ORIGINAL PAPER

Occurrence and field densities of Coleoptera in the maize herb layer: implications for Environmental Risk Assessment of genetically modified *Bt*-maize

Stefan Rauschen · Frank Schaarschmidt · Achim Gathmann

Abstract Beetles (Coleoptera) are a diverse and ecologically important group of insects in agricultural systems. The Environmental Risk Assessment (ERA) of genetically modified *Bt*-crop varieties with insect resistances thus needs to consider and assess the potential negative impacts on non-target organisms belonging to this group. We analysed data gathered during 6 years of field-release experiments on the impact of two genetically modified *Bt*-maize varieties (*Ostrinia*-resistant MON810 and *Diabrotica*-resistant MON88017) on the occurrence and field densities of Coleoptera, especially the two families Coccinellidae and Chrysomelidae. Based on a statistical analysis aimed at establishing whether *Bt*-maize varieties are equivalent to their near-isogenic counterparts, we discuss the limitations of using field experiments to assess the effects of *Bt*-maize on these two beetle families. The densities of most of the beetle families recorded in the herb layer were very low in all growing seasons. Coccinellidae and Chrysomelidae were comparatively abundant and diverse, but still low in numbers. Based on their role as biological control agents, Coccinellidae should be a focus in the ERA of *Bt*-plants, but given the large natural variability in ladybird densities in the field, most questions need to be addressed in low-tier laboratory tests. Chrysomelidae should play a negligible role in the ERA of *Bt*-plants, since they occur on-crop as secondary pests only. Species occurring off-crop, however, can be addressed in a similar fashion as non-target Lepidoptera in Cry1Ab expressing *Bt*-maize.

S. Rauschen (✉)
Department of Plant Physiology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
e-mail: Rauschen@Bio3.RWTH-Aachen.de

F. Schaarschmidt
Faculty of Natural Sciences, Institute of Biostatistics, Leibniz Universität Hannover, Herrenhaeuser Straße 2, 30419 Hannover, Germany
e-mail: schaarschmidt@biostat.uni-hannover.de

A. Gathmann
Institute of Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
e-mail: achim.gathmann@bvl.bund.de

Introduction

One of the major applications for genetically modified (GM) crops is insect pest control based on the expression of proteins derived from *Bacillus thuringiensis* (Berliner). This ubiquitous soil bacterium forms crystalline protein inclusion bodies upon sporulation, which are toxic to specific insect taxa

(Schnepf et al. 1998; Glare and O'Callaghan 2000). Genes coding for these Cry-proteins are transformed into plants that in turn express *Bt*-proteins as plant-incorporated protectants in different concentrations in all tissues. In Europe, only *Bt*-maize lines resistant against the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) based on the expression of the protein Cry1Ab have been cultivated. All of these maize lines are derived from the transformation event MON810.

Another trait provided by the *Bt*-technology is resistance of corn plants to the Western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae). The WCR has been accidentally introduced to Europe from the United States approximately 15 years ago and is generally regarded as a serious threat to European maize production. It has spread considerably over the last years, now covering already vast areas of Europe (Hummel 2003; Kiss et al. 2005; Miller et al. 2005). Resistance of the currently available GM maize plants to *D. virgifera virgifera* is conferred by the expression of some very different Cry proteins (Ostlie 2001). Maize lines based on the transformation events MON863 and MON88017 (Monsanto Co.; Vaughn et al. 2005) both express Cry3Bb1, MIR604 (Syngenta Seeds Inc.; Walters et al. 2008) expresses a modified Cry3A, and DAS-59122-7 (DOW Agro-Sciences LLC; Gao et al. 2004) produces both Cry34Ab1 and Cry35Ab1. For most of these maize lines an application for cultivation has been submitted in the European Union (EU).

Under EU legislation a pre-market risk assessment of genetically modified crops is mandatory (EC 2001, 2002; EFSA 2006). Among the scientific community there is consensus that the focus of biosafety research should be on organisms that are (1) exposed to a specific *Bt*-crop's toxin because of their association with the host plant and (2) potentially sensitive to the transgene product (the *Bt*-protein) due to their phylogenetic relationship to the target organism. The nature of an organism's association to the host plant can in general be either herbivorous, i.e. exposure by directly feeding on plant tissue, and/or predacious, i.e. exposure via the consumption of herbivorous insects which have previously been exposed to a Cry-protein, or both in cases of omnivorous species (see figure 1 in Romeis et al. 2006 for exposure routes). The choice of non-target

species to be tested in a tiered risk-assessment approach is governed by a variety of factors, e.g. ecosystem function, representativeness for other non-target organisms under the surrogate species concept, and amenability to testing (Romeis et al. 2008; Todd et al. 2008). In order to make an informed choice of sensible non-target species for insect-resistant *Bt*-maize, fundamental information on the general occurrence and field-densities of potential candidate organisms is needed for the different geographic areas where the genetically modified maize will be planted.

Because of their close phylogenetic relationship, other Coleoptera are a main focus in the assessment of the potential negative environmental impact of WCR-resistant *Bt*-maize varieties. *Bt*-proteins are generally regarded as very specific in their action, with a very limited impact on non-target organisms (Roh et al. 2007), meaning that only species closely related to a targeted pest can reasonably be hypothesised to be at some risk of experiencing harm. Three families within the Coleoptera are of special interest: (1) other chrysomelid beetles, since they are most likely to be affected by the toxic action of *Diabrotica*-specific Cry-proteins; (2) coccinellid and (3) carabid beetles, which serve important biological control functions as predators of aphids and other herbivores (Kromp 1999; Romeis et al. 2006).

In this work, we present data gathered on beetles and especially the two beetle families Chrysomelidae and Coccinellidae during field-release experiments with genetically modified maize varieties based on the transformation events MON810 and MON88017, performed from 2001 through 2003 and from 2005 to 2007, respectively. The studies were conducted in two different regions of Germany. In the first study, beetles were mostly recorded on a higher taxonomic level only (mostly order, in one case family), since they were not assumed to be sensitive to the Cry1Ab protein expressed in MON810 and hence not a special focus. In the second study, however, they were identified to the species level whenever possible, since the Cry3Bb1 protein expressed in MON88017 directed against *Diabrotica* was hypothesised to potentially have an impact on other Coleoptera. We analysed these data in order to establish equivalence between the genetically modified varieties and their respective near-isogenic counterparts. These analyses showed the limitations of using higher-tier field-release

experiments for assessing the potential impact of *Bt*-maize varieties on Coccinellidae and other beetles. Additionally, we discuss literature data on the occurrence of Coleoptera in maize fields, their exposure to Cry proteins and the potential impacts of *Bt*-plants to make a comprehensive analysis of the accompanying implications for Environmental Risk Assessment.

Materials and methods

The field-release experiment with MON810

Three maize treatments were compared in a field study from 2001 to 2003: (1) *Bt*-maize (*Bt*; transformation event MON810, cultivar Novelis) expressing the Cry1Ab protein, (2) the near-isogenic maize line without insecticide treatment (ISO, cultivar Nobilis) and (3) the isogenic maize treated with a synthetic pyrethroid insecticide (INS; cultivar Nobilis treated with 750 ml Baythroid 50 per ha; active ingredient 50 g Cyfluthrin/l; emulsifiable concentrate) representing the conventional cultivation strategy. The experimental site was located near Bonn (North Rhine-Westphalia, Germany) and consisted of two maize fields approximately 500 m apart. One field measured 182 by 248 m with 15 plots (five replications of each treatment arranged in three rows of five plots), the other measured 178 by 186 m with nine plots (three replications arranged in three rows of three plots). The size of each plot was 0.25 ha. The plots were arranged in a randomized complete block design. The field was planted on May 20 (calendar week CW 21) in 2001, on May 17 (CW 20) in 2002 and on April 28 (CW 18) in 2003. The experiment was performed in three successive years, whereby the location of the plots with their respective maize lines was identical to ensure similar abiotic parameters. A mixture of Callisto (900 ml per ha; active ingredient Mesotrione) and Gardobuc (900 ml per ha; active ingredients Terbuthylazin and Bromoxynil) herbicides was applied 4 weeks after planting. The insecticide treatment depended on European corn borer phenology in the first half of July.

The field-release experiment with MON88017

Within an area of approximately 4 ha four different maize lines were planted in a systematically randomized plot design with eight replicates each, at a field site near Würzburg (Lower Frankonia, Germany). Apart from *Bt*-maize DKc5143-*Bt* (event MON88017) and its near isogenic line DKc5143 (ISO; both Monsanto Co.) two conventional maize lines, Benicia (Pioneer HiBred, Johnston, Iowa, USA) and DK315 (Monsanto Co.), were used for the experiment. Individual plots measured 40.5 by 31.5 m (0.13 ha) and contained 42 maize rows with 75 cm distance between them and 15 cm between individual plants. The plots were aligned in four parallel rows of eight plots each, with a 4.5 m wide clearance between neighbouring rows for easy access. The experimental field was surrounded by a 4.5 m clearance strip followed by a 10 m wide perimeter of conventional maize (Gavott, KWS Saat, AG, Einbeck, Germany). The experiment was performed in three successive years, whereby the location of the plots with their respective maize lines was identical to ensure similar abiotic parameters. The field was planted on May 27 (CW 21) in 2005, on May 9 (CW 19) in 2006 and on May 21 (CW 21) in 2007. In 2005, a mixture of 0.8 l Motivell (BASF AG, active ingredient Nicosulfuron), 0.8 l Spectrum (BASF AG, active ingredient Dimethenamid-p) and 2.0 l Artett (BASF AG, active ingredients Terbuthylazine and Bentazon) were used to control weeds during the 2 to 8 leaf stadium. In 2006 and 2007, a mixture of 3.0 l Gardo Gold (Syngenta Co., active ingredients S-Metolachlor and Terbuthylazine) and 0.8 l Callisto (Syngenta Co., active ingredient Mesotrione) was used for this purpose.

