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Deliverable 6.2 Report on risks of GM crops to bee pollinators and pollination services

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Introduction

The issue of the possible effects of genetically modified plants (GMPs) on pollinators has been the subject of several reviews. Malone and Burgess (2009) concluded that none of the commercially available GM crops expressing herbicide tolerance or insect resistance traits have deleterious impacts on pollinators. Using the approach of a meta-analysis considering 25 independent laboratory studies Duan *et al.* (2008) concluded that Cry toxins do not negatively affect the survival of either honey bee larvae or adults in laboratory settings. Arpaia *et al*., (2011) argue that the inclusion of additional measurement endpoints, e.g. foraging behaviour, are needed to assess possible effects not strictly linked to the toxic properties of the transgene.
The EFSA Guidance Document (GD) on environmental risk assessment of GMPs (EFSA, 2010) requires that the protection of species richness and ecological functions should be considered in the ERA. Particularly for field trials, estimation of ecosystem functions and services could complement or replace data on focal species.
The main aim of this deliverable is therefore to suggest methods to be used for estimating at field level differences and similarities of pollinator diversity and biology when foraging on GM crops in comparison to their conventional counterparts. These will be used to highlights potential direct risks of GM crops on focal pollinator species. The results of the activities reported here should provide support to environmental risk assessment and post market monitoring for pollinators and flower visiting insects, which constitute a relevant group of non-target organisms, as well.

In this document, we firstly reviewed and evaluated a series of existing methods used in the European scientific literature to estimate the overall pollination service, then for selected GM crops in different study regions, pollinator diversity was monitored with standardized methods developed in the EU-project ALARM.
As planned, we also report on the results of a field experiment setup in Germany and related to the possible effects of a stacked insect resistant maize event.
Further, special emphasis was given to bumble bees as an important group of non-target pollinators, which are purposefully added to commercial cultivation as enhancers of natural insect crop pollination and therefore could be particularly exposed to GM crops in commercial conditions. We evaluated flower visiting behaviour and possible preferences of GM crops or their isogenic conventional counterparts; this was studied with the specific goal of analyzing direct interactions between pollinators and transgenic plants. Finally, a new method for field assessment of possible exposure of bumble bees in a diversified landscape is proposed.

It was planned to anaylse a potential GM impact on pollination services and colony performance of bumble bees, by adopting methods from the EU-projects ALARM and STEP and honey bee risk assessment procedures developed by WUE in a previous national project, however we detected no significant effects of GM crop pollen on honey bees and thus the preconditions were not given.

Chapter 1. Reviewing the methods to assess pollination under field conditions

Ecosystem services are the benefits provided to humans by natural ecosystem processes and the species that are related to them (MEA 2005). The expansion of agriculture and particularly the use of pesticides produced negative consequences on the environment and human health (e.g. Pimentel, 2005). Pollinators are among the functional groups for which an environmental risk assessment is expected to be conducted by the applicants according to EFSA (2010).

To quantify ecosystem services, several different methods have been used. Traditionally, the "provider density reflect function intensity" approach dominated, which concerns the composition of various guilds or communities to measure changes in species densities within ecosystems and hence estimate the services associated with the ecosystem service in question. Thus, the efficiency of pollination was traditionally estimated by conducting census of pollinating insects. This method works under the assumption that the magnitude of pollination service will be proportional to the quantity of pollinating insects. However, the relationship between these is not linear, and this is prevailing to most ecological effects. Limitations of this approach were also highlighted by Bos *et al*. (2007). On the other hand, in a particular assemblage, the abundance of any species naturally fluctuates and the decline of a certain population might be compensated by another species within the same guild without adversely affecting functionality (Naranjo, 2005a, b). For example, the overall predation rate of a guild of predatory species could be selected as an assessment endpoint in field trials (Arpaia *et al*., 2009).

This review maps methods used to quantify pollination under field conditions in agricultural habitats in Europe.

Selection criteria

All experiments considered were field experiments, and all of them were conducted in an agro-environmental habitat. The few which were conducted in a semi-natural habitat consisted of grasslands which were being or had been grazed, and thus were also classified as agricultural land. One study was conducted in a garden of an experimental farm. Target plants that were not necessarily a crop were accepted, as long as they were placed in an agricultural context. The experiments were all located in Europe. All the selected studies used a method of measuring pollination or a proxy for pollination such as flower visitation.

Search strategy

Searches were done using the *Web of Science* database which also includes other databases such as CABI, Web of ScienceTM Core Collection, and the BIOSIS Citation IndexSM.

Two different searches were conducted, and the second one after a revision of the search string. The first search delivered 111 hits from which 52 were chosen for further evaluation (after the reading of abstract). The search terms were subsequently modified and the second search yielded 92 hits from which 34 were chosen for further evaluation.

The first assessment about the relevance of the paper was based on the titles. Those which referred to a measurement of pollination or pollinators were included. However, titles which referred to a non-crop such as ornamental trees and studies which were conducted outside Europe were excluded. In case of doubts the article was retained for further scrutiny.

The next evaluation step was made by reading the abstracts. If the studies were conducted in an agricultural environment and otherwise met the former selection criteria, they were included in the next step.

The next and last step of exclusion was after reading the full text of the articles. If the selection criteria were met the studies were included in the review. Several articles were excluded at this point as many of the selection criteria were not identified after reading the whole paper. After eliminating duplicates and performing the multi-step relevance evaluation, 20 papers were retained for this review.

Parameters measured. The studies all had various research questions which were grouped into seven groups: pollination effect on yield, habitat effects on pollinators/pollination, pollination affected by pesticides, the effect of abundance of pollinators on pollination, pollinator conservation, pollinator influences on or influenced by flower traits, and bumblebees and diet breadth.
Thirty different response variables were identified. They were classified as either indirect or direct measures of pollination.
Indirect measures of pollination included: no. of pollinators, visitation rates, no. of visits, no. of pollen grains on pollinator or stigma/anthers, no. of pollen collectors, no. of nectar collectors, nectar collection relative to no. of individual pollinators, pollen collection relative to no. of visits, and chance of pollen transfer.
The direct measures of pollination included: no. of seeds per fruit/pod, no. of fruits per flower, no. of seeds per plant, no. of seed sets, fruit mass, fruit mass/100 seeds, fruit mass/plant, fruit mass/capsule, or fruit mass/pod, no. of fruit sets, no. of pods or beans/plant, the proportion of fruit set, proportion of fruit set of flowers (%) or of visits (%), pod set (%), proportion of damaged ovules, and mean seed mass/ pod.
The 30 response variables (table 1) were further pooled according to their subject of interest: Visitation, abundance of pollinators, fruit mass, no. of seeds/fruits, no. of pollen grains, no. of pollen/nectar collectors, damaged ovules, chance of pollen transfer, nectar collection relative to no. of individual pollinators, pollen collection relative to no. of visits.

Results. In Table 1 the research questions of the studies are grouped to ease the comparison. Two papers focused on pollination effect on yield, eight papers focused on habitat effects on pollinators/pollination. One paper investigated pollination affected by pesticides and two others the effect of abundance of pollinators on pollination. The pollinator conservation was investigated by two studies. Pollinator influence on or influenced by flower traits was the main focus for three papers, and the last two studies focused on bumblebees and diet breadth.
Ninety per cent of the papers (18 papers) used an indirect measurement as a response variable to answer their research question. Fifty per cent used only an indirect response variable, 40 % used both direct and indirect measures (8 papers), while 10 % (2 papers) only used a direct measurement to answer their research question.

Table 1. A list of research questions, response variables and studies included in the review.

Research question	Response variables	Studies
Pollination effect on yield	Visitation	[2, 5]
	No. of seeds or fruits	[2]
	Fruit mass	[5]
Habitat effects on pollinators/pollination	Visitation	[1, 6, 8, 17, 20]
	No. of seeds or fruits	[1, 3, 6, 17, 18]
	Abundance of pollinators	[6, 14, 18, 20]
	Fruit mass	[6, 18]
	Chance of pollen transfer	[20]
	Nectar collection relative to individual pollinators	[20]
	Pollen collection relative to no. of visits	[20]
Pollination affected by pesticides	Damaged ovules	[15]
	No. of seeds or fruits	[15]
The effect of abundance of pollinators on pollination	Visitation	[12, 13]
	Abundance of pollinators	[12]
	No. of pollen grains	[13]
Pollinator conservation	Visitation	[9, 19]
	No. of seeds or fruits	[9, 19]
	Fruit mass	[9, 19]
	No. of pollen grains	[19]
Pollinator influences on or influenced by flower traits	Visitation	[4, 7, 16]
	Abundance of pollinators	[7]
Bumblebees and diet breadth	Visitation	[11]
	No. of pollen/nectar collectors	[10]

[1] (Albrecht et al., 2007); [2] (Albrecht et al., 2012); [3] (Andersson et al., 2014); [4] (Barbir et al., 2015); [5] (Bartomeus et al., 2014); [6] (Brittain et al., 2010); [7] (Campbell et al., 2012); [8] (Ebeling et al., 2011); [9] (Garratt et al., 2014); [10] (Goulson and Darvill, 2004); [11] (Goulson et al., 2008); [12] (Hanley et al., 2011); [13] (Hayter and Cresswell, 2006); [14] (Kohler et al., 2007); [15] (Lundin et al., 2013); [16] (Nuttman and Willmer, 2003); [17] (Power and Stout, 2011); [18] (Samnegard et al., 2011); [19] (Stanley et al., 2013); [20] (Woodcock et al., 2013).

Methods for collection of pollinators. This method was used in eight papers. The methods used were pan traps (3 papers), sticky traps (1 paper), plot collection (3 papers), and transect surveys (1 paper). Five papers only used one of the methods [1, 4, 6, 14, and 18], while two used two of them [7, 19].

Other methods to collect insects included the use of sweep nets, butterfly nets, and/or aspirators, and were conducted only during the day under weather conditions favourable to pollinating insects. Direct observations were used in 15 papers. The collection area was either a plot or transect of various lengths. The methods used were transect walks (7 papers), circular searches (2 papers), plot observation in open field (5 papers), and cage observation (1 paper). All papers only used one of the above techniques.

Exclusion of pollinators. Eight studies (40 %) used this method as a control treatment. The measured endpoint was insect pollination (two studies) [1, 17], difference in seed set [1, 6, 18] difference in fruit mass [5, 6] or a combination of the above (nine studies).

Pollinator guilds. Pollinators were grouped in various ways in the studies (Table 2). Sixteen studies recorded the pollinators by their order or species, while others (8 studies) grouped them according to different criteria (e.g. functional groups, sizes, or as morpho-species). Four of the studies focused on a species or an order, but still recorded other pollinators. Eleven studies recorded honey bees (*Apis mellifera*), 15 focused on bumblebees (*Bombus spp.*), 7 addressed solitary bees, and one study used the criterion of sociality. Hoverflies (Diptera) were recorded in 10 studies, whereas butterflies (Lepidoptera) were only recorded in 5 of them. In four studies flower visiting Coleoptera were detected. During two studies wasps were recorded. Bees in general were a main focus in three studies. Seven studies also recorded other pollinators or groups of pollinators such as Diptera other than hoverflies, other wild bees, and moths.

Table 2. The list of studies and the number of pollinator groups considered.

Number of pollinator species or groups recorded	Study
1	[3, 10, 11, 12, 15]
2	[13, 14]
3	[7, 16, 17, 19, 20]
4	[4, 5, 6]
>4	[1, 2, 8, 9, 18]

[1] (Albrecht et al., 2007); [2] (Albrecht et al., 2012); [3] (Andersson et al., 2014); [4] (Barbir et al., 2015); [5] (Bartomeus et al., 2014); [6] (Brittain et al., 2010); [7] (Campbell et al., 2012); [8] (Ebeling et al., 2011); [9] (Garratt et al., 2014); [10] (Goulson and Darvill, 2004); [11] (Goulson et al., 2008); [12] (Hanley et al., 2011); [13] (Hayter and Cresswell, 2006); [14] (Kohler et al., 2007); [15] (Lundin et al., 2013); [16] (Nuttman and Willmer, 2003); [17] (Power and Stout, 2011); [18] (Samnegard et al., 2011); [19] (Stanley et al., 2013); [20] (Woodcock et al., 2013).

The investigation of the effect of abundance of pollinators on pollination, found that twice as many bumblebees visited crops adjacent to mass flowering crops while no significant difference was found in bumblebee visitation between crops in the post-flowering period [12].

Discussion. The twenty studies evaluated in this review have used various methods to measure pollination. Collection and observation of insects were classified as indirect measurements of pollination, whereas collection of fruits and seeds were considered direct measurements.

Collection of pollinators can be done by using traps or by sweep nets, a more time consuming method. These methods allow the description of abundance and diversity of pollinators. Direct observation of pollinators can be very time consuming dependent on the number and sizes of observation areas, and the time needed to effectively monitor specimen. The method of collection by trapping is thorough in collecting species that are difficult to observe, but on the other hand it has the disadvantage that it collects also species that are not effective pollinators.

Direct measures of pollination cannot be obtained before the fruits begin to develop. Collection of fruits for examination should be done before they ripe and the seeds get dispersed. There a various response variables connected to measures on fruits. It could be on fruit mass or number of fruits or seeds. Proportions can be calculated to find out how many flowers developed fruits or how many seeds was developed per fruit.

It is noteworthy that only a few of the twenty studies [2, 3, 5, and 15] in this review adopted exclusively direct measurements of pollination.

Due to the adoption of many different methods to measure pollination, it is rather difficult to compare measurements taken applying different methods and across habitats. This is the reason why a standardized method is needed. This attempt was initiated by Meyer *et al*, (2015) who proposed a Rapid Ecosystem Function Assessment (REFA), which describes a series of methods developed for measuring ecosystem functions in a rapid, easy to use, low-tech, repeatable and cost efficient way. The authors summarized this approach in a review of numerous studies of ecosystem functions, to suggest the ones that are most applicable for REFA. The selected REFA proxy for pollination is, however, not a direct measurement of pollination. Pollinator abundance is in fact the approach chosen to estimate pollination in REFA.

Recommendations

1. Indirect measurements of pollination.

The relative performances of methods for collecting pollinators in field conditions have not been systematically evaluated and compared. In response to the strong need to record ongoing shifts in pollinator diversity and abundance, global and regional pollinator initiatives must adopt standardized sampling protocols when developing large-scale and long-term monitoring schemes. Standardized methods from the EU-project ALARM (Westphal *et al*. 2008) for the assessment of pollinators in the field, were selected by analysing data sets from different European biogeographic regions (see also AMIGA Deliverable 6.3). Westphal *et al*. (2008) systematically evaluated the performance of different sampling methods (observation plots, pan traps, standardized and variable transect walks, trap nests with reed internodes or paper tubes) that have been commonly used across a wide range of geographical regions in Europe in two habitat types (agricultural and semi-natural). The authors focused on bees since they represent the most important pollinator group worldwide. Several characteristics of the methods were considered in order to evaluate their performance in assessing bee diversity: sample coverage, observed species richness, species richness estimators, collector biases (identified by subunit-based rarefaction curves), species composition of the samples, and the indication of overall bee species richness (estimated from combined total samples). The most efficient method in all geographical regions, in both the agricultural and semi-natural habitats, was the pan trap method (Fig 1). The method had the highest sample coverage, collected the highest number of species, showed negligible collector bias, detected similar species as the transect methods, and was the best indicator of overall bee species richness.

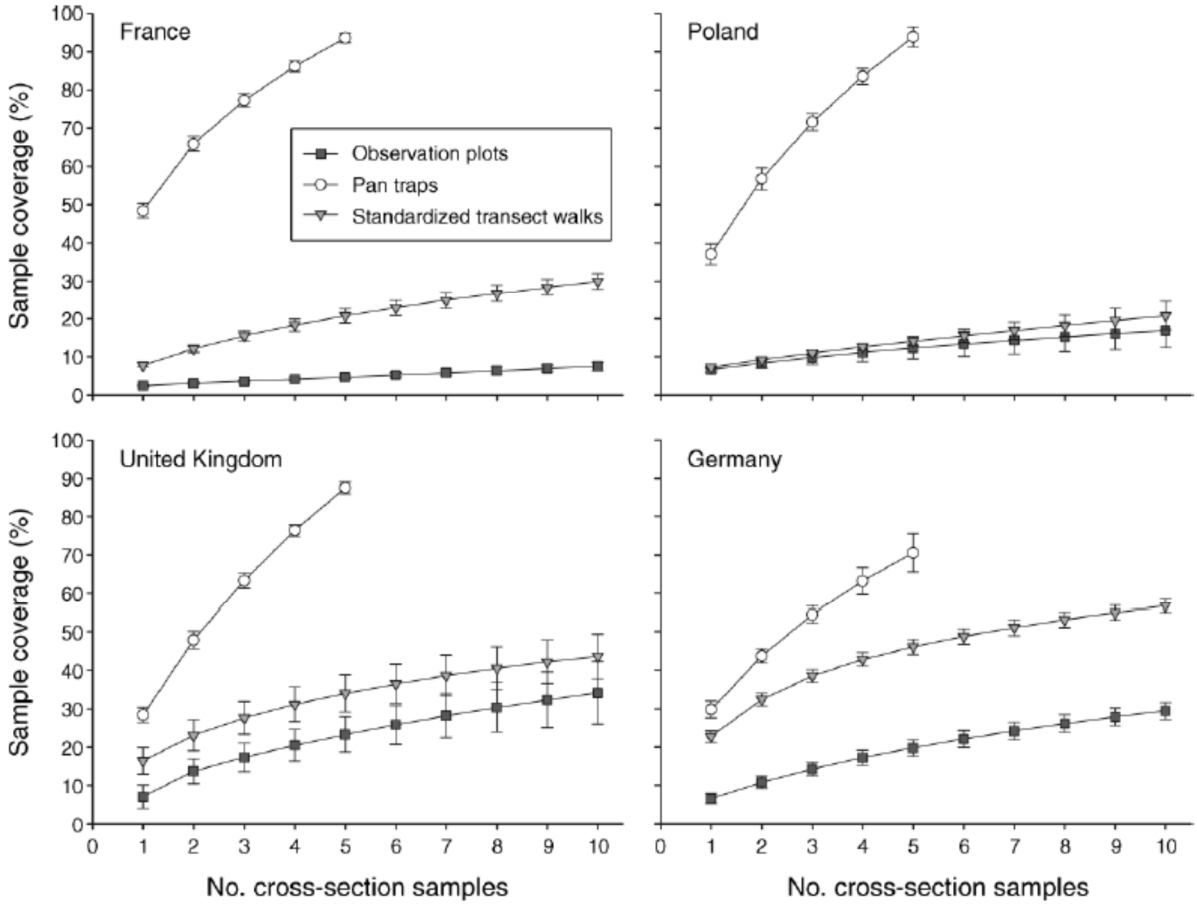

Fig. 1: Subunit-based rarefaction curves for the observation plots, pan traps, and standardized transect walks that were tested in the agricultural study sites (N = 4 sites) of four European countries. The sample coverage of rarefied cross-section samples is given (mean 6 ± SE). One cross-section sample represents the cumulated species numbers found during all surveys in one specific subunit (i.e., specific plot, 5-min interval, or pan trap cluster; reprint from Westphal et al., 2008).

The transect methods were also relatively efficient, but they had a significant collector bias. The observation plots showed poor performance. As trap nests are restricted to cavity-nesting bee species, they had a naturally low sample coverage. However, both trap nest types detected additional species that were not recorded by any of the other methods.
Monitoring data on focal pollinator species of different European agricultural landscapes are also included in Deliverable 6.3
For large-scale and long-term monitoring schemes we therefore recommend pan traps as the most efficient, unbiased, and cost-effective method for sampling bee diversity. In addition, relatively low level of expertise is needed. Trap nests with reed internodes could be used as a complementary sampling method to maximize the numbers of collected species. Transect walks are the principal method for detailed studies focusing on plant–pollinator associations. They could be also used in monitoring schemes, but an accurate training of the surveyors is needed to obtaining personnel with the required skills (Westphal *et al*., 2008).

The applicability of this monitoring method was also proved during an additional study, providing baseline quantitative data on bee abundance and diversity in agro-ecosystems, was performed in Scania, Sweden. These variables, were investigated using pan traps in conventional maize fields in Scania, as well as in Amiga trial sites for non-target organisms and for Integrated Pest Management with GM and conventional maize. Since maize

represents a rather new crop for Sweden, this list constitutes the first report of pollinating species active in maize agro-ecosystems in the country.

The study was performed in 2012, 2013 and 2014 in the Scania region, south Sweden. The fields were spread across the county in order to cover a broad range and represent the variety of landscape. In 2013 and 2014 respectively 5 and 3 conventional maize fields were selected for the experiment. In 2012-2014 the NTO trial was investigated, and in 2013 and 2014 the IPM trial. The GM varieties were maize DKC 4442 YG in 2012 and maize DKC 3872 YG in 2013 and 2014.

SITES	GPS coordinate		Field	Mais	2012	2013	2014
BORGEBY	55.7530496	13.0481294	GM Trial	Silage	x	x	x
ANDERSLÖV	55.4139156*	13.3487309*	GM Trial	Silage		x	x
VIKHÖG	55.7417495	12.9931158	Conv	Silage		x	x
BERGSJÖHOLM	55.4540111	13.7729222	Conv	Silage		x	x
GISLÖVSHAMMAR	55.4880813	14.3023396	Conv	Silage		x	x
DALBY	55.6866538	13.322281	Conv	Silage		x	x
VANSTAD	55.611662	13.8053454	Conv	Sweet maize		x	x
HÖKÖPINGE / VELLINGE	55.4983611	13.0535526	Conv	Silage		x	
LÖBERÖD	55.7570648	13.5388121	Conv	Silage		x	

*Table 3: List of the conventional sites (Conv) and genetically modified trials (GM) including their GPS location and type of maize grown. * In 2014 the field was moved 500 meter away from the previous year 55.413901; 13.345492.*

Pan traps were set up in 10 out of the 20 plots (5 in GM and 5 in non-GM maize) in the NTO-trial (Borgeby in Tab. 3). The design was maintained for 2013 and 2014. In the IPM trial (Anderslöv in Tab. 3), 6 pan traps were set up in 2013 and 10 pan traps in 2014. (During 2013 the GM-IPM plots were only available for the June and July trapping dates.) Pan traps were also set up in 7 conventional maize fields in 2013 and in 5 conventional fields in 2014. The fields in Bergsjöholm, Dalby, Gislövshammar, Vanstad and Vikhög were sampled both years whereas Löberöd and Vellinge were only sampled in 2013. Each conventional field had a single window trap located in the middle of the field. In the trial fields, the window traps were placed in the middle of each randomly selected plot.
Each pan trap consisted of a 40 × 20 cm transparent plastic sheet supported by 2 wooden poles (Fig. 4). The upper edge of the window was at a height of approximately 180 cm. A rectangular white plastic container (pan) with 50-70% propylene glycol was suspended below the window.
The sampling period was from 23 July to 22 October 2012 with 2 week trapping periods. In 2013 and 2014 the sampling period was standardized from June to September with 1 week trapping periods. The samplings were from 01 July to 12 September 2013 and from 17 June to 12 September 2014. In total 6 samplings were collected in 2012 and 4 samplings per year in 2013 and 2014.
The collected bees were sorted and fixed in 70% ethanol and identified to the genus level under a dissecting microscope according to Baldock and Collins (2008). Once identified and sexed bees were pinned in insect boxes, until their identification to the species level.

Results

A total of 890 bees representing 10 genus and 31 species were collected during the 3 years sampling (Table 4). *Apis mellifera* was the most abundant species, accounting for 49,33% of the total collection. The other 3 most abundant species were *Bombus terrestris* (24,04%),

Lasioglossum morio (7,42%) and *Lassioglossum leucopus* (4,72%).

Table 4: Overview of the abundance and the number of species and genus caught by the window traps during the 3 years experiment in Scania, Sweden; all locations included.

Genus	#sp	Species	2012	2013	2014	
Apis	***1***	*Apis mellifera*	298	80	62	
Andrena	***5***	*Andrena fucata*	0	0	1	
		Andrena haemorrhoa	0	0	4	
		Andrena nigroaenea	0	0	27	
		Andrena semilaevis	0	1	0	
		Andrena semilaevis	0	0	1	
Bombus	***7***	*Bombus hortorum*	1	2	1	
		Bombus hypnorum	1	0	0	
		Bombus lapidarius	2	5	5	
		Bombus muscorum	0	1	0	
		Bombus pascuorum	0	2	2	
		Bombus sylvarum	2	2	1	
		Bombus terrestris	34	63	117	
Colletes	***1***	*Colletes daviesanus*	1	1	0	
Halictus	***2***	*Halictus rubicundus*	0	0	2	
		Halictus tumulorum	2	2	0	
Heriades	***1***	*Heriades truncorum*	0	1	0	
Hylaeus	***2***	*Hylaeus angustatus*	1	5	0	
		Hylaeus brevicornis	0	1	0	
Lasioglossum	***10***	*Lasioglossum aeratum*	0	0	4	
		Lasioglossum calceatum	0	0	22	
		Lasioglossum lativentre	0	0	9	
		Lasioglossum leucopus	12	8	22	
		Lasioglossum minutissimum	1	0	0	
		Lasioglossum morio	2	33	31	
		Lasioglossum nitidiusculum	0	1	0	
		Lasioglossum semilucens	0	1	0	
		Lasioglossum villosulum	0	0	1	
		Lasioglossun calceatum	0	10	0	
Sphecodes	***1***	*Sphecodes pellucidus*	0	0	1	
Sphecidae	***1***	*Sphecidae crossucens*	0	1	0	
TOTAL		**31**	**357**	**220**	**313**	**890**
		Species diversity	**12**	**19**	**18**	

The abundance of bees differed between years. In the Non-target Organism trial site (Borgeby), more bees were caught in the early season in 2012 than in 2013 or 2014 (Fig. 2). This could probably be explained by the high abundance of *Apis mellifera* in 2012 (Fig. 2, Tab. 4).

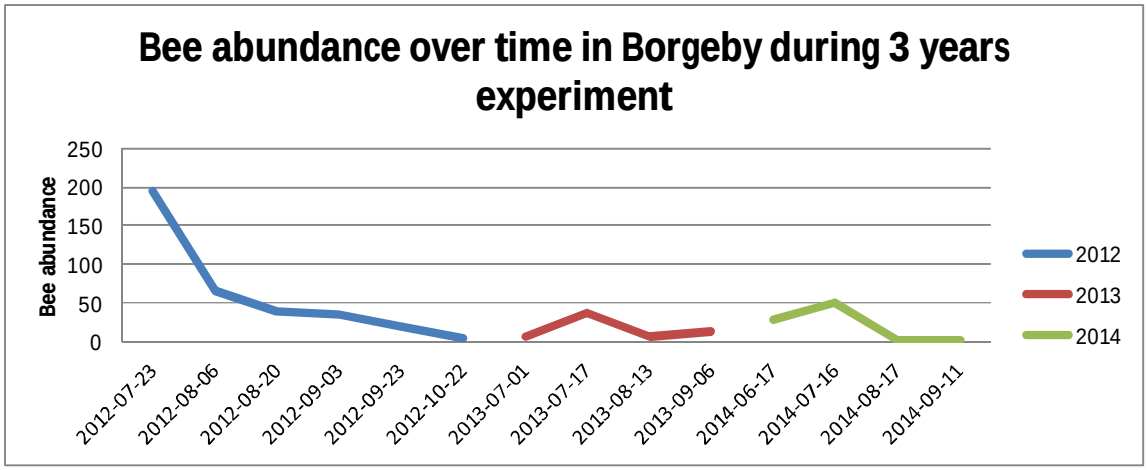

Figure 2: Total bee abundance (no. individuals) in 10 window traps in the Non-Target organism trial at Borgeby, Sweden.

In the conventional fields, 209 bees representing 19 different species were caught in the pan traps (Fig. 3). In both years, fewer bees were caught in August and September. There was clear variation between years, and in particular more bees were caught in July 2013 than in July 2014 (Fig. 3).

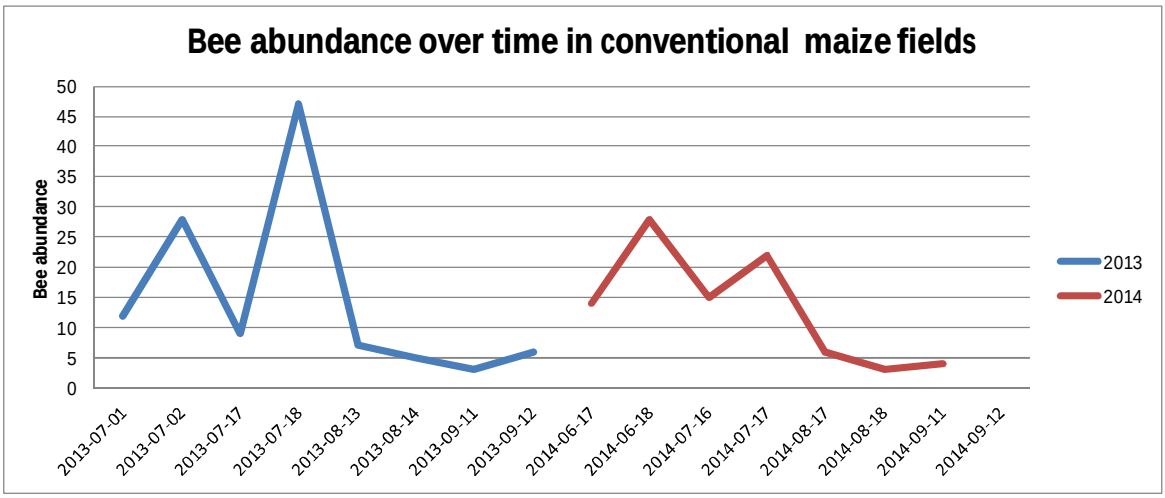

Figure 3: *Figure 3:* Abundance of bees in conventional fields in Scania, Sweden in 2013 and 2014.

Figure 4: View of pan trap in Amiga maize field in Scania, Sweden.

The recommendations of this report therefore suggest the use of pan traps as an indirect measurement of pollination activities and in this respect we agree with the conclusions by Meyer *et al*. (2015). Moreover, this method, being cost effective and with limited need of specialized field biologists, could be easily transferred to support post market environmental monitoring activities.

2. Direct measurements of pollination.

In an environmental risk assessment, following the outcomes of the problem formulation, and where data from several field trials available, it might be useful to consider endpoints directly targeted to measure pollination activity.
Considering the results of the review, we propose a method which compares data from open pollinated plots and control plots where insect pollination has been excluded. The exclusion however should be provided with nets with mesh-sizes that do not impair wind pollination.
As a reliable measurement endpoint related to pollination, several authors suggested the use of fruit biomass. Within the advantages of using this variable, there is the practicability of the method for which there are no requirements to record the size or the numbers of seeds.
the selection of standardized methods create a need for collecting empirical data which should ideally be made available from databases to constitute a historical record of baseline variability, and therefore make future review studies easier to perform(Meyer *et al*., 2015).

Chapter 2. Monitoring foraging behavior of pollinators

Introduction.

According to Malone and Burgess (2009) GM crops currently grown have no negative impacts on pollinators. Available scientific literature however, is mostly based on tests aimed at detecting acute or lethal effects. While mortality is obviously a main life history factor to be measured, it is important to consider that sub-lethal effects alone can even drive arthropod populations to extinction (Hallam *et al.*, 1993). The commonly used median lethal dose calculated during acute toxicity tests, may then represent only a partial measure of possible deleterious effects due to the tested compounds, since it is known that, e.g., pesticides, can adversely affect learning performance, behaviour and neurophysiology (Desneux *et al*., 2007).

Enzyme inhibition with physiological effects on honey bees due to pesticides were demonstrated, e.g., by Pilling *et al* (1995), Bendahou *et al* (1999). Behavioural perturbations may be particularly harmful for social hymenoptera like honey bees or bumble bees; for instance negative effects of deltamethrin were detected by Vandame *et al*. (1995) in the homing ability of honey bee foragers. Disruption in the ability to locate food source may occur because chemicals may reduce olfactory capacity in *Apis mellifera* L. adults (Decourtye and Pham-Dèlegue, 2002).

Therefore, measurement endpoints such as development, growth, fecundity, fertility, feeding behaviour, etc. need to be considered to predict possible environmental effects on pollinators and complement risk assessment (Andow *et al*. 2006).

The assessment of sub-lethal effects of transgenic products on honey bees was addressed in Babendreier *et al*. (2005) who used the development of the hypopharyngeal glands as measurement endpoint. Authors found that there was no difference in diameter and in weight between bees fed either Bt pollen or Bt-containing sugar solutions and their respective controls.

Ramirez-Romero *et al*. (2008) indicated that feeding behaviour of honey bees was affected only when exposed to extremely high concentrations of Cry1Ab toxin (5000 ppb), inducing bees to slowly imbibe the contaminated syrup. However, results may be different when different transgenic products are expressed in GM plants. For instance Han *et al* (2010) discovered sublethal effect on feeding behaviour of honey bees fed Cry1Ac + CpTI-expressing cotton pollen (event CCRI41) during 7-day oral exposure.

Social bees collect relevant amounts of pollen as a food for their colonies from several crop plants which may contain, in case of insect-resistant GM plants, sensible amounts of toxins (Hellmich *et al.* 2001; Mendelsohn *et al.* 2003). The most recent European guideline for assessing possible effects of field release of GM plants on the wider biodiversity indicates that it is necessary to identify the ecosystem functions and services and the guilds of species providing these services in production systems (EFSA 2010). A particular requirement introduced in the EFSA Guidance Document is the consideration of the GM plant, in addition to the introduced traits, as a potential environmental stressor. Therefore the interactions between GM plants and non-target organisms need to be evaluated in experimental setups where plants or their parts are included. For this reason, the collection of relevant data on GM plants-pollinators interactions in a pre-commercial phase may offer important information in estimating the potential impact of GM plants on this functional guild.

Arpaia *et al*. (2011) found a tendency of bumblebees to prefer flowers on GM eggplants compared to the near isogenic line. Their results indicate that those plants represented an attractive food source for the selected pollinators and that control and modified plants might be “perceived” as different.

In this study the feeding behaviour of bumblebees was monitored in two different conditions (laboratory observations and experimental field) to validate the reliability of this method for environmental risk assessment.

2.1 Laboratory studies

The availability of a standardized method for measuring pollinators' behaviour in controlled conditions, could allow early detection of possible disturbance of pollination activity during environmental risk assessment of new genetically modified events. In order to test the effectiveness of laboratory methods for this scope, our approach was the selection of known attractors for bumblebee foragers to be assessed in different arenas to verify the sensitivity of the experimental setup.
Laboratory experiments were performed using three different methodologies:

1. *In vitro* walking behaviour in presence of odour source.
2. Observation on micro colonies in wind tunnel.
3. Observation of individual flight behaviour in wind tunnel.

The first two methods were discarded due to the limitations already described in the second periodic report (cfr. Deliverable 1.2). In the same report, we also described the preliminary experiments conducted in wind tunnel and the necessity to improve bees' responsiveness.
In the successive months, experiments for monitoring flight behavior in wind tunnel were conducted and the tunnel was modified in order to allow a different access to the arena for individual foragers which remained connected to their colony during assays (Figure XXX).
Two types of experiments were conducted where the attracting source was constituted by: a) artificial flowers with the addition of odour and food, or b) flowering plants of different species known to be visited by bumblebees in field conditions (e.g. *Salvia officinalis, Brassica* spp., *Lavandula* spp., *Verbena* spp.,).

Each experiment was carried out with a single bumblebee (*Bombus terrestris* L.) colony for 10 days. Daily observations were conducted, one hour in the morning and one hour in the afternoon. The colony was open to allow bees to freely enter the wind tunnel and closed when 6 bees were present in the arena simultaneously in order to have a manageable number of specimen on which direct observations could be conducted. The bees were then separated by the colony so that additional observations were conducted on unexperienced individuals.

Artificial flowers were made from yellow paper. Six flowers were present in the tunnel, in three of them (treatment) an Eppendorf vial (2ml) filled with a sugar solution was put in the centre and a few drops of an alcoholic solution of limonene were added to the petals. Three artificial flowers without food and odour represented the alternative choice (control).

In experiments with flowering plants, two individual plants of each of the species indicated above were introduced in the wind tunnel and weedy flowerless plants provided the alternative choice for foragers.

Foraging behaviour was analysed with the use of a laptop on site as an event recorder, the sequence of behaviours was analysed using the software "The observer XT" (Noldus Information Technology, The Netherlands). The observation method chosen was of live observations and a continuous observation method. The independent variables recorded were the observer, the date and time.

The list of behaviours is presented in table 5

Table 5. List of behaviours monitored.

Type of behaviour	Further specification	Mutually exclusive
Walking		Yes
Flying	Towards plants or Opposite direction	Yes
Landing on plants	Moving or Still	Yes
Landing on flowers	Feeding or Still	Yes
Continue feeding	Same flower, Different flower or Different plant	No

The initial state was set by default as: still. In experiments where artificial flowers were used (with or without addition of scents on petals) the landing behaviour was recorded without further distinctions.
Our measurement endpoint was that of *oriented flights*, which were defined as those events during which flying behaviour was directed toward plants (or artificial flowers), and concluded with landing on them without intermediate stops. The count of oriented flight was done at the end of all the observation period, analysing the single records for the entire period and counting those events in which an oriented flight was noted.

Results.

The overall response of bumblebees was rather poor. In 32% of the cases (experiments with plants) or 14% in the case of artificial flowers a bee reached its target. Moreover, less than 20% of these were classified as oriented flights. In most cases, in fact bees ended up on target after stopping, or by walking or changing direction. Due to the overall low response, no further examination of single behavioural elements was therefore performed.

2.2 Field studies

Experiments were conducted in July 2015. Two bumblebee (*Bombus terrestris* L.) colonies were released in the centre of the potato experimental field in Carlow (IE). The experimental field design was a randomized block with three treatments 5 replicates/treatment, plot size (20-30m x 30m). Treatments were the following:

- No spraying against potato late blight;
- Current practice i.e., weekly application of preventive fungicides for late blight control;
- IPM 2.0 control strategy, low fungicide input, monitoring virulence and treatments according to the outcomes of a Decision Supporting System based on input from observations on sentinel plants and meteorological data.

Potato plots started flowering in the last decade of July. Behavioural observations were conducted as indicated for laboratory experiments and the list of behaviours was slightly modified as indicated in Table 6. Monitoring was conducted on single plots with a “double blinded” approach; observations started from 10 AM every day and each plot was observed for 30’. Field observations were conducted in three consecutive weeks.

Table 6. List of behaviours monitored.

Type of behaviour	Further specification	Mutually exclusive
Approaching flower		Yes
Flying	Towards plants or Opposite direction	Yes
Landing on plants	Moving or Still	Yes
Landing on flowers	Feeding or Still	Yes
Continue feeding	Same inflorescence, Different inflorescence or Different plant	No

The initial state was set by default as: no bees on the plot. The measurement endpoint was the *number of visiting bees* recorded on each plot in the 30' time limit.

Results.

Bumblebees responded poorly to all different treatments. Activity was generally low, also due to the low air temperature, and never more than 3-4 bees flying over the entire field were observed in an observation round. The majority of active foragers preferred visiting flowers of *Trifolium*, almost the unique alternative food source in the area during potato flowering. The mean number of visits in potato plots was 0,085 (±0,012) with no apparent differences between treatments, though the low number of data does not allow a correct statistical comparison.

Conclusions.

The analysis of foraging behaviours of bee colonies is considered an important indication of colony health and activity. However, the response of such observations is very variable according to the plant species used as food source, the weather conditions and the environment where observations are being conducted. Potato plants used in this study proved to be a poor attractor for foraging *B. terrestris* individuals while the same experimental trials in choice situations where performed in the past with the same bumblebee species and different crop plants (i.e. Arpaia *et al*., 2011; 2012).

Studies in laboratory conditions were apparently not suitable to trigger the usual foraging activity of the colonies. The laboratory setup of wind tunnel showed technical flows and therefore its applications are not reflecting the natural foraging behaviours of bumblebees.

One possible reason of the low response in the field, might be due to the limited attractiveness of potato plants themselves, as observed also in behavioural experiments with parasitoids, during work package 5.

We conclude that the experiments conducted here aimed at evaluating foraging behaviour of bumblebees on genetically modified crops, can not be considered for routine environmental risk assessment programs. The response might be different in other plant/traits combinations when the crop is known to be highly attractive for pollinators (e.g.

tomato, canola, eggplant, etc.); therefore in those cases if previous phases of ERA have indicated a potential threat to this group of non-target organisms ad hoc experiments might be planned.

Chapter 3. Honey bee colonies exposed to flowering Bt maize: the impact on nurse bees and their gut bacteria

One of the aims of this task was to move from laboratory or mesocosm studies to some measurement under real field conditions. To do so, we chose to evaluate possible risks for honeybees in a field experiment using a Bt-expressing maize event.
Biosafety research on genetically modified crops rarely considers effects on nurse bees from intact colonies, even though they receive and primarily process the largest amount of pollen. The objective of this study was to analyse the response of nurse bees and their gut bacteria to pollen of flowering Bt maize expressing three different insecticidal Cry proteins (Cry1A.105, Cry2Ab2, and Cry3Bb1).

Materials and Methods

AMIGA partner von Thunen Institute established a 6-ha experimental maize field in Braunschweig Germany, which consisted of 40 randomized plots (30 m x 42 m) of which 24 were used in this study. These plots were part of a randomized plot design and included three different maize varieties (''treatments''). The genetically modified Bt maize was the hybrid MON 89034 6 MON 88017 (indicated here as ''treatment'' BT) in the genetic background of the conventional variety DKC 5143. The other two maize varieties were DKC 5143 with no genetic modification (treatment DKC) and Benicia (BEN). Five days before the onset of anthesis (August 1st, BEN; August 8th, BT and DKC), artificial swarms of *Apis mellifera* carnica were prepared from one breeding line (Institute for Apiculture Celle). Each new colony contained one queen with approximately 1,100 workers (122.9 g bee biomass 67.2 SD, n= 49 colonies). All queens were sisters mated with a controlled drone population.
Colonies of *Apis mellifera carnica* were kept during whole anthesis in flight cages on field plots with either the Bt maize, or the two different conventionally bred maize varieties, and without cages, 1-km outside of the experimental maize field to allow ad libitum foraging to mixed pollen sources.

Results

During their 10-days life span, the consumption of Bt maize pollen had no effect on survival rate, body weight and rates of pollen digestion compared to the conventional maize varieties (Figure 5). As indicated by ELISA-quantification of Cry1A.105 and Cry3Bb1, more than 98% of the recombinant proteins were degraded. Bacterial population sizes in the gut were not affected by the genetic modification. Bt-maize, conventional varieties and mixed pollen sources selected for significantly different bacterial communities which were, however, composed of the same dominant members, including Proteobacteria in the midgut and Lactobacillus sp. and Bifidobacterium sp. in the hindgut. Surprisingly, Cry proteins from natural sources, most likely *B. thuringiensis*, were detected in bees with no exposure to Bt maize. The natural occurrence of Cry proteins and the lack of detectable effects on nurse bees and their gut bacteria give no indication for harmful effects of this Bt maize on nurse honey bees.

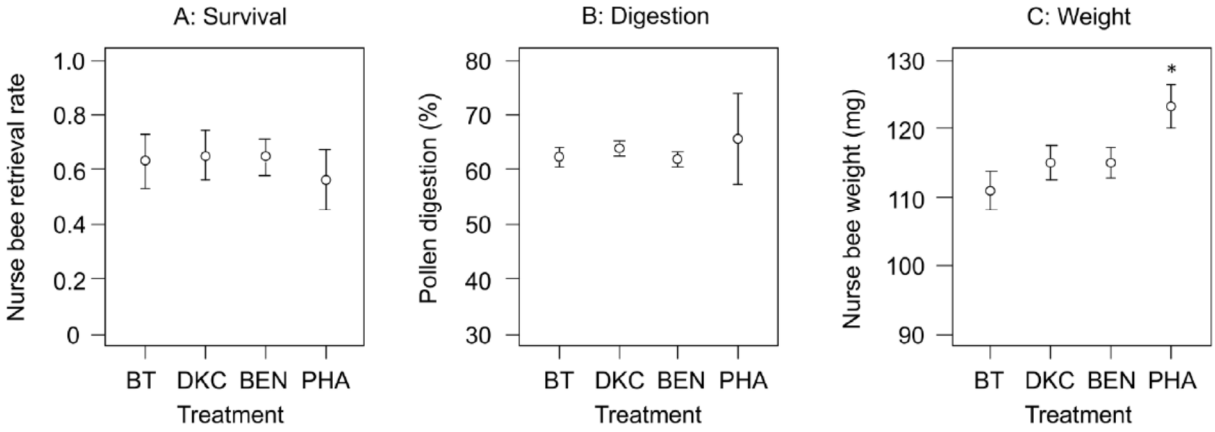

*Figure 5:Response of nurse bees after a 9 d exposure period either to flowering Bt maize (treatment BT), or two conventional maize cultivars (DKC, BEN), or controls with ad libitum access to different pollen sources from colonies kept at a Phacelia field (PHA). The survival (A) was indicated by the retrieval rate of marked bees, their weight (B) was determined at the moment of their retrieval. Microscopic analysis of bee hindguts was performed to calculate a weighted average degree of maize pollen digestion (C). The error bars indicate 95% confidence intervals. *indicates significant difference of a specific treatment. (Reprint from Hendriksma et al. 2013).*

Conclusion

This study shows that honey bee nurses which were forced to cover their full protein demand by pollen from a stacked Bt maize showed no apparent effects on survival rates, body weight and pollen digestibility in a short term study. The community structure of the gut bacteria significantly responded to the different pollen diets, but differences found with the Bt maize pollen were in the range of those occurring between pollen from conventionally bred varieties or mixed pollen sources. The relatively low Cry protein concentration measurements compared to the high exposure of nurse bees indicate that the recombinant proteins were actively digested. The natural occurrence of Cry proteins in the gut of nurse bees with no exposure to Bt maize and the lack of detectable effects on nurse bees and their gut bacteria give no indication for harmful effects of this Bt maize on honey nurse bees (from Hendriksma *et al*. 2013).

The detection of Cry proteins and the effects of different diets on microbial gut content also indicates that this method is sensitive enough to provide information related to the colony (nurse bees in this particular case) in field experiments.

Chapter 4. A new field method to support environmental risk assessments for bumble bees

In this chapter, we describe an adapted pollen trapping method for bumble bees suitable to measure exposure risks of bumble bee colonies to GM crop pollen. Pollen trapping is suggested as a useful tool for environmental risk assessments of GM crops as well as other stressors for bumblebees (e.g. evaluation of exposure to pesticides through pollen).

Introduction

Most bees have morphologic adaptations like external body structures specialized for carrying pollen e.g. the body hair on the bees is finely branched to grasp pollen while foraging (Abrol, 2011). With their legs they push the pollen into their modified hind tibia, the corbiculae (Michener *et al*., 1978), and form with nectar pellet-like loads of pollen (Thorp, 2000).

Honey bees in general are highly polylectic and generalist foragers in all habitats (Koppler *et al*., 2007). This is classified as having a generalistic and opportunistic foraging strategy which they share with many, but not all bumble bees. Bumble bees collect higher quality pollen compared to pollen collected by honey bees, which could be due their different strategies of optimized foraging. Bumble bees use their ability to perceive and judge food quality in order to get the best pollen in terms of quality whereas honey bees maybe invest more in quantity, which makes the recruitment of a large troop of foragers necessary (Leonhardt and Bluthgen, 2012).

Additionally *Bombus terrestris*, as a short-tongued species, visits a broader spectrum of forage plants than honey bees and a narrower one than *Bombus pascorum*, a long-tongued species (Leonhardt and Blüthgen, 2012). The analysis of collected pollen can give an idea of the spectrum of the visited plants and the pollen itself. Collecting pollen loads from bees than *Apis mellifera* spec. nevertheless can be difficult because of a lack of easy practical methods. Due to the uniform phenotype of worker bees of *Apis mellifera* a common approach in gathering pollen is practicable among beekeepers and scientists. In this approach of a pollen trap a thin plate with holes with a diameter of 5 mm, is placed in a small box which is connected in front of the hive entrance. The foraging honey bees have to pass through this additional gate to return to their hive. In the process of passing through the hole the pollen loads often drop off the bees and fall through a grid in the bottom of the pollen trap box. Collecting pollen from bumble bees is not as simple as it is from honey bees. Manual extraction of the pollen loads of each incoming bumble bee is an option but the process is very time consuming and handling the bees might disturb their normal behaviour. Another method is the analysis of leftover pollen grains in cocoon walls of *Bombus* sp. and thereby investigating the pollen sources, but this method gives no information about time or even day of the individual pollen foraging activity. The intention of this study was to develop a method with which pollen loads can automatically be obtained from foraging bumblebees. Using a pollen trap to collect pollen loads could provide reliable estimates of exposure of bees to pollen, to support the environmental risk assessment for this group of pollinators.

Material and Methods

Three different prototypes (Figure 6) were tested:

- Type A: The standard honey bee adaptation

A standard honey bee pollen trap for observation hives with the dimensions of 13 cm (length), 8.6 cm (width) and 11 cm (height) and an implemented drawer with the volume of 0.082 liter as seen in figure 6 is a standard device used to collect pollen from honey bees. The drawer is separated from the pollen trap by a metal grid, with a mesh size of about 2.5 mm for honey bees which was altered to 4 mm for bumble bees because their pollen tended to be too big to fall through the grid. The trap was modified with a few updates assuming that bumble bees in general are larger than honey bees. As mentioned earlier, for honey bees a thin plate with holes with a diameter of 5 mm is usual. For almost all bumble bees this diameter is too small to pass. Only the smallest bumble bees pass easily but especially the bigger ones tend to forage more often than the smaller ones. To find a better size several diameters were tested. A diameter of 7.3 mm or bigger was the most reasonable choice since most of the bumble bees, even the biggest worker bees, could just pass the hole.

- Type B: test tube cleaning brush and hair roller tube

The idea of this prototype was inspired by a carwash facility. A commercial hair roller was freed of its outer layer of a Velcro structure. The left tube with a diameter of 25 mm has a grid structure with holes of about 4 mm and a length of 6 cm. On two opposite sides of the grid a bar with the width of 1 cm was removed. Test tube cleaning brushes were added along the created openings on the tube. With a piece of wire the construction was tied and the length of the bristles of the test tube cleaning brushes was adjusted. Later test tube cleaning brushes with harder bristles were chosen to improve efficiency. Also the sticks of the brushes were moved to the inside of the tube to increase density of the bristles in the passage. At both ends of the tube some bristles were shortened to create an opening to the passage with the diameter of about 5 mm the bees are able to spot.

Type C: hair roller with layer of Velcro structure

For this trap type hair rollers with a diameter of 15 mm similar to the hair roller of type B were chosen. The outer Velcro layer was first extracted, then shortened and placed inside the tube.

Pollen traps were connected by a PVC-tube to the entrance of the bumble bee box. This PVC tube contains a locking slide as seen in order to stop passing bees, if necessary. This feature provided the spectator with the possibility to assess whether pollen foragers had lost their pollen loads in the trap.

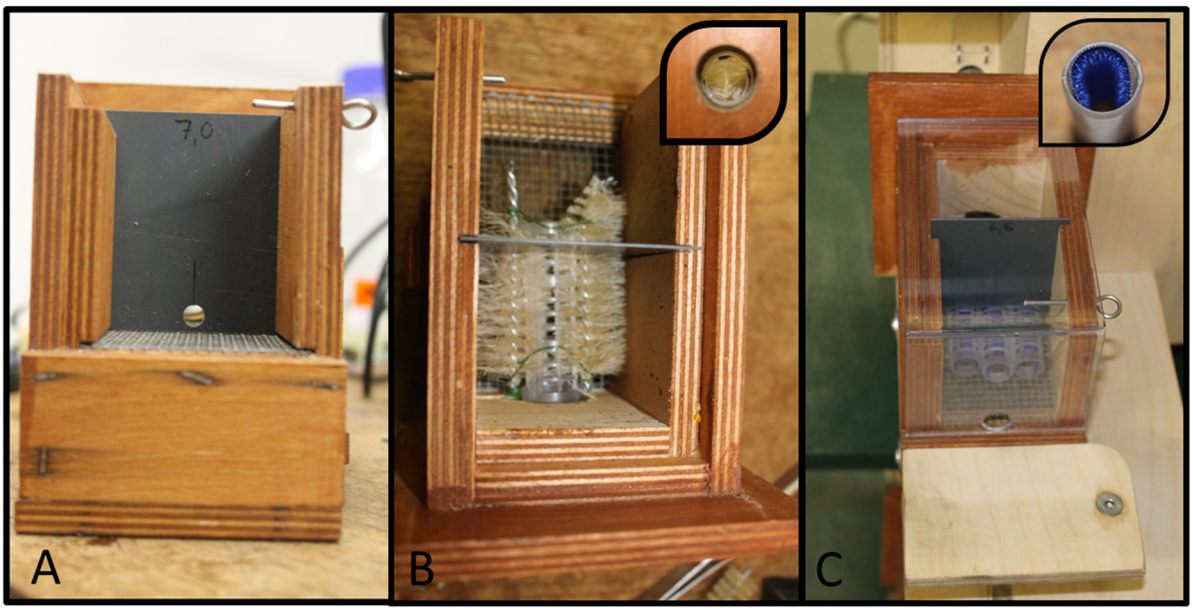

Figure 6. Pollen trap prototypes. Trap A The bumble bee simply crawls through, similar as to how a honey bee pollen trap works, Trap B The bumble has to crawl through a tube with sturdy brushes at both sides, Trap C Instead of round brushes, Velcro is used to brush of pollen pellets.

Results

Pollen traps situated at the entrance sometimes deterred bumblebees from returning to their hives and bumblebees then 'switched' to another hive. Therefore, we compared numbers of foragers successfully returning to the colony (Fig.7). Trap B had the highest number of foragers (n=348) passing through ($\chi 2 = 113.73$, df = 2, $p < 0.0001$).

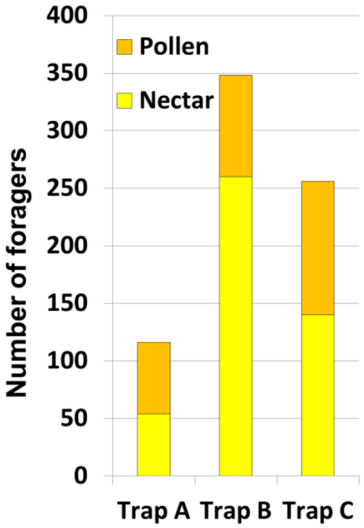

Fig 7 Bees successfully entering the hive through the trap

Trap B was also the most efficient trap (Fig. 8), intercepting almost a quarter (23.9%) of all the pollen pellets (GLMM: $\chi 2 = 14.236$, $p < 0.0001$).

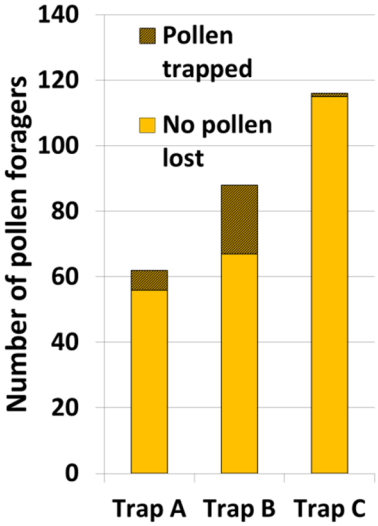

Fig 8. Pollen loss in the trap in relation to the overall number of pollen foragers

Pollen trap B was further improved up to a catch rate of 80 % by using sturdier brushes that were placed closer to each other (data not shown).

In a comparative study pollen samples were collected every 2 hours from a bumble bee and a honey bee colony. Pollen pellets were then identified In the laboratory to morphospecies level. In the same landscape, honey bees collected pollen from more plant species for most part of the day, but the overall Simpson diversity index was not significantly different (Mann-Whitney-U: W = 875, p = 0.1078, Fig 9). On average, honey bees collected 392 pollen pellets and bumble bees 8, however the standardised average weight per pollen load was higher for bumble bees (Mann Whitney-U: W = 990, p = 0.0046, Fig 9).

Data show that pollen samples from bumble bees and honey bees differed in various aspects. *Apis mellifera* collected more pollen species and a considerably larger amount of pollen loads, which can be explained through their much bigger colony size. When investigating the diversity using the Simpson index, the relative size of the colony is taken into account and *Bombus terrestris* reaches on average higher values, which might be due to their lower flower constancy. Furthermore, bumble bees collected heavier pollen loads which might be due to their different morphology.

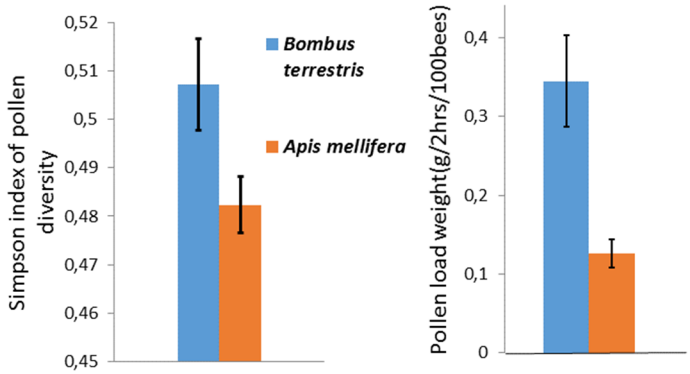

Fig 9. Results of the comparative study

Conclusions

The newly developed pollen trap for bumble bee hives is a useful tool that can provide estimates of exposure of bumble bees to pollen in a landscape as a first step of environmental risk assessment. It is expected that, similarly to the case of honey bees, this method can also be useful for carrying out monitoring programs as well as fundamental and applied research regarding bumble bee foraging activity.

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