“Biosafety research into *Diabrotica*-resistant Bt-maize: Indicator organisms and monitoring methods”

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Meinen Eltern.

The genetic modification of crop plants holds many possibilities for the improvement of different plant characteristics, or for the simplification of agricultural practices. The most common modifications are resistances against different insect pests on the basis of *Bacillus thuringiensis* proteins, as well as tolerances against the herbicidal compounds glyphosate and glufosinate.
EU directive 2001/18/EC and regulation 1829/2003/EC stipulate an environmental risk assessment for and a monitoring of genetically modified plants. In each single case, a tiered case-by-case assessment has to be carried out, before a GM-plant can be marketed.
A development with future relevance for the European market are GM-maize varieties with resistance against the Western corn rootworm *Diabrotica virgifera* virgifera. This chrysomelid beetle has been introduced from the United States and poses a dire threat to the maize production in Europe. The resistance of maize plants is based on the expression of the Bt-protein Cry3Bb1 in all green tissued. This protein is supposed to be specific against leaf beetles (Chrysomelidae).
The potential impact of the cultivation of the Bt-maize variety MON88017 on non-target arthropods in the maize herb-layer was assessed. The non-deregulated MON88017, its near-isogenic line DKC5143 and the two conventional hybrids DK315 and Benicia were cultivated on a field-release site. Sweep netting, sticky traps, cob and panicle samples were used to survey the herb layer fauna, and were evaluated for their applicability in the context environmental monitoring. The exposure of selected arthropods to Cry3Bb1 was assessed with an immunological assay (DAS-ELISA).
A very diverse community of arthropods was found in the herb layer, which in part could be identified as being bound to maize based on published data. Two herbivorous arthropods were of special interest because of their high densities during the 3 field seasons: the maize leafhopper *Zyginidia scutellaris* and the rice leaf bug *Trigonotylus caelestialium*. Both species are considerably exposed to the Bt-proteins expressed in genetically modified maize, because of their mode of feeding. This and their wide distribution over the whole of Europe make them candidates for surrogate non-target species in future assessments of genetically modified maize.
The statistical analyses showed no negative impact of MON88017 on the selected non-target organisms. For *T. caelestialium*, a strong and consistent varietal effect could be shown, however: the two conventional maize lines DK315 and Benicia were significantly different with regard to the observed field densities of *T. caelestialium*. This underlines the necessity for ecologically meaningful baselines as references in the assessment of the enviromental impacts of the cultivation of genetically modified plants. This is a challenge for future biosafety research.
The methods employed are all suitable for monitoring and surveillance purposes, however, some special aspects need to be considered and merit attention.

Die gentechnische Veränderung von Kulturpflanzen bietet eine Fülle von Möglichkeiten zur Optimierung verschiedener Eigenschaften, sowie der Vereinfachung der Kulturführung. Die bislang bedeutsamsten Veränderungen sind verschiedene Insektenresistenzen auf Basis von *Bacillus thuringiensis* Proteinen, sowie eine Herbizidtoleranz gegenüber den Wirkstoffen Glyphosat und Glufosinat.
Die EU Richtlinien 2001/18/EC und 1829/2003/EC schreiben die Bewertung und Umweltbeobachtung von gentechnisch veränderten Pflanzen vor. Für jede Kulturpflanze, für die ein Antrag zur Inverkehrbringung in der EU gestellt wird, muss eine stufenweise Einzelfallbewertung vorgenommen werden.
Eine zukünftig für den europäischen Markt relevante Entwicklung sind gentechnisch veränderte Maispflanzen mit einer Resistenz gegenüber dem Westlichen Maiswurzelbohrer *Diabrotica virgifera virgifera*. Dieser Blattkäfer wurde aus den Vereinigten Staaten eingeschleppt und stellt eine ernste Bedrohung für den Maisanbau in Europa dar. Die Resistenz der Maispflanzen basiert auf der Expression des Bt-Proteins Cry3Bb1, das in allen grünen Pflanzenteilen gebildet wird und als spezifisch gegen Blattkäfer (Chrysomelidae) gilt.
Die möglichen Auswirkungen des Anbaus der Maissorte MON88017 auf Nichtzielorganismen der Krautschicht in Mais wurden untersucht. Auf einem Versuchsfeld wurden im Rahmen einer Freisetzung die noch nicht in der EU zugelassene Sorte MON88017, die zu ihr nah-isogene Linie DKC5143, sowie die beiden konventionellen Sorten DK315 und Benicia angebaut. Es wurden Kescherfänge, Klebetafeln, Kolbenproben und Blütenklopfproben zum Fang der Krautschichtfauna angewandt und hinsichtlich ihrer Eignung für die Umweltbeobachtung evaluiert. Für ausgewählte Arthropoden wurde ihre Exposition gegenüber dem Cry3Bb1 Protein mittels immunologischen Nachweisverfahren (DAS-ELISA) untersucht.
Es konnte eine sehr diverse Gemeinschaft an Arthropoden in der Krautschicht des Maises festgestellt werden, die mit Hinblick auf bisherige Arbeiten eine spezielle, teilweise an den Mais gebundene Artengemeinschaft erkennen läßt. Zwei herbivore Arthropoden waren auf Grund ihrer Dichten über die 3 Versuchsjahre von besonderer Bedeutung: die Maisblattzikade *Zyginidia scutellaris* und die Weichwanze *Trigonotylus caelestialium*. Beide Arten sind auf Grund ihrer Ernährungsweise gegenüber den im gentechnisch veränderten Mais exprimierten Bt-Proteinen in hohem Maße exponiert. Aus diesem Grund und wegen ihrer weiten Verbreitung in ganz Europa werden diese beiden Spezies als Stellvertreterarten für zukünftige Untersuchungen mit gentechnisch verändertem Mais vorgeschlagen.
Die statistische Analyse ergab, dass ein negativer Einfluss von MON88017 auf die untersuchten Nichtzielorganismen nicht nachweisbar ist. Für die Weichwanze *T. caelestialium* konnte aber ein deutlicher, über die Jahre konsistenter Sorteneffekt gezeigt werden: die im Freisetzungsversuch angebauten konventionellen Sorten DK315 und Benicia unterschieden sich hinsichtlich der Dichten der in ihnen vorkommenden *T. caelestialium* statistisch signifikant. Dies veranschaulicht die Notwendigkeit von ökologisch sinnvollen Basislinien als Referenz in der Bewertung der Umweltauswirkungen des Anbaus von gentechnisch veränderten Pflanzen. Dies stellt eine Herausforderung für die zukünftige Biosicherheitsforschung dar.
Die angewandten Methoden eignen sich prinzipiell alle für die Umweltbeobachtung von gentechnisch verändertem Mais. Einige Aspekte erfordern aber besondere Aufmerksamkeit.

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1. Abstract

1.1 Thematic overview

The genetic modification of crops for insect resistance derived from *Bacillus thuringiensis* (Berliner) genes is a prominent technology in current agriculture. Most common Bt-crops are cotton and maize, with Bt-varieties of potato, tomato and eggplant also available. Even rice, the most important crop worldwide has been successfully modified using the Bt-trait. In Europe, only Bt-maize resistant against the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) has been commercialised so far. The only event of this kind currently eligible for planting in the European Union is MON810.

A novel trait provided by the Bt-technology is resistance to the Western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) based on the expression of the coleopteran-specific protein Cry3Bb1. This pest has been introduced to Europe from the United States and has spread rapidly, already reaching economic thresholds in some Balkan states, especially Serbia. *Diabrotica* is generally regarded as a serious threat to European maize production, because of its feeding on plant roots as well as silk and corn cobs. Severe measures have been put forward by the European Union (EU) Commission to prevent the further spread of *Diabrotica*. Genetically modified maize may become a key strategy to contain or eliminate WCR populations, since these are not readily controlled by current plant protection practices including crop rotation.

Under EU legislation, authorisation based on Environmental Risk Assessment (ERA) of genetically modified plants is mandatory before these are placed on the market. Additionally, directive 2001/18/EC stipulates a monitoring plan for the continuous observation of potential environmental impacts, which have not necessarily been identified in the risk assessment stage, after a new GMO crop is placed on the market. This monitoring comprises two parts: 1) the hypothesis-driven Case Specific Monitoring, which is only necessary if certain risks have been identified in the ERA that warrant further observation, and 2) the General Surveillance, which focuses on possible adverse effects not foreseen in the environmental risk assessment and which is open to how a GMO may affect the environment.

One of the possible adverse effects of the cultivation of genetically modified plants is the negative impact on the occurrence and density of non-target organisms (NTO) on the cultivated land or the surrounding crop margins, as well on the local as on the regional scale. This impact can be direct - uptake of Bt-protein by feeding on parts of the genetically modified plant or through Bt-protein laden prey and subsequent toxic action of the protein against the NTO - or indirect - reduced quality and abundance of susceptible prey and therefore for example reduced parasitoid and predator densities. The potentially affected NTOs of significance, the relevant exposure pathways, the potential effects, the relevance of the subsequent ecological changes in a given agro-ecosystem and the (experimental) ways to assess the likelihood and magnitude of such changes are identified in the first step of the ERA, which is called Problem Formulation. In Problem Formulation, available data and expert opinion are taken into account to identify areas of concern or uncertainty and thus to streamline and purposefully target the following ERA process on relevant and important management or protection goals.

In risk assessment it is important to quantitate the two factors constituting risk: first hazard, the nature and magnitude of the negative effect of a Bt-protein on an NTO; second exposure, the way, amount, probability and circumstance under which an NTO comes into contact with a Bt-protein. Hazard can most conveniently and accurately be quantitated in first tier laboratory tests, with defined and controlled conditions. If a hazard is identified under worst-case conditions, semi-field and field tests are needed to fully assess the probability of a hazard under more realistic circumstances of exposure. In general, a risk assessment is only sensible for species that are exposed to the Cry protein in the field. Without exposure, no risk can reasonably be anticipated, even if a possible hazard has been identified in the laboratory. On the other hand, exposure to a Bt-protein does not automatically imply the existence of a risk: the documented specificity of Cry proteins restricts their toxicity to certain groups of insects. The non-susceptibility of an NTO negates the existence of a risk despite exposure in the field. However, exposed insects can be a link to higher trophic levels, and therefore it is of paramount importance to assess exposure under field conditions. In addition, the mode of action of a specific Cry protein is not always fully understood, so that the possibility of a non-target action cannot be completely discarded. For these reasons, arthropods that are exposed to transgenic proteins based on their mode of feeding and the plant substrates they

utilise are a reasonable choice in the assessment of environmental non-target effects.

There is an ongoing discussion about the merits of field versus laboratory experiments and the kind of species that should be selected for early tier tests in risk assessment. Field experiments have the major advantage of testing the possible impact of a genetically modified plant under the most realistic conditions. This asset, however, is bought with an increase in variance in the data. This leads to an increase in uncertainty over whether an observation is indeed based on an effect or rather the consequence of a variable that could not be controlled under field conditions (such as weather, soil parameters, immigration of insects from surrounding crops). Laboratory tests on the other hand allow robust and reproducible observations on clearly defined endpoints under controlled conditions. The ecological relevance of findings under these conditions often remains questionable, however, based on the artificial environment, unrealistically high exposure and oversimplified trophic interactions. Field experiments give room to assess such interactions and the ecological functions of non-target organisms in the environment, yielding information that are indispensable in the wider risk analysis procedure. Furthermore, such experiments put the whole plant under scrutiny. This allows the investigation of possible unintended, pleiotropic effects, based on the insertion of the transgenic sequence or positional influences. These will be of significance if they lead to changes in the metabolism of the plant that have an influence on the associated organisms. Another major difference between laboratory and field experiments is the fact than in the first only a small number of surrogate species can be tested, while in the latter a whole range of non-target organisms can be studied. On the other hand, however, species for early tier tests can be chosen based on a number of defined criteria, while the species in field experiments are those naturally occurring at the geographic location of the experimental site.

1.2 Aims and scope of this work

The genetically modified Bt-maize line MON88017 underwent Environmental Assessment by the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS). Based on the history of safe use of maize, the substantial equivalence of MON88017 to conventional maize varieties and its

similarity to the already commercialised MON863, the USDA-APHIS determined that MON88017 will not have any significant impacts on the human environment. In the European Union, MON88017 is currently under evaluation by the European Food Safety Authority (EFSA) according to Regulation (EC) No 1829/2003. The application for authorization aims at this maize line to be eligible for import and processing and as animal feed and human food, but not for cultivation. Nonetheless, the cultivation of genetically modified maize lines with WCR resistance within the EU is probable for the near future, since Cry3Bb1 expressing maize lines are currently the only reasonable long-term strategy to control WCR populations. Based on the need for the continuous observation of possible unintended environmental effects of such cultivation, a national, multi-partner project sponsored by the German Federal Ministry of Education and Research (BMBF) was assembled. The general aims of this project were to confirm the previous assertion, that the cultivation of MON88017 does not pose any harm to the environment, to evaluate methods with which environmental impacts can be assessed, and thus to refine ERA and monitoring approaches.

Within this project, the work on hand aimed at

1) Identifying organisms that are exposed to Cry3Bb1 from MON88017. Only arthropods that are exposed to the transgenic protein under field conditions may be affected and will pass their Bt-protein payload on to higher trophic levels. They can therefore be reasonably used in the General Surveillance of possible environmental impacts of the cultivation of Bt-maize. Generally, they are also interesting candidates for ERA protocols in relation to future genetically modified maize lines. This part helps to strengthen the initial problem formulation stage of ERA.
2) Evaluating field sampling methods with respect to their feasibility in the assessment and monitoring of possible non-target effects. Methods need to be cost- and time-effective, since financial resources will be a limiting factor in the continuous monitoring of genetically modified plants, especially when it is aimed at detecting unforeseen effects during General Surveillance. Time is an important factor also with regard to informed regulatory decisions that need to be reached in a reasonable timeframe to satisfy the interests of all

stakeholders (the public, regulators, industry). Additionally, methods should be easy to use and yield data good enough for a robust statistical analysis.

3) Assessing possible non-target effects of the cultivation of MON88017. In order to classify potential differences between the Bt-variety and its near-isogenic line two conventional hybrids were integrated into the experiment. This allowed for multiple comparisons between different maize lines and for an assessment of the natural variation between them. Multiple comparators are a prerequisite for the ecological interpretation of the biological relevance of statistically significant results in highest tier ecotoxicological tests, i.e. release field experiments under natural conditions.

A number of criteria were set up, defining valuable non-target organisms with respect to their use as possible indicators in the General Surveillance of genetically modified maize:

a) The geographic range of the organism should cover at least central Europe. This allows for a specific monitoring and ERA of one or a few key species under many different cropping regimens, climatic conditions and agricultural practices.
b) The relationship to maize as the host plant should be close, so that the organism can be expected where- and whenever maize is grown with a high degree of certainty.
c) The density in the field should be reasonably high throughout the growing season of maize to ensure that a valid statistical analysis is possible.
d) The organism should experience exposure to the novel protein from the genetically modified maize. Without exposure, no hazard and subsequently no risk can reasonably be expected.
e) The species should be amenable to being surveyed and quantitated in the field and feasible methods should exist for these purposes. Additionally, it should be easy to unambiguously identify the organism.

Four different field sampling methods were used and evaluated in this study:

I. Sweep nets to survey the aerial and foliage community of the herb layer in maize
II. Custom made sticky traps for the assessment of the Auchenorrhyncha assemblage in maize over the growing season
III. Panicle samples, in which the community of insects occurring on the male inflorescences can be surveyed
IV. Whole cob samples to investigate the trophically linked assemblage of arthropods on maize cobs

1.3 Major results

The arthropod community in maize

A highly diverse community of arthropods was sampled in maize, comprising a wide range of different insect orders and trophic gilds. Most abundant were herbivores, with plant- and leafhoppers (Auchenorrhyncha), plant bugs (Heteroptera: Miridae), thrips (Thysanoptera) and aphids (Homoptera: Aphidae) being the most prevalent groups. Plant beetles (Coleoptera: Chrysomelidae), the direct relatives of the target organism of MON88017, were caught only infrequently. They do not reach high densities in the field and their taxonomic diversity in maize is limited. This calls into question their relevance in the ERA of coleopteran-resistant Bt-maize lines directed against the Western corn rootworm.

Predators were represented by damsel bugs (Heteroptera: Nabidae), flower bugs (Heteroptera: Anthocoridae), lady beetles (Coleoptera: Coccinellidae), lacewings (Neuroptera) and spiders (Arachnida).