Cob samples

Cob sampling is described in Eckert et al. (2006). In the study with MON88017 the methodology was slightly changed: only 10 cobs (instead of 20) were sampled per plot, by slipping a small plastic bag over the cobs and then cutting them off the plants. The plastic bags were tightly closed and all ten sample bags from a plot were then put together into a larger bag. This ensured that arthropods would not get away during sampling or subsequent transport to the laboratory. Samples were stored at −20°C until analysis. Additional data from Eckert et al. (2006) on beetles sampled from cobs are given here to compare them to the data gathered in the MON88017 project.

Sweep net samples

Sweep netting (net diameter 40 cm, mesh width 1.5 mm) was carried out in a transect-wise manner along the maize rows as previously described in Rauschen et al. ([2008](#)). In the study with MON810 sweep netting was performed in five transects in 2002 and 2003. In the study with MON88017 it was done in four transects in all 3 years. This is due to the different plot sizes in the two field-release experiments. Beginning at approx. 5 m into the plot, to avoid edge effects, thirty large steps were quickly made whilst holding the sweep net vertically in front of the chest at a height of approximately 90 cm above the ground. After each transect, captured insects were transferred into glass vials with 70% ethanol. Sweep net sampling was done on August 26 (CW 35) in 2002, August 8 (CW 32) in 2003, July 13 and August 24 (CW 28 and 34) in 2005, on July 19, August 16 and September 6 (CW 29, 33 and 36) in 2006, and on July 18, August 14 and September 4 (CW 29, 33 and 36) in 2007. Collected specimen were identified in the laboratory under a binocular microscope.

Visual assessments

Visual assessments of the Coleoptera on plants were applied in 2002 and 2003 and performed as follows: Four plants per plot were randomly chosen and assessed 7 times during the growing seasons at 2 week intervals from June to September (26 June to 17 September 2002, and from 4 June to 1 September 2003). Different parts of the plants—leaves, stalks, cobs and panicles—were scanned individually. Plants were neither damaged, nor were insects removed from them during the assessment.

Panicle samples

In each plot, five combined samples were taken by shaking the flowers of five adjacent plants into a plastic funnel. The maize anthers and all insects on and in them were collected in a glass vial with a snap-on lid connected to the end of the funnel. In this way, 25 flowers per plot were sampled during the peak of anthesis (usually from the end of July to beginning of August). In 2001, 2002 and 2007 samples were taken during anthesis once for each treatment, while in 2003, 2005, 2006 samples were taken twice. This was

due to differences in flowering phenology during the years and the prevalent weather conditions during anthesis.

Moericke traps

The use of Moericke traps was restricted to the experiment performed from 2001 to 2003 (MON810 project). One plastic coloured Moericke trap (bowl measuring 340 mm by 260 mm, half of the trap bottom white and half yellow) was mounted in the middle of each plot onto a wooden pole at a height of approximately 1.2 m. Traps were filled with water and a small amount of detergent and were set up for 1 week, for 7 intervals in 2001, 6 in 2002 and 2 in 2003. After this time, the caught insects were immediately transferred to glass vials with snap-on lids and stored in 70% ethanol until being sorted and identified in the laboratory under a stereomicroscope.

Statistical analyses of field abundances

Since our objective is to assess environmental safety of a novel treatment, we want to use statistical procedures such that the consumer's or environmental risk is directly controlled by the type-I-error α at a low, pre-specified level, chosen by convention at 5%. This can be achieved by applying a test of equivalence (e.g. Andow [2003](#); Wellek [2003](#); EFSA [2009](#)) instead of using customary tests on difference. In comparisons of abundance between a GM and a conventional variety, a test of equivalence defines the null-hypothesis as the state of either unacceptable increase or unacceptable decrease of abundance in the GM relative to the conventional treatment. The alternative hypothesis defines the state where slight changes in abundance are allowed, but a severe increase or decrease does not occur.

Among other possible ways, confidence limits might be used to decide upon such hypothesis. The procedure is the following:

1. Define the margin of relevant decrease and relevant increase r_L, r_U, preferably in the scale of the ratio of mean abundances in GM relative to the mean abundance in the conventional treatment.
2. Calculate a lower 95% confidence limit and an upper 95% confidence limit for the ratio of mean

abundances based on the data at hand. Note that both limits together usually constitute a 90% confidence interval.

3. If, and only if, both the lower confidence is larger than the r_L and the upper confidence limit is smaller than r_U, we can reject the null hypothesis and conclude that GM is equivalent to ISO.

This procedure protects the consumers or environmental risk at 5% because in only 5% of the cases one erroneously concludes for equivalence, when indeed abundance is relevantly increased or decreased in only 5% of the cases. The inherent problem of this approach is the choice of r_L and r_U, i.e., the definition of which changes in abundance are considered relevant and which changes are considered irrelevant. It is of fundamental importance that these choices of r_L and r_U need to be made by ecological reasoning and background knowledge.

When applying the test of equivalence (or any other significance test) for abundance data, one faces the problem that uncertainty concerning the true effect (and the width of confidence intervals) becomes large for low abundances, a small number of replications and large residual variation. Counting of individuals scattered randomly in the observational windows of a study would yield data following the Poisson distribution. However, large residual variation in abundance data often occurs due to the clustering of individuals, termed extra-Poisson variation or over-dispersion in the statistical context (McCullagh and Nelder 1989). Then, the probability to show equivalence if indeed GM has no effect on the abundance of a species may be very low, if rare species or species with high variability in local or temporal abundance or activity are investigated with a low number of replications. However, a secondary aim in safety assessment of a new technique should be to keep the producer's risk at an acceptable level. That is, if indeed a GM would be harmless, the risk of erroneously doubting its harmlessness (i.e. not rejecting the null-hypothesis of an equivalence test) should not be too high. In other words, one should try to keep the type-II-error β of the equivalence test at a low level. This can only be achieved, roughly, by experimental design. That is, planning trials with a sufficient number of replications, based on available prior information concerning the mean abundance and variability of the observations.

For this second purpose, we first analyse available datasets of coccinellids and other Coleopterans concerning their mean abundance and their variability. In a second step, we simulate abundance data for different choices of mean abundance, variability and experimental design and subsequently compute the confidence limits for these data sets. Finally, we display the ranges in which 90% confidence intervals are completely included (i.e. one can conclude for equivalence) in 50 or 80% of the cases. It is fundamental to note that the presented ranges should not be the basis of the definition of r_L and r_U. Conversely, these should be defined based on ecological reasoning. However, the figures might be used to roughly assess, for which choices of $\{r_L, r_U\}$ it is not realistic to expect that equivalence can be shown even for a completely harmless GM.

Both trials were designed as randomized complete block designs and repeated over 3 years. Although performed at the same locations, the consecutive years are included as independent in the analyses. In order to assess relevant values of variability, a generalized linear model with log-link assuming the Negative binomial distribution (McCullagh and Nelder 1989) was fitted to each data set, using the implementation in the R-package MASS (Venables and Ripley 2002). For each taxon separately, a model was fitted including effects for the blocks, treatments, years and a treatment-year interaction. After adjusting for these effects (subtracting fitted values from the data on the log-scale), the residual variance was recorded for each data set.

The major objective of the study was the quantitation of the effect of Bt-maize on the relative densities of Coleoptera. In this respect, the comparison of arthropod densities between the Bt-maize varieties (BT) and the near-isogenic lines (ISO) is of primary interest, as it potentially is the best indicator for any differences caused by the Bt-protein. Based on the mean and variance parameters estimated in the models described above, Wald-type confidence intervals for the ratio of mean abundance were calculated (McCulloch and Searle 2001).

Simulation study

In order to assess the power of a test of equivalence, a Monte Carlo simulation study was performed. Throughout the study we assumed the most favorable

case, i.e. that the *Bt*-varieties and their near-isogenic lines have identical mean abundances. To simulate insect count data, random data were drawn from the following model:

$$\log(\mu_{ij}) = \delta_i + \varepsilon_j + (\delta\varepsilon)_{ij}$$
$$Y_{ijk} \sim \text{NegBin}(\mu_{ij}, \tau)$$

In this model, δ_i, $i = 1,2$ describes the log-abundance between the GM (δ_1) and the conventional variety (δ_2). The term ε_j describes the mean log-abundance in J different sites or years, indexed by $j=1, \ldots, J$. The actual values of ε_j are drawn from a normal distribution with expected value 0. The term $(\delta\varepsilon)_{ij}$ describes the treatment- environment site (or treatment-year) interaction, with values drawn form a normal distribution with expected value 0. The counts Y_{ijk} are simulated from a negative binomial distribution, where the parameter τ models the extra-Poisson variability following the relation

$$V(Y_{ijk}) = \mu_{ij} + \tau\mu_{ij}^2$$

The index $k = 1, \ldots, K$ identifies the K replications of each treatment within each site.

All combinations of the following parameter settings are considered: In all settings the mean abundance is chosen equal in GM and conventional treatment $\exp(\delta_1) = \exp(\delta_2)$ and is set to 0.5, 1, 5, 10, 50. The extra-Poisson variability is chosen $\tau = 0.001$, 0.1, 1. The following combinations (J, K) for the number of sites and the number of replications J within each site K are chosen: (3,8), (2,12), (5,10), (10,10). For each combination of parameter settings, 10,000 data sets were drawn and for each data set, a model of the form above was fitted, assuming the negative binomial distribution and obtaining estimates for δ_i, ε_j, $(\delta\varepsilon)_{ij}$, τ from the data, using the implementation by Venables and Ripley (2002) in the R package MASS. Large sample confidence intervals for the average ratio of mean abundance are calculated following McCulloch and Searle (2001) by

$$[C_L; C_U] = \big[\exp(d_1 - d_2 - z_{1-\alpha}s_{d_1-d_2});$$
$$\exp(d_1 - d_2 + z_{1-\alpha}s_{d_1-d_2})\big],$$

where d_i denotes the estimates for δ_i, $z_{1-\alpha}$ denotes the $(1-\alpha)$ quantile of the standard normal distribution and s_{d1-d2} denotes the estimated standard error of the difference $d_1 - d_2$ obtainable from the model fit. All simulations and subsequent computations were

performed in the free software R-2.6.2 (R Development Core Team 2008). To summarize the 10,000 simulation results of one parameter setting we search for an $r_L < 1$, $r_U = 1/r_L$ such that $P(r_L < C_L \cap C_U < r_U) = 1 - \beta$, where β is the type-II-error. That is, we search for that value r_L, for which $(1 - \beta) \times 10,000$ of the 10,000 simulated confidence intervals $[C_L; C_U]$ are completely included in the margins of relevant change r_L, r_U.

Results

General overview of beetle species in maize

A total of 6,529 individuals from 9 families of beetles was caught over the 6 growing seasons at the two field release sites (Table 1), represented by 29 genera or species. Most insects were caught with Moericke traps, followed by panicle and cob samples, and the sweep net samplings, although this differed among the years. The most diverse groups appeared to be the ladybirds (Coccinellidae) and the leaf beetles (Chrysomelidae), with eight and six identified genera, respectively. These two families were also the 2nd and 3rd most abundant, in the years a more accurate identification was performed. Minute brown scavenger beetles (Lathridiidae) represented by the single species *Cortinicara gibbosa* (Herbst) were the family with the highest number of individuals, although this species was almost exclusively sampled from maize cobs (see also Eckert et al. 2006) and only single specimen were ever sampled from the foliage.

Staphylinids were only identified in 2005 and not further investigated due to their very low abundances in all samples taken from the herb-layer with the different methods.

Within the ladybirds, eight species were identified: *Propylea 14-punctata* (L.) and *Coccinella 7-punctata* (L.) were the most abundant (Table 2). However, during the course of the release experiment with MON88017 there seemed to be an increase in the number of the non-native multicoloured Asian lady beetle *Harmonia axyridis* (Pallas) in the field. In 2007, most coccinellid larvae collected from maize foliage appeared to belong to this species, based on the presence of the conspicuous, coloured and branched appendages on the abdominal flanks of the larvae of this species.

Table 1 Overview of Coleoptera caught in the two field-release experiments during the periods from 2001 to 2003 and from 2005 to 2007 with the different sampling methods

	2001	2002	2003	2005	2006	2007	Total
Coleoptera	1,187	2,351	2,102	271	437	181	6,529
caught with							
Moericke traps	1,034	1,947	1,251	–	–	–	4,232
Panicle samples	111	228	708	101	129	8	1,285
Visual assessments	–	126	52	–	–	–	156
Sweep netting		10	20	106	157	75	368
Cob samples[a]	47	40	94	64	151	98	494
Anthicidae							
Anthicus spp.				0	1	0	1
Notoxus monoceros				2	4	3	9
Cantharidae							
Cantharis spp.				1	0	0	1
Carabidae							
Demetrias sp.				0	16	0	16
Chrysomelidae							
Chaetocnema spp.				9	8	0	17
Longitarsus spp.				0	0	1	1
Oulema lichenis				26	4	0	30
Oulema melanopa				2	2	1	5
Phyllotreta spp.				48	98	18	164
Psylliodes chrysocephala				0	0	1	1
Coccinellidae							
Adalia bipunctata				2	3	1	6
Adonia variegata				2	3	1	6
Calvia 10-guttata		10					10
C. 14-guttata			10				10
Coccinella 7-punctata			10	26	4	5	45
Harmonia axyridis				0	2	15	17
Propylea 14-punctata				77	117	30	224
Tytthaspis sedecimpunctata				0	2	2	4
Curculionidae							
Apion spp.				2	13	2	17
Barynotus spp.				0	5	3	8
Sitona spp.				0	4		4
Lathridiidae							
Cortinicara gibbosa[a]	47	40	94	64	151	98	494
Malachiinae							
Axinotarsus spp.				1	0	0	1
Staphylinidae							
Aleochara spp.				1	–	–	1
Oxypoda spp.				1	–	–	1
Oxytelus spp.				6	–	–	6
Philonthus spp.				1	–	–	1
Syntomium spp.				0	–	–	0

Table 1 continued

	2001	2002	2003	2005	2006	2007	Total
Tachyporus spp.				1	–	–	1
Total	47	50	114	272	437	181	1,101

[a] Data for 2001 to 2003 taken from Eckert et al. (2006)

Table 2 Overview of coccinellid beetles and total numbers caught with two different methods during the field-release experiment from 2005 to 2007

	Sweep nets	Panicle samples	Total
Propylea 14-punctata			
2005	24	53	77
2006	66	51	117
2007	28	2	30
Coccinella 7-punctata			
2003	10	–	10
2005	3	23	26
2006	2	2	4
2007	5	0	5
Calvia 10-guttata			
2002	10	–	10
C. 14-guttata			
2003	10	–	10
Harmonia axyridis			
2005	0	0	0
2006	1	1	2
2007	11	4	15
Tytthaspis sedecimpunctata			
2005	0	0	0
2006	2	0	2
2007	2	0	2
Adonia variegata			
2005	0	2	2
2006	0	3	3
2007	0	1	1
Adalia bipunctata			
2005	0	2	2
2006	0	3	3
2007	0	1	1
Coccinellidae larvae			
2005	1	3	4
2006	1	1	2
2007	29	3	32

Three species of this family were only caught with either of the two samplings methods employed: *Tytthaspis sedecimpunctata* (L.) was only caught with sweep nets in the foliage, while *Adonia variegata* (Goeze) and *Adalia bipunctata* (L.) were only sampled from flowering tassels (Table 2).

During the first experiment with MON810 from 2001 to 2003 Coccinellidae were only seldomly recorded in sweep net or panicle samples. This family was quite abundant at the field site, however, with 105 and 147 individuals caught in Moericke traps in 2002 and 2003, respectively. Ladybirds were not individually counted in 2001, so it is unclear how many of the 1,034 beetles caught in yellow traps in that year were in fact coccinellids.

The most abundant out of the six leaf beetle genera recorded at the field site in Bavaria in the time from 2005 to 2007 were *Phyllotreta* spp. and *Oulema lichenis* (Voet) (Table 3). Their densities were uniformly low over the different growing seasons in comparison to other beetle groups, however, despite being recorded on a regular basis. Specimens of the other four genera or species recorded were caught on only few occasions.

The sampling methods differed again with respect to the species that were caught: both *Oulema* species and the *Chaetocnema* spp. were only sampled from foliage. The most abundant chrysomelid genus, *Phyllotreta*, on the other hand, was sampled from both maize foliage and flowering tassels.

The minute brown scavenger beetles (Lathridiidae) are saprovorous/mycetophagous and could regularly be found between the husk leaves of maize cobs. They were one of the few groups that could always be found in considerable densities over all 6 growing seasons, although there were large differences between the years and the field-release sites.

However, they might be regarded as a typical member of the community of insects occuring in maize.

Table 3 Overview of chrysomelid beetles and total numbers caught with different methods during the field-release experiment from 2005 to 2007

	Sweep nets	Panicle samples	Total
Oulema lichenis			
2005	26	0	26
2006	4	0	4
2007	0	0	0
Oulema melanopa			
2005	2	0	2
2006	2	0	2
2007	1	0	1
Phyllotreta spp.			
2005	27	21	48
2006	45	53	98
2007	18	0	18
Chaetocnema spp.			
2005	9	0	9
2006	8	0	8
2007	0	0	0
Psylliodes spp.			
2005	0	0	0
2006	0	0	0
2007	1	0	1
Longitarsus spp.			
2005	0	0	0
2006	0	0	0
2007	1	0	1

Further statistical analysis of the Coleoptera data

Figure 1 summarizes the analysis of coleopteran data from field trials with respect to the relation between mean abundance and residual variation. For counts of single species, abundances are usually very low, rarely larger than 1 but often in the range around 0.1. For those rare counts, there is no hint to extra-Poisson variability. Conversely, the observed residual variability is usually slightly below the mean abundance. This effect is probably due to an over-fit by the model that includes block, treatment, year effects and interactions, which are not necessary to explain effects in the sparse data. Summarized counts on the family or order level show mean abundances between 1 and 50. Especially for high abundances we observed substantial extra-Poisson variation, most prominently for the data of all coleopterans.
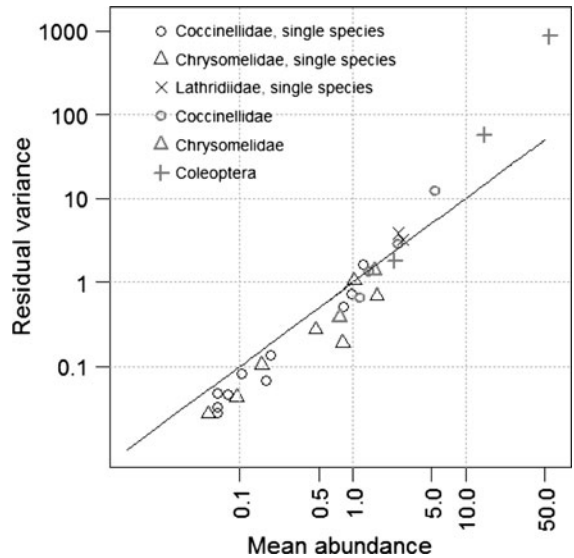
Fig. 1 Plot of residual variance versus estimated mean abundance in logarithmic scale for 28 abundance data sets from field trials in 2001–2003 and 2005–2007. *Bold grey symbols* indicate data that are summarized at the level of families or orders, while *black symbols* indicate data of single species. *Different symbols* indicate different families (or summary of all coleopteran counts). The *solid line* indicates the theoretic 1:1 relation of variance and mean for the Poisson distribution

With respect to the final aim of assessing the power of a test of equivalence for abundance data, we conclude: very low abundances may be relevant when single species are considered. In the present cases, considering species of the Coccinellidae, Chrysomelidae and *Cortinicara* in a very restricted number of locations, we found no evidence for over-dispersion on the species level. However, for different species or different locations, over-dispersion may of course be present on the species level as well. When considering counts summarizing the abundance of higher taxonomic levels, extra-Poisson variation is clearly present in the data at hand. Hence it should be considered when analysing the power of a test of equivalence.

Results of the simulation study

In the simulation study, the case is considered that abundance in GM does not differ from that in the conventional standard. Figures 2 and 3, respectively depict the ranges in which 80 and 50% of 10,000 simulated confidence intervals for the ratio of mean

abundance (GM/conventional) are fully included. These ranges are shown for different experimental designs, mean abundance and variability. That is, the study provides estimates for the narrowest choice of equivalence margins {r_L, r_U} for which one could expect to successfully show equivalence in 80% (or 50%) of trials with a given design, mean abundance and variability. First, consider the relatively wide equivalence margins {0.5; 2.0} (outer, dotted, vertical lines) and the experimental setup used in the two projects, with 8 replications per treatment, performed over 3 years ("site" here means one field-year combination, Figs. 2a, 3a). In this situation, it is only possible to show equivalence in at least 80% of the cases (Fig. 2a) if the mean abundances are larger than 1 and there is no extra variation in the data (light bars). When taxa with substantial extra variation (dark bars), or mean abundances below 1 are considered, yielded confidence intervals are much wider. Hence the chance to be included in the equivalence margins {0.5, 2.0} is below 80% in these cases, even though the abundance in the GM and conventional variety are exactly equal. For showing equivalence for rare taxa or taxa with substantial extra variability with a high probability of success

(80%), much higher experimental effort would be needed, e.g. 5 sites, with 10 replications per site or 10 sites with 10 replications per site (Fig. 2c, d). For taxa with strong extra-Poisson variation one will be successful in just about 50% of the cases even with mean abundances as high as 50 individuals per observational unit.

If a stricter definition of equivalence, i.e. margins of {0.8; 1.25} (inner, dashed, vertical lines), was to be used with a design with 3 years and 8 replications within a year, the chance to correctly establish equivalence is below 50% when the mean abundance is about 5 or below, or the taxon exhibits any extra-Poisson-variability (Fig. 3a). In this strict case, even with the largest trial shown in Fig. 2d, one could not expect to establish equivalence in at least 80% of the cases, except for very abundant species (mean abundance about 50 or above) without strong extra variation.

Confidence intervals for a test of equivalence for the 14 most abundant taxa

As a last step, we analysed the most abundant taxa obtained from the trials with MON810 (2001–2003)
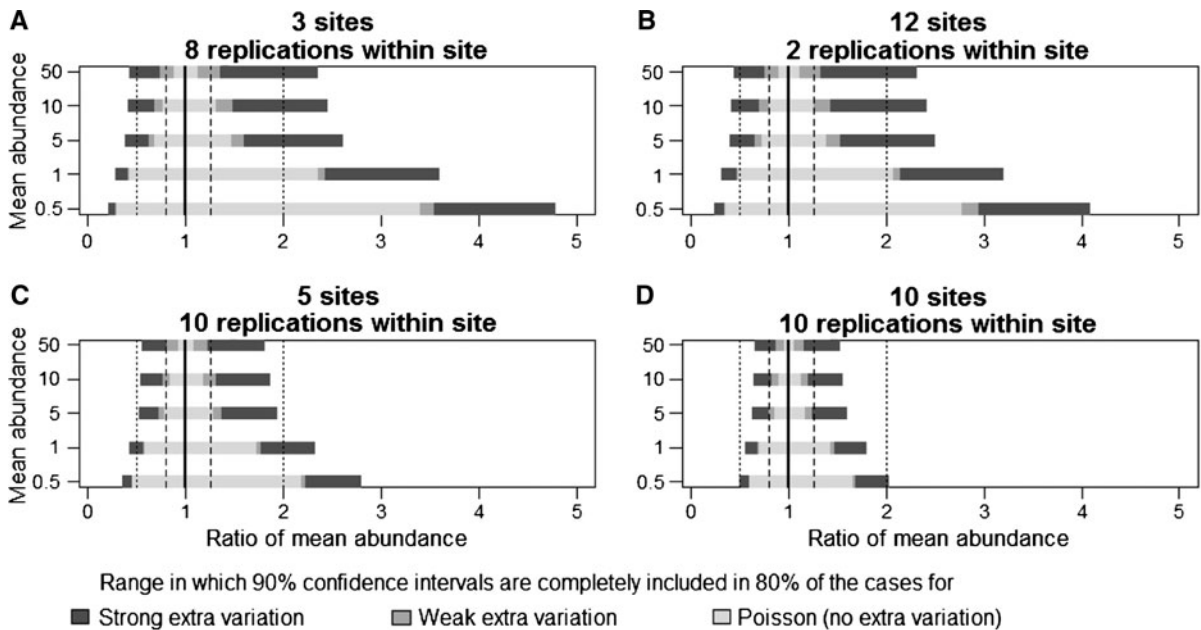
Fig. 2 Range in which 90% confidence intervals are included in 80% of 10,000 simulated cases for extra-Poisson variation $\tau = 1$ (strong extra variation, *dark grey bars*), $\tau = 0.1$ (weak extra variation) and $\tau = 0.001$ (nearly no extra-variation, *light*

grey bars), and various levels of mean abundance. The *dotted vertical lines* indicate equivalence margins {0.5, 2.0}, *dashed lines* show margins {0.8, 1.25}
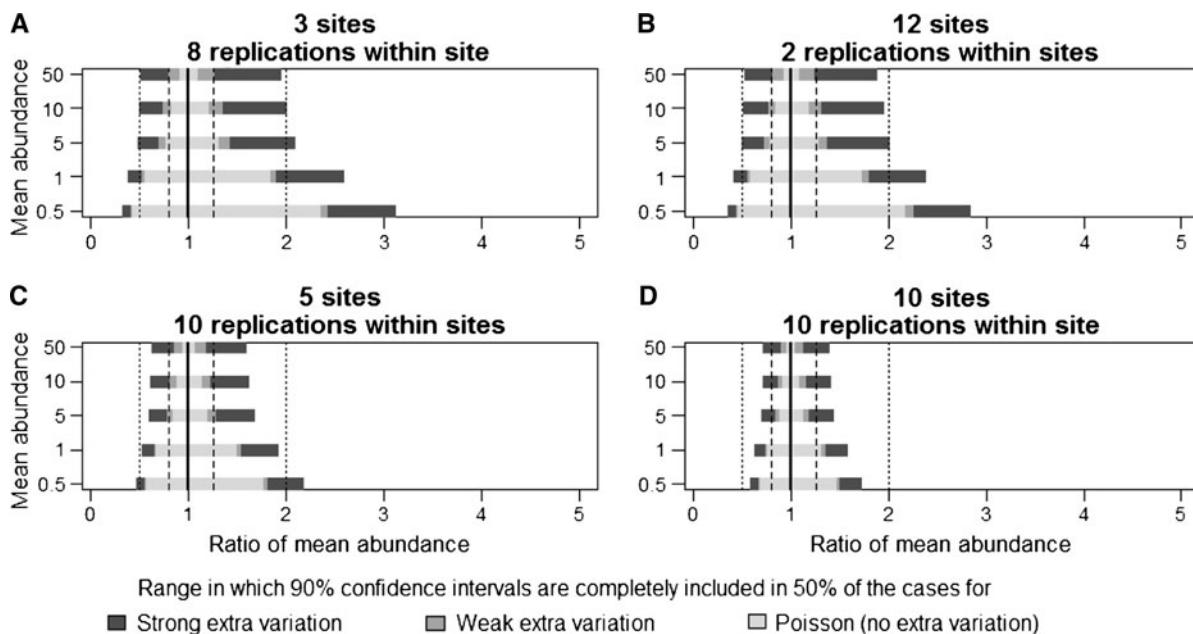
Fig. 3 Range in which 90% confidence intervals are included in 50% of 10,000 simulated cases for extra-Poisson variation $\tau = 1$ (strong extra variation, *dark grey bars*), $\tau = 0.1$ (weak extra variation) and $\tau = 0.001$ (nearly no extra variation, *light grey bars*), and various levels of mean abundance. The *dotted vertical lines* indicate equivalence margins {0.5, 2.0}, *dashed lines* show margins {0.8, 1.25}

and with MON88017 (2005–2007) using the confidence interval approach described above. The models fitted are slightly more complex than the one used for the simulations, since both trials additionally contained blocks. Lower 95% and upper 95% confidence limits, suitable for a test of equivalence with 5% error probability per taxon are considered and shown in Fig. 4. For the four species included in the analysis (*Oulema lichenis*, *Phyllotreta vittula*, *Propylea 14-punctata*, *Cortinicara gibbosa*, assessed by cob sampling and sweep netting) one can show equivalence with equivalence margins defined $r_L = 0.5$, $r_U = 2.0$ only for *C. gibbosa*. Using this definition of equivalence for the remaining 8 taxa (being summarized counts at the family or order level) showing equivalence is successful for six of them, but fails for two (Coccinellidae counts by visual assessment in MON810 and for the sum of all coleopterans obtained in MON810 by cob sampling).

If we would base the decision of equivalence on much narrower margins {0.8, 1.25}, i.e. considering a *Bt*-abundance decreasing to 80% or increasing to 125% of the abundance in the near-isogenic variety as an already problematic event, it would not be possible to reject the null-hypothesis of no equivalence for a

single taxon considered in this study. However, we cannot decide whether failing to conclude for equivalence is due to a true effect or a high uncertainty due to low abundance, low sample size and high variability.

Discussion

Occurrences and densities of Coleoptera in maize

The total number of 29 genera or species recorded and 6,529 individuals caught from the herb-layer in both field-release experiments was rather low in comparison to other non-target organism groups at the same field sites during the same study years:

The community of plant- and leafhoppers (Auchenorrhyncha) was surveyed during the MON810 project in 2002 and 2003, resulting in 5 species and a total number of 3,037 individuals recorded. Of these, the maize leafhopper *Zygindia scutellaris* (Herrich-Schäffer) alone accounted for 2,480 individuals (Rauschen et al. 2008). In the MON88017 project, 17 Auchenorrhyncha species were identified, with a total of 10,474 individuals over 3 years
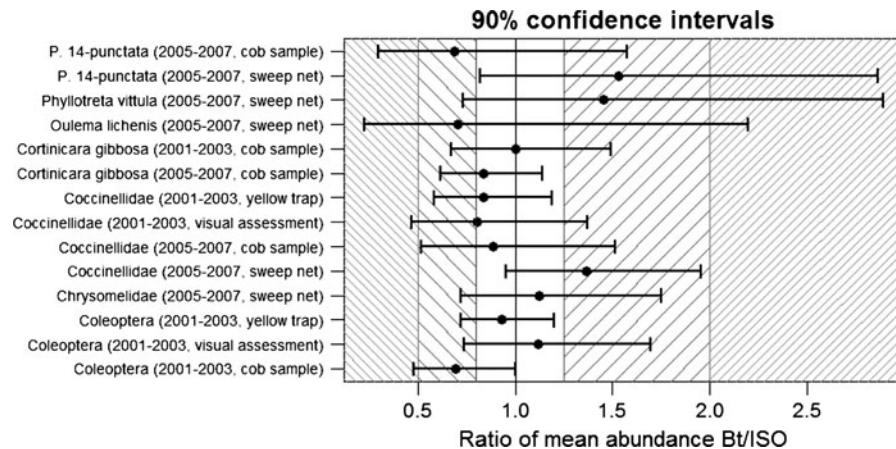
Fig. 4 90% confidence intervals for the ratio of mean abundance in *Bt* relative to the mean abundance in the corresponding near-isogenic conventional line for the 14 most abundant taxa in the two field trials. *Dots* are the estimated ratios of mean abundances (pooled over the years), *bold solid* lines extending below and above the point estimates depict lower and upper confidence intervals. *Densely and less densely shaded areas* indicate two possible definitions of equivalence margins {0.8; 1.25} and {0.5; 2.0}

and *Z. scutellaris* accounting for 6,746 of these (Rauschen, unpublished data). The heteropteran family Miridae was surveyed in the same experiment, with 6 genera and 3,592 insects caught over three years. *Trigonotylus caelestialum* (Kirkaldy) was the most abundant species, with 3,361 individuals (Rauschen et al. 2009). Looking at other non-target Coleoptera only, soil-surface dwelling groups were also much more abundant. Carabidae were much more diverse and exhibited much higher densities (scored as "activity abundances") than all the other beetle families: Toschki et al. (2006) counted a total of 57 species of carabids during the MON810 project, amounting to 142,120 individuals. The carabids were sampled with pitfall traps that were active during a course of 13 to 14 weeks during all three years. Here, *Pterostichus melanarius* (Illiger) was eudominant, with between 80.94 and 92.82% of all individuals caught belonging to this species. A comparatively large number and diverse range of carabids was also present at the MON88017 field-release site. During the course of 2005 and 2006, a total of 12,235 beetles in 63 different species was caught (Priesnitz 2008). The most abundant species was *Calathus fuscipes* (Goeze) with around 50% of all individuals. A further important beetle family are rove beetles (Staphylinidae). They can be caught in similar densities as carabids (e.g. Jasinski et al. 2003), but were not particularly considered in the analysed studies. These species can rarely be found in the maize herb-layer, however. Overall, the densities of other non-target organisms in the same field-release experiments put the numbers obtained for the herb-layer Coleoptera into perspective.

Most herb-layer beetles were caught with the Moericke traps. Between 5 and 10% of the catch belonged to the Coccinellidae. The other beetle families were not individually scored for this method. The number of beetles sampled during the second field-release experiment would have been higher, if Moericke traps had also been employed. This was not done, however, since a large amount of bycatch, mainly flies, midges and thrips, is caught in the yellow traps which results in time-consuming work.

Depending on the organisms to be assessed in the field, a method specifically tailored to these should be used in order to minimise the workload and hence the monetary expenses, while at the same time yielding robust data. Based on the experiences from the first experiment, only methods with which beetles from the herb-layer could more specifically be targeted were used in the second field-trial. This proved to be sensible with respect to the intended identification of specimen to the species level, but reduced the total number of caught insects considerably. This has to be taken into consideration with respect to the subsequent statistical evaluation of the field data.

Coccinellidae

Ladybirds are important key species for biological control of various pests (Obrycki and Kring 1998). They have therefore been the test organisms in various laboratory assays for pesticide registration since a long time, but also for different Cry and other proteins conditioning pest resistance, and also frequently quantitated in field experiments with *Bt*-plants (Wold et al. 2001; Al-Deeb and Wilde 2003; Candolfi et al. 2004; Bhatti et al. 2005b; Daly and Buntin 2005; de la Poza et al. 2005; Dively 2005; Lundgren et al. 2005; McManus et al. 2005; Ahmad et al. 2006; Bai et al. 2006; Romeis et al. 2006; Ferry et al. 2007). They have been shown to be exposed to proteins from genetically modified plants via their prey, provided the prey takes up these proteins (Ferry et al. 2003; Harwood et al. 2005; Obrist et al. 2006; Álvarez-Alfageme et al. 2008). In maize, this is not the case for aphids which are considered to be the most prominent prey item of coccinellids (Raps et al. 2001; Lundgren and Wiedenmann 2005). Feeding on aphids will therefore hold no risk whatsoever for Coccinellidae in maize. In other crop plants however, this must not necessarily be true, since is has been shown for cotton that aphids may transfer *Bt*-proteins to their coccinellid predators (Zhang et al. 2006). The same may be true for *Bt*-oilseed rape which carries Cry-protein in the phloem (Burgio et al. 2007). Moreover ladybirds are not solely carnivorous, but may also consume pollen (Cottrell and Yeargan 1998; Lundgren et al. 2005). Feeding on pollen might lead to considerable exposure, depending on the amount of pollen consumed and the expression of the respective *Bt*-protein in it. Given that in the widely cultivated MON810, the only *Bt*-maize event cultivated in Europe, the expression of Cry1Ab in pollen is very low with a maximum of less than 100 ng/g (Nguyen and Jehle 2007), ladybirds will only be exposed to negligible amounts of Cry-protein via this pathway. For event MON88017, higher amounts of Cry3Bb1 have been observed in pollen, with around 3.7 μg/g (Nguyen and Jehle 2009).

There are conflicting reports on the toxicity of *Bt*-proteins to Coccinellidae, exemplified by two recent publications: Schmidt et al. (2009) report on acute toxic effects of Cry1Ab and Cry3Bb1 on *Adalia bipunctata*. This study has met strong criticism, however, based on a number of flaws (Rauschen

2009; Ricroch et al. 2009). Dhillon and Sharma (2009) report effects of Cry1Ab and Cry1Ac on *Cheilomenes sexmaculatus*, but conclude that negative impacts in the field will probably be negligible. Generally, the literature so far indicates no effect of Cry proteins on Coccinellidae: Álvarez-Alfageme et al. (2008) show an absence of any effect of Cry1Ab on *Stethorus punctillum*. Wold et al. (2001) found a trend for fewer *C. maculata* in Cry1Ab expressing maize, but only in 1 year and also no impact on other coccinellids. Bai et al. (2005; 2006), who investigated the potential effect of Cry1Ab expressing GM-rice pollen and Cry1Ab containing prey on *Propylea japonica* found no indication of a negative impact. The results of Ahmad et al. (2006) show no effect of pollen from Cry3Bb1 expressing corn or of exposed prey items on *Coleomegilla maculata*. There has been no definitive report on a consistent negative impact on ladybirds in field studies with genetically modified *Bt*-plants: Wolfenbarger et al. (2008) in their meta-analysis demonstrated effects of *Bt*-cotton on Coccinellidae as a whole group. For *Bt*-maize, however, they found higher densities of *Coleomegilla*. Such differences were also observed by Marvier et al. (2007). Naranjo (2005) detected a negative impact of *Bt*-cotton on *Hippodamia convergens*, but found no effect on Coccinellidae as a group. For *Bt*-eggplants, Arpaia et al. (2007) found no effect on either *Coccinella* or *Hippodamia* beetles, nor on ladybird larvae.

For future developments of *Bt*-transgenic and other genetically modified crops expressing different entomotoxic proteins, exposure of and hazard for Coccinellidae should foremost be investigated in low-tier laboratory tests, possibly in combination with analyses of protein activities, the concentration of the protein at the molecular target site and other factors and parameters that influence toxicity. Only if some degree of hazard can be detected for an exposure pathway with relevance in the field, further semi-field and field tests should be considered. In these, a set of specialised sampling methods should be employed, with which ladybirds can be reliably detected and enumerated in the field. These experiments should also take into account (1) the natural variability of conventional plant lines, since these might generally have a considerable influence on the field densities of non-target organisms—as shown for the mirid *Trigonotylus caelestialium* (Rauschen et al. 2008), and (2)

the impact of conventional management strategies (CMS) like herbicide and insecticide sprayings. The latter have been shown to have a clear detrimental impact on beneficial insects (Marvier et al. 2007). The CMS potentially to be replaced need to be considered to fully assess the risks and especially the benefits of the wide-spread adoption of a new GM-cultivar and the accompanying crop management.

On another note, it might be worthwhile to include *Harmonia axyridis* (Pallas) into the list of Coccinellidae to be tested for the maize agro-ecosystem, since this species can now be found regularly in central Europe and can be regarded as established.

Chrysomelidae

Non-target leaf beetles are regularly found in maize (Kiss et al. 2002, undated; Daly and Buntin 2005) and are exposed to the *Bt*-proteins based on their herbivorous feeding, as shown for the genera *Chaetocnema* (Harwood et al. 2005) and *Oulema* (Obrist et al. 2006). An impact of the cultivation of *Diabrotica*-resistant *Bt*-maize can reasonably be expected, depending on the uptake of Cry-protein(s), the amount of these proteins reaching the insect gut in bioactive form and the susceptibility of the individual species to the toxic action. This scenario must not necessarily be of concern, however, since the leaf beetles occurring in the maize field can be considered to be minor or secondary pests. In Hungary, for example, they often reach densities that can result in visible damage to maize plants (J. Kiss, personal communication). A reduction of their densities therefore would rather be an added benefit of *Diabrotica*-resistant *Bt*-plants than an environmental concern. Other non-target chrysomelid beetles living on their host plants outside and around maize fields may be exposed to Cry-proteins through pollen deposited on the plant surfaces during anthesis. For this scenario to be of concern, a number of factors must act jointly together: (1) the close proximity of a leaf beetle's host plant to maize stands; (2) the occurrence of susceptible life-stages of the beetle during the time of anthesis; (3) the amount of pollen deposited on a given leaf area of the host plant; (4) the size of leaf area consumed by the susceptible life stage; (5) the amount of toxin per gram of pollen; (6) the level of susceptibility of the life stage in question; (7) the severity of the impact on exposed individuals;

(8) the percentage of affected individuals of the local population; (9) the relevance of this impact in comparison to other environmental, biotic and abiotic factors. This is generally in line with assessments and risk characterisations for Lepidoptera and Cry1Ab expressing *Bt*-maize (Sears et al. 2001; Gatehouse et al. 2002). Given the low densities of Chrysomelidae at the two field-release sites, the relevance of the leaf beetles in the Environmental Risk Assessment of genetically modified *Bt*-maize at least in central Europe seems to be negligible.

Lathridiidae

The importance and role of the minute brown scavenger beetles within the maize agro-ecosystems remain unclear. They have been reported in Eckert et al. (2006) from maize cobs and were therefore investigated in the second field-release experiment. They are mycetovorous/saprovorous and may therefore play a role in organic matter degradation. However, their contribution to the organic matter cycling in maize and the overall degradation in this crop is not known.

Based on their feeding habit, it can be assumed that they will be exposed to only low amounts of Cry-proteins. This leads to the conclusion that a negative impact on these beetles will have a low probability.

There is a large gap in knowledge, however, that could be addressed by further research.

Carabidae

In the herb-layer, only the genus of *Demetrias* was observed with a few individuals. However, ground beetles are an important group of biological control agents (Lang et al. 1999; Kromp 1999) and exposed to *Bt*-proteins due to both feeding on exposed prey and on plant materials (Meissle et al. 2005; Zwahlen and Andow 2005; Priesnitz et al. 2007), and are therefore interesting in Environmental Risk Assessment. Despite this exposure, field studies have shown an absence of any negative impact of the cultivation of *Bt*-plants on these arthropods, for MON810 (Szekeres et al. 2006; Toschki et al. 2006; Floate et al. 2007), for *Bt*-transgenic vegetables (Leslie et al. 2007), for MON88017 (Priesnitz et al. 2007), for MON863 (Bhatti et al. 2005a). In laboratory assays,

there has been no direct toxic effect of *Bt*-crops observed on selected carabids, neither in potato (Ferry et al. 2007), nor in *Bt*-canola (Ferry et al. 2006), nor in *Bt*-corn (Ahmad et al. 2006). It has also been shown, that carabids must not necessarily take up *Bt*-proteins via their prey, even if the prey contains them (Harwood et al. 2006).

Again, the published literature points to an absence of a direct toxic effect of *Bt*-plants to carabids, even in the case of *Diabrotica*- or *Leptinotarsa*-resistant cultivars. Field studies can help to identify regionally representative species of ground-dwelling carabids that might serve as surrogates in early-tier laboratory tests. An integration of our knowledge on the occurrence and distribution of carabids in maize in Europe is direly needed to establish geographically adapted lists of non-target ground-beetles to be assessed in future experiments.

Implications for Environmental Risk Assessment

Beetles are a diverse and important group of insects in agricultural systems. They comprise both important biological control agents (Coccinellidae, Carabidae) and economically important pests (Chrysomelidae). Other families (Staphylinidae, Lathridiidae, Nitidulidae, Anthicidae, Curculionidae, Cantharidae) have diverse ecological functions, but are mostly caught sporadically and in very low densities in maize. In Environmental Risk Assessment it is appropriate to especially consider those groups of non-target organisms that (1) serve anthropogenically valued purposes (both tangible and intangible), (2) have important ecological functions within the ecosystem, (3) are widespread and occur regularly and (4) can be tested in the laboratory and also assessed in the field.

Coccinellidae and Carabidae meet all of these characteristics. However, the results of the simulation studies show that it is extremely difficult to show equivalence between a *Bt* and its near-isogenic variety in terms of the densities of Coccinellidae, if the mean densities of the species/group are low and they show some extra variability. Testing risk hypotheses for Coccinellidae in the field and using their mean densities as an assessment endpoint requires a large number of replicates and long experimental times, if mean densities are low. It is questionable if recommendations that experiments should be designed to detect differences of 10%

between treatments (e.g. Andow and Hilbeck 2004) are realistic for field experiments, given the natural variability and differences between conventional cultivars. Furthermore, detecting differences of 20–30% will also be hardly achievable for most other species, in particular foliage foraging predators, which often occur in low densities. This means that only species occurring in large numbers can be reasonably tested in the field and should therefore be the focus of research. Other species should be thoroughly tested in early tier laboratory tests to comprehensively assess the potential risks of *Bt*-maize varieties. If higher tier tests were warranted, these would require greater efforts than are currently undertaken in field experiments. For herb-layer Carabidae it is obvious based on the presented data, that an assessment of potential negative impacts of *Bt*-maize in the field does not prove to be a fruitful endeavour. If these conclusions hold true for ground-dwelling Carabidae remains to be assessed.

Chrysomelidae do not meet all of the above mentioned characteristics for relevant non-target organisms, as these are mostly pests within the agricultural landscape. Therefore, their value as indicator species in field tests is limited.

Other coleopteran groups, like the Staphylinidae serve important functions (Albajes et al. 2003). However, predatory rove beetles may occur inconsistently at a given site or in only very low densities. Saprophagous Staphylinidae require special sampling techniques (e.g. soil emergence traps, soil bait cylinders, pitfall arenas) because of their obscure lifestyle. While they may be amenable to testing in the laboratory, results cannot easily be confirmed or refuted in field experiments. Also, there are difficult to determine to the species level and require a substantial expertise.

The significance of Lathridiidae remains unclear. Given their mode of feeding and the obtained results so far, it appears that they are and will probably not be affected by *Bt*-maize plants.

In conclusion, by integrating the presented data and the literature reports we propose to concentrate the Environmental Risk Assessment for insect resistant *Bt*-maize plants on herb-layer ladybirds and ground beetles. The majority of the assessment should be performed in controlled laboratory experiments, since higher-tier field-scale experiments will need disproportional effort to reach robust and sound

conclusions on potential impacts of genetically modified maize varieties on species occurring in low densities.

References

Ahmad A, Wilde GE, Whitworth RJ, Zolnerowich G (2006) Effect of corn hybrids expressing the coleopteran-specific Cry3Bb1 protein for corn rootworm control on above-ground insect predators. Econ Entomol 99:1085–1095

Albajes R, López C, Pons X (2003) Predatory fauna in corn-fields and response to imidacloprid seed treatment. Econ Entomol 96:1805–1813

Al-Deeb MA, Wilde GE (2003) Effect of Bt corn expressing the Cry3Bb1 toxin for corn rootworm control on above-ground nontarget arthropods. Environ Entomol 32:1164–1170

Álvarez-Alfageme F, Ferry N, Castañera P, Ortego F, Gatehouse AMR (2008) Prey mediated effects of Bt maize on fitness and digestive physiology of the red spider mite predator Stethorus punctillum Weise (Coleoptera: Coccinellidae). Transgenic Res 17:943–954

Andow DA (2003) Negative and positive data, statistical power, and confidence intervals. Environ Biosafety Res 2:1–6

Andow DA, Hilbeck A (2004) Science-based risk assessment for nontarget effects of transgenic crops. Bioscience 54:637–649

Arpaia S, Di Leo GM, Fiore MC, Schmidt JEU, Scardi M (2007) Composition of arthropod species assemblages in Bt-expressing and near isogenic eggplants in experimental fields. Environ Entomol 36:213–227

Bai YY, Jiang MX, Cheng JA (2005) Effects of transgenic rice pollen on fitness of Propylea japonica. J Pest Sci 78:123–128

Bai YY, Jiang MX, Cheng JA, Wang D (2006) Effects of Cry1Ab toxin on Propylea japonica (Thunberg) (Coleoptera: Coccinellidae) through its prey, Nilaparvata lugens (Stål) (Homoptera: Delphacidae), feeding on transgenic Bt rice. Environ Entomol 35:1130–1136

Bhatti MA, Duan J, Head GP, Jiang C, McKee MJ, Nickson TE, Pilcher CL, Pilcher CD (2005a) Field evaluation of the impact of corn rootworm (Coleoptera: Chrysomelidae)—protected Bt corn on ground-dwelling invertebrates. Environ Entomol 34:1325–1335

Bhatti MA, Duan J, Head GP, Jiang C, McKee MJ, Nickson TE, Pilcher CL, Pilcher CD (2005b) Field evaluation of the impact of corn rootworm (Coleoptera: Chrysomelidae)—protected Bt corn on foliage-dwelling arthropods. Environ Entomol 34:1336–1345

Burgio G, Lanzoni A, Accinelli G, Dinelli G, Bonetti A, Marotti I, Ramilli F (2007) Evaluation of Bt-toxin uptake by the non-target herbivore, Myzus persicae (Hemiptera: Aphididae), feeding on transgenic oilseed rape. Bull Entomol Res 97:211–215

Candolfi MP, Brown K, Grimm C, Reber B, Schmidli H (2004) A faunistic approach to assess potential side-effects of genetically modified Bt-corn on non-target arthropods under field conditions. Biocontrol Sci Technol 14:129–170

Cottrell TE, Yeargan KV (1998) Effect of pollen on Coleomegilla maculata (Coleoptera: Coccinellidae) population density, predation, and cannibalism in sweet corn. Environ Entomol 27:1402–1410

Daly T, Buntin GD (2005) Effect of Bacillus thuringiensis transgenic corn for lepidopteran control on nontarget arthropods. Environ Entomol 34:1292–1301

de la Poza M, Pons X, Farinós GP, López C, Ortego F, Eizaguirre M, Castañera P, Albajes R (2005) Impact of farm-scale Bt maize on abundance of predatory arthropods in Spain. Crop Prot 24:677–684

Dhillon MK, Sharma HC (2009) Effects of Bacillus thuringiensis-endotoxins Cry1Ab and Cry1Ac on the coccinellid beetle, Cheilomenes sexmaculatus (Coleoptera, Coccinellidae) under direct and indirect exposure conditions. Biocontrol Sci Technol 19:407–420

Dively GP (2005) Impact of transgenic VIP3A x Cry1Ab Lepidopteran-resistant field corn on the nontarget arthropod community. Environ Entomol 34:1267–1291

EC (2001) Directive 2001/18/EC of the European parliament and of the council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing council directive 90/220/EEC. L 106/1 of 4/17/2001

EC (2002) Council decision of 3 October 2002 establishing guidance notes supplementing annex VII to directive 2001/18/EC of the European parliament and of the council on the deliberate release into the environment of genetically modified organisms and repealing council directive 90/220/EEC. L 280/27 of 10/18/2002

Eckert J, Schuphan I, Hothorn LA, Gathmann A (2006) Arthropods on maize ears for detecting impacts of Bt maize on nontarget organisms. Environ Entomol 35:554–560

EFSA (2006) Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed. EFSA J 99:1–100

EFSA (2009) Scientific opinion on statistical considerations for the safety evaluation of GMOs. EFSA J 1250

Ferry N, Raemaekers RJM, Majerus MEN, Jouanin L, Port G, Gatehouse JA, Gatehouse AMR (2003) Impact of oilseed rape expressing the insecticidal cysteine protease inhibitor oryzacystatin on the beneficial predator Harmonia axyridis (multicoloured Asian ladybeetle). Mol Ecol 12:493–504

Ferry N, Mulligan EA, Stewart CN, Tabashnik BE, Port GR, Gatehouse AMR (2006) Prey-mediated effects of transgenic canola on a beneficial, non-target, carabid beetle. Transgenic Res 15:501–514

Ferry N, Mulligan EA, Majerus MEN, Gatehouse AMR (2007) Bitrophic and tritrophic effects of *Bt* Cry3A transgenic potato on beneficial, non-target, beetles. Transgenic Res 16:795–812

Floate KD, Cárcamo HA, Blackshaw RE, Postman B, Bourassa S (2007) Response of ground beetle (Coleoptera: Carabidae) field populations to four years of lepidoptera-specific *Bt* corn production. Environ Entomol 36:1269–1274

Gao Y, Schafer BW, Collins RA, Herman RA, Xu X, Gilbert JR, Ni W, Langer VL, Tagliani LA (2004) Characterization of Cry34Ab1 and Cry35Ab1 insecticidal crystal proteins expressed in transgenic corn plants and *Pseudomonas fluorescens*. J Agric Food Chem 52:8057–8065

Gatehouse AMR, Ferry N, Raemaekers RJM (2002) The case of the monarch butterfly: a verdict is returned. Trends Genet 18:249–251

Glare TR, O'Callaghan M (2000) *Bacillus thuringiensis*: biology, ecology and safety. Wiley, Chichester

Harwood JD, Wallin WG, Obrycki JJ (2005) Uptake of *Bt* endotoxins by nontarget herbivores and higher order arthropod predators: molecular evidence from a transgenic corn agroecosystem. Mol Ecol 14:2815–2823

Harwood JD, Samson RA, Obrycki JJ (2006) No evidence for the uptake of Cry1Ab *Bt*-endotoxins by the generalist predator *Scarites subterraneus* (Coleoptera: Carabidae) in laboratory and field experiments. Biocontrol Sci Technol 16:377–388

Hummel HE (2003) Introduction of *Diabrotica virgifera virgifera* into the Old World and its consequences: a recently acquired invasive alien pest species on *Zea mays* from North America. Commun Agric Appl Biol Sci 68:45–57

Kiss J, Szentkirályi F, Tóth F, Szénási A, Kádár F, Árpás K, Szekeres D, Edwards CR (2002) *Bt* corn: impact on non-targets and adjusting to local IPM systems. In: Lelley T, Balázs E, Tepfer M (eds) Ecological impact of GMO dissemination in agro-ecosystems. Facultas, Wien, pp 157–172

Kiss J, Edwards CR, Berger HK, Cata P, Cean M, Cheek S, Derron J, Festic H, Furlan L, Igrc-Barcic J, Ivanova I, Lammers W, Omelyuta V, Princzinger G, Reynaud P, Sivcec I, Sivicek P, Urek G, Vahala O (2005) Monitoring of western corn rootworm (Diabrotica virgifera virgifera LeConte) in Europe 1992–2003. In: Vidal S, Kuhlmann U, Edwards CR (eds) Western corn rootworm: ecology and management. CABI Publishing, Wallingford, pp 29–39

Kiss J, Szentkirályi F, Szénási A, Tóth F, Szekeres D, Kádár F, Árpás K, Edwards CR (undated) Effect of transgenic *Bt*-corn on biodiversity of non-target insects in corn fields. Excerpt from the report on Contract Number QLK3-CT-2000-00547 (project "*Bt*-BioNoTa") within the 5th EU framework programme provided by József Kiss

Kromp B (1999) Carabid beetles in sustainable agriculture: a review on pest control efficacy, cultivation impacts and enhancement. Agric Ecosyst Environ 74:187–228

Lang A, Filser J, Henschel JR (1999) Predation by ground beetles and wolf spiders on herbivorous insects in a maize crop. Agric Ecosyst Environ 72:189–199

Leslie TW, Hoheisel GA, Biddinger DJ, Rohr JR, Fleischer SJ (2007) Transgenes sustain epigeal insect biodiversity in diversified vegetable farm systems. Environ Entomol 36:234–244

Lundgren JG, Wiedenmann RN (2005) Tritrophic interactions among *Bt* (Cry3Bb1) corn, aphid prey, and the predator *Coleomegilla maculata* (Coleoptera: Coccinellidae). Environ Entomol 34:1621–1625

Lundgren JG, Huber A, Wiedenmann RN (2005) Quantification of consumption of corn pollen by the predator Coleomegilla maculata (Coleoptera: Coccinellidae) during anthesis in an Illinois cornfield. Agric For Entomol 7:53–60

Marvier M, McCreedy C, Regetz J, Kareiva P (2007) A meta-analysis of effects of *Bt* cotton and maize on nontarget invertebrates. Science 316:1475–1477

McCullagh P, Nelder JA (1989) Generalized linear models. Chapman & Hall/CRC, Boca Raton

McCulloch CE, Searle SR (2001) Generalized, linear and mixed models. Wiley, New York

McManus BL, Fuller BW, Boetel MA, French BW, Ellsbury MM, Head GP (2005) Abundance of *Coleomegilla maculata* (Coleoptera: Coccinellidae) in corn rootworm-resistant Cry3Bb1 maize. Econ Entomol 98:1992–1998

Meissle M, Vojtech E, Poppy GM (2005) Effects of *Bt* maize-fed prey on the generalist predator *Poecilus cupreus* L. (Coleoptera: Carabidae). Transgenic Res 14:123–132

Miller N, Estoup A, Toepfer S, Bourguet D, Lapchin L, Derridj S, Kim KS, Reynaud P, Furlan L, Guillemaud T (2005) Multiple transatlantic introductions of the western corn rootworm. Science 310:992

Naranjo SE (2005) Long-term assessment of the effects of transgenic *Bt* cotton on the abundance of nontarget natural enemies. Environ Entomol 35:1193–1210

Nguyen HT, Jehle JA (2007) Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize MON810. J Plant Dis Prot 114:82–87

Nguyen HT, Jehle JA (2009) Expression of Cry3Bb1 in transgenic maize MON 88017. J Agric Food Chem 57:9990–9996

Obrist LB, Dutton A, Albajes R, Bigler F (2006) Exposure of arthropod predators to Cry1Ab toxin in *Bt* maize fields. Ecol Entomol 31:143–154

Obrycki JJ, Kring TJ (1998) Predaceous Coccinellidae in biological control. Annu Rev Entomol 43:295–321

Ostlie K (2001) Crafting crop resistance to corn rootworms. Nat Biotechnol 19:624–625

Perry JN, ter Braak C, Dixon PM, Duan JJ, Hails R, Huesken A, Lavielle M, Marvier M, Scardi M, Schmidt K, Toth-meresz B, Schaarschmidt F, van der Voet H (submitted) Statistical aspects of environmental risk assessment of GM plants for effects on non-target organisms

Priesnitz KU (2008) Biologische Sicherheitsforschung an einem *Bt*-Mais mit integrierter Maiswurzelbohrer-Resistenz: Mögliche Auswirkungen des Anbaus von MON88017 auf epigäische Raubarthropoden. Jahresbericht 2007 der Bayerischen Landesanstalt für Landwirtschaft, Institut für Pflanzenschutz. www.lfl-bayern.de

Priesnitz KU, Benker U, Roß-Nickoll M (2007) Impact of cole-opteran-specific *Bt* maize on carabid beetles: results after two years of field and laboratory research. Talk held at the IOBC/wprs EIGMO meeting, 23–25 May 2007, Warsaw, Poland

R Development Core Team (2008) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org

Raps A, Kehr J, Gugerli P, Moar WJ, Bigler F, Hilbeck A (2001) Immunological analysis of phloem sap of Bacillus thuringiensis corn and of the nontarget herbivore Rhopalosiphum padi (Homoptera: Aphididae) for the presence of Cry1Ab. Mol Ecol 10:525–533

Rauschen S (2009) A case of "pseudo science"? A study claiming effects of the Cry1Ab protein on larvae of the two-spotted ladybird is reminiscent of the case of the green lacewing. Transgenic Res doi: 10.1007/s11248-009-9301-0

Rauschen S, Eckert J, Schaarschmidt F, Schuphan I, Gathmann A (2008) An evaluation of methods for assessing the impacts of Bt-maize MON810 cultivation and pyrethroid insecticide use on Auchenorrhyncha (Planthoppers and Leafhoppers). Agric For Entomol 10:331–339

Rauschen S, Schultheis E, Pagel-Wieder S, Schuphan I, Eber S (2009) Impact of Bt-corn MON88017 in comparison to three conventional lines on Trigonotylus caelestialium (Kirkaldy) (Heteroptera: Miridae) field densities. Transgenic Res 18:203–214

Ricroch A, Bergé J B, Kuntz M (2009) Is the German suspension of MON810 maize cultivation scientifically justified? Transgenic Res doi: 10.1007/s11248-009-9297-5

Roh JY, Choi JY, Li MS, Jin BR, Je YH (2007) Bacillus thuringiensis as a specific, safe, and effective tool for insect pest control. J Microbiol Biotechnol 17:547–559

Romeis J, Meissle M, Bigler F (2006) Transgenic crops expressing Bacillus thuringiensis toxins and biological control. Nat Biotechnol 24:63–71

Romeis J, Bartsch D, Bigler F, Candolfi MP, Gielkens MMC, Hartley SE, Hellmich RL, Huesing JE, Jepson PC, Layton R, Quemada H, Raybould A, Rose RI, Schiemann J, Sears MK, Shelton AM, Sweet J, Vaituzis Z, Wolt JD (2008) Assessment of risks of insect-resistant transgenic crops to nontarget arthropods. Nat Biotechnol 26:203–208

Schaarschmidt F, Gathmann A (submitted) Assessing equivalence for non-target Lepidoptera in field trials

Schmidt JEU, Braun CU, Whitehouse LP, Hilbeck A (2009) Effects of activated Bt transgene products (Cry1Ab, Cry3Bb) on immature stages of the ladybird Adalia bipunctata in laboratory ecotoxicity testing. Arch Environ Contam Toxicol 56:221–228

Schnepf E, Crickmore N, van Rie J, Lereclus D, Baum J, Feitelson F, Zeigler DR, Dean DH (1998) Bacillus thuringiensis and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Sears MK, Hellmich RL, Stanley-Horn DE, Oberhauser KS, Pleasants JM, Mattila HR, Siegfried BD, Dively GP (2001) Impact of Bt corn pollen on monarch butterfly populations: a risk assessment. Proc Natl Acad Sci USA 98:11937–11942

Szekeres D, Kadar F, Kiss J (2006) Activity density, diversity and seasonal dynamics of ground beetles (Coleoptera: Carabidae) in Bt-(MON810) and in isogenic maize stands. Entomol Fenn 17:269–275

Todd JH, Ramankutty P, Barraclough EI, Malone LA (2008) A screening method for prioritizing non-target invertebrates for improved biosafety testing of transgenic crops. Environ Biosafety Res 7:35–56

Toschki T, Hothorn LA, Roß-Nickoll M (2006) Effects of cultivation of genetically modified Bt maize on epigeic arthropods (Araneae; Carabidae). Environ Entomol 36:967–981

Vaughn T, Cavato T, Brar G, Coombe T, DeGooyer T, Ford S, Groth M, Howe A, Johnson S, Kolacz K, Pilcher C, Purcell J, Romano C, English L, Pershing J (2005) A method of controlling corn rootworm feeding using a Bacillus thuringiensis protein expressed in transgenic maize. Crop Sci 45:931–938

Venables WN, Ripley BD (2002) Modern applied statistics with S, 4th edn. Springer, New York

Walters FS, Stacy CM, Lee MK, Palekar N, Chen JS (2008) An engineered Chymotrypsin/Cathepsin G site in domain I renders Bacillus thuringiensis Cry3A active against western corn rootworm larvae. Appl Environ Microbiol 74:367–374

Wellek S (2003) Testing statistical hypotheses of equivalence. Chapman & Hall/CRC, Boca Raton

Wold SJ, Burkness EC, Hutchison WD, Venette RC (2001) In-field monitoring of beneficial insect populations in transgenic corn expressing a Bacillus thuringiensis toxin. J Entomol Sci 36:177–187

Wolfenbarger LL, Naranjo SE, Lundgren JG, Bitzer RJ, Watrud LS (2008) Bt crop effects on functional guilds of non-target Arthropods: a meta-analysis. PLoS One 3:e2118

Zhang G-F, Wan F-H, Lövei GL, Liu W-X, Gui J-Y (2006) Transmission of Bt toxin to the predator Propylea japonica (Coleoptera: Coccinellidae) through its aphid prey feeding on transgenic Bt cotton. Environ Entomol 35:143–150

Zwahlen C, Andow DA (2005) Field evidence for the exposure of ground beetles to Cry1Ab from transgenic corn. Environ Biosafety Res 4:113–117