetation adjacent to agricultural fields are expected to be exposed regularly to spray drift concentrations of herbicides. Hence, reduction in floral resources for pollinators due to herbicide spray drift is likely to be widespread in agricultural areas. Decline of wildflowers and, consequently, food limitation of flower-visiting insects is a major concern and is thought to be an important driver of historical pollinator decline of flower visiting insects incl. pollinators (Biesmeijer et al. 2006; Potts et al. 2010).

6. Flowering of herbaceous species as response to herbicide exposure – monitoring of flowering in stream buffers

6.1 Introduction

The effect of distance to the cultivated field and hereby exposure to drifting herbicides on flowering of non-target terrestrial plant species was studied in buffer strips between fields and streams. The following hypotheses were tested: 1) Flowering of herbaceous species in natural and semi-natural habitats adjacent to fields treated with herbicides is affected by sub-lethal dosages of herbicides drifting into the habitats. 2) Herbicide exposure of non-target terrestrial plants reduces and delays flowering of herbaceous plants in natural and semi-natural habitats. 3) Flowering of herbaceous species in natural and semi-natural habitats such as the habitat type 6430; Hydrophilous tall herb fringe communities of plains (Habitats Directive 1992) is applicable as indicator of herbicide exposure.

In addition, the data may indicate, to what extent buffer strips along streams with habitat type 6430 are at risk of receiving drifting pesticides.

6.2 Material and methods

6.2.1 Study sites and registration method

Fifteen study sites located along two streams (13 buffer strips along the stream Skjold Å and 2 buffer strips along Rårup Å) in a restricted geographical area in the Mideast part of Jutland, Denmark, were used in the study (Fig. 19). The area was characterized by intensive agriculture and the selected buffer strips were next to cultivated fields (12 fields with cereals, two with grass seeds and one with rape). All fields were treated with herbicides in the year of the study. The study was carried out in a 100 m transect of the buffer strips and delineated to the area spanning from the stream (width of stream 1-4 m) to the nearby field with crop rotation. The width of the buffer strip varied between 3 and 12 m (Fig. 20).

To cover a gradient in the potential drift of pesticides from the fields and ensure an even distribution of recorded plants throughout the buffer strips from the stream to the field, buffer strips were divided into three equally wide zones (1-4 m) (Fig. 20). In each zone, 15 individuals of each of the five most common flowering herbaceous plants (excluding grasses and sedges) were registered and the numbers of flowers on each individual were counted; giving an optimal total of 225 individuals (5 species x 15 individuals x 3 zones) in each buffer strip (Fig. 20). Both individuals with and without flowers were registered. For species having flowers in heads, umbels and other dense inflorescences, where it was hard to count the individual flowers, the number of inflorescences rather than the number of flowers was counted. For each individual, the distance to the stream and to the field was measured. The same five species were registered each time, but not necessarily the same individuals.

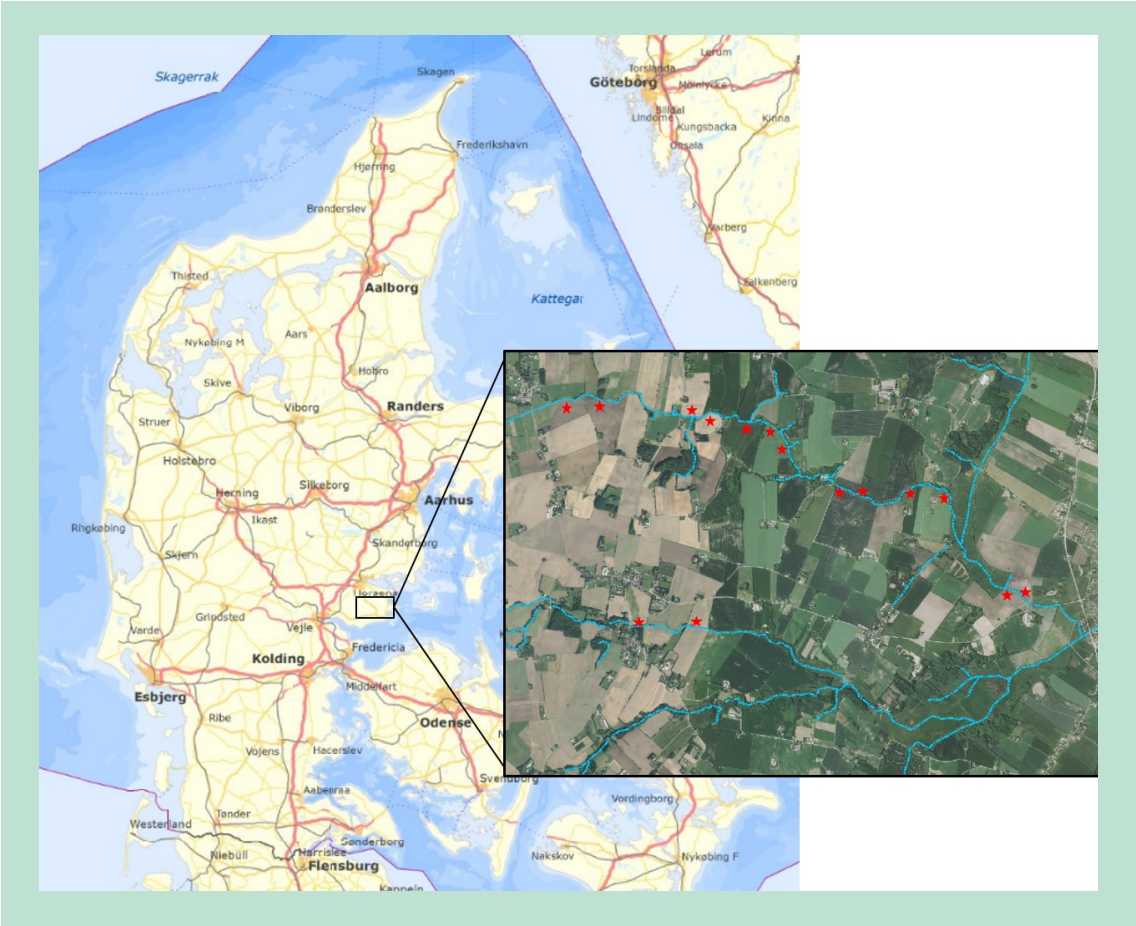

FIGURE 19. Geographical location of the 15 buffer strips. The area was characterized by intensive cultivation

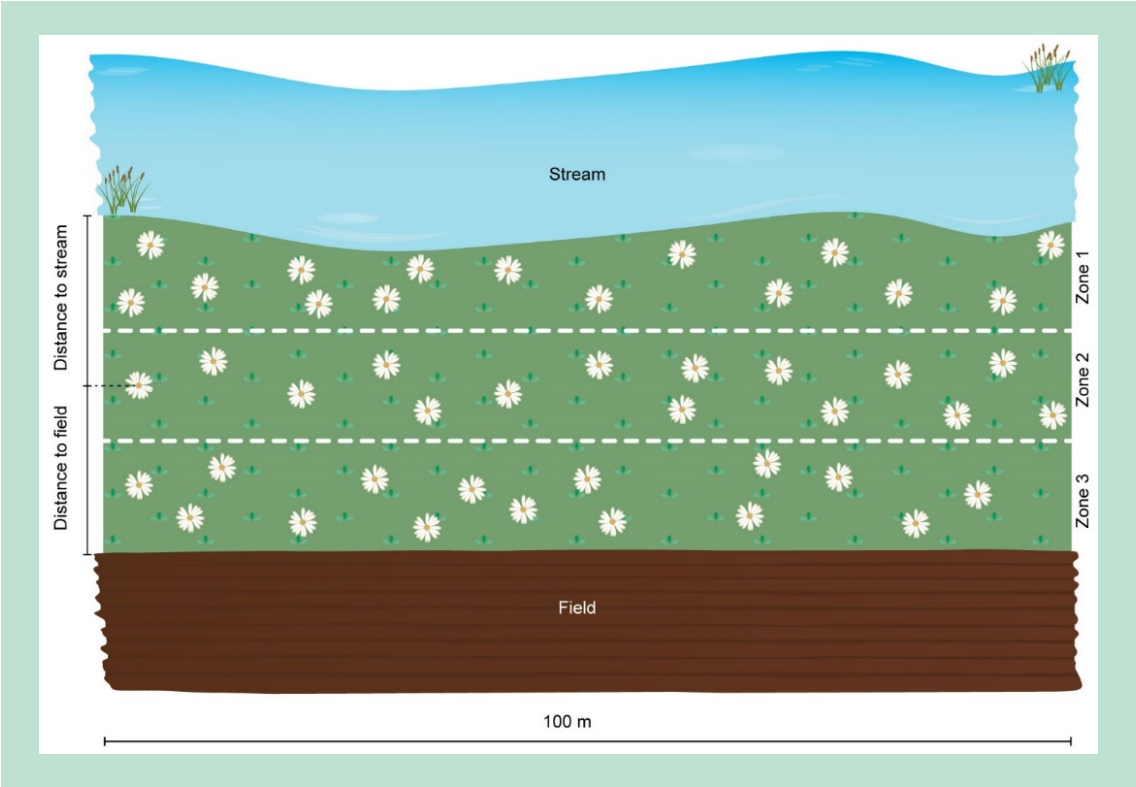

FIGURE 20. Schematic view of a study site. In each zone (1-3) 15 individuals of five species were registered, distance to the field and stream was measured and the number of flowers were counted

The registration of individuals was “approximated random”, but depended on the abundance of the species: If many individuals were present in a zone (e.g. *Cirsium arvense*), an individual was registered for approximately each 6.5 m. If few individuals of the species were present (e.g. *Cirsium vulgare*), all individuals in the buffer strip were registered.

In general, the buffer strips between stream and cultivated fields were species poor, and for six buffer strips it was not possible to find five flowering species, which occurred in all three zones, thus, only four species were registered here. In addition, it was not possible to register 45 individuals of all species in some buffer strips. Here, the maximum possible number was registered.

Registrations were performed five times during the period from mid-May to late August 2015. However, in two buffer strips, the vegetation was harvested after two registrations and in another two after four registrations.

Flowering of twenty species were recorded and within these species, 10,445 individuals were recorded and 10,083 flowers were counted (Table 19). The table gives information on the flowering period of the species (Hansen, 1985) and information on the number of buffer strips with the species present, the number of individuals sampled per species per buffer strip, the number of individuals sampled in total and the total number of flowers registered. Based on the flowering period, we divided the species into early, intermediate and late flowering species.

TABLE 19. Summary of species and flower occurrences in the sampled buffer strips. Only species occurring at more than one site and species registered more than twice were included in the species-specific analyses. These species are denoted with an asterisk (*)

Species	Time of flowering	Time of flowering-category	Registration in no. of sites	No. of individuals sampled/site	Total no. of individuals sampled (all sites)	Total no. of flowers registered (all sites)
Geranium dissectum	June-July	intermediate	1	219	219	188
Epilobium hirsutum*	July-September	late	2	212-225	437	427
Cirsium vulgare*	July-August	late	4	74-153	475	232
Ranunculus repens*	May-July	early	6	117-225	1043	516
Taraxacum sp.*	May-June	early	8	90-225	1358	527
Cirsium oleraceum*	July-August	late	8	93-225	1327	1255
Rumex crispus	July-August	intermediate	1	151	151	130
Rumex obtusifolius*	July-August	intermediate	5	90-223	759	1524
Trifolium repens*	June-August	intermediate	3	132-225	582	318
Trifolium pratense*	May-September	intermediate	2	166-214	380	50
Plantago major*	June-August	intermediate	2	121-160	281	10
Aegopodium podagraria	June-July	intermediate	1	90	90	0
Achillea millefoilum	June-October	late	1	90	90	0
Vicia cracca	June-August	intermediate	1	90	90	0
Heracleum sphondylium	June-July	intermediate	1	198	198	36
Symphytum x uplandicum	June-August	intermediate	1	121	121	2014
Stachys sylvatica	June-August	intermediate	1	149	149	97
Cirsium arvense*	June-September	late	15	90-225	2886	1619
Angelica sylvestris*	July-August	late	2	128-196	324	38
Anthriscus sylvestris*	May-June	early	4	90-218	593	3567

6.3 Statistics

Analyses were performed with the statistical software SAS. The number of flowers was square root transformed prior to the analyses to achieve normality.

Effects of distance to the edge of the field on the number of flowers was tested by linear regression analysis with buffer strip as random effect.

First, the effect was tested on groups of species based on flowering period (early, intermediate and late flowering species). Second, the effect was tested on single species. Some of the species occurred in only one site, some were registered less than twice. These species were therefore withdrawn from the species-specific statistical analysis (Table 19).

6.4 Results

Cirsium arvense was abundant and the only species occurring in all fifteen buffer strips. The second most common species were *Taraxacum* sp. and *Cirsium oleraceum*, which were present in eight buffer strips each. Eight species were only present in one buffer strip.

6.4.1 Flowering period

We found no effect of distance to field when grouping the plants into early, intermediate and late flowering species. Figure 21 shows the average number of flowers in two species of Cirsium and in Ranunculus repens, within the three zones. Both *Cirsium* species are late-flowering, but the effect of distance to the edge of the field was opposite for the two species; *C. arvense* had the highest number of flowers per individual close to the stream, i.e. where herbicide doses was expected to be lowest, whereas *C. oleraceum* had highest number of flowers close to the field. The early flowering species *R. repens* had, like *C. arvense*, the highest number of flowers close to the stream. We, thus, decided to study the effect on single species instead of grouping into period of flowering.

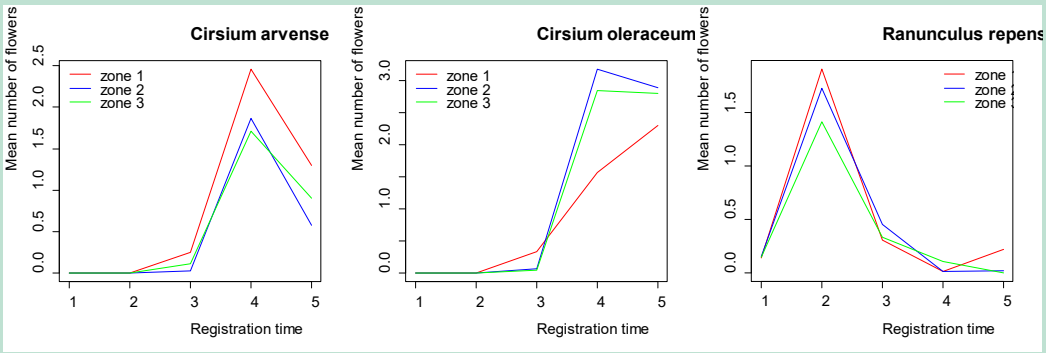

FIGURE 21. Mean number of flowers in two late-flowering species (*C. arvense* and *C. oleraceum*) and one early-flowering species (*R. repens*) in the three zones. Zone 1 is close to the stream, zone 3 is close to the field

6.4.2 Effect of distance to the edge of field on the flowering of individual species

Modelling the effect of distance to the neighbouring field on the number of flowers, we found significant effect for four species, but only for *Epilobium hirsutum* and *Ranunculus repens* we found the expected positive effect of increasing distance to the field, whereas the response was opposite for *Cirsium vulgare* and *Anthriscus sylvestris* (Table 20).

TABLE 20. Effects of distance from neighbouring field on the number of flowers for species registered in more than one buffer strip and with more than two occurrences in the sampling period. Significant regression models are given in bold. Positive estimates indicate that flowering increases with increasing distance to the field, whereas negative estimates indicate the opposite pattern

	Intercept	Estimate	F	P
Epilobium hirsutum	**1,2846**	**0,1694**	**14,42**	**0,0003**
Cirsium vulgare	**1,9207**	**-0,1008**	**7,60**	**0,0074**
Ranunculus repens	**1,2628**	**0,02828**	**5,99**	**0,0151**
Taraxacum sp.	1,3837	-0,01445	1,72	0,1911
Cirsium oleraceum	1,7900	-0,03386	2,89	0,0901
Rumex obtusifolius	4,0317	-0,06577	0,34	0,5615
Trifolium repens	1,2509	-0,01250	1,56	0,2132
Trifolium pratense	1,0270	0,02733	1,49	0,2308
Plantago major	1,0023	0,01097	0,07	0,8066
Cirsium arvense	7,7023	-0,00912	0,29	0,5924
Angelica sylvestris	2,0873	-0,1097	0,31	0,5951
Anthriscus sylvestris	**4,5552**	**-0,2381**	**27,00**	**<0,0001**

6.5 Discussion

We observed contrasting responses of flowering of the different species in the buffer strips in relation to distance to herbicide treated fields. For two species, *Epilobium hirsutum* and *Ranunculus repens,* we found that flowering increased with increasing distance to the field, whereas the opposite response was observed for another two species, *Cirsium vulgare* and *Anthriscus sylvestris.* We are not aware of the mechanism behind these different responses. However, we expect that the impact from applied pesticides and nutrients decline with increasing distance to the field edge and, therefore, the response might relate to agricultural practice. Moreover, it is likely that different tolerances towards herbicides can explain the observed pattern, whereas the role of nutrients may be inferior. Thus, all four species are highly productive and benefit from increasing nutrient availability (Nygaard et al. 2009, Ellenberg et al. 1991). The investigated buffer strips were generally species poor, but some species, which are considered characteristic of hydrophilous tall herb fringe communities (habitat type 6430; Habitats Directive 1992) were found, e.g. *E. hirsutum, C. oleraceum* and *A. podagraria.* Consequently, agricultural practice may be a threat for the existence of this habitat type along Danish streams.

7. Plant flowering as an indicator for herbicide exposure – line of evidence

7.1 Introduction

In order to test the project hypothesis (6) "Flowering of herbaceaous species in natural and seminatural habitats are applicable as an indicator of herbicide exposure"; we will assess the quality of flowering of NTTPs growing in habitats adjacent to conventional fields where herbicides are used as an indicator of the herbicide exposure of the habitat, i.e. habitat herbicide exposure is the indicandum.

Based on an extensive literature review, Heink and Kowarik (2010b) listed nineteen criteria that could be used to evaluate the quality of a given ecological parameter as an indicator of the phenomenon in question. The criteria could be categorized belonging to five categories: 1) specificity (relation between indicator and indicandum), 2) sensitivity, 3) relevance, 4) feasibility for analysis and interpretation, and 5) stakeholder perception. We will evaluate plant flowering as an indicator based on these criteria but focus on four first mentioned categories specificity, sensitivity, relevance and feasibility for analysis and interpretation.

7.2 Specificity of plant flowering

Several studies have demonstrated that plant flowering is sensitive to herbicides exposure (Marrs et al. 1991a, Schmitz et al. 2013, 2014; Boutin et al. 2014; Bohnenblust et al. 2016). Within the PENTA project, we have observed that the response of individual plant species to low herbicide doses varies among species. This was the case both in lab tests (Chapter 3), in the field experiment (Chapter 5) and in the stream buffer zones (Chapter 6). Former studies of flowering of herbaceous species at hedgerow grounds also demonstrated this individual response (Strandberg et al. 2013). Using flowering of individual species within a habitat as an indicator for herbicide exposure therefore seems doubtful.

However, establishment of an herbicide-free buffer zone along hedgerows improved flowering for the majority of hedgerow ground species: four species only flowered where a buffer zone was present, and for eleven out of the remaining twenty-five species flowering was significantly increased by the establishment of a herbicide-free buffer zone (Strandberg et al. 2013). This gave us the idea of using flowering of all herbaceous species present in a habitat as an indicator for herbicide exposure.

Such an indicator, I, compiles flowering data for all individual species within a habitat exposed to herbicides (spray) and within habitats not being exposed (non) into a measure of pesticide impact:

$$I = \frac{\sum_{i=1}^{N}\left[\sum_{j=1}^{M}\left[\sum_{k=1}^{O}\left[\sum_{z=1}^{P}\left[\sum_{f=1}^{Q}\left[F_{i,j,k,z,f}^{non} - F_{i,j,k,z,f}^{spray}\right]\right]\right]\right]\right]}{\sum_{i=1}^{N}\left[\sum_{j=1}^{M}\left[\sum_{k=1}^{O}\left[\sum_{z=1}^{P}\left[\sum_{f=1}^{Q}\left[F_{i,j,k,z,f}^{non} + F_{i,j,k,z,f}^{spray}\right]\right]\right]\right]\right]}$$

where $F_{i,j,k}^{non}$ and $F_{i,j,k}^{spray}$ is the number of flowers on herbaceous plant species within habitats adjacent non sprayed and sprayed fields, respectively, at the i'te region in Denmark, at the j'te crop type at the k'te sample. The indicator I range between -1 and 1. I=1 when no flowering are seen in habitats adjacent to sprayed fields, I=0 when flowering within habitats adjacent to

non-sprayed fields are equal to flowering in habitats adjacent to sprayed fields, and I=-1 when flowing only occurs in habitats adjacent to sprayed (unlikely, but mathematical possible).

7.3 Sensitivity of plant flowering

In order to evaluate the relationship between flowering and herbicide exposure (present or not present) we reanalyzed data on plant flowering within hedgerow ground vegetation (see Strandberg et al. 2013). The study was carried out in nine Danish hedgerows adjacent to herbicide treated fields at different farms. Each field and hedgerow was 400 m or longer. At the field border a 20-24 m wide herbicide-free buffer zone (BZ) was established along half of hedgerow (Figure 22). This setup was used to ensure that the vegetation within the two stretches was as similar as possible with respect to species composition, other field treatments (e.g. fertilization), soil and climatic conditions. At the hedgerow ground 15 permanent plots, each 50 X 50 cm, where selected for estimation of plant cover and flowering intensity of all herbaceous species. Thus, the study included 135 plots exposed to spray drift (no BZ) and 135 exposed to a much reduced deposition due to the buffer-zone (BZ).

For each plant species, i, four characteristic values was defined:

N_{bz}^{F}: The total number of flower units counted for all BZ-plots located at the ground adjacent to a herbicide-free buffer zone,

$N_{no\ bz}^{F}$: The total number of flower units counted for all no BZ-plots located at the ground adjacent to a normally sprayed field,

N_{bz}^{P}: The total number of BZ-plots where the species *i* was present, and

$N_{no\ bz}^{P}$: The total number of no-BZ-plots where the species *i* was present.

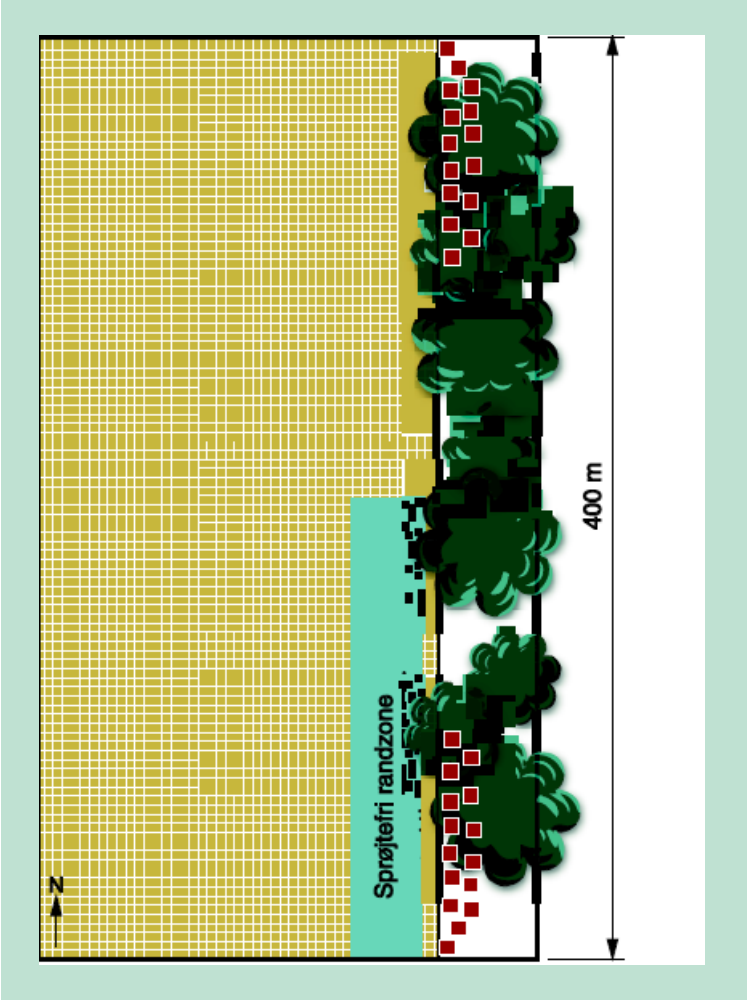

FIGURE 22. Design of a study was carried out in nine Danish hedgerows adjacent to herbicide treated fields at different farms (Strandberg et al. 2013). Each field and hedgerow was 400 m or longer. At the field border a 20-24 m wide herbicide-free buffer zone was established along half of hedgerow. At the hedgerow ground 15 permanent plots, each 50 X 50 cm, where selected for estimation of plant cover and flowering intensity of all herbaceous species over a three-year period

When occurrence (red symbols and line, Figure 23) and flowering (blue symbols and line) of each species within BZ and no-BZ plots, respectively, are plotted, then the fitted line x=y (i.e. *a*=1 and *b*=0) shows the situation where herbicide exposure due to spray drift does not affect plant occurrence and flowering. This seems actually to be the case for the red line (Fig. 23) for the pair $\left(N_{bz}^{P}, N_{no\ bz}^{P}\right)$, where *a* = 1.05 and b = -1.54. The pair $\left(N_{bz}^{F}, N_{no\ bz}^{F}\right)$ is also fitted to the blue line as y=*a*x+*b*, where *a* = 0.68 and *b* = -3.01. Using a General Linear Model, the parameter *b* is not significantly different form 0 (*p*=0.6) but the parameter *a* is strongly significantly different from one (p<1e-9). Thus, the slope of the blue line, *a*=0.68 shows a deviation from one and it can be concluded that the herbicide-free buffer zone results in increased flowering including all species at the hedgerow ground.

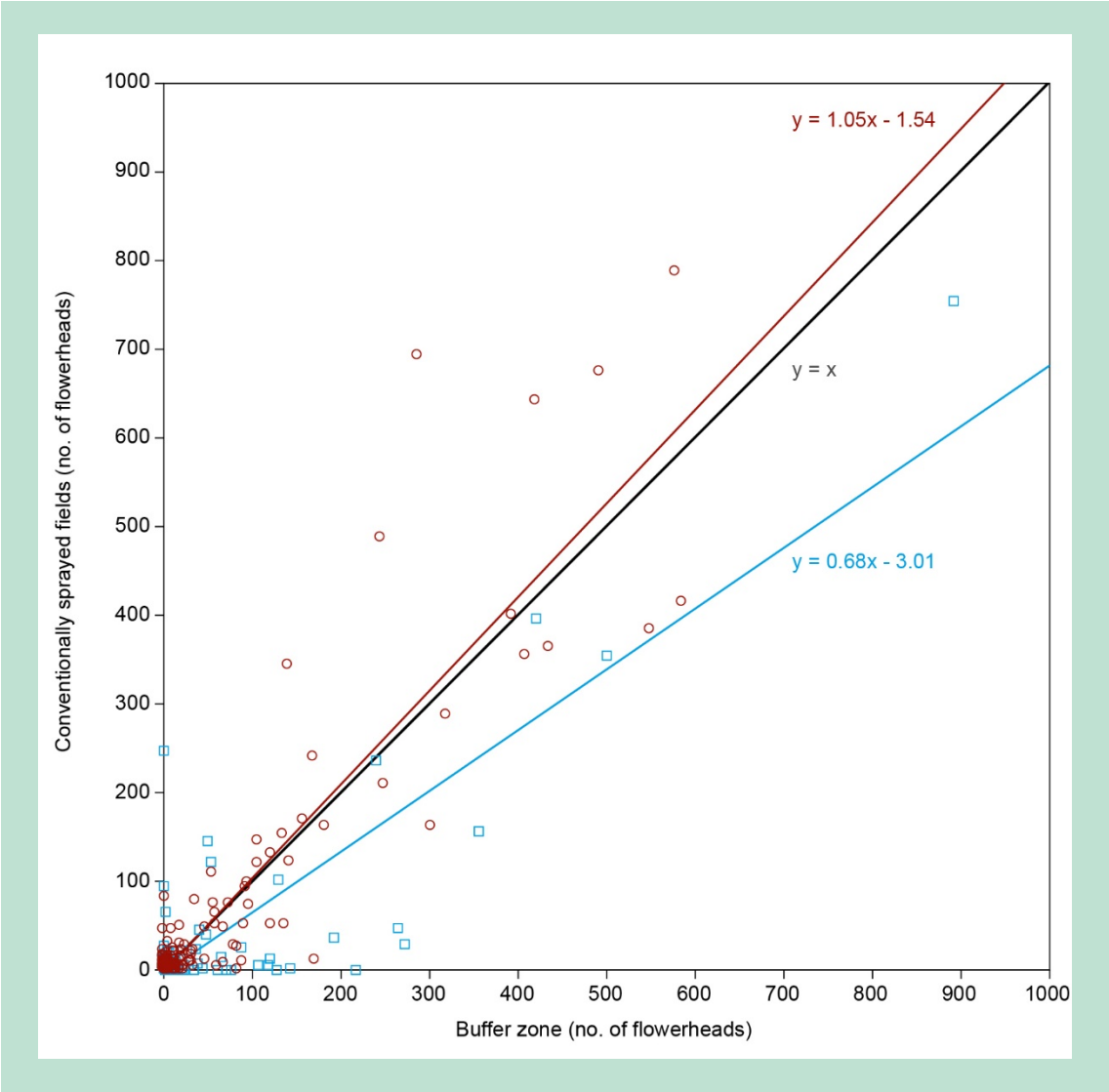

FIGURE 23. Occurrence (red symbols and line) and flowering (blue symbols and line) of plant species at hedgerow grounds adjacent to either conventionally sprayed fields (y-axis) (no bz) or a 20-24m wide buffer zone (x-axis) (bz). The red and blue lines and the equations are least square fittings of the two data sets

The changes of plant flowering that was already found the year of establishment of the buffer zone (Strandberg et al. 2013). Species composition, however, was relatively un-changed over the three-year study period indicating that longer time without or with much reduced herbicide exposure are needed for species to (re)establish. Time lags in species response to environmental changes is well-known as species often need more time to fully respond to environmental alterations than is required for the environmental changes themselves (Essl et al. 2015, Takkis et al. 2013, Kuussaari et al. 2009, Tilman et al. 1994). They occur both when biodiversity decreases, i.e. extinction debt, and when it raises, i.e. immigration credit (Jackson and Sax 2009). Whereas, time lag is well-documented in relation to climate change, biological invasions, habitat fragmentation and resource exploitation (Fahrig 2003, Thomas et al. 2004, Vellend et al. 2006, Sax and Gaines 2008, Brooks et al. 1999), time lag in species responses to pesticides has not received much attention. However, reduction in species richness occurs as consequences of pesticide spray drift to field boundaries and other habitats adjacent to agricultural fields (e.g. Kleijn and Snoeojing 1997, Kleijn and Verbeek 2000, Storkey et al. 2012), and immigration of plant species may be expected following reduction in herbicide exposure (e.g. Aude et al., 2003; Boutin et al., 2008). Contrary to the fast increase in plant diversity in

agricultural fields in response to conversion to organic farming, i.e. cessation of pesticide exposure (Petersen et al., 2006; Jonason et al., 2011), the increase in species richness has been shown to be a slow process in semi-natural habitats and equilibrium was not reached within thirty years (Strandberg et al., 2013). The fast response of plant flowering relative changes in herbicide exposure makes it a promising indicator.

Based on flowering data from the buffer zone experiment (Strandberg et al. 2013) a herbicide effect index (*HI*) can be defined:

$$HI = \frac{N_{bz}^{F}}{N_{bz}^{F}+N_{no\ bz}^{F}} \quad (1)$$

$HI \sim 0$ Indicates that a plant species is only flowering when it is exposed to herbicides. $HI \sim ½$ indicates that a plant species flowers equally well with and without exposure to herbicides, and $HI \sim 1$ indicates that a plant species is highly sensitive to herbicide exposure.

Some plant species only had a few or no flowers. The relationship between herbicide exposure and flowering of these species may be rather uncertain. To avoid the strongest influence from this random uncertainty, calculation of *HI* was only done for plant species for which the sum $N_{bz}^{F} + N_{no\ bz}^{F}$ was ≥ 30. Figure 24 shows the herbicide effect index, *HI*, for plant species commonly flowering within the nine studied Danish hedgerows with and without herbicide-free buffer zone. Within these hedgerows, there were more species with a high *HI*-index. However, a few species seems to flower more intensively without herbicide-free buffer zone. Therefore, *HI* for all commonly occurring species seems to give a good indication of whether the habitat was exposed to herbicides or whether exposure are absent or very limited.

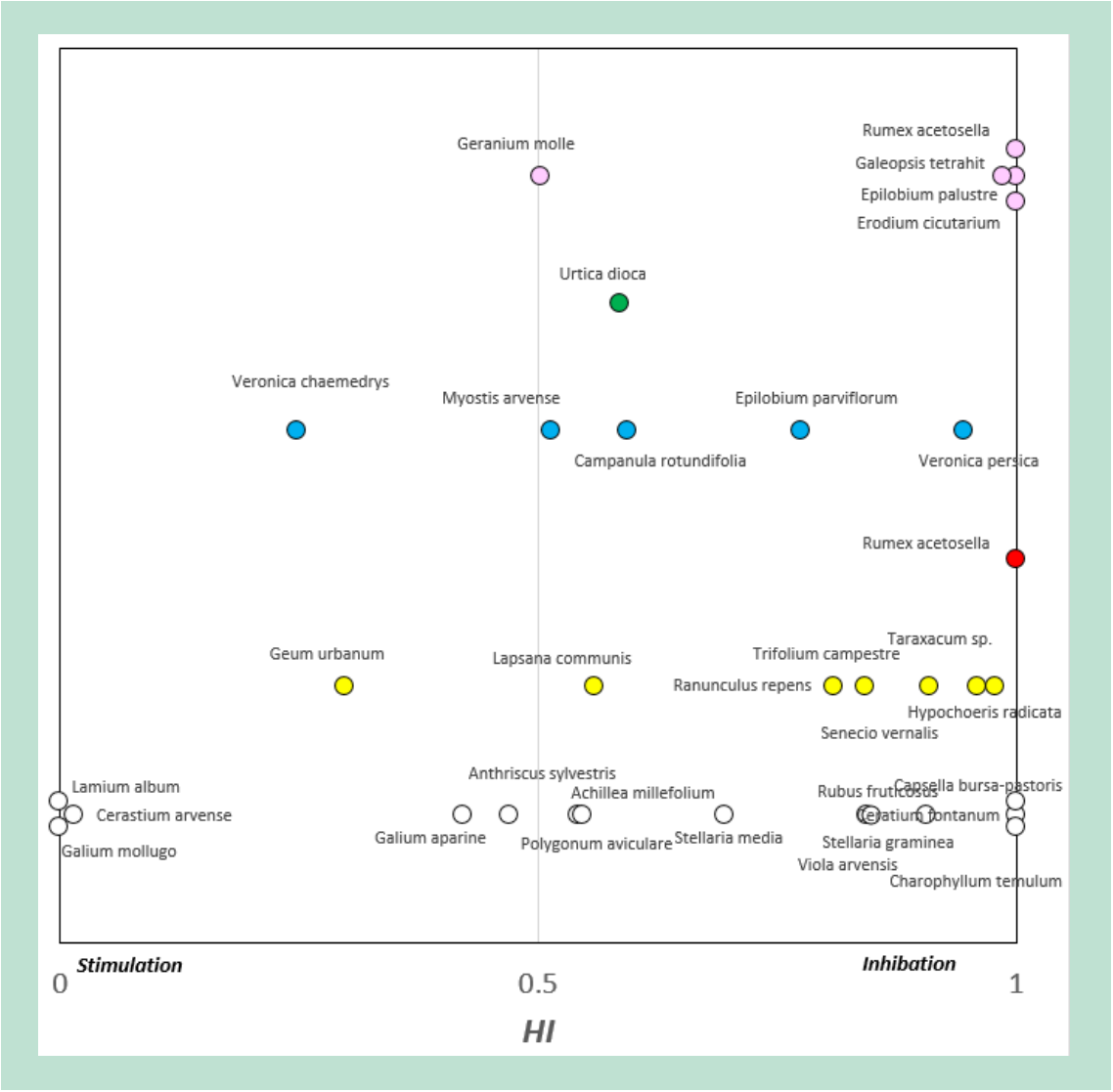

FIGURE 24. The herbicide effect index, *HI*, calculated for commonly occurring hedgerow ground species within nine Danish hedgerows (data from Strandberg et al. 2013). The colour of the dots indicates flower colour

Changes in number of flower units can be a result of either a change in plant abundance or a change in the flowering intensity. If the number of flowers relative to the plant abundance for each species in plots with buffer zone (bz) (x-axis) and plots without herbicide-free buffer zones (no bz) (y-axis) (Figure 25) then the data point will be placed close to the line if flowering relative to abundance is about the same with and without an herbicide-free buffer zone. However, most plant species is found below the line indicating that the reduced flowering is reinforced by a reduced intensity of flowering for the plants still being present.

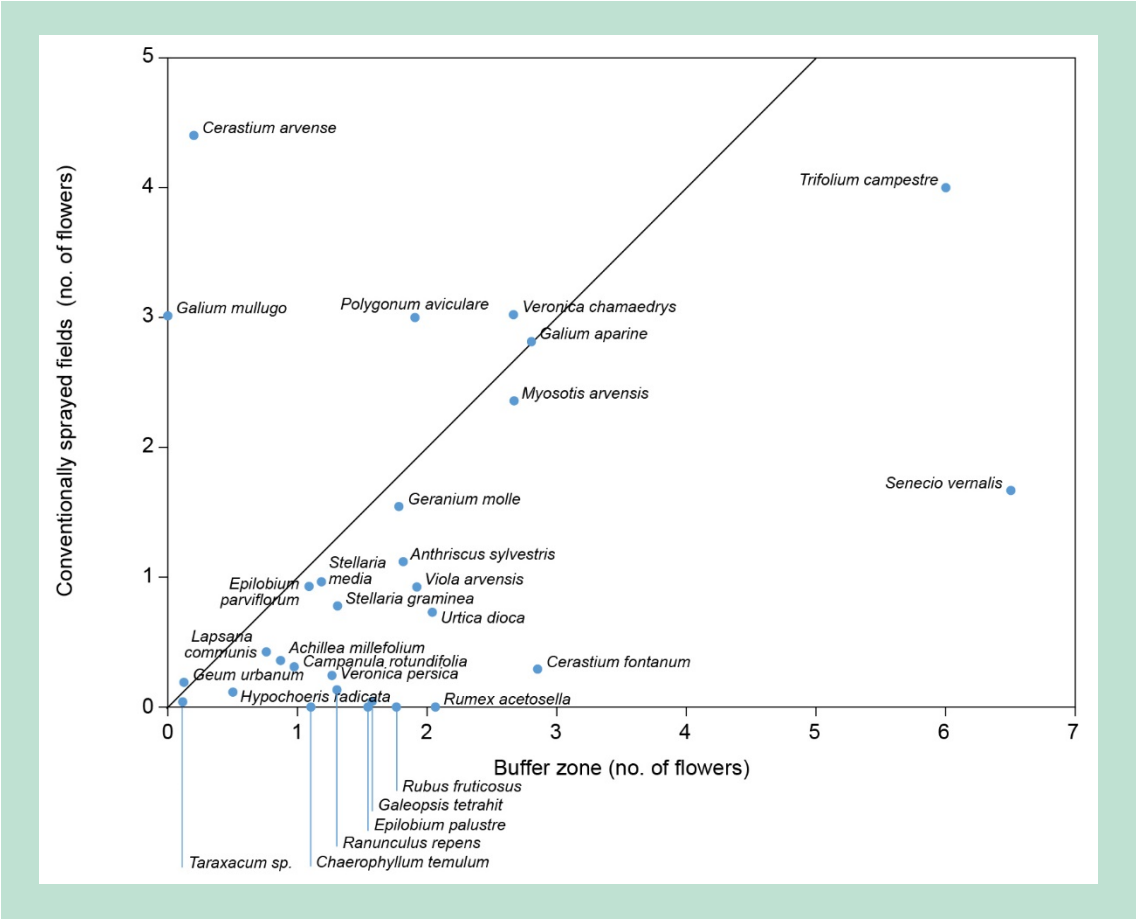

FIGURE 25. Number of flowers relative to plant abundance for each species in plots either adjacent to a buffer zone on x-axis or conventional field without buffer zone on the y-axis

The collection of data to support the indicator need to be as efficient as possible for larger areas. It is therefore important to assess the uncertainty of the indicator for different strategies of data collection.

7.4 Relevance

Plant flowering is highly relevant both for plant reproductive fitness and for flower visiting insects such as pollinators, see Chapter 3.5.

7.5 Feasibility for analysis and interpretation

The key property of an indicator is that the data need has to be realistic to fulfill for available resources. The benefit of flowers is that they are rather easy to identify by image analysis. This will especially be true for the flowers that are “not white” but has a distinct color to be identified with, however, it seems actually to be a benefit to exclude while flowers in the indicator. This is illustrated in Figure 26 (data from Strandberg et al. 2013), where the number of flower units is summed up for respectively all plant species and only for plants species that are not flowering as white flowers. The ratio between the number of flower units for respectively sprayed zone (SFZ) and no-spray zone (nSFZ) is seen to increase when white flowers are excluded. This indicates a more specific and thus certain indicator based on non-white flowers. If area data are collected based on image analysis then such information can turn out to be highly relevant in order to improve the indicator.

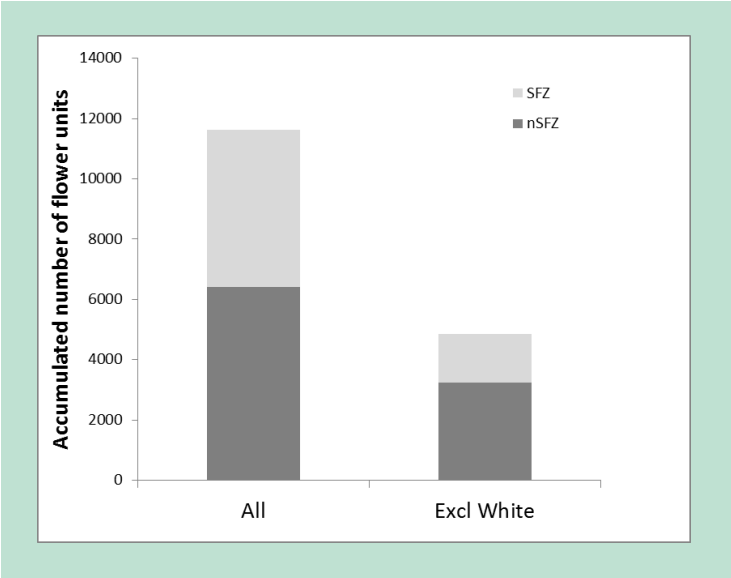

FIGURE 26. The total number of flower units summed up for respectively all plant species and only for plants species that are not flowering as white flowers for respectively sprayed zone (SFZ) and no-spray zone (nSFZ). Data from Strandberg et al. (2013).

Automatic counting of flowers is illustrated in the following by identification of reed clover. A picture of mixed red clover community is show in Figure 27 (left) and the identified flower heads are shown in Figure 27 (right). The flower identification is done using a free standard image analyzing software ImageJ as illustrated in Figure 28.

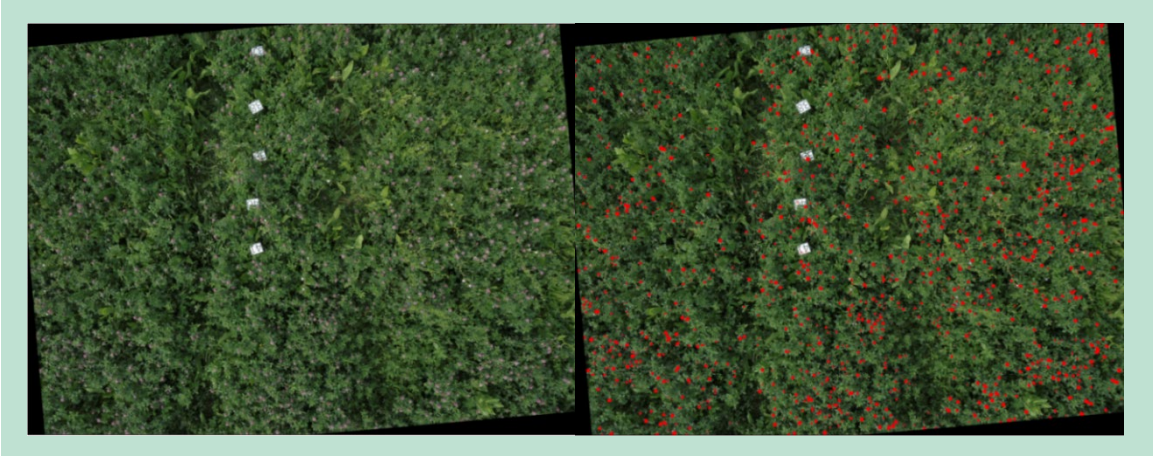

FIGURE 27. Mixed red clover community is (left) and the automatic identified flower heads (right)

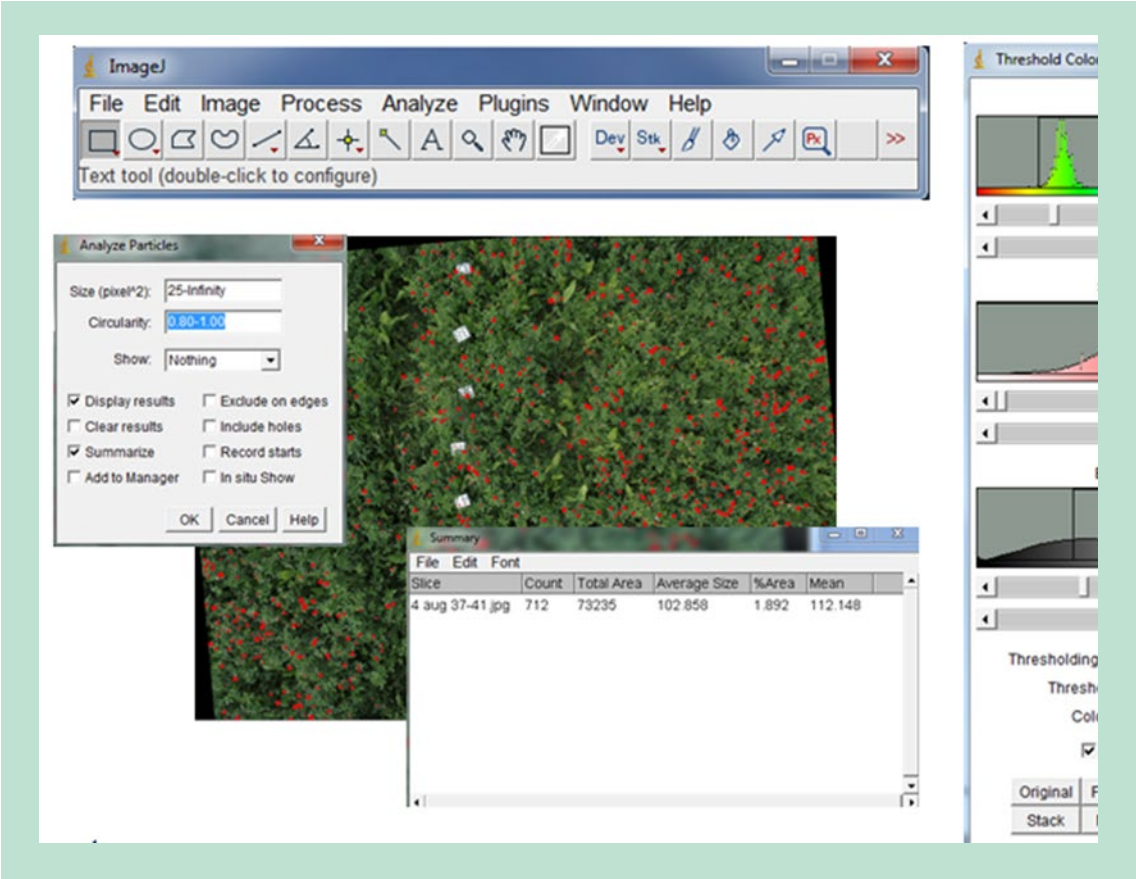

FIGURE 28. Flower identification by use of the free software program ImageJ. The threshold color using the HSB system is combined with a particle analyzer that defines the shape and size. The summary shows the flower head number, the average size and the fraction of total area occupied with flower heads

The output panel “Summary”, shown in the figure shows the counted number (712) of flower heads in the frame and the average size of each flower head in pixels (102.858) and the area fraction covered by flowers (1.892 %). The number of flowers in the frame and the size of them will obviously be dependent on several factors as the height above ground from where the picture is taken, then optics and the tilt (deviation from vertical). While the area fraction covered by flowers will be much more robust than number of flowers by being not so dependent on those factors. Consulting the pictures, it seems like the image analysis is rather successful to identify the flowers, however, this has to be validated by manually counted flowers. The image analysis may miss flowers or identify false flowers due to the misinterpretation in the algorithm. Furthermore, there can be hidden flowers in the vegetation. It is therefore important to validate image analyses to see how well a proxy the analysis is to manually counting’s. The result of a ground truthing using the red clover image analysis is shown in Figure 29.

A ground truthing will depend on many factors such as flower colour, light conditions and flower geometry of the plant structure that carries the flower. A ground truthing procedure should therefore be an integrated part of the data collections that delivers data to the indicator.

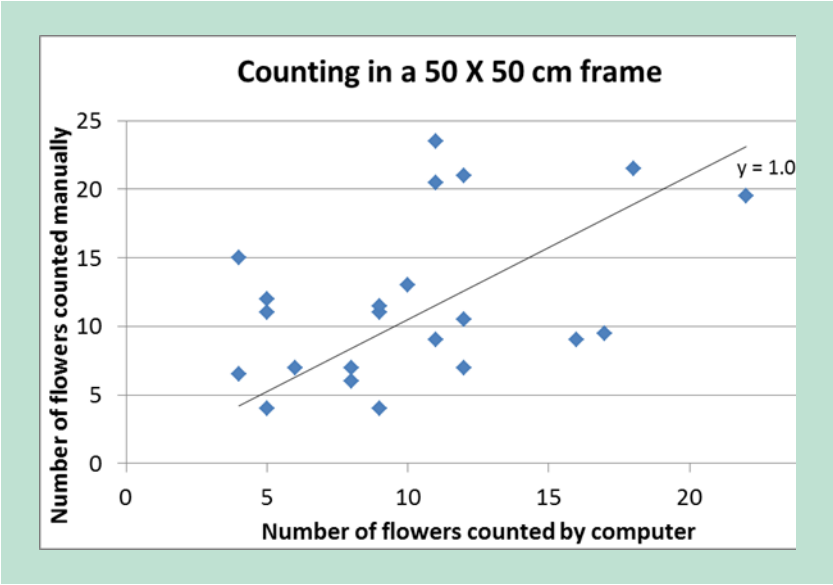

FIGURE 29. Ground truthing of flower heads counted by simple image analysis and manually in field

The number of flowers counted in a frame will depend of the area covered by the frame and this will again depend on the distance from where the frame is taken, the angel for the photographing and the optic of the camera. It will be rather complicated to secure standards for such recordings under the field conditions, so the flower numbers are instead converted into the fraction of area in the frame that is considered to be covered by flowers. This will be a much more robust measure to collect routinely in the field.

7.5.1 Drafting indicator inventory resources

The specific numbers in this paragraph is tentative in order to assess the magnitude of the effort, while a more detailed design should be made before application. Using the mandatory database for agricultural practice and the electronically spraying database, it will be possible to select field edges for the indicator, where a 200 m long edge could be standard. Two groups have to be selected: (1) field edges without pesticide application and (2) Field edges with pesticide application. The selection can be stratified into most grown crop types and region in the country. In each combination of crop type and region, a series of spraying (conventional) and non-spraying (organic) field edges are selected. A numeric example could be to divide into 5 regions and 5 crop types yielding 25 combinations. For each combination 10 edges are randomly selected as respectively non-sprayed and sprayed. This makes 500 field edges to be analyzed for flowering. A smaller investigation area (each of around 1000 km2) is selected in each of the five regions to represent the region in order to limit the need for driving. The field edges are selected to be rather easy assessable by a van.

An Unmanned Air System (UAS)-van is equipped for each investigation area and the sequence in recording is design to remove time bias in flowering phenology illustrated in Table 21.

TABLE 21. Systematic for the sequence of measuring to eliminated bias due to flowering phenology

Sequence	Crop j	
	Spray	Non spray
1	50 pictures	50 pictures
2	50 pictures	50 pictures

The field edge of 5 m X 200 m = 1000 m^2 can be measured. When flower intensity is recorded as a simple fraction of a flowering area flowering the image analysis will be robust without a need for stitching and/or creation of orthophoto so there is no need for a high degree of picture overlap. A realistic resolution of 1 mm^2 will generate need of 10^9 pixels to cover 1000 m^2. Thus a standard 20 MP camera can cover the 1000 m^2 by 50 pictures and it will take a UAS 10 minutes to do that operation in the air. The pictures in raw format will take 50 MB storage each, so one site will take 2.5 GB and the storage need for the whole country (500 sites) will be 1.25 TB. Each of the pictures will be analyzed to determine the area fraction on the picture of defined flowering themes. A flowering theme could be based on color or size/shape.

The UAS collection of pictures shall be followed by a procedure for ground truthing, where a number of frames, e.g. 10 frames of 1 X 1m is placed having unique Id visible on the recorded pictures and distributed in a way that there are covering different types of flowering. For each frame the number of flower heads is counted for each plant species. All other plant species in the frame are not recorded just the flowering species. The frame will be visible in the pictures and used for ground truthing and validation purposes. A string of a strong color is used to mark the precise edge of field and subsequently image analysis will cut away the field area on the frames before application of image analysis of flower identification in order to avoid counting flowers on the sprayed field area.

The following is rough time estimates: A realistic average time budget for two persons to drive to the field edge, set up 10 frames and string to mark field edge, count the flowers manually in the frames and let the UAS do an automatic flight in fixed highness to take pictures is 2 hours. So data from about three field edges can be collected by two persons in one day. The image analysis can define flowering themes (clustered in colors and shapes). Having the image analysis procedure the image analysis can be done every year on a standard computer for image processing.

7.6 Discussion

There are strong evidence for plant flowering being a promising indicator and the study confirms the tested hypothesis. The response is sensitive to herbicide exposure and it seems attractive to measure flowering intensity. However, more investigations are needed before a full indicator is ready. The need of ground thruthing has to be estimated based on pilot project data sets. The automated image analysis has to be developed based on existing methods. Therefore, the next steps should be a pilot project specifically addressing the capability of the indicator, and a more qualified design for the data collection based on the pilot investigations and subsequent analysis of image data to estimate flowering. The outline in this report is a first step illustrating the relevance of such a pilot project to develop an outstanding new indicator that addresses real and full-scale effects.

8. Overall discussion

8.1 Comparison of endpoints

For tests performed following standard test guidelines (OECD 2006a,b; USEPA 2012) biomass is the most frequently measured endpoint (EFSA PPR Panel 2014). However, when herbicides are applied to crops, NTTPs may be at vegetative or reproductive stages, and effects may be immediate or delayed. Based on available phytotoxicity studies (Olszyk et al., 2009, Riemens et al., 2009, Carpenter and Boutin, 2010, Strandberg et al, 2012, Carpenter et al., 2013) it has been concluded that reproductive endpoints (e.g. flower and seed production), in general, may be more sensitive than biomass, and thus effects on the whole life cycle have to be considered to properly assess effects on NTTPs (EFSA PPR Panel 2014, Boutin et al. 2014). Furthermore, when plants are exposed at developed/late growth stages (reproductive stage) vegetative growth has often ceased and therefore the sensitivity measured as effects on biomass results in unrealistically high ER10 or ER50 values (Strandberg et al. 2012).

The meta-analysis (Table 7) performed on data from the present study covering a broad spectrum of NTTPs (3 annuals, 6 perennials; belonging to 6 plant families) and herbicides (4 herbicides with different modes of action, 1 and 5% of label rates) confirmed that plants are generally most sensitive to herbicides at early life stages. For plants exposed at the 6-8 leaf stage compared to plants exposed at the bud stage, significant effects were found for biomass ($p = 0.007$) and number of germinable seeds ($p = 0.0099$) with effect size, Hedges' g, being larger for biomass (0.84) than for number of germinable seeds (0.40). However, this overall conclusion covers many different response patterns for individual cases (plant*herbicide) and it is evident that herbicide effects on many NTTPs may not be fully covered by short-term standard tests using vegetative endpoints, mainly biomass, as the only measured endpoints. For example significant effects were obtained on number of seeds but not on biomass for glyphosate and metsulfuron-methyl applied to V. arvensis at the bud stage. Furthermore, it has to be noted that plant flowering, was not included in the meta-analysis. Therefore, it is not possible to conclude about the sensitivity of this endpoint relative to other endpoints. See below for discussion of herbicide effects on flowering of NTTPs.

Additionally, the relevance of using biomass as an endpoint for environmental risk assessment (ERA) also needs to be questioned. Whereas risk assessment currently is carried out at the individual species level and only includes short term effects, the protection goals are defined at the population and ecosystem level, thus encompassing short-term (acute) as well as long-term (chronic) effects (EU 2009b, Nienstedt et al. 2012; EFSA PPR Panel 2014). In ERA, tests are performed in a tiered approach, with tests increasing in complexity with tier progression; simple single species tests are first conducted under controlled conditions using standard guidelines (OECD 2006 a, b; USEPA 2012). At higher tiers, multispecies community tests (i.e. microcosms, mesocosms or field trials) are requested on a case-by-case basis even though no standard protocol currently exists (EFSA PPR Panel 2014; Arts et al. 2015). In reality, higher tier testing is rarely required, nor provided, for ERA. Therefore, at the moment very little is known about the long-term herbicide effects on plant populations and communities, but see discussion below, and no measures to fulfil the gap between data and protection goals exists (EFSA PPR Panel 2014). A recent mechanistic model, IBC grass, developed by J. Reeg and co-workers at Bayer (Reeg et al. 2017) states that their model presents a promising approach to bridging the gap by extrapolating standardized pot experiments to the community level. However, the model has not been developed to include herbicide effects and thereafter need

validation. Until this is done, it seems questionable whether a model relying solely on individual species biomass responses can accurately simulate the many effects that may occur at the community level other than the effects on biomass of individual species.

8.2 Population level effects – plant competition matters

The fact that herbicide effect is influenced by intra- and interspecific competition, as observed in the present study for two annual NTTPs growing in conspecific and interspecific competition, demonstrates that single species tests should be used with caution in ERA. Results with individual species demonstrated that both S. noctiflora and C. cyanus were affected at the young vegetative stage and in their reproduction primarily when 5% glyphosate or metsulfuron methyl were applied. From the individual species results, however, it could not be ascertained or predicted that S. noctiflora was more sensitive than C. cyanus when growing jointly at different densities, and that the latter would likely outcompete the former when herbicide spray was added as an additional stressor. In fact, there is limited evidence that results from single-species tests can be used to make extrapolations to ecosystem responses (Cairns 1984). Therefore, including competitive interactions in ERA would improve protections of plant populations by predicting ecologically relevant outcomes following exposure.

While further experiments would be beneficial, this study along with a few others (Humphry et al. 2001; Riemens et al. 2008, 2009; Damgaard, et al. 2008, 2014; Dalton and Boutin 2010) suggests that the ERA guidelines, testing individually potted plants or monocultures (several individuals per pot), are not appropriate for making predictions on the effect of herbicides on wild plant populations and communities as a whole, as these tests cannot predict possible changes in community structure that may arise as a consequence of changes in, for example, competitive interactions.

Single species tests for protecting the environment are used because they are inexpensive, quick and simple, and they demonstrate clear dose-response patterns that are relatively straightforward to interpret. They are also easily standardized and more practical than tests involving multiple species. Although individual plants or single species tests may not provide ecologically meaningful results, there are still several limitations for requesting higher tier tests to be performed. Multispecies tests in microcosms, mesocosms or field trials are both time and resource consuming, and although the overall realism is increased, the ability to replicate and standardize the tests decreases (Dalton & Boutin 2010). Competition experiments using two species, as presented here, may be an acceptable compromise. In addition, this study and several others mentioned above demonstrated that measuring the effects solely at the young vegetative stage (biomass) was not sufficient to reveal the full impact of herbicides, and that effects on reproductive endpoints should be considered. Regulatory agencies should therefore work to establish a standard protocol involving plant-plant interactions in long-term experiments for higher tier testing to improve risk assessment. While EFSA is currently working towards developing higher tiered guidance, amending extrapolation factors to account for competition as well as the sensitivity of reproductive endpoints would also be useful; however, there is currently not enough information available to be able to adequately calculate such a factor (EFSA PPR Panel 2014).

8.3 Herbicide effects on plant flowering

Within the present study, herbicide effects on flowering of NTTPs were quantified under a variety of test conditions including:

1. Pot-grown experiments in greenhouses (9 NTTPs, 4 herbicides, 1% and 5% of label rate) (Chapter 3),
2. Annual NTTPs (two species, 2 herbicides, 1% and 5% of label rate) growing in conspecific and interspecific combinations exposed to two herbicides (Chapter 4),

3. Four perennials in an experimentally established vegetation exposed to glyphosate spray drift (Chapter 5), and
4. Plants (five most common herbaceous species) in stream buffer zones adjacent to herbicide treated agricultural fields (distance to field is used as proxy for exposure) (Chapter 6).

Cumulative number of flowers and time of peak flowering were used as response variables in studies 1 and 3, in study two only cumulative number of flowers and number of flowers per plant (measured five times during the season) was used in study four. Within all studies, significant reductions in flower production were observed as a response to the low herbicide doses evaluated. Though slightly more difficult to assess than total flower production, delays to peak flowering were present either as a statistically significant delay (study 1) or as a trend (studies 1 and 3) for individual cases (species*herbicide).

Overall, many species responded negatively to herbicide exposure, although, variability in individual species responses to herbicides in terms of total flower production and timing of flowering were observed in all studies. For study 1, significant negative effects on total flower production or non-significant trends (>10% reduction) were found in 57% of all cases with reductions spanning from 4% to 100%. Delays to peak flowering were present either significantly or as trends towards a delay in 40% of all cases. Across all life-stages the longest delays at 5% herbicide exposure were 9, 27, 22, 15 and 24 days for bromoxynil, ioxynil+bromoxynil, metsulfuron-methyl, clopyralid and glyphosate, respectively, for plants that survived. Simultaneous effects on both flower production and time of peak flowering occurred in approximately 25% of all study cases. Similar to what was observed for other endpoints, flower production and time of peak flowering were most affected when plants were exposed at the 6-8 leaf stage compared to the bud stage. In study 2, plants growing in two-species mixture with no herbicide revealed that *C. cyanus* was a better competitor than *S. noctiflora*. With herbicide (glyphosate) exposure, however, the competitive effect of both species was weakened and altered. Flower production, which could only be measured in *C. cyanus,* increased with an enhanced presence of S. *noctiflora* (higher ED10) but was little affected by *C. cyanus* conspecific interactions. It is as though, albeit less competitive than *C. cyanus, S. noctiflora* was protecting the other species from the effect of the herbicide glyphosate, likely due to overlapping of exposed areas and change in morphology of interacting species at different densities.

Although the effects of spray drift has been the focus of other studies (e.g. Marrs et al. 1991a; Gove et al. 2007; Boutin et al. 2014; Schmitz et al. 2013, 2014; Bohnenblust et al. 2016), the drift spray experiment (study 3) conducted as part of the PENTA project, to our knowledge, is the first study that includes 1) exposure to real spray drift, 2) assessment of exposure levels, and 3) effects on NTTPs. Herbicide exposure in the above-mentioned studies, has either involved direct overspray with low doses of herbicide (Schmitz et al. 2013, 2014) or real spray drift without accurate assessment of exposure (Marrs et al. 1991a, Gove et al. 2007, Boutin et al. 2014, Bohnenblust et al. 2016). In accordance with these studies, we found that the cumulative number of flowers was significantly reduced for two species (*T. pratense*, *L. corniculatus*) out of the four test species. Additionally, we observed a trend towards delayed flowering for three species (*T. pratense*, *L. corniculatus*, *C. intybus*). The sprayer was equipped with drift reducing nozzles, and consequently, the highest glyphosate dose deposited at the experimental area was 2.8% of the label rate (1440 g a.i. ha^{-1}). Despite the low glyphosate concentrations, we demonstrated that plant flowering was significantly affected by drift.

The results of the individual studies are discussed in detail in Chapters 3.5, 4.5.3, 5.5 and 6.5.

Although flowering of individual NTTPs responded differently, a growing body of evidence is showing effects of sub-lethal herbicide doses on plant flowering. Schmitz et al. (2013, 2014)

observed an 85% reduction in the flower intensity of *Ranunculus acris* within meadow vegetation, as well as negative effects on flowering of *Lathyrus pratensis* and *Vicia sepium,* when exposed to drift relevant doses of sulfonylureas herbicide formulation Atlantis WG containing mesosulfuron-methyl and iodosulfuron-methyl-sodium. In pot experiments, Boutin et al. (2014) found significant reductions in the cumulative number of flowers of *T. pratense* and *Taraxacum vulgare* that were exposed to low doses of fluroxypyr, as well as significant delays in flowering of a number of species including the previous and *Capsella bursa-pastoris*, *Anagallis arvensis*, *Helianthus strumosus*, and *Lobelia inflata*. The four latter plant species were exposed to glufosinate ammonium or chlorimuron ethyl. Only *Chenopodium album* responded differently by shortening the period to flowering at higher doses (Boutin et al. 2014). In addition to non-target species and weeds, the flowering of crops has also been shown to be negatively affected by sub-lethal herbicide exposure. For instance, Londo et al. (2014) found that glyphosate (56 and 112 g. a.i. ha^{-1}) delayed flowering of *Brassica* spp. and impaired plant reproduction.

The altered flowering of plants exposed to sub-lethal herbicide doses through spray drift may ultimately have negative consequences for individual plant populations as well as communities. Potential consequences including reduced seed production and thereby negative effects on plant reproductive fitness, altered species compositions, as well as cascading negative consequences for flower visiting insects, including pollinators, are thoroughly discussed in Chapter 3.5.

8.4 Using plant flowering as an indicator for herbicide exposure

The stream invertebrate indicator (Lies et al. 2005) was a first step towards a new generation of pesticide indicators where the indicators (species distribution of invertebrates) are directly responding to pesticide exposure. Flowering of all NTTPs within a habitat seems to be a sensitive and specific indicandum, i.e. effect measure responding to herbicide exposure, see Chapter 7.3 for discussion. One of the strengths of utilizing flowering of all species within a habitat as an indicator, is that this indicator is not dependent on the occurrence of specific species but instead on the functional response of the species currently present. One of the major problems of using invertebrates or other fauna elements as indicators is that they are mobile; species both move away as a response to the pesticide and then recolonize the habitat rapidly. Plants are much less mobile and also show a much slower resilience to disturbances (e.g. Milchunas and Lauenroth 1995). Therefore, responses are more easily detected in plant communities. Another advantage of flowering as an indicator is the ease in measuring flowering. Most plant species display their flowers above the canopy in order to attract pollinators, which can facilitate the counting of flowers either manually or by photometric tools.

Time-lag in responses to environmental change is well-known primarily as extinction debt following forcing events such as fragmentation or land-use intensification (Tilman et al., 1994; Jackson and Sax, 2010). However, earlier studies of plant flowering as a response to the establishment of herbicide-free buffer zones demonstrated that plant flowering responded positively the year of reestablishment (Strandberg et al. 2013).

9. Research perspectives

In accordance with most other studies, the PENTA project find that early vegetative stages (4-6 or 6-8 leaf stages), generally, seems to be the most sensitive growth stage. Additionally, this study also shows that biomass in many cases was as sensitive an endpoint as seed production and number of germinable seeds. However, depending on the plant, the herbicide and the growth stage at exposure we also show that reproductive endpoints including plant flowering were significantly affected in many cases. Furthermore, plant competition also was shown to affect species sensitivity. The importance of these findings for plant populations and ecosystems still need attention. However, the focus of future studies can be moved from detection of most sensitive growth stages towards investigating the importance of reduced number of flowers, seeds and fruit and reduced seed germinability for long-term effects on plant populations and communities. Additionally, the importance of reduced flowering for sustainable pollination and for pollinators need focus.

Expectedly, future ERA will also be based on standard plant tests. Therefore, it is important to focus on if and how data from standard test can be used for assessment of effects on populations and ecosystems. Recently, a mechanistic model that use sensitivity data from standard plant test to assess community effects have been developed (Reeg et al. 2017). Such models need focus: 1) they need to be validated using data from experimental studies (mesocosm and other multi-species experiments) or from natural- and semi-natural habitats, and 2) the challenging question about how effects on biomass can be translated into effects on plant reproduction also need attention.

The fact that plant flowering was affected by the low glyphosate concentrations in the spray drift experiments makes it likely that the exposure by small droplets in the spray drift has to be taken serious in future investigations. The glyphosate spray drift experiment also revealed the importance of revisiting exposure models as we found that distance to the edge of the field was not always a good proxy for the exposure of the adjacent habitat. Previously, flowering of hedgerow ground vegetation at conventional farms have been shown to be negatively affected presumably by herbicide spray drift (Boutin et al. 2014). The spray drift experiment support this assumption. However, assessment of plant flowering in several other habitats are needed to generalize about the effect. Furthermore, the importance of the reduced flowering for pollinating insect in agricultural landscapes also need attention. Additionally, flowering as an indicator for herbicide exposure need more focus to assess the quality of the indicator and to develop the feasibility of the analysis and interpretation.

10. Relevance for administration

One of the main drivers for the PENTA project was research questions formulated by EFSA (EFSA PPR Panel 2014) and the need for harmonised pesticide indicators formulated by EU in The EU Directive for Sustainable Use of Pesticides, SUD stk. 20, (EU, 2009a). Additionally, the lack of floral resources that are recognized as one of the major drivers for the continuous decline of pollinator diversity including bees, hoverflies and butterflies in agricultural landscapes have been an important motivation for the study of herbicide impacts on plant flowering.

The main results of the project, therefore, have direct relevance for administration of pesticides both at the national level and in the EU including authorities responsible for the ERA, i.e. EFSA and EU Directorate-General (DG) Agri and DG Environment, but also stakeholders in the fields of biodiversity and pollination e.g. The Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (*IPBES*).

The finding that low herbicide doses of herbicides have significant negative effects on NTTPs and biodiversity is important for the on-going revision of the Guidance Document on Terrestrial Ecotoxicology developed under Directive 91/414/EEC. Furthermore, it is highly relevant to include the project findings in the current work on landscape-level approaches to risk assessment.

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Appendix 1. Supplemental information

TABLE 2. Logistic data for each experiment

			Seedling stage		Bud stage		
Country	**Plant species**	**Sowing**	**Application**	**Harvest DW**	**Application**	**Harvest DW**	**Watering terminated**
DK	CENCY	12.06.2015	03.07.2015	31.07.2014	10.07.2015	07.08.2015	27.08.2015
DK	SILNO	24.06.2014	22.07.2014	12.08.2014	06.08.2014	28.08.2014	05.09.2014
DK	VIOAR	22.08.2014	29.09.2014	21.10.2014	13.10.2014	06.11.2014	02.12.2014
DK	TAROF	11.09.2014	31.10.2014	28.11.2014	13.03.2015	15.04.2015	16.06.2015
DK	CERAR	17.09.2014	12.11.2014	18.12.2014	27.03.2015	23.04.2015	27.05.2015
DK	KNAAR	20.10.2014	27.11.2014	23.04.2014	27.03.2015	23.04.2015	03.08.2016
DK	EPIMO	17.07.2015	03.09.2015	01.10.2015	14.09.2015	15.10.2015	22.10.2016
DK	CIRAR	05.09.2015	27.10.2015	04.12.2015	03.06.2016	06.07.2016	25.07.2016
CA	CENCY-CA	18.05.2016	07.06.2016	28.06.2016	20.06.2016	11.07.2016	06.09.2016
CA	CENCY-DK	10.11.2014	08.12.2014	29.12.2014	22.12.2014	12.01.2015	26.02.2015
CA	SILNO	24.11.2014	07.01.2015	28.01.2015	19.02.2015	12.03.2015	01.05.2015
CA	VIOAR	03.03.2015	07.04.2015	28.04.2015	15.04.2015	06.05.2015	15.06.2015
CA	TAROF	16.06.2015	09.07.2015	30.07.2015	09.03.2016	30.03.2016	09.05.2016
CA	CERAR	21.08.2015	17.09.2015	08.10.2015	01.10.2015	22.10.2015	24.11.2015
CA	TRFPR	21.04.2016	24.05.2016	14.06.2016	06.06.2016	27.06.2016	11.08.2016
CA	EPIMO	11.12.2015	07.01.2016	28.01.2016	02.02.2016	23.02.2016	08.03.2016
CA	CIRAR	08.12.2015	07.01.2016	28.01.2016	31.05.2016	21.06.2016	09.09.2016

Pesticide effects on non-target terrestrial plants at individual, population and ecosystem level (PENTA)

The aim of the project was to gain knowledge on which effects low doses of herbicides have on non-target plants, and which consequences this may have on populations and ecosystems.

The project consisted of four individual studies consisting of both lab. and field studies.

The lab. studies suggested that low doses of herbicides have significant effect on biomass, flowering, seed production, and germination. A reduction of the total number of flowers and a delayed time of flowering were observed.

The competition experiment suggested that competition among plants affected the pesticide sensibility. These effects were seen both for inter- and intraspecies competition.

The field experiment on wind induced pesticide drift using a field sprayer mounted with low-drift nozzles suggested that the low doses of glyphosate that were deposited in the neighboring habitat resulted in a significant reduction of the total number of flowers in two of tested four plant species. Furthermore, there was a tendency to a delayed time of flowering in three of the four tested plant species.

However, more information and additional studies are necessary before it will be possible to extrapolate from lab trials to effects on population levels. In addition, there is a need for more information on whether competition and delayed time of flowering may influence the ecosystems the plants inhabit.

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