Results and conclusions for ERA

There were no indications of a negative impact of MON88017 on any of the studied non-target organisms. The densities of selected NTO in the genetically modified and the near-isogenic line were always similar and fell well within the range of the natural variability between the conventional maize lines. For the plant bug *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae), a cultivar effect could be observed,

with significantly less individuals in one than in the other of the two conventional maize lines. This was especially true for nymphal stages and could generally be observed in all three study years. This result suggests that the experimental setup was sensitive enough to reveal differences between the maize lines, and that it is appropriate for the assessment of the biological relevance of differences between maize lines within ERA. The genetically modified cultivar MON88017 and its near-isogenic line are equivalent. There are major differences between the two conventional maize cultivars used in the field experiment, especially with regard to the densities of *T. caelestialium*. This difference between the two conventional cultivars was not consistently observed for other studied NTOs, such as thrips, plant- and leafhoppers or predatory bugs.

Species selection of NTOs: representative herbivores

Two species were identified that match the criteria for candidate indicator organisms agreed on in risk assessment including problem formulation and monitoring: the maize leafhopper *Zyginidia scutellaris* (Herrich-Schäffer) (Auchenorrhyncha: Cicadellidae) and the plant bug *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae). Both species have a close bond to maize as their host plant, are exposed to the Cry3Bb1 protein from MON88017, have a wide geographic distribution and can easily be surveyed and quantitated in the field using sweep nets.

Evaluation of sampling methods and strategies

Sweep net catches appear to be the best method as they realistically reflect the densities of plant- and leafhoppers during the whole growing season and have the added benefit of catching a wider range of herb-layer arthropods. For plant- and leafhoppers in general, custom made sticky traps can also be reasonably used. Sweep nets and sticky traps differ in their capability of quantitating the in-field populations of Auchenorrhyncha species. Panicle and whole cob samples give a clear view on small sections of the maize biocoenosis, but require a large amount of work. For panicle samples, good weather during the sampling period is essential, while whole cob samples are easily hampered by high densities of aphid populations. Both points reduce the practical effectiveness of these two methods.

2. Introduction

"In theory, there is no difference between theory and practice. But, in practice, there is."
Jan L. A. van de Snepscheut

2.1 Genetically modified maize with Bt-resistance genes

Insect resistance based on the Bt-technology is the most prominent application of genetic modification in current agriculture. The ubiquitous soil bacterium *Bacillus thuringiensis* (Berliner) forms crystalline protein inclusion bodies upon sporulation that exhibit specific toxicity against single systematic groups of insects (Schnepf et al., 1998; Glare & O´Callaghan 2000). There are well over a hundred different Cry proteins that are classified according to their sequence homology (Crickmore et al., 1998). Important protein groups are the Cry1 family with activity against Lepidoptera and the Cry3 family with activity against Coleoptera. The mode of action has long been hypothesized to solely rely on the interaction of proteolytically activated Bt-proteins with specific receptors in the insect midgut, subsequent pore formation that leads to lysis of the epithelial wall and ultimately septicaemia resulting in the death of the insect (Bravo et al., 2007). Experimental work has shown, however, that the interactions are far more complex, involving a number of receptor molecules like cadherin, the function of enzymes like aminopeptidases, the oligomerization of Cry-protein fragments to larger structures and in at least some susceptible insect species the presence of certain micro-organisms in the gut (Crickmore 2005; Broderick et al., 2006; Siqueria et al., 2006; Bravo et al., 2007; Soberón et al., 2007; Rodrigo-Simón et al., 2008; Ibiza-Palacios et al., 2008).

Through transformation with genes encoding for specific Cry proteins plants gain resistance against a pest or a group of pests susceptible to these proteins. Most common Bt-crops are cotton and maize, with Bt-varieties of potato, tomato and eggplant also available (AGBIOS; James 2006). Even rice, the most important crop worldwide has been successfully modified. Commercialisation of Bt-rice has not yet been achieved, due to various concerns (High et al., 2004). Europe has so far only seen cultivation of Bt-maize resistant against the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Resistance against this pest is conferred by the protein Cry1Ab. The only event of this kind eligible for planting in the European Union is MON810. In Germany, an area of 2,751 hectares was planted to this Bt-maize in 2007, an increase of almost 200% over 2006. For 2007, an acreage of around 3,500 hectares is expected (BVL 2008).

A novel trait provided by the Bt-technology is resistance to the Western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae). This pest has been introduced to Europe from the United States approximately 15 years ago (Miller et al., 2005; Kiss et al., 2005) and has spread rapidly, reaching economic thresholds in some Balkan states) (Hummel 2003). The most current data on the distribution of *Diabrotica* in Europe can be seen in Figure 1 (taken from http://www.entm.purdue.edu/wcr).

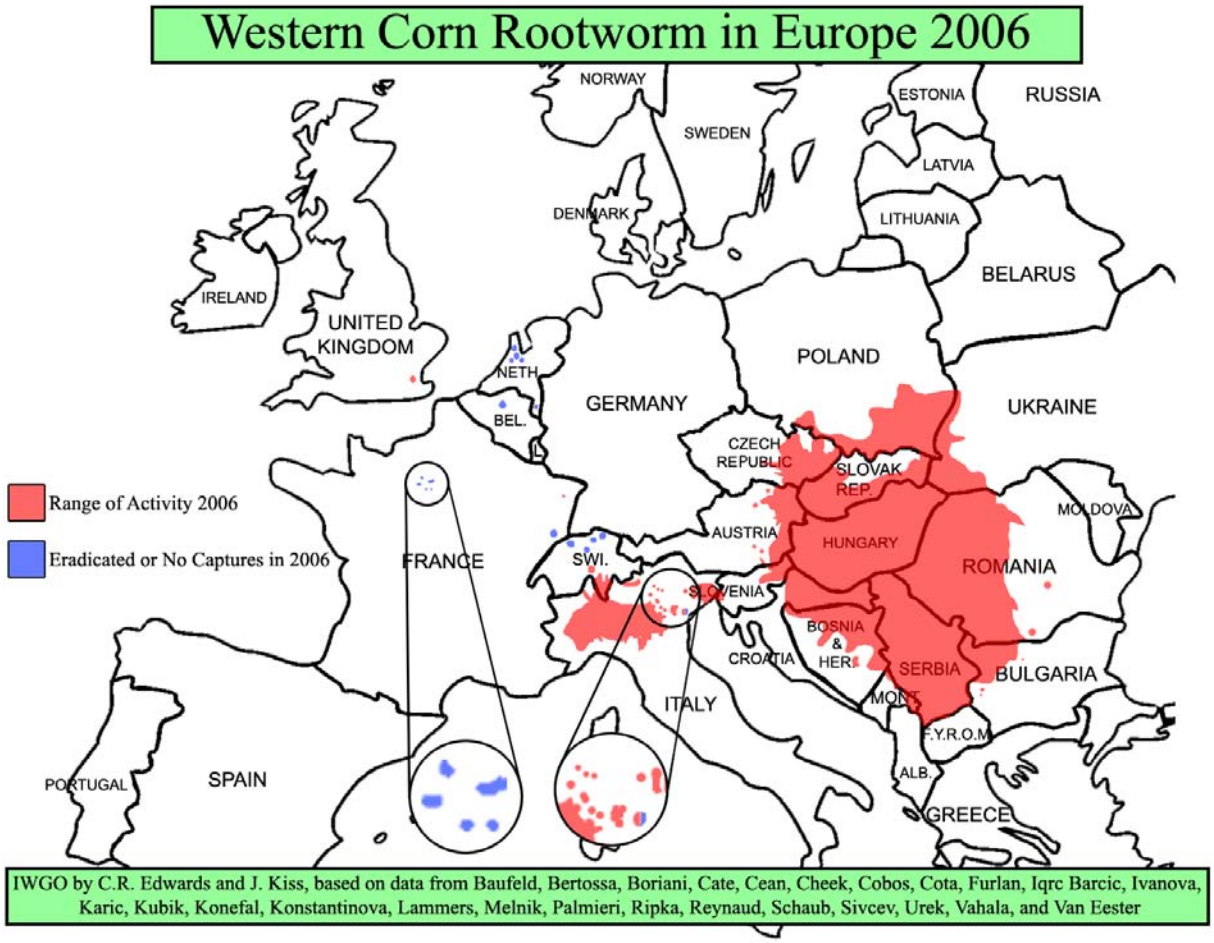


Figure 1: Distribution of *D. virgifera virgifera* in Europe

2.2 *D. virgifera virgifera* **and its significance for European maize production**

Larvae of the Western corn rootworm hatch from eggs that have overwintered in the soil and feed on newly developed and young roots of maize plants (EPPO data sheet on quarantine pests; Moeser & Hibbard, 2005). Damage to the root system (“root

pruning", Figure 2) can lead to severe crop losses, especially due to plant lodging. The pupation of WCR takes place in the soil. The emerging adults move onto maize plants and mainly feed on leaves, pollen and silk. Feeding on silk ("silk clipping") may lead to reduced cob filling (Figure 3), an important factor in seed and sweet maize production. *Diabrotica* is therefore generally regarded as a serious threat to European maize production, and severe measures have been put forward by the EU Commission to prevent the further spread of *Diabrotica* within the European Union (EC 2003). The cultivation of genetically modified maize may become a key strategy to contain or eliminate WCR populations, since these are not readily controlled by current plant protection practices (Furlan et al., 2006). Until only recently, crop rotation strategies, i.e. rotating maize and soybeans, were used to reduce the pest pressure. These have failed since the establishment of a rotation resistant strain of the Western corn rootworm (Spencer et al., 2005). In the future, Bt-maize lines that express the Cry3Bb1 protein and that are thus resistant against the WCR will become important for the European market. These genetically transformed maize plants have an antibiotic and repellent effect against first instar larvae of *Diabrotica* (Clark et al., 2006), but exhibit only a moderate acute toxicity, with mature beetles remaining vastly unaffected by the uptake of Cry3Bb1 (Siegfried et al., 2005; Nowatzki et al., 2006). The mode of action of Cry3Bb1 against susceptible beetle species is primarily based on the formation of ion-channels in the insect gut by single membrane-bound Cry3Bb1 or by oligomers of this protein (Galitsky et al., 2001).



Figure 2: Injuries and damages caused by Western corn rootworm. Left: root pruning (http://ipmworld.umn.edu/chapters/maize.htm); middle: silk clipping and right: reduced ear filling (http://entomology.unl.edu/pmguides/crwadult.htm)

2.3 Herbicide tolerance based on CP4 EPSPS

The use of herbicides for weed control is common practice in conventional agriculture. Herbicides can be either selective, i.e. with action against certain groups of plants only, based on a variety of factors conferring resistance; or broad-spectrum, killing almost all groups of plants equally well. There are different modes of action of the active compounds in herbicides, e.g. inhibition of photosynthesis and the respiratory chain; disruption of plant hormone regulation; interruption of biosynthetic pathways; corrosive damaging of plant surfaces.

One of the most widely used herbicidal compounds is glyphosate [N-(phosphonomethyl)-glycine], marketed under the trade name Roundup by Monsanto Company. The target enzyme of glyphosate is the 5-Enol-pyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS catalyses an important step in the biosynthetic pathway of aromatic amino acids. Disruption of this pathway not only creates a deficiency in protein synthetic precursors, but it also affects many other plant cell components that are derived from intermediates and derivatives of this pathway (Heck et al., 2005). This ultimately leads to plant stunting and eventually death.

Tolerance against glyphosate is based on the integration of a gene derived from the *Agrobacterium* sp. strain CP4 coding for a variant of EPSPS that is not affected by glyphosate into the plant genome. Important crop plants, namely canola *Brassica napus* (L.), cotton *Gossypium hirsutum* (L.), soybean *Glycine max* [(L.) Merr.] and maize *Zea mays* (L.), have been genetically modified with CP4 EPSPS and are grown on large acreages worldwide (James, 2006; Cerdeira & Duke, 2006). In recently developed genetically modified crop plants herbicide tolerance is usually combined with one or more insect resistance genes.

2.4 Environmental Risk Assessment

Under EU legislation an authorisation based on an environmental risk assessment (ERA) of genetically modified plants is mandatory before these are placed on the market. The ERA is to be designed on a case by case basis to identify and evaluate possible direct, indirect, immediate and delayed adverse effects of the deliberate release of genetically modified organisms on the environment and human health.

Additionally, directive 2001/18/EC stipulates a monitoring plan for the continuous observation of possible environmental impacts, which have not necessarily been identified in the risk assessment stage, after a new GMO crop is placed on the market (EC 2001, 2002; EFSA 2006). This monitoring comprises two parts: 1) the hypothesis-driven Case Specific Monitoring, which is only necessary if certain risks have been identified in the ERA that warrant further research, and 2) the General Surveillance, which focuses on possible adverse effects not foreseen in the environmental risk assessment and which is open to how a GMO may affect the environment. One of the possible adverse effects of the cultivation of genetically modified plants is the negative impact on the occurrence and abundance of non-target organisms (NTO) on the cultivated land or the surrounding crop margins, as well on the local as the regional scale. This impact can be direct (uptake of Bt-protein by feeding on parts of the genetically modified plant or through Bt-protein laden prey and subsequent toxic action of the protein against the NTO) or indirect (reduced quality and abundance of susceptible prey and therefore for example reduced parasitoid and predator densities) (Romeis et al., 2006). The potentially affected NTOs of significance, the relevant exposure pathways, the potential effects, the relevance of the subsequent ecological changes in a given agro-ecosystem and the (experimental) ways to assess the likelihood and magnitude of such changes are identified in the first step of the ERA, which is called Problem Formulation. In Problem Formulation, available data, expert opinion and stakeholder deliberations are taken into account to identify areas of concern or uncertainty and thus to streamline and purposefully target the following ERA process on relevant and important management or protection goals (Romeis et al., 2008). Starting with Problem Formulation (Figure 3), relevant fields of research are identified and suitable experiments are designed, and the tiered risk assessment approach is passed through beginning with first tier laboratory tests. If necessary, based on the results and remaining uncertainties, more complex (i.e. multitrophic, multi-species, semi-field or field) higher tier tests follow (in Figure 3: arrow from decision points D_x to the following tier) or a conclusion is reached on whether to reject or approve an application of a genetically modified crop (from decision points D_x to ERA). This means that not all tiers have necessarily to be passed through to reach a decision, resulting in a concise, selective and purposeful implementation of the risk

assessment procedure, making use of all available data and using resources responsibly.

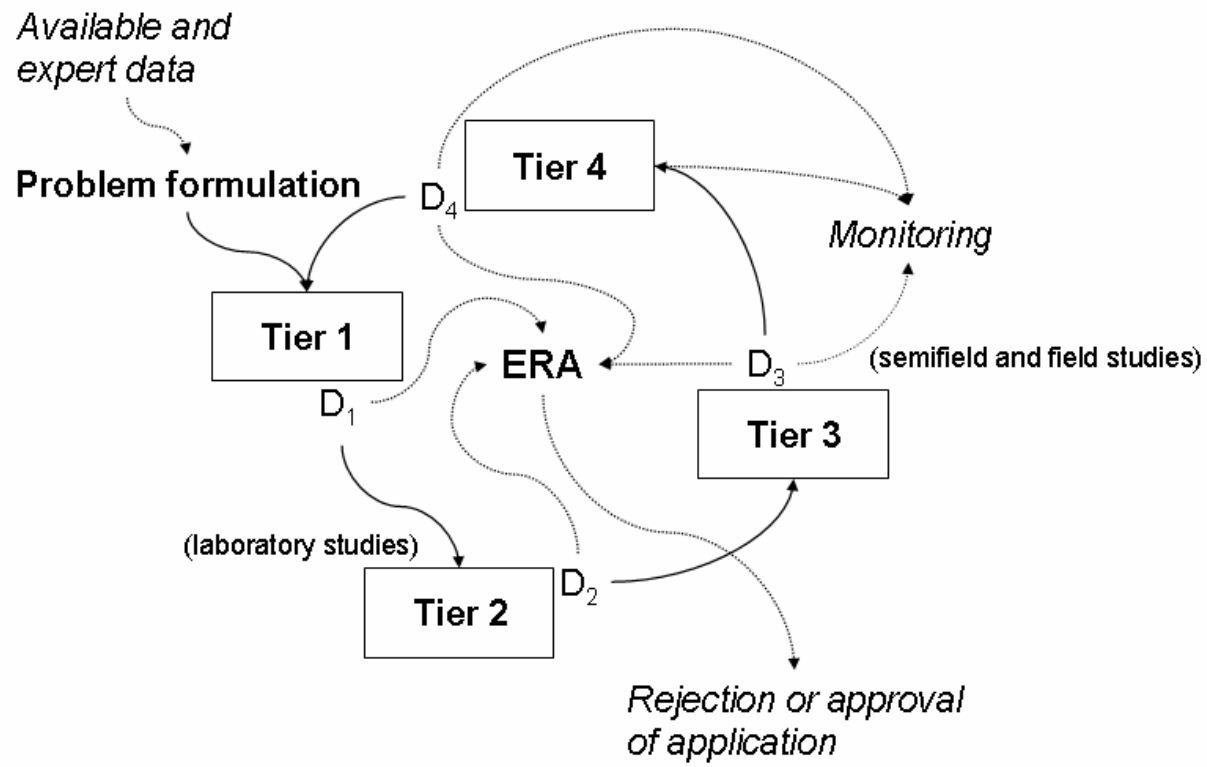


Figure 3: Schematic for the tiered Environmental Risk Assessment (ERA) approach, indicating the preparatory step of Problem Formulation and the subsequent tiers of experiments increasing in complexity from laboratory studies to field experiments. Note that not all tiers have to be passed through to either reject or approve an application of a GMO (D_x denotes decision points in the tiered approach).

In risk assessment it is important to quantitate the two factors constituting risk: firstly hazard, the nature and magnitude of the negative effect of a Bt-protein on an NTO, given that the species is susceptible; secondly exposure, the way, amount, probability and circumstance under which an NTO comes into contact with a Bt-protein (Johnson et al., 2006; Raybould, 2006). Hazard can most conveniently and accurately be quantitated in first tier laboratory test, with defined and controlled conditions. If a hazard is identified under worst-case conditions, semi-field and field tests are needed to fully assess the probability of a hazard under more realistic circumstances of exposure (Garcia-Alonso et al., 2006; Romeis et al., 2006, Romeis

et al., 2008). In general, a risk assessment is only sensible for species that are exposed to the Cry protein in the field. Without exposure, no risk can reasonably be anticipated, even if a possible hazard has been identified in the laboratory. On the other hand, exposure to a Bt-protein does not automatically imply the existence of a risk: the documented specificity of Cry proteins restricts their toxicity to certain groups of insects, while all other groups remain unaffected. The non-susceptibility of an NTO negates the existence of a risk despite exposure in the field. Exposed insects can be a link to higher trophic levels however (Figure 3), and therefore it is of paramount importance to assess exposure under field conditions.

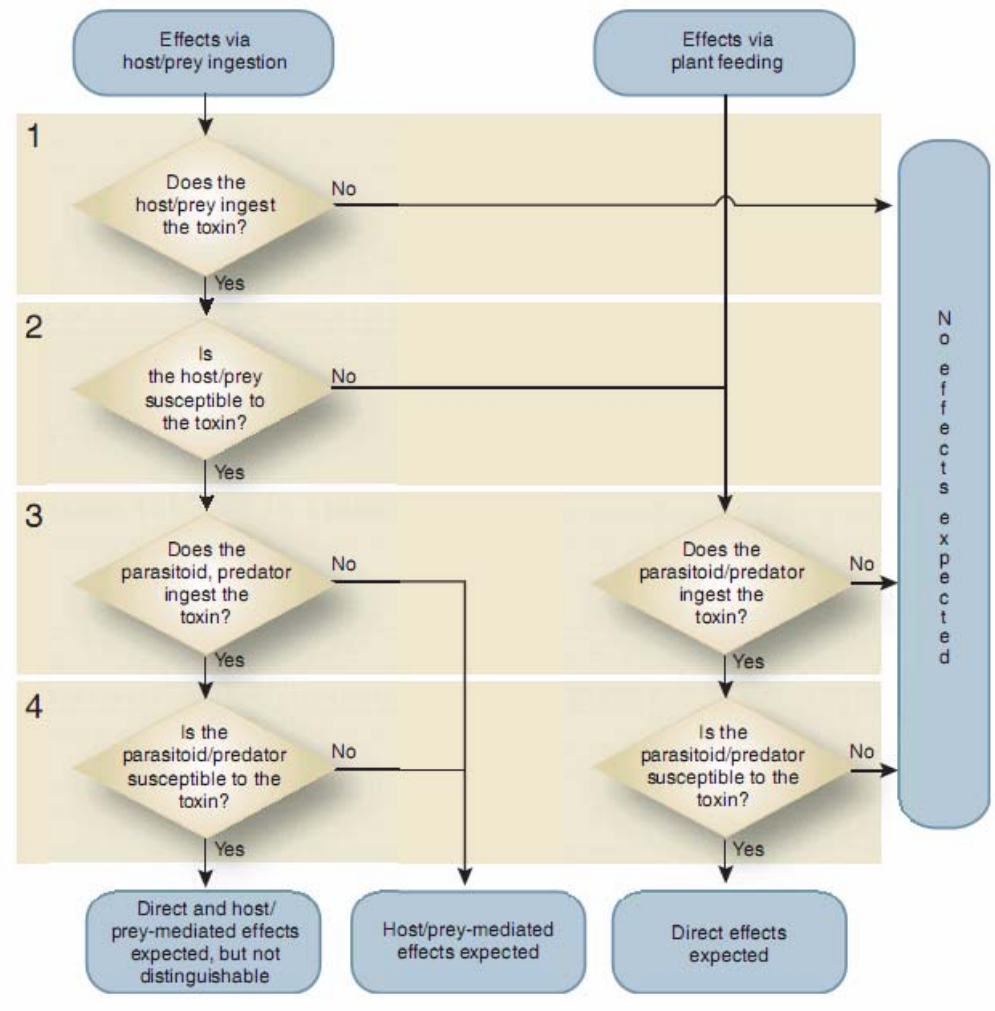


Figure 4: Possible exposure pathways and sources of direct and indirect effects of Bt-proteins to higher trophic levels (taken from Romeis et al., 2006)

The mode of action of a specific Cry protein is not always fully understood, so that the possibility of a non-target action cannot be completely discarded. For these

reasons, arthropods that are exposed to transgenic proteins based on their mode of feeding and the plant substrates they utilise are a reasonable choice in the assessment of environmental non-target effects. Arthropods that are not exposed to the transgenic protein may react to possible changes in a variety of plant physical, visual and olfactory cues, but will not be affected by the transgenic trait itself. This has been described for the aphid *Rhopalosiphum padi* on Bt-176 maize (Lumbierres et al., 2004). Another species of the same genus, *R. maidis*, also performed better on Bt-maize, presumably due to differences in the amino acid content and composition of the transgenic maize lines tested (Faria et al., 2007). The positive effect on the aphids even led to a higher parasitisation of caterpillars by the parasitoid *Cotesia marginiventris*. Adults of this braconid utilise the honeydew of aphids as food source. Bt-maize lines may therefore also have a positive impact on biological control. Generally, aphids are not exposed to Cry proteins in transgenic maize, since these are not translocated into the phloem sap (Raps et al., 2001; Head et al., 2001). Therefore, phloem feeding arthropods are not sensible candidates in the risk assessment of non-target effects of genetically modified maize. For Bt-oilseed rape, however, it has recently been shown that the Cry1Ac protein is present in the phloem and can also be detected in aphids (Burgio et al., 2007). In this case, aphids may serve as reasonable model organisms in risk assessment and the evaluation of multi-trophic links.

There is an ongoing discussion about the merits of field versus laboratory experiments and the kind of species that should be selected for early tier tests in risk assessment (Andow & Hilbeck 2004; Raybould 2006; Romeis et al., 2006; Lang et al., 2007; Romeis et al., 2007, 2008). Field experiments have the major advantage that they test the possible impact of a genetically modified plant under the most realistic conditions. This asset, however, is bought with an increase in variance in the biological data obtained. This leads to an increase in uncertainty over whether an observation is indeed based on an effect or rather the consequence of a variable that could not be controlled under field conditions (such as weather, soil parameters, immigration of insects from surrounding crops). Laboratory tests on the other hand yield robust and reproducible observations on clearly defined endpoints under controlled conditions. The ecological relevance of findings under these conditions often remains questionable, however, based on the artificial environment, unrealistically high exposure and oversimplified trophic interactions. A reasonable

combination of laboratory, semi-field and confirmatory field tests is needed to fully and accurately assess the risk a genetically modified plant poses for a given non-target organism, an ecosystem function or the environment. Notwithstanding, field experiments give room to assess trophic interactions and ecological functions of non-target organisms in the environment, yielding information that may be very useful in the wider risk analysis procedure (Johnson et al., 2006). Such experiments yield a large set of ecological data needed to quantitate the significance of a potential negative impact of a Bt-crop plant. Prey choice and preference, for instance, is an important factor in assessing the exposure of a predatory arthropod in the field. When prey that was previously exposed to a Cry protein constitutes only a marginal part of a predator's diet and the main part is made up of species that are unexposed to the Bt-protein, the predator will only be at low risk of suffering from potential negative effects. When, on the other hand, under certain circumstances prey preference shifts towards exposed prey species, the risk for a negative impact may increase considerably. Field experiments are therefore indispensable in assessing and confirming the full range of possible trophic interactions and the associated exposure pathways and shares in total risk.

Until now, only few field experiments have been conducted on possible non-target effects of maize expressing Cry3Bb1. Al-Deeb & Wilde (2003) assessed possible effects on a diverse range of non-target arthropods. They found no significant effect on *Orius insidiosus* (Say) (Anthocoridae), selected ladybird beetles and arthropods caught in pitfall traps. McManus et al. (2005) found no negative impact on the coccinellid *Coleomegilla maculata* De Geer. Ahmad et al. (2006) investigated effects of Cry3Bb1 on aboveground insect predators, namely minute pirate bugs (Anthocoridae), ladybird beetles (Coccinellidae) and carabid beetles (Carabidae). They found no effect of the transgenic plant material on surrogate species from these three beneficial insect groups and conclude that the Bt-maize variety used could be part of an integrated pest management system. Devare et al. (2004) found no deleterious effect of growing corn rootworm resistant Bt-maize on the soil microbial biomass, activity or the bacterial community structure.

All of these experiments were conducted with MON863 (or derived maize lines), and moreover all in the agro-ecosystem in northern American maize growing regions, namely the US corn belt. For the new Bt-maize line MON88017 it has only recently

been reported that its forage and grain are compositionally equivalent to forage and grain from control and conventional maize hybrids (McCann et al., 2007) and that broilers and rats fed diets containing MON88017 show a similar performance to animals fed diets prepared from the near-isogenic line and other conventional maize varieties (Taylor et al., 2005; Healy et al., 2008). There are currently no other published data on this maize variety, especially so for the European situation. Since risk assessment is to be carried out in the EU on a case-by-case basis, there is a need for research addressing questions specifically for this maize line.

2.5 The arthropod community in maize

A wide variety of insects with a broad spectrum of feeding modes and utilised plant substrates occurs in maize (Schmitz & Bartsch, 2001; Bourguet et al., 2002; Kiss et al., 2002; Dively & Rose, 2003; Jasinski et al., 2003; Candolfi et al., 2004; Musser et al., 2004; Rauschen et al., 2004; Daly & Buntin, 2005; Harwood et al., 2005; Scholte & Dicke, 2005; Eckert et al., 2006). Certain plant- and leafhoppers (Auchenorrhyncha), herbivorous plant bugs (Heteroptera: Miridae), thrips (Thysanoptera) and leaf beetles (Coleoptera: Chrysomelidae) are of interest, because they have been demonstrated to be exposed to Cry proteins from Bt-maize (Zwahlen et al., 2000; Dutton et al., 2004; Harwood et al., 2005; Obrist et al., 2005, 2006a). Generalist, e.g. the web spider *Theridion impressum* (Árpás et al., 2005) or the heteropteran genera *Nabis* and *Orius* (Harwood et al., 2005; Obrist et al., 2006a; Torres & Ruberson, 2007), and specialised predators and parasitoids of these species will be exposed to Bt-proteins and might therefore be directly affected.

2.6 Aims and scope

Based on published literature, the focus of research were herbivores from the herb-layer known or presumed to be exposed to the transgenic protein, their general and specialised antagonists and the communities of arthropods on maize cobs (Daly & Buntin, 2005; Eckert et al., 2006) and male inflorescences. The general aims of this work were to confirm the previous assertion, that the cultivation of MON88017 does not pose any harm to the environment, to evaluate methods and species with which

environmental impacts can be assessed, and thus to refine ERA and monitoring approaches.
This was done by:

1) Identifying organisms that are exposed to Cry3Bb1 from MON88017. Only arthropods that are exposed to the transgenic protein under field conditions may be affected and will pass their Bt-protein payload on to higher trophic levels. They can therefore be reasonably used in the General Surveillance of possible environmental impacts of the cultivation of Bt-maize. Generally, they are also interesting candidates for ERA protocols in relation to future genetically modified maize lines. This part helps to strengthen the initial problem formulation stage of ERA.
2) Evaluating field sampling methods with respect to their feasibility in the assessment and monitoring of possible non-target effects. Methods need to be cost- and time-effective, since financial resources will be a limiting factor in the continuous monitoring of genetically modified plants, especially when it is aimed at detecting unforeseen effects during General Surveillance. Time is an important factor also with regard to informed regulatory decisions that need to be reached in a reasonable timeframe to satisfy the interests of all stakeholders (the public, regulators, industry). Additionally, methods should be easy to use and yield data good enough for a robust statistical analysis.
3) Assessing possible non-target effects of the cultivation of MON88017. In order to classify potential differences between the Bt-variety and its near-isogenic line two conventional hybrids were integrated into the experiment. This allowed for multiple comparisons between different maize lines and for an assessment of the natural variation between them. Multiple comparators are a prerequisite for the ecological interpretation of the biological relevance of statistically significant results in highest tier ecotoxicological tests, i.e. release field experiments under natural conditions.

A number of criteria were set up, defining interesting non-target organisms with respect to their use as possible indicators in the monitoring and general surveillance of genetically modified maize:

a) The geographic range of the organism should cover at least central Europe. This allows for a specific monitoring and ERA of one or a few key species under many different cropping regimens, climatic conditions and agricultural practices.
b) The relationship to maize as the host plant should be close, so that the organism can be expected where- and whenever maize is grown with a high degree of certainty.
c) The density in the field should be reasonably high throughout the growing season of maize to ensure that a valid statistical analysis is possible.
d) The organism should experience exposure to the novel protein from the genetically modified maize. Without exposure, no hazard and subsequently no risk can reasonably be expected.
e) The species should be amenable to being surveyed and quantitated in the field and feasible methods should exist for these purposes. Additionally, it should be easy to unambiguously identify the organism.

Four different sampling methods were used and evaluated in this study:

I. Sweep nets to survey the aerial and foliage community of the herb layer in maize (Rauschen et al, 2004).
II. Custom made sticky traps for the assessment of the Auchenorrhyncha assemblage in maize over the growing season (Rauschen et al., 2004).
III. Panicle samples, in which the community of insects occurring on the male inflorescences can be surveyed.
IV. Whole cob samples to investigate the trophically linked assemblage of arthropods on maize cobs (Eckert et al., 2006).

To assess possible differences in the occurrence and abundance of non-target organisms in MON88017, the primary comparison is between the genetically modified maize line and its near-isogenic parental line. To range in these differences into the natural variability, multiple comparators – i.e. other conventional maize lines – are needed. This experimental design establishes the biological relevance of possible statistically significant findings.

3. Materials and Methods

3.1 The genetically modified maize MON88017

Plants of the Bt-maize variety MON88017 (Monsanto Company, St. Louis, Missouri, USA) contain a transgenic cassette with two genes: one encoding for a variant of the *Bacillus thuringiensis kumamotoensis* coleopteran active Cry3Bb1 protein directed against the Western corn rootworm *Diabrotica virgifera virgifera* (trademark YieldGard® Rootworm); and another for the *Agrobacterium* sp. strain CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), which confers tolerance against glyphosate, the active ingredient of the herbicide Roundup (trademark Roundup Ready®) (AGBIOS, 2007). Recombination of maize cells was performed by the *Agrobacterium* mediated transfer of the plasmid vector PV-ZMIR39 (McCann et al., 2007).

MON88017 underwent Environmental Assessment by the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS, 2005): Based on the history of safe use of maize, the substantial equivalence of MON88017 to conventional maize varieties and its similarity to the already commercialised MON863, the USDA-APHIS determined that MON88017 will not have any significant impacts on the human environment. In the European Union, MON88017 is currently under evaluation by the European Food Safety Authority (EFSA) according to Regulation (EC) No 1829/2003. The application for authorization aims at this maize line to be eligible for import and processing and as animal feed and human food, but not for planting (Monsanto Co., 2005).

3.2 Experimental design

At a field site located near Würzburg in Lower Franconia (Bavaria), four different maize varieties were planted in a systematic plot design with 8 replications each, (Figure 1): Two conventional hybrids, Benicia (Pioneer HiBred International, Inc.) and DK315 (Monsanto Co.), the Cry3Bb1 expressing Bt-maize MON88017 (BT; Monsanto Co.) and its near isogenic line DKC5143 (ISO). Plots measured 40.5 m by 31.5 m (i.e. 42 rows of maize plants with a distance of 15 cm between plants and 75 cm between rows), yielding an area of 0.13 ha. The plots were arranged in four

columns of eight. A distance of 4.5 m was left free between neighboring columns for easier access and orientation, but not between adjacent plots within the same column. The experimental field was surrounded by a perimeter of at least 10 m width, planted with the conventional hybrid Gavott (KWS SAAT AG). A strip of 4.5 m was left free between the plot columns and the perimeter to ensure the same distance to planted areas for all plot columns. In all three experimental years, the location of the plots and the respective maize varieties planted on them were identical. This was to ensure that (i) the respective abiotic parameters (i.e. soil structure, moisture content, pH etc.) remained fixed over the whole experimental time for each replication; (ii) the medium-term impacts of the continuous cultivation of the Bt-maize variety on the same sites could be assessed over time; (iii) arthropods with a low capacity of dispersal would be exposed to the Bt-maize over successive generations.

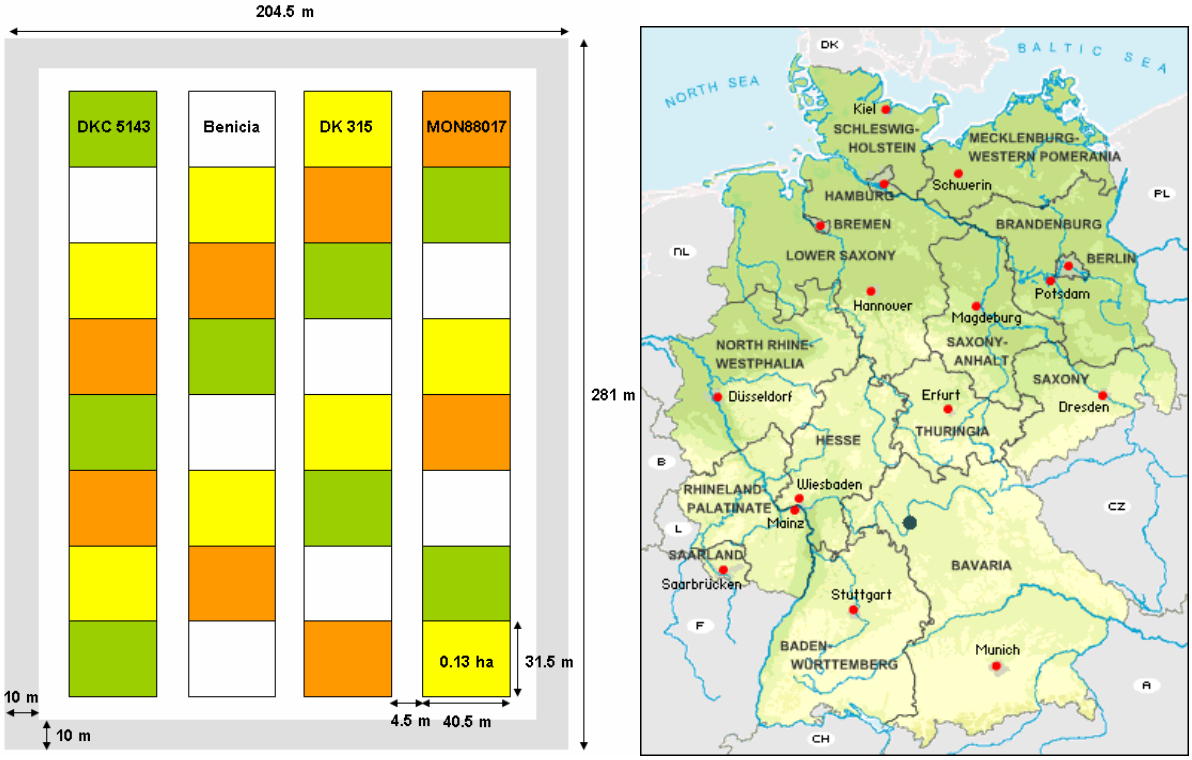


Figure 5: Schematic of the field design and geographic location of the experimental site (indicated with a blue circle)

The field was planted on May 27 in 2005 (calendar week CW 21), on May 9 in 2006 (CW 19) and on May 21 2007 (CW 21). 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 per hectare to control weeds during the 2 to 8 leaf stadium. In 2006 and 2007, a mixture of 3.0 liters 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.

3.3 Sampling methods

The occurrence and abundance of the systematically and functionally diverse range of non-target arthropods (i.e. herbivores, pollinators, predators, parasitoids and saprovores) was assessed using four different sampling methods that had different focuses with respect to selected groups of non-target organisms.

Sweep netting

Catching with a sweep net (diameter 40 cm, mesh width 1.5 mm) was carried out in each plot on 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. Beginning at about 5 m into the space between two rows of maize plants, thirty one-meter steps (approx. 3 ms^{-1}) were taken while holding the sweep net vertically in front of the body at a height of approximately 90 cm above ground. In this manner, four linear transects were covered in each plot, between rows 5 and 6, 15 and 16, 25 and 26, 35 and 36. After each transect, captured insects were directly transferred into glass vials with snap-on lids and stored in 70% ethanol. Identification of species was done under a binocular microscope in the laboratory.

With this method, all insects occurring in the herb layer that belong to the foliar community in maize were caught. Special focus lay on the plant- and leafhoppers, the plant bugs and their antagonists.

Sticky traps

Sticky traps were constructed from bamboo sticks (length approx. 1.22 m, diameter around 10 mm), a rectangular wire frame and a clear office plastic folder (300 mm by 240 mm, DIN A4) that was slipped over the frame. The surfaces of the folder were

covered with insect glue (Temmen GmbH, Hattersheim, Germany). One glue trap was placed in the centre of each plot, the top of the folder reaching a height of around 1 m above ground. Traps were set up for 1 week in time frame parallel to the sweep netting. After this interval, the folders were covered with cling film, removed from the wire frame and stored frozen at -20°C, until the caught insects were carefully detached from the glued surfaces with a fine needle. The recovered specimens were transferred to Eppendorf tubes with 70% ethanol.
This method was specifically targeted against the Auchenorrhyncha assemblage in maize, as described in Rauschen et al. (2004).

Panicle samples

The sampling of panicles during anthesis was performed as follows: 5 neighbouring plants were haphazardly chosen. Their panicles were consecutively bend into a funnel and shaken forcibly. At the end of the funnel a glass was attached in which all insects were caught that had been present on the panicles or the anthers of the sampled plants. This procedure was repeated, so that in each plot 5 samples representing 25 plants were taken. Samples were taken at two times in the years 2005 and 2006, during the peak of anthesis (CW 31) and towards its end one week later. In 2007, samples were only taken once at the high-point of anthesis.
Insects caught with this method are either feeding on pollen or on the anthers of maize or predators. The whole community occurring in the male inflorescences was the object of research.

Whole cob sampling

Towards the end of the maize growing season and shortly before harvest, single cobs were sampled to survey and quantitate the community of insects occurring on them. This method was modified from Eckert et al. (2006) in such a way that plastic bags were quickly slipped over the cobs before these were detached from the plants. This prevented agile insect species from escaping from the cob during sampling. 10 cobs per plot were sampled, frozen at around -20°C and the insects recovered from them by destructive sampling. The number of husk leaves and the lengths and weights of cobs were measured to test for effects of these parameters on the structure and density of the insect community.

The assemblage of arthropods on maize cobs is tightly linked trophically and can therefore be used to assess influences of transgenic maize on different trophic levels with a single sampling method (Eckert et al., 2006).

3.4 Exposure assessment

To assess the exposure of selected arthropod species, double-antibody enzyme linked immunosorbent assay (DAS-ELISA) tests were performed to measure the internal concentration of Cry3Bb1. For these experiments, the commercially available quali-plate kit by Agdia Inc. (Elkhart, IN, USA; purchased via Linaris GmbH, Wertheim-Bettingen, Germany) was used. Insect samples were weighed on a microbalance, ground under liquid nitrogen and extracted with 2 ml of extraction buffer. Extracts of single specimens and insect bulk samples had to be diluted with buffer to different ratios. All steps of the test procedure were carried out following the manufacturer's instructions. After colour development, 50 µl of 3M sulphuric acid stop solution (Agdia, sold separately) were added to each testwell and plates were measured at 450 nm using a MRX plate reader (Dynatech). To allow quantitation of the Cry3Bb1 content, the positive control of 40 ng Cry3Bb1/ml provided with the kit was used to create a standard curve with concentrations of 0.1, 0.2, 0.4, 0.8, 1.25, 2.5 and 5.0 ng Cry3Bb1 ml^{-1}. According to the manufacturer, the test kit shows no cross-reactivity with CP4 EPSPS.

The internal concentrations of Cry3Bb1 in tested insects were taken as evidence for their exposure to the transgenic protein, either by feeding on different parts of the maize plants or through predation of exposed arthropods. ELISA test kits are routinely used to detect proteins from transgenic plants for this purpose (Dutton et al., 2004; Obrist et al., 2006; Rauschen et al., 2007) and in a variety of other contexts (Hopkins & Gregorich, 2003; Baumgarte & Tebbe, 2005; Lutz et al., 2005a; Rauschen & Schuphan, 2006). They have the major drawback, however, that they do not only quantitate complete intact proteins, but also immunoreactive breakdown products (Lutz et al., 2005b). It is therefore impossible to decide whether an insect with a detectable content of Cry3Bb1 degraded the protein and whether it was exposed to the full-length protein in the first place. Another drawback of this method is the variation in the measurements. A single sample, measured multiple times on the same plate or on different plates, can yield results with a variance of up to 30%

(Crespo et al., 2007; Nguyen et al., in press). Results obtained with an ELISA test therefore have to be interpreted carefully.

3.5 Environmental factors

Weather data

In close proximity to the experimental site (Figure 2), the weather station Number 39 of the Bavarian State Research Center for Agriculture (LFL Bayern) continuously records a set of climatic data. These were obtained for the three growing periods from the webpage of the Agricultural Meteorology Service at the following URL: http://www.lfl.bayern.de/agm/lflinclude.php?url=/cgi-bin/agm/agmabruf.cgi?statnr=39

Average and minimal and maximal temperatures, air temperature at 20 cm and 200 cm above ground, relative humidity, wind velocity at 250 cm above ground and the amount of rain (in millimetres) were measured daily and taken into consideration in the interpretation of the entomological field data.

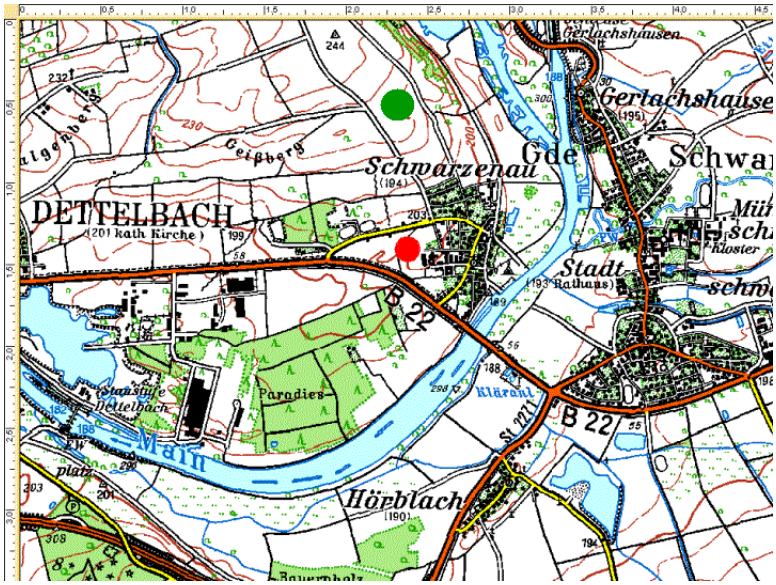


Figure 6: location of the field site (green circle) and weather station Number 39 (red circle)

Soil parameters

A working group from the Institute of Applied Biotechnology in the Tropics at the University of Göttingen (working group Dr. Frank Gessler) analysed the soil at the field site as part of the BMBF-project. With regard to possible influences of the soil on

the field-densities of plant bugs, a number of soil parameters were determined. Samples from the topsoil (0-20 cm) and the subsoil (40-60 cm) were taken from each plot. Field-moist samples were air-dried and sieved through a 2-mm mesh. In order to determine the particle size distribution, organic matter was removed from air-dried samples with 30% H_2O_2. Sodium pyrophosphate (0.04 M $Na_4P_2O_7$) was used to reach a complete dispersion of the soil samples. Clay fractions (< 2 µm) and silt fractions (2 µm-63 µm) were determined by the sedimentation and pipette method. The sand (63 µm-2 mm) was fractionated by wet sieving. The pH of the air-dried samples was measured potentiometrically at a soil to solution ratio of 1:25 using 0.01 M $CaCl_2$. According to the method of van Reeuwijk (2002), the cation exchange capacity (CEC) of the air-dried samples was determined by using silver thiourea. The amount of organic C and N was measured by dry combustion in oxygen using a CNS-analyser (CNS-2000, Leco, St. Joseph, MI, USA). To obtain the amount of total mineralized nitrogen (N_{min}), field-moist samples were sieved through a 5-mm mesh and leached with 0.0125 M $CaCl_2$. In the leachates, mineralized NO_3^- and NH_4^+ were determined with ion chromatography (ICS-90, Dionex, Sunnyvale, CA, USA).

3.6 Statistical analyses

One objective of the study was the quantitation of the effect of Bt-maize on selected non-target organisms. Special focus was put on the plant- and leafhoppers as well as the plant bugs. The comparison of abundance between the Bt-maize MON88017 (BT) and the near-isogenic line DKC5143 (ISO) is of primary interest. Additionally, the two conventional hybrids DK315 and Benicia were compared with both Bt-maize and the near-isogenic line and also to each other. This aimed at assessing the variability between different hybrid lines, at testing for possible hybrid effects and at ranging in differences between BT and ISO.

The number of insects in adult or nymphal stage per plot (n=8 per sampling date) were analysed separately using generalized linear models (GLM) in R_{GUI} (R Development Core Team, 2007). The abundance of a life stage in a given plot was expressed in a linear dependence of the different soil parameters and the different maize varieties that together characterise the habitat variability. The Akaike Information Criterion (AIC) was used to identify significant covariables for the models.

This first step was performed to reduce the variance in the field-count data, which allowed more stringent conclusions based on the subsequent analyses. For these, the package multcomp (Hothorn et al., 2007) was used to calculate Tukey Contrasts for multiple comparisons between the maize varieties based on the results of the GLMs. Here, the density data corrected on the basis of the GLMs were compared between all 6 possible pairings of the four maize varieties (i.e. BT vs ISO; BT vs DK315; BT vs Benicia; ISO vs DK315; ISO vs Benicia; DK315 vs Benicia).

4. Results

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny..'" Isaac Asimov

The non-target organisms caught in the field with the different sampling methods can be classified into three groups: the community of foliage-dwelling arthropods, the community of arthropods living on panicles and the assemblage of arthropods occurring on maize cobs. In the following, the results on these groups will be described consecutively.

4.1 Foliage-dwelling arthropods

With the sweep net a highly diverse community of arthropods from the herb-layer was sampled (Table 1). 15 major taxonomic groups with 65 families or super-families were identified, representing a variety of feeding modes, utilised plant substrates and trophic levels and interactions (Schmitz & Bartsch, 2001; Scholte & Dicke, 2005). Arthropods that are interesting based on their mode of feeding (non phloem feeding herbivores), their close systematic relationship to the target pest of MON88017 (Coleoptera in general, Chrysomelidae in particular), their abundance or their ecological functions within the food-web (major herbivores, their predators) were identified to the genus or species.

The major herbivorous groups in terms of abundance were the plant bugs (Heteroptera: Miridae) with the rice leaf bug *Trigonotylus caelestialium* (Kirkaldy) as the most prominent representative, and the leafhoppers (Auchenorrhyncha: Cicadellidae) with the maize leafhopper *Zyginidia scutellaris* (Herrich-Schäffer) as the absolute most abundant single species. Both arthropods are mesophyll feeders. The leaf beetles (Coleoptera: Chrysomelidae), close relatives to the target organism of MON88017, *D. virgifera virgifera*, were present with five genera, of which those belonging to the flea beetle subgroup (Halticinae), *Chaetocnema* spec. and *Phyllotreta* spec., were the most abundant. The genus *Oulema* was present with *O. lichenis* and *O. melanopus*. The chrysomelids in general did not have high densities in the field, with only single to few individuals caught in total over the growing season (Table 2).

Predatory arthropods were represented by damsel bugs (Nabidae), minute pirate bugs (Anthocoridae), lady beetles (Coccinellidae), green and brown lacewings (Neuroptera) and spiders.

Table 1: Community of foliage-dwelling arthropods of the herb layer (major taxa in bold)

Acarina			Diptera	(contd.)	
Araneida	Araneidae			Syrphidae	
	Thomisidae	*Xysticus cristatus*		Tachinidae	
Coleoptera	Anthicidae	*Anthicus antherinus*		Tephritidae	
		Notoxus monoceros		Tipulidae	
	Anthribidae		Ephemeroptera		
	Byrrhidae		Heteroptera	Anthocoridae	*Orius* spec.
	Cantharidae	*Cantharis* spec.		Lygaeidae	
	Carabidae	*Demetrias atricapillus*		**Miridae**	*Megalocoleus* spec.
	Chrysomelidae	*Chaetocnema* spec.			*Notostira erratica*
		Phyllotreta spec.			*Lygus* spec.
		Oulema spec.			*Stenodema* spec.
	Coccinellidae	*Coccinella 7-punctata*			***Trigonotylus caelestialium***
		Harmonia axyridis		Nabidae	*Nabis pseudoferus*
		Propylea 14-punctata		Pentatomidae	*Rhaphigaster nebulosa*
		Tytthaspis sedecimpunctata		Piesmatidae	
	Curculionidae	*Apion* spec.	Homoptera	Aphidae	
		Barynotus spec.		**Auchenorrhyncha**	*Anoscopus albifrons*
		Sitona spec.			*Balclutha punctata*
	Lathridiidae	*Cortinicara gibbosa*			*Dicranotropis hamata*
	Malachiinae	*Axinotarsus pulicarius*			*Empoasca pteridis/affinis*
	Staphylinidae				*Euscelidius variegatus*
Collembola					*Hardya tenuis*
Dermaptera	Forficulidae	*Forficula auricularia*			*Javesella pellucida*
Diplopoda					*Laodelphax striatella*
Diptera	Agromyzidae				*Macrosteles laevis*
	Anthomyiidae				*Megadelphax sordidula*
	Anthomyzidae				*Neophilaenus campestris*
	Cecidomyiidae				*Psammotettix alienus*
	Chironomidae				*Ribautodelphax albostriata*
	Chloropidae				***Zyginidia scutellaris***
	Conopidae		Hymenoptera	Chalcidoidea	
	Culicidae			Cynipoidea	
	Dolichopodidae			Dryinidae	
	Empididae			Formicidae	
	Ephydridae			Ichneumonidae	
	Fanniidae			Proctotrupoidea	
	Helcomyzidae			Sphecidae	
	Lauxaniidae			Tenthredinidae	*Athalia* spec.
	Milichiidae			Vespidae	
	Muscidae		Lepidoptera	Crambidae	*Ostrinia nubilalis*
	Opomyzidae		Planipennia	Chrysopidae	*Chrysoperla* spec.
	Phoridae				*Micromus* spec.
	Pipunculidae		Psocoptera		
	Platypezidae		Thysanoptera	Aeolothripidae	*Aeolothrips* spec.
	Sciaridae			Phlaeothripidae	*Haplothrips* spec.
	Sciomyzidae			Thripidae	*Stenothrips* spec.
	Sepsidae				*Anaphothrips* spec.
	Sphaeroceridae				*Chirothrips* spec.
	Stratiomyidae				*Frankliniella* spec.
					Limothrips spec.

Table 2: Total number of chrysomelid beetles caught in all maize lines during the three growing seasons

	year		
taxon	**2005**	**2006**	**2007**
Phyllotreta spec.	27	38	18
Oulema lichenis	26	4	-
Oulema melanopa	2	2	1
Chaetocnema spec.	9	9	9
Psylliodes spec.	-	-	1
Longitarsus spec.	-	-	1

Given their high densities in the field and their exposure against Cry3Bb1 based on their mode of feeding, the rice leaf bug and the maize leafhopper were chosen as focal organisms.

Density pattern of *T. caelestialium*

The rice leaf bug *Trigonotylus caelestialium* (Kirkaldy) was by far the most abundant of six species of plant bugs found on the experimental site (Table 3). The genus *Stenodema* was the second most common and present with the two species *St. laevigatum* (Linnaeus) and *St. calcaratum* (Fallén), followed by the genus *Lygus*. *Megalocoleus spec. and Notostira erratica* (Linnaeus) were not consistently detected during the three years and only caught as single individuals.

Table 3: Total counts of Miridae caught on the experimental field

	total count		
species	2005	2006	2007
Trigonotylus caelestialium (Kirkaldy)	1216	1614	531
Lygus rugulipennis (Poppius)	38	5	17
Stenodema laevigatum (Linnaeus)	17	65	72
Stenodema calcaratum (Fallén)	4	2	5
Megalocoleus spec.	2	-	1
Notostira erratica (Linnaeus)	-	3	-

Abundance of T. caelestialium in different maize hybrids

The rice leaf bug was present on the maize field with two generations per year: In July, the abundance of adult *T. caelestialium* of the first generation (hatched from overwintered eggs in spring, Wheeler & Henry, 1985) was highest, with no or only a few nymphs of the second generation caught (Table 4). The following months were characterized by a decrease in the abundance of adults and, conversely, an increase in the number of nymphal stages. Generally, there was an increase in the density of the rice leaf bug from 2005 to 2006. In 2007, lower densities were recorded.

Most adults of *T. caelestialium* were found in July and the highest densities were recorded in MON88017 and the near-isogenic line. The lowest numbers of adults were found in Benicia. This pattern could be observed in all three experimental years (Figure 7). In August, the pattern changed. Most adults were now caught in Benicia and fewest in DK315. MON88017 and the near-isogenic line showed densities intermediate between the two conventional lines. Overall, the number of adults was reduced when compared to July. In September, the density of adults had further declined.

Table 4: Mean number (± 1 standard deviation) over 8 plots of *Trigonotylus caelestialium* adults and nymphs listed for the different hybrids and sampling dates (calendar week of sampling dates given in parenthesis) (a dash indicates that no sampling was carried out)

		date 1 (CW 28 & 29)		date 2 (CW 33 & 34)		date 3 (CW 36)	
year	hybrid	adults	nymphs	adults	nymphs	adults	nymphs
2005	DK315	17.75 ± 8.41	0	1.38 ± 1.06	8.75 ± 3.99	-	-
	Benicia	13.63 ± 6.46	0	3.63 ± 2.33	29.75 ± 8.61	-	-
	MON88017	16.63 ± 8.31	0	3.25 ± 3.41	19.00 ± 6.76	-	-
	DKC5143	20.00 ± 8.86	0	2.63 ± 2.20	15.63 ± 2.83	-	-
2006	DK315	20.88 ± 9.83	0.13 ± 0.35	1.50 ± 1.93	3.38 ± 4.34	1.00 ± 0.93	3.75 ± 1.28
	Benicia	17.88 ± 10.38	0.63 ± 0.92	7.38 ± 5.24	18.38 ± 10.77	3.13 ± 1.55	8.88 ± 3.27
	MON88017	31.13 ± 19.85	0.25 ± 0.46	7.38 ± 6.0	10.75 ± 6.56	1.13 ± 0.99	4.75 ± 2.60
	DKC5143	37.25 ± 24.63	0.38 ± 0.52	5.25 ± 2.19	11.38 ± 8.47	1.33 ± 1.06	3.75 ± 3.11
2007	DK315	6.63 ± 4.37	0.50 ± 0.76	0.63 ± 0.74	3.25 ± 1.67	0.50 ± 0.53	1.25 ± 0.89
	Benicia	5.25 ± 3.28	1.13 ± 0.99	1.00 ± 1.07	9.63 ± 3.54	0.50 ± 0.76	2.38 ± 1.19
	MON88017	7.38 ± 2.62	0.38 ± 0.52	1.50 ± 1.51	5.63 ± 1.92	0.38 ± 0.52	0.38 ± 0.52
	DKC5143	7.63 ± 3.16	0.50 ± 0.76	1.75 ± 1.98	6.50 ± 2.39	0.13 ± 0.35	0.88 ± 0.64

Nymphs of the rice leaf bug also showed consistent patterns during the three years: in August, most nymphs could be found in Benicia and fewest in DK315. MON88017 and the near-isogenic line showed similar densities of rice leaf bug nymphs that lay in between those of the conventional hybrids (Figure 8). In September, a similar pattern could be observed, with again overall lower abundances

Statistical analyses

The generalized linear models for the analyses of *T. caelestialium* data showed that the different maize lines had a considerable influence on the density of the rice leaf bug. Hybrid types as covariables explained from 13 to over 66 % of the variance in single models based on the density data of a certain life-stage (nymph or adult) at a single sampling date (Table 5). Combining life stages or sampling dates revealed no conclusive results, due to different trends for adults and nymphs at different times during the growing season. There was no single soil parameter that had a consistent influence on the density of the rice leaf bug in experimental plots. This was true for both the upper and lower soil compartment. Generally, the upper soil compartment had a marked influence on the densities of the rice leaf bug, whereas the lower compartment did not. Varying combinations of upper soil compartment parameters explained between nil to 46 % of the variance in individual models (Table 5).

Differences in the abundances of *T. caelestialium* between the two conventional hybrids DK315 and Benicia were statistically significant in almost all cases (Table 6, Figure 7 & 8). There were significantly more adults in DK315 in July and in Benicia in August in the years 2005 and 2006. This distribution could, however, not be observed in 2007. For nymphs, there were significantly higher numbers recorded in Benicia at all samplings but one. In September 2007 the differences were not statistically significant. There was only one significant difference between MON88017 and the near-isogenic line: in August 2006 more adults were caught in Bt-maize than in the near-isogenic line. Differences between DK315 and Benicia on the one hand and MON88017 and ISO on the other hand were significant in some instances, but not consistently so.

Table 5: Results of the statistical analysis of the density data of *T. caelestialium* using generalized linear models (GLM). The different maize hybrids and parameters of the upper soil compartment for individual plots were used as covariables. Marked are the soil parameters that contributed to the reduction of deviance in a specific model ("-" indicates that no soil parameters were retained in the model; "n.c." means not computable due to too small abundances).

sampling date		life stage	% deviance explained by hybrids	% deviance explained by soil parameters	soil parameters in model									
					pH	C	N	CN	Nmin	CECclay	CEC	sand	silt	clay
2005	July	adults	19	32		X	X	X			X	X	X	X
	Aug.	adults	28	28		X	X		X		X	X	X	
		nymphs	66	6						X	X			
2006	July	adults	13	46		X	X			X		X	X	X
	Aug.	adults	49	9	X	X	X	X			X			X
		nymphs	45	32	X	X	X	X		X				
	Sept.	adults	34	2					X					
		nymphs	39	-										
2007	July	adults	23	15							X	X	X	
	Aug.	adults	30	30	X			X	X			X	X	X
		nymphs	57	8					X		X			
	Sept.	adults	n.c.	n.c.										
		nymphs	40	-										

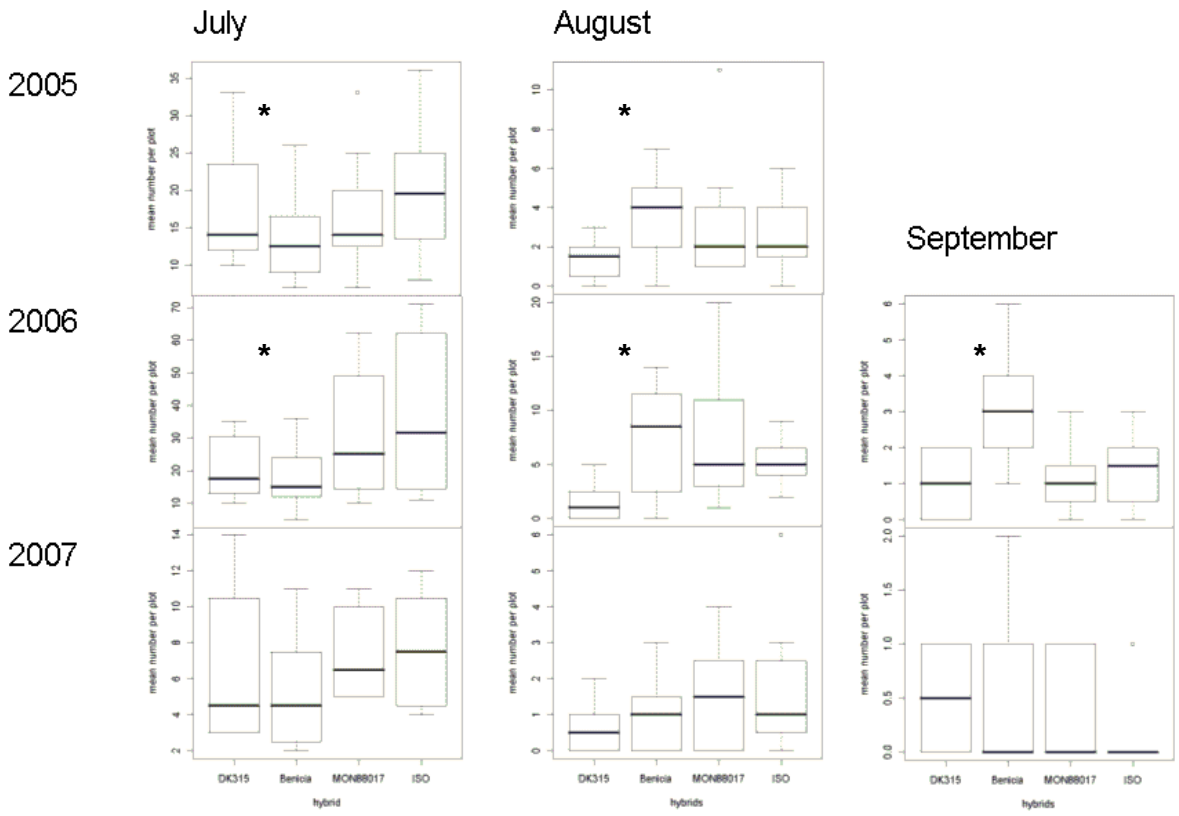


Figure 7: Mean number per plot of *T. caelestialium* adults caught in the different maize hybrids. “ISO” denotes the near-isogenic hybrid. Box plots show the median and the interquartile range; outliers are marked as circles. Statistically significant differences between the two conventional hybrids are marked with an asterisk.

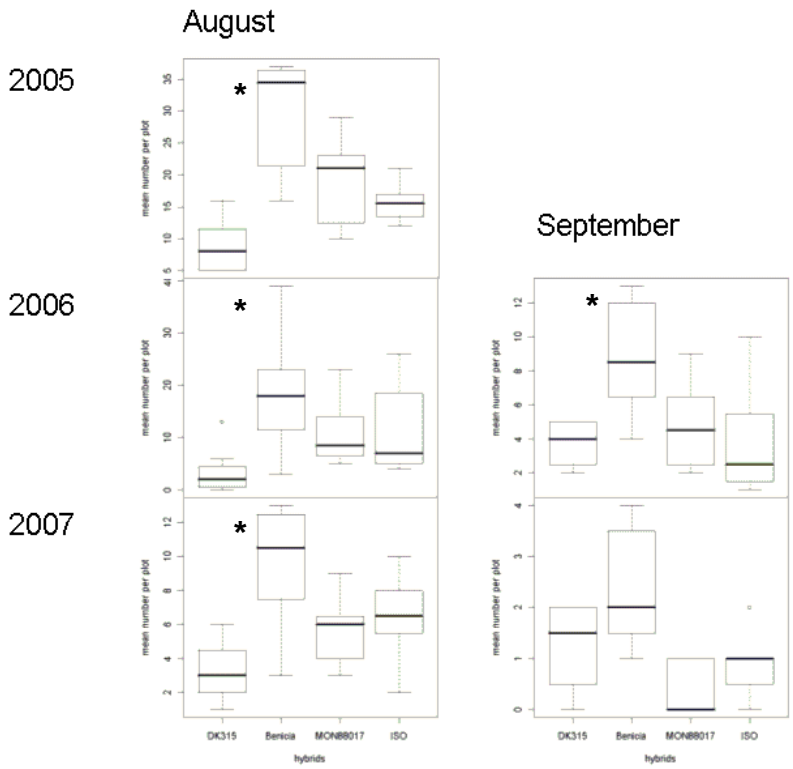


Figure 8: Mean number per plot of *T. caelestialium* nymphs caught in the different maize hybrids. “ISO” denotes the near-isogenic hybrid. Box plots show the median and the interquartile range; outliers are marked as circles. Statistically significant differences between the two conventional hybrids are marked with an asterisk.

Table 6: Tukey contrasts for multiple comparisons of the abundances of the life stages of *T. caelestialium* at different sampling periods between the four maize hybrids. Given are z- and p-values for the respective comparions. Statistically significant differences between MON88017 and the near-isogenic line are highlighted.

2005			**Benicia**	**MON88017**	**ISO**
July	**DK315**	adults	z=-3.270, p=0.00617		
	ISO	adults	z=4.459, p<0.001		
August	**DK315**	adults	z=3.508, p=0.00253		
		nymphs	z=8.904, p<0.001		
	Benicia	nymphs		z=-4.589, p<0.001	z=-6.273, p<0.001
	MON88017	nymphs	z=4.989, p<0.001		
	ISO	adults	z=-3.719, p=0.00115		
		nymphs	z=3.408, p<0.004		
2006					
July	**DK315**	adults	z=-2.712, p=0.03342	z=3.467, p=0.00295	z=2.930, p=0.01747
	Benicia	adults		z=5.980, p<0.001	z=5.623, p<0.001
August	**DK315**	adults	z=6.799, p<0.001	z=5.379, p<0.001	z=3.357, p=0.00417
		nymphs	z=8.722, p<0.001	z=4.646, p<0.001	z=4.218, p<0.001
	Benicia	adults		z=-2.880, p=0.01949	z=4.340, p<0.001
		nymphs		z=-5.708, p<0.001	z=-5.738, p<0.001
	MON88017	adults			**z=-2.560, p=0.04833**
September	**DK315**	adults	z=0.0252, p=2.809		
		nymphs	z=3.956, p<0.001		
	Benicia	adults		z=-2.929, p=0.0173	
		nymphs		z=-3.110, p=0.0101	z=-3.956, p<0.001
2007					
July	**Benicia**	adults		z=2.826, p=0.02421	z=3.282, p=0.00559
August	**DK315**	adults		z=2.604, p=0.0442	
		nymphs	z=5.131, p<0.001		z=2.860, p=0.02150
	Benicia	adults		z=2.831, p=0.0233	z=2.871, p=0.0207
		nymphs		z=-3.474, p=0.00299	z=-2.713, p=0.03290
September	**Benicia**	nymphs		z=-2.971, p=0.0148	

Internal concentration of Cry3Bb1 in T. caelestialium

In 2005, 43 and 60 *T. caelestialium* were caught in plots with transgenic and near-isogenic maize, respectively. The bulk sample collected from MON88017 had a content of around 7 µg Cry3Bb1/g fresh sample, the one from the near-isogenic maize 43 ng Cry3Bb1/g. The contents correspond to 18 and 0.06 ng Cry3Bb1 per individual insect.

In 2006, 50 and 64 insects were sampled from plots with Bt-maize and 36 and 30 from conventional maize. Bulk samples from Bt-plots had contents of 10 and 5 µg Cry3Bb1/g fresh sample, while the two samples from conventional maize were

negative for Cry3Bb1. Mean contents per individual were 22 and 10 ng Cry3Bb1 per individual in positive samples, respectively. A bulk sample of 29 nymphs of different stages collected in a Bt-plot had concentrations of 4 µg Cry3Bb1/g, corresponding to 8 ng Cry3Bb1 per individual.

In addition to the bulk samples, 45 adult insects, 21 female and 24 male, were analyzed individually for their exposure to Cry3Bb1 in 2006 and 2007. Females had a significantly higher mean body weight and contained significantly more Cry3Bb1 protein than males (Table 7). While males had a mean content of 3 ng Cry3Bb1, females contained 17 ng of transgenic protein. Calculated on a gram basis, this corresponds to a payload of 2 µg to 5 µg Cry3Bb1, respectively. 7 male and 4 female *T. caelestialium* caught in a plot adjacent to two Bt-plots were all tested negative for Cry3Bb1.

Table 7: Mean weight and Cry3Bb1 protein content (±1 standard deviation) for *Trigonotylus caelestialium* adult single specimens. Values in the same column followed by different letters are significantly different.

sex	number of individuals	mean body weight mg[1]	mean Cry3Bb1 content ng[2]
females	21	3.27 ± 0.75 a	16.82 ± 16.61 a
males	24	1.32 ± 0.26 b	2.95 ± 1.40 b

[1]: Welch t-test: t=7.2623, df=18.562, p<0.001

[2]: Welch t-test: t=2.4994, df=12.184, p=0.028

The Auchenorrhyncha assemblage in maize

The Auchenorrhyncha assemblage at the experimental site consisted of 5 species from the planthopper family Delphacidae and 12 species from the two leafhopper families Aphrophoridae and Cicadellidae (Table 8). Phloem is the predominant resource utilised. Only leafhoppers from either the family Aphrophoridae or the Typhlocybinae subgroup of the family Cicadellidae feed on xylem and mesophyll, respectively. According to the literature, various grasses are the host plants for most of these species, with *Z. scutellaris*, *L. striatella* and *J. pellucida* being the only species for which maize is expressly given as a host.

In terms of total numbers, 10,474 individual insects were caught over the three experimental years. 99.3% of these specimens belong to only 7 different species: *Z. scutellaris* (6,746 individuals; 64.4%), *P. alienus* (956; 9.1%), *J. pellucida* (638; 6.1%), *E. atropunctata* (576; 5.5%), *E. pteridis* (531; 5.1%), *M. laevis* (527; 5.0%) and *L. striatella* (430; 4.1%). The other 10 species combined account for 70 individuals (0.7%). Only the maize leafhopper *Z. scutellaris* was present in numbers high enough for a robust statistical analysis.

Table 8: Systematic overview of the Auchenorrhyncha species assemblage found in maize with mention of the utilised plant substrates and the host plants.

Suborder	Family (Subfamily)	Species	feeding on[1)]	host plants[1)]
Fulgoromorpha (planthoppers)	Delphacidae	*Laodelphax striatella* (Fall.)	phloem	grasses, maize
		Javesella pellucida (F.)	phloem	grasses, maize
		Dicranotropis hamata (Boh.)	phloem	grasses
		Ribautodelphax albostriata (Fieb.)	phloem	*Poa*
		Megadelphax sordidula (Stal)	phloem	*Arrhenatherum*
Cicadomorpha (leafhoppers)	Aphrophoridae	*Philaenus spumarius* (L.)	xylem	herbs
		Neophilaenus campestris (Fall.)	xylem	grasses
	Cicadellidae (Aphrodinae)	*Anoscopus albifrons* (L.)	phloem	grasses
	(Typhlocybinae)	*Zyginidia scutellaris* (H.-S.)	mesophyll	grasses, maize
		Empoasca pteridis (Dhb.)	mesophyll	herbs
		Eupteryx atropunctata (Goeze)	mesophyll	Lamiaceae
	(Deltocephalinae)	*Psammotettix alienus* (Dhb.)	phloem	grasses
		Macrosteles laevis (Rib.)	phloem	grasses, herbs
		Hardya tenuis (Germ.)	phloem	grasses
		Euscelidius variegatus (Kbm.)	phloem	unknown
		Artianus interstitialis (Germ.)	phloem	grasses
		Balclutha punctata (F.)	phloem	grasses, trees

[1)] according to Nickel, 2003

The relative proportion of the most abundant species changed over the three growing seasons. On sticky traps, the percentage of *Z. scutellaris* increased from 43% to 70% of all captured individuals, while the fraction of *J. pellucida* dropped from 33% to 1% (Figure 9). The percentages of the other species changed from year to year.

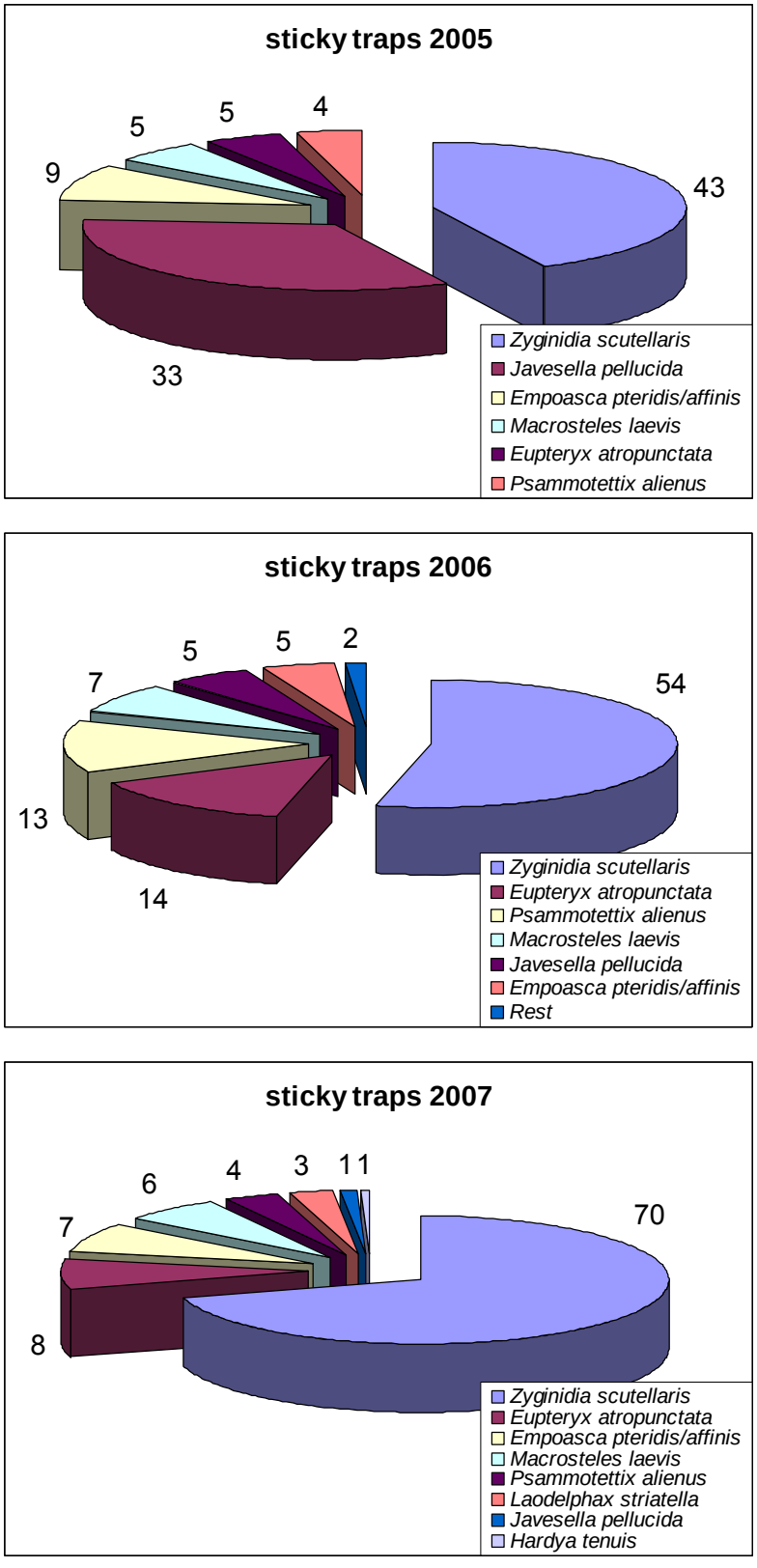


Figure 9: Per cent proportions of the most abundant Auchenorrhyncha species on sticky traps

In sweep net catches the proportions changed more dramatically: the percentage of *Z. scutellaris* ranged from 50% to 78%, while the fractions of *L. striatella* and *J. pellucida* dropped considerably from the 2005 to the 2007 growing season (Figure 10). The percentages of the other species changed from year to year, as for the sticky traps.

While there were differences between the two sampling methods with respect to the proportions of the most abundant species during single as well as during all three years, both methods identified the same 7 species as the most abundant. The number of species caught with sweep nets and sticky traps was also similar. In 2005, a total of 9 species were caught with the sweep nets, while 8 species were found in sticky traps. In 2006, the numbers of species increased to 15 and 12, while in 2007 they declined to 9 and 8, respectively. Some species were caught as single individuals and only with one of the sampling methods.

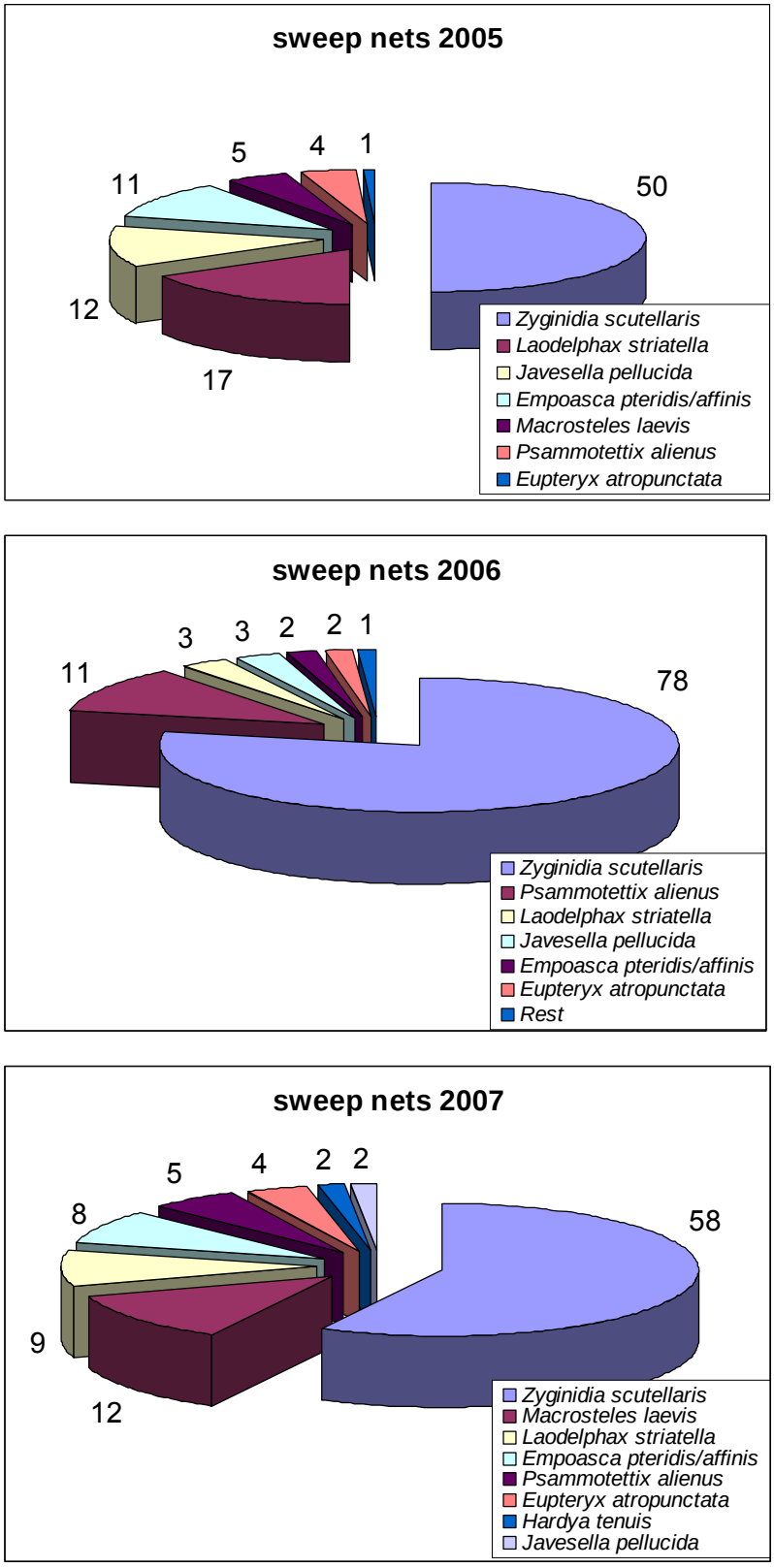


Figure 10: Per cent proportions of the most abundant Auchenorrhyncha species in sweep net catches

Abundance of Z. scutellaris *over the growing season*

There was an increase in the density of the maize leafhopper over the growing season based on the data obtained with sweep netting (Table 9). In 2005, the densities increased in all maize hybrids 6-fold at average from July to August, while in 2006 there was increase from 4- to 7-fold, depending on the variety, from July to September. In 2007, however, this increase could not be observed. Conversely, the population densities decreased steadily over the growing season from a high level in July, reaching only a fraction of that level in September.

The sticky traps in general showed a converse pattern to the sweep netting, with most insects caught early in the season and lower densities at later sampling dates. In July, more *Z. scutellaris* were caught with sticky traps, whereas in August and September the sweep netting yielded more individuals. This was not true in 2007, however, when less leafhoppers could be caught with the sticky traps than with the sweep nets at all sampling dates.

Generally, the maize leafhopper was more abundant in 2006 than in 2005. In 2007, fewer individuals than in the two previous years were caught, although in July a high abundance of *Z. scutellaris* was detected with the sweep nets.

Sex ratios of Z. scutellaris recorded with the sweep nets and sticky traps

The two methods reflected different sex ratios for *Z. scutellaris* over the growing season in all years (Table 9): The sweep nets always yielded a higher proportion of females ranging from 58.5% to over 86%. The percentage of males increased from around 15% in July towards 33.5-41.5% later in the season. There were always more female maize leafhoppers caught in July, and a clear tendency to an approximate ⅔ female to ⅓ male sex ratio in the course of the growing season.

On sticky traps females accounted for 31.6% to 71% of individuals caught. The sex ratios fluctuated around an approximate ⅓ female to ⅔ male sex ratio over of the whole growing season. This is in direct contrast to the results of the sweep netting. In 2007 however, the sticky traps followed the trend of the sweep netting and exhibited a similar sex ratio over all three sampling dates.

Table 9: Numbers of females and males caught with the two sampling methods at different sampling dates and percent fractions of total individuals caught. Numbers are pooled over all maize hybrids.

2005	**sweep netting**								
	1st sampling (July)			2nd sampling (August)					
	females	males	sum	females	males	sum			
total	39	6	45	166	102	268			
percent	86.7	13.3		61.9	38.1				
	sticky traps								
	females	males	sum	females	males	sum			
total	119	150	269	17	26	43			
percent	44.2	55.8		39.5	60.5				
2006	**sweep netting**								
	1st sampling (July)			2nd sampling (August)			3rd sampling (September)		
	females	males	sum	females	males	sum	females	males	sum
total	300	62	362	548	389	937	1286	671	1957
percent	82.9	17.1		58.5	41.5		65.7	34.3	
	sticky traps								
	females	males	sum	females	males	sum	females	males	sum
total	278	430	708	72	78	150	48	104	152
percent	39.3	60.7		48.0	52.0		31.6	68.4	
2007	**sweep netting**								
	1st sampling (July)			2nd sampling (August)			3rd sampling (September)		
	females	males	sum	females	males	sum	females	males	sum
total	496	81	577	203	102	305	26	14	40
percent	86.0	14.0		66.6	33.4		65.0	35.0	
	sticky traps								
	females	males	sum	females	males	sum	females	males	sum
total	207	85	292	123	68	191	11	10	21
percent	70.9	29.1		64.4	35.6		52.4	47.6	

Statistical analysis of** Z. scutellaris **data

The use of the different soil parameters and the four different maize lines as covariables in the generalized linear models reduced the variance in the field-data by 10.7 – 76.5%. Different models contained from 1 up to 10 different covariables, but there were no consistent patterns. The hybrids were one of the variables retained most often, but were present in only half of the 16 different models, 6 times in models with sweep net data and 2 times in models with sticky trap data.

The Tukey contrasts for comparisons between the different maize cultivars based on the results of GLM showed significant but inconsistent differences between the four maize lines with regard to the abundance of *Z. scutellaris* assessed with the two sampling methods (Table 10). Only comparisons between MON88017 and the near-isogenic line on the one hand, and the two conventional lines on the other hand are

shown, because these are most informative. All significant results but one were obtained with the sweep nets, while the sticky traps only yielded one significant difference between the two conventional hybrids.

In 5 comparisons for sweep netting data at different sampling dates there were significant differences between DK315 and Benicia. In 2 of these cases, *Z. scutellaris* was more abundant in the former; in the other 3 cases it was more abundant in the latter cultivar. The differences ranged from 19.4 – 296.8%. There were 3 significant differences between MON88017 and the near-isogenic line. One in the 2005 and two in the 2006 growing season. In two of these cases, there were 2.9% and 3.9% more *Z. scutellaris* in the Bt-maize cultivar. In the other, the abundance of the maize leafhopper was reduced in MON88017 by 16.7% when compared to the ISO cultivar.

In most cases with the sticky traps, the different hybrids were not retained as covariable in the generalized linear models. There was only one significant difference, with 136.4% more leafhoppers in DK315 than in Benicia.

Table 10 Statistically significant differences between the conventional hybrids DK315 and Benicia and between Bt-maize MON88017 and the near-isogenic line according to Tukey contrasts based in the GLMs analyses of abundance data of *Z. scutellaris*. Differences between MON88017 and the near-isogenic line are highlighted.

method	sampling period		hybrids		mean	z value	p value
sweep nets	2005	August	Benicia	vs	7.75		
			DK315		9.25	0.2129	0.02156
			ISO	**vs**	**8.38**		
			MON88017		**8.63**	**-2.618**	**0.04319**
	2006	July	Benicia	vs	11.00		
			DK315		13.13	-2.787	0.0272
		August	Benicia	vs	53.13		
			DK315		29.75	12.119	<0.001
			ISO	**vs**	**18.88**		
			MON88017		**19.63**	**0.1219**	**0.00402**
		September	Benicia	vs	89.38		
			DK315		60.63	7.023	<0.001
			ISO	**vs**	**55.25**		
			MON88017		**46.00**	**3.418**	**0.00356**
	2007	September	Benicia	vs	2.50		
			DK315		0.63	3.234	0.00645
sticky traps	2007	July	Benicia	vs	5.50		
			DK315		13.00	-5.211	<0.001

4.2 Community of arthropods living on panicles

The most abundant taxa occurring on male inflorescences during flowering were thrips and aphids and their shared principle predator, minute pirate bugs from the *Orius* complex (Table 11, 12). Most other taxa were representatives from the foliage-dwelling community, with a few exceptions: for example, the coccinellids *Adalia 2-punctata* and *Adonia variegata* were only found in panicle samples.

Table 11: Systematic overview of arthropods found on maize panicles. Major taxa (most abundant/prevalent) are highlighted.

Araneida	Araneidae		**Heteroptera**	**Anthocoridae**	***Orius* spec.**
	Opilionida			**Miridae**	***Lygus* spec.**
	Thomisidae	*Xysticus cristatus*			*Megalocoleus* spec.
Coleoptera	Anthicidae	*Notoxus monoceros*			*Psallus* spec.
	Byrrhidae				*Stenodema* spec.
	Carabidae	*Demetrias atricapillus*			***Trigonotylus caelestialium***
	Chrysomelidae	*Phyllotreta* spec.		Nabidae	*Nabis pseudoferus*
		Oulema spec.		Pentatomidae	*Carpocoris* spec.
	Coccinellidae	*Adalia 2-punctata*	**Homoptera**	**Aphidae**	
		Adonia variegata		Auchenorrhyncha	*Empoasa pteridis*
		Coccinella 7-punctata			*Eupteryx atropunctata*
		Harmonia axyridis			*Javesella pellucida*
		Propylea 14-punctata			*Laodelphax striatella*
	Lathridiidae	*Cortinicara gibbosa*			*Macrosteles laevis*
	Nitidulidae	*Glischrochilus hortensis*			*Psamotettix alienus*
	Staphylinidae				*Zyginidia scutellaris*
Dermaptera	Forficulidae	*Forficula auricularia*	Hymenoptera	Chalcidoidea	
Diplopoda				Cynipoidea	
Diptera	Anthomyiidae			Formicidae	
	Braconidae			Ichneumonidae	
	Calliphoridae			Proctotrupoidea	
	Cecidomyiidae			Tenthredinidae	*Athalia* spec.
	Chironomidae		Lepidoptera	Crambidae	*Ostrinia nubilalis*
	Chloropidae				
	Dolichopodidae		Planipennia	Chrysopidae	*Chrysoperla* spec.
	Empididae		Psocoptera		*Micromus* spec.
	Phoridae		**Thysanoptera**	**Aeolothripidae**	***Aeolothrips* spec.**
	Scatophagidae			**Phlaeothripidae**	***Haplothrips* spec.**
	Sciaridae			**Thripidae**	***Stenothrips* spec.**
	Synneuridae				***Anaphothrips* spec.**
	Syrphidae				***Chirothrips* spec.**
	Tephritidae				***Frankliniella* spec.**
	Tipulidae				***Limothrips* spec.**
Ephemeroptera					

Table 12: Densities (mean abundance in one pooled sample of 5 panicles ± 1 SD) of the major taxa found on maize inflorescences.

non-target organism			year	sampling	maize cultivar			
family	**genus**	**stage**			**Dk315**	**Benicia**	**Bt MON88017**	**DKC5143 ISO**
Anthocoridae	*Orius*	adult	2005	1	9.14 ± 4.53	8.57 ± 4.89	-	-
				2	1.13 ± 1.25	1.50 ± 1.20	2.13 ± 1.46	4.38 ± 2.92
				3	-	-	2.50 ± 1.51	3.25 ± 1.83
			2006	1	2.68 ± 2.52	2.18 ± 1.47	3.21 ± 1.84	3.26 ± 2.27
				2	1.75 ± 1.79	2.23 ± 2.93	2.93 ± 2.42	2.78 ± 1.59
			2007	1	0.30 ± 0.52	0.80 ± 1.16	-	-
				2	-	-	0.90 ± 1.00	0.98 ± 0.89
		nymph	2005	1	4.86 ± 2.54	4.86 ± 1.57	-	-
				2	2.38 ± 2.00	1.88 ± 1.25	2.88 ± 2.47	3.38 ± 2.67
				3	-	-	2.25 ± 0.89	1.88 ± 1.64
			2006	1	0.35 ± 0.53	0.42 ± 0.55	0.74 ± 0.99	0.67 ± 0.84
				2	0.33 ± 0.62	0.35 ± 0.58	0.45 ± 0.81	0.45 ± 0.71
			2007	1	0.25 ± 0.44	0.23 ± 0.42	-	-
				2	-	-	0.43 ± 0.59	0.23 ± 0.53
Miridae	*Trigonotylus*	adult	2005	1	4.71 ± 3.35	4.00 ± 1.91	-	-
				2	0.50 ± 0.53	1.50 ± 0.93	0.63 ± 0.92	0.88 ± 0.99
				3	-	-	0.25 ± 0.46	0.63 ± 1.06
			2006	1	3.75 ± 2.91	2.10 ± 1.67	4.18 ± 3.58	5.56 ± 4.35
				2	0.75 ± 0.98	0.88 ± 0.97	1.03 ± 1.15	1.15 ± 1.14
			2007	1	0.23 ± 0.53	0.13 ± 0.40	-	-
				2	-	-	0.23 ± 0.62	0.05 ± 0.22
		nymph	2005	1	1.71 ± 1.80	2.57 ± 1.51	-	-
				2	1.13 ± 1.64	2.00 ± 2.14	0.63 ± 0.92	0.75 ± 0.89
				3	-	-	1.13 ± 0.64	0.63 ± 0.74
			2006	1	0.13 ± 0.33	0.49 ± 0.79	0.18 ± 0.45	0.13 ± 0.34
				2	0.33 ± 0.62	0.55 ± 0.85	0.20 ± 0.41	0.30 ± 0.72
			2007	1	0.05 ± 0.22	0.08 ± 0.27	-	-
				2	-	-	0.08 ± 0.27	0.08 ± 0.27
	Lygus	adult	2005	1	4.86 ± 3.13	2.43 ± 1.99	-	-
				2	1.00 ± 0.76	0.63 ± 0.52	0.88 ± 0.99	0.63 ± 0.74
				3	-	-	0.13 ± 0.35	0.50 ± 0.76
			2006	1	0.18 ± 0.38	0.10 ± 0.31	0.15 ± 0.43	0.26 ± 0.55
				2	0.13 ± 0.33	0.15 ± 0.36	0.15 ± 0.42	0.15 ± 0.36
			2007	1	0.00 ± 0.00	0.00 ± 0.00	-	-
				2	-	-	0.08 ± 0.27	0.05 ± 0.22
		nymph	2005	1	4.14 ± 4.06	3.00 ± 4.55	-	-
				2	0.88 ± 1.13	0.50 ± 0.76	1.25 ± 1.49	1.75 ± 1.58
				3	-	-	0.63 ± 0.74	1.00 ± 0.76
			2006	1	0.03 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.22
				2	0.00 ± 0.00	0.03 ± 0.16	0.03 ± 0.16	0.00 ± 0.00
			2007	1	0.05 ± 0.22	0.00 ± 0.00	-	-
				2	-	-	0.03 ± 0.16	0.00 ± 0.00

Table 12 (continued): Densities (mean abundance in one pooled sample of 5 panicles ± 1 SD) of the major taxa found on maize inflorescences.

non-target organism	year	sampling	maize cultivar			
family			Dk315	Benicia	Bt MON88017	DKC5143 ISO
Thripidae	2005	1	3.25 ± 2.38	5.50 ± 3.89	-	-
		2	4.00 ± 2.07	5.38 ± 3.50	1.38 ± 1.06	1.63 ± 1.30
		3	-	-	3.38 ± 1.77	3.88 ± 1.46
	2006	1	1.73 ± 2.51	1.82 ± 1.75	1.59 ± 1.71	1.36 ± 1.20
		2	1.55 ± 1.43	1.60 ± 1.66	1.58 ± 1.50	2.08 ± 3.43
	2007	1	1.00 ± 1.13	1.28 ± 1.26	-	-
		2	-	-	1.05 ± 1.08	1.18 ± 0.84
Phlaeothripidae	2005	1	27.00 ± 10.19	28.14 ± 10.02	-	-
		2	10.50 ± 4.99	10.88 ± 4.12	13.38 ± 5.76	11.88 ± 5.41
		3	-	-	21.75 ± 7.32	22.63 ± 9.23
	2006	1	75.53 ± 49.12	96.97 ± 90.86	81.77 ± 53.69	66.33 ± 52.48
		2	20.30 ± 14.66	24.18 ± 22.22	32.40 ± 17.96	30.18 ± 18.87
	2007	1	3.30 ± 2.08	4.15 ± 2.59	-	-
		2	-	-	4.48 ± 2.92	3.48 ± 2.24
Aeolothripidae	2005	1	3.14 ± 2.73	2.43 ± 2.07	-	-
		2	4.13 ± 2.90	3.88 ± 1.73	2.13 ± 1.55	2.13 ± 1.13
		3	-	-	1.00 ± 1.20	0.50 ± 0.76
	2006	1	6.13 ± 4.44	6.64 ± 5.12	5.82 ± 7.09	3.00 ± 2.18
		2	1.38 ± 1.48	1.10 ± 1.71	1.40 ± 1.63	1.48 ± 1.43
	2007	1	1.13 ± 0.97	2.13 ± 1.99	-	-
		2	-	-	0.70 ± 0.94	0.75 ± 0.89
Aphidae	2005	1	54.29 ± 29.77	26.29 ± 26.76		
		2	19.63 ± 14.33	11.13 ± 4.99	16.25 ± 7.78	33.75 ± 31.60
		3	-	-	10.88 ± 4.94	13.88 ± 5.30
	2006	1	2.18 ± 2.33	0.64 ± 1.32	5.72 ± 9.35	13.89 ± 18.50
		2	0.03 0.16	0.10 ± 0.50	1.45 ± 2.50	1.23 ± 3.43
	2007	1	4.50 ± 4.99	4.33 ± 4.68	-	-
		2	-	-	5.65 ± 5.57	5.78 ± 4.62

In 2005, statistically significant differences in the densities of adult *Orius* bugs and Thripidae existed between either or both of BT (MON88017) and ISO (near-isogenic) and the two conventional hybrids (Figure 11). There were more minute pirate bugs on the near-isogenic maize than on DK315 (z=3.517, p=0.00253) and Benicia (z=3.111, p=0.00996). There were no differences for nymphs of the *Orius* genus or for adults and nymphs of the rice leaf bug.

Densities of Thripidae were significantly lower on BT and ISO when compared to Benicia (for BT: z=-3.635, p=0.00158; for ISO: z=-3.408, p=0.00341). DK315 also had more Thripidae, but the differences were not statistically significant.

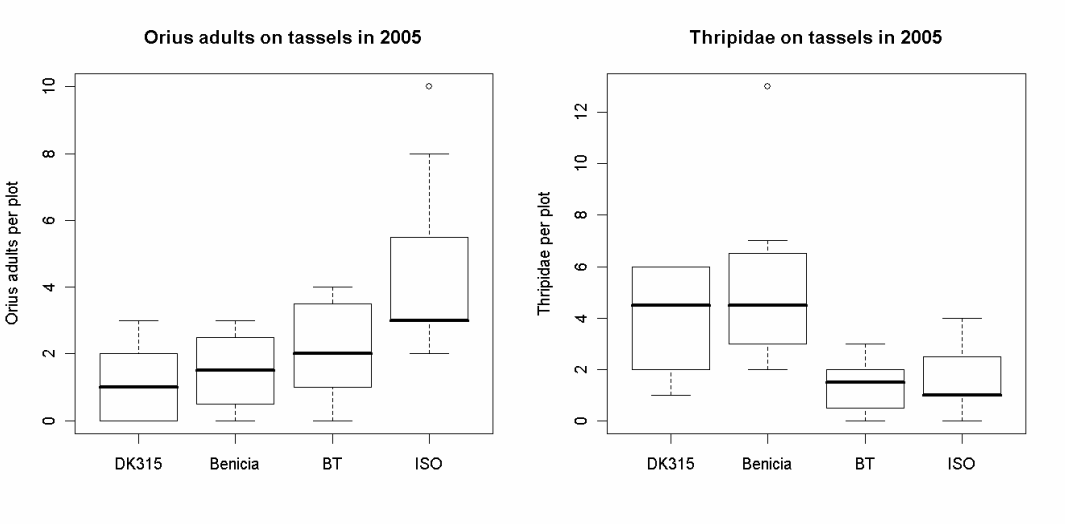


Figure 11: Densities of Orius adults and Thripidae on panicles in 2005

For Phlaeothripidae, Aeolothripidae and aphids, no statistically significant differences between the four hybrids were found.

In 2006, there were no differences in the densities of both *Orius* spec. adults and nymphs between the hybrids. For the rice leaf bug, however, there were some statistically significant differences: at the first sampling during early anthesis (Figure 12), there were more adult *Trigonotylus* on ISO than on Benicia (z=3.071, p=0.0114), while more nymphs could be found on Benicia than on any other hybrid (vs DK315: z=3.742, p=0.00104; vs BT: z=-3.207, p=0.00728; vs ISO: z=-3.742, p=0.00106). At the later sampling, there were no differences for adult *Trigonotylus*. Most nymphs could be found on MON88017, however, with differences between BT and both DK315 and ISO being statistically significant (Figure 13; vs DK: z=3.271, p=0.00568; vs ISO: z=-3.388, p=0.00425).

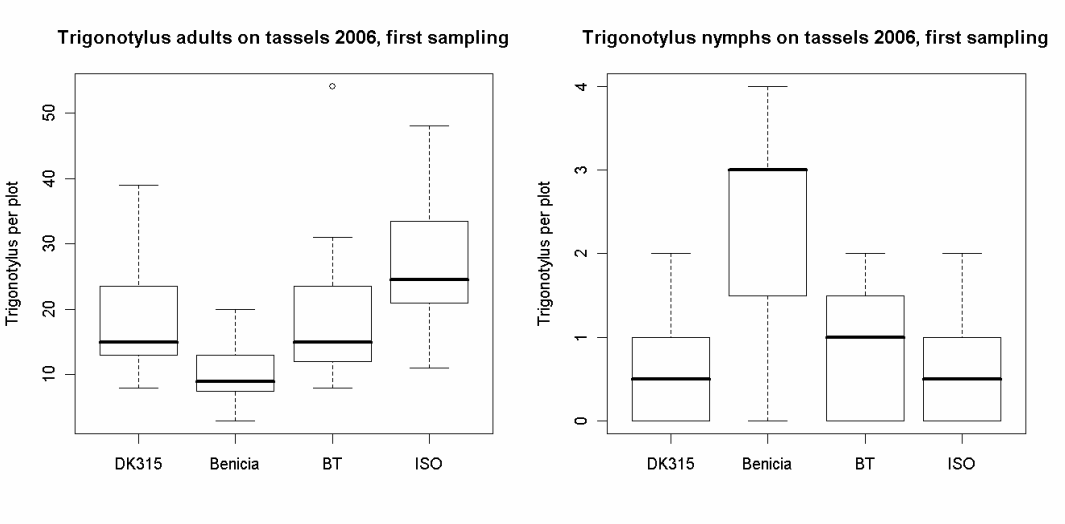


Figure 12: Adults and nymphs of the rice leaf bug on panicles at early anthesis

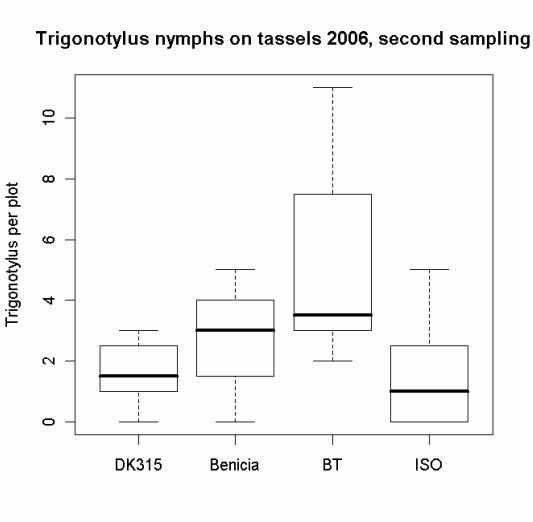


Figure 13: Nymphs of the rice leaf bug on panicles at late anthesis

For Phlaeothripidae, Aeolothripidae and Thripidae, there were no differences. For aphids, highest densities were recorded during early anthesis on the near-isogenic line (vs DK315: $z=4.263$, $p<0.001$; vs Benicia: $z=4.844$, $p<0.001$; vs MON88017: $z=2.989$, $p=0.0145$), and on MON88017 during late anthesis (vs DK315: $z=2.917$, $p=0.0185$; vs Benicia: $z=2.917$, $p=0.0185$) (Figure 14). Differences between the near-isogenic line and the two conventional hybrids were not statistically significant (vs DK315: $z=2.457$, $p=0.0667$; vs Benicia: $z=2.457$, $p=0.0666$).



Figure 14: Aphids on panicles during early and late anthesis 2006

There were statistically significant differences between samples taken during early and late anthesis in 2006: there were more adults of *Trigonotylus* in earlier samples than in the later samples ($t=6.5668$, $p<0.001$), while the opposite was true for nymphs ($t=-3.3211$, $p=0.002$). Phlaeo- and Aeolothripidae were also more abundant during early anthesis ($t=5.5878$, $p<0.001$ and $t=6.6351$, $p<0.001$, respectively). For Thripidae and for *Orius* adults and nymphs there were no differences ($t=-0.5066$, $p=0.6142$; $t=1.1647$, $p=0.2488$ and $t=1.3914$, $p=0.1693$, respectively).

In 2007, there were statistically significant differences for two groups of arthropods: Adults of the *Orius* complex were more abundant on the near-isogenic maize than on DK315 ($z=2.585$, $p=0.0481$) (Figure 15). This is in accordance with results from the 2005 growing season. Aeolothripidae were more abundant on Benicia than on MON88017 and the near-isogenic line (vs MON88017: $z=-3.481$, $p=0.00299$; vs ISO: $z=-3.359$, $p=0.00416$).

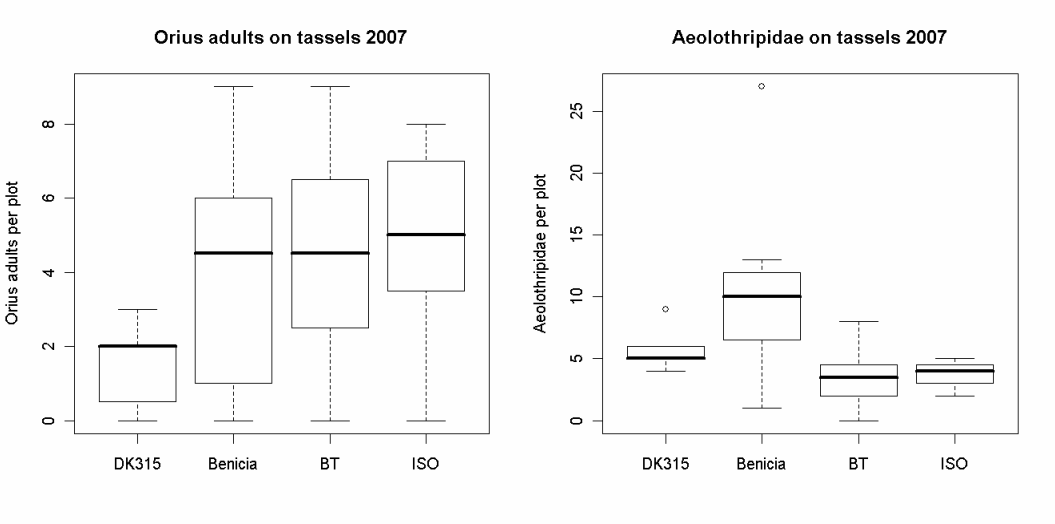


Figure 15: Adults of the Orius complex (left) and Aeolothripidae (right) on panicles during anthesis 2007

There were large between-year variations in the abundances of the major taxa in panicle samples: Thysanoptera were extremely abundant in 2006, with Phlaeothripidae reaching mean densities of up to 400 individuals in one sample (consisting of 5 male inflorescences). This was by a factor of 10 to 40 more than was observed in the previous and the following year. The same held true, albeit to a lesser extent, for Aeolothripidae and Thripidae, for the *Orius* bugs and *T. caelestialium*, and for aphids.

4.3 Community of arthropods living on maize cobs

A systematically and functionally diverse assemblage of arthropods was found on maize cobs, although the majority of individuals belonged to only 6 taxa (Table 13): most abundant in terms of numbers were aphids and thrips, followed by the minute pirate bug complex of the genus *Orius* (Table 14). The next most frequently found taxon was the minute brown scavenger beetle *Cortinicara gibbosa*.
Statistical analysis focussed on these taxa, since the numbers obtained for the other groups were inadequate for a reasonable evaluation.

Table 13: Systematic overview of the arthropod assemblage found on maize cobs. Major taxa (most abundant/prevalent) are highlighted.

Araneida	Araneidae		**Heteroptera**	**Anthocoridae**	***Orius* spec.**
	Thomisidae	*Xysticus cristatus*		Lygaeidae	
Coleoptera	Anthicidae	*Anthicus antherinus*		Miridae	*Lygus* spec.
		Notoxus monoceros			*Trigonotylus caelestialium*
	Bruchidae			Nabidae	*Nabis pseudoferus*
	Byrrhidae			Pentatomidae	*Rhaphigaster nebulosa*
	Carabidae	*Demetrias atricapillus*	**Homoptera**	**Aphidae**	
		Trechus spec.		Auchenorrhyncha	
	Chrysomelidae	*Phyllotreta* spec.			*Empoasa pteridis*
		Oulema spec.			*Psamotettix alienus*
	Coccinellidae	*Coccinella 7-punctata*			*Zyginidia scutellaris*
		Propylea 14-punctata	Hymenoptera	Chalcidoidea	
	Curculionidae	*Apion* spec.		Formicidae	
	Lathridiidae	***Cortinicara gibbosa***		Ichneumonidae	
	Staphylinidae			Proctotrupoidea	
Collembola				Tenthredinidae	*Athalia* spec.
Dermaptera	Forficulidae	*Forficula auricularia*	Lepidoptera	Crambidae	*Ostrinia nubilalis*
Diplopoda			Planipennia	Chrysopidae	*Chrysoperla* spec.
Diptera	Anthomyiidae				*Micromus* spec.
	Chloropidae		Psocoptera		
	Phoridae				
	Sciaridae		**Thysanoptera**	Aeolothripidae	*Aeolothrips* spec.
	Syrphidae			**Phlaeothripidae**	***Haplothrips* spec.**
	Tipulidae			**Thripidae**	***Stenothrips* spec.**
					***Anaphothrips* spec.**
					***Chirothrips* spec.**
					***Frankliniella* spec.**
					***Limothrips* spec.**

Table 14: Densities (mean per cob ± 1 SD) of the major taxa found on maize cobs

non-target organism		year	maize cultivar			
	stage		Dk315	Benicia	Bt MON88017	DKC5143 ISO
Orius spec.	adult	2005	0.51 ± 0.68	0.80 ± 0.91	0.53 ± 0.78	0.73 ± 0.89
(Anthocoridae)		2006	0.83 + 0.96	0.34 ± 0.57	0.62 ± 0.80	0.55 ± 0.71
		2007	0.48 ± 0.73	0.75 ± 0.96	0.93 ± 1.04	0.75 ± 0.89
	nymph	2005	0.13 ± 0.35	0.27 ± 0.46	0.11 ± 0.32	0.13 ± 0.51
		2006	0.26 ± 0.65	0.19 ± 0.51	0.16 ± 0.40	0.23 ± 0.78
		2007	0.15 ± 0.36	0.10 ± 0.34	0.39 ± 0.58	0.36 ± 0.60
Thripidae		2005	1.36 ± 1.43	0.92 ± 1.10	0.51 ± 0.71	0.70 ± 1.05
		2006	2.23 ± 1.94	2.33 ± 1.72	1.23 ± 1.33	1.21 ± 1.11
		2007	0.84 ± 1.10	1.15 ± 1.39	0.43 ± 0.67	0.43 ± 0.69
Phlaeothripidae		2006	2.04 + 2.15	2.09 ± 2.06	1.60 ± 1.45	1.05 ± 1.15
Cortinicara gibbosa		2005	0.15 ± 0.33	0.16 ± 0.37	0.21 ± 0.49	0.23 ± 0.50
(Lathridiidae)		2006	0.35 + 0.78	0.46 ± 0.75	0.47 ± 0.79	0.38 ± 0.62
		2007	0.25 ± 0.58	0.11 ± 0.36	0.20 ± 0.43	0.43 ± 0.67
Aphidae		2005	42.05 ± 56.43	66.49 ± 64.52	95.44 ± 71.85	89.67 ± 68.98
		2006	1.01 ± 4.97	3.28 ± 13.25	27.32 ± 54.75	11.48 ± 21.65
		2007	93.61 ± 99.57	75.26 ± 95.16	117.58 ± 86.69	113.48 ± 90.44

For aphids, there were statistically significant differences between the different maize lines (Figure 16): In 2005, there were fewer aphids in DK315 than in MON88017 and its near-isogenic parental line (vs BT, $z=5.139$, $p<0.001$; vs ISO, $z=4.457$, $p<0,001$). The difference between Benicia and MON88017 were also significant ($z=2.744$, $p=0.0306$). There were no differences between the genetically modified and the near-isogenic line ($z=-0.551$, $p=0.9462$).

In 2006, there were statistically significant differences between both DK315 and Benicia against MON88017 (vs DK315, $z=4.535$, $p<0.001$; vs Benicia, $z=4.424$, $p<0.001$), as well as between MON88017 and ISO ($z=-2.768$, $p=0.0288$). There were more aphids present on MON88017 than on samples from the near-isogenic maize (Table 14). Generally, there were far fewer aphids present in 2006 than in 2005.

In 2007, densities of aphids increased over the values of 2005. Fewest aphids were found in Benicia and this was statistically significant when compared to both MON88017 ($z=2.854$, $p=0.0227$) and ISO ($z=2.577$, $p=0.049$). There was no difference between the transgenic and the near-isogenic maize.

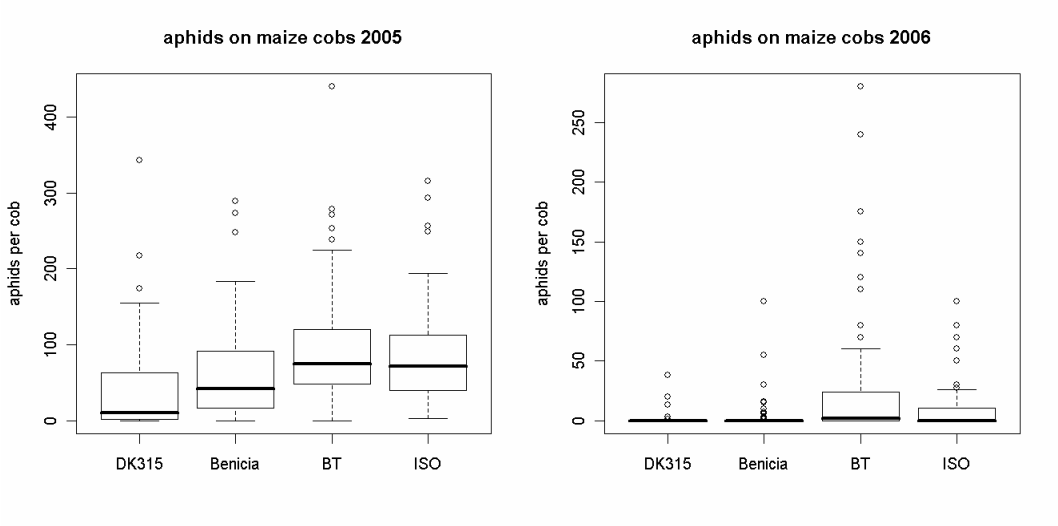


Figure 16: Mean number of aphids per cob on the four different maize lines in 2005 and 2006

Densities of *C. gibbosa* were uniformly low in the three experimental years with a slight increase in 2006 (Table 14). There were no statistically significant differences between the four maize hybrids, except in the last year. There were fewer *C. gibbosa* in MON88017 than in ISO ($z=2.707$, $p=0.0342$). Lowest densities were recorded in Benicia, however (vs ISO: $z=3.759$, $p<0.001$).

The number of individuals from the Orius complex changed over the three years in different directions in the four maize lines (Table 14): In DK315 and MON88017 a slight increase was observable, while in Benicia and the near-isogenic line the densities of minute pirate bugs decreased. For statistical analysis, data for adults and nymphs were pooled. Significant differences were only observed in the last year, with DK315 having lower densities than both MON88017 ($z=4.131$, $p<0.001$) and ISO ($z=2.929$, $p=0.0176$). The difference between Benicia and BT were also significant ($z=2.779$, $p=0.0278$).

The densities of thrips were variable over the years (Figure 17, Table 14). Thripidae were the single group of Thysanoptera found except for 2006, when also Phlaeothripidae were observed, and then in high densities. In all three years, there were no statistically significant differences between MON88017 and the near-isogenic line, for both Thripidae and Phlaeothripidae Statistically significant differences were found, however, between BT and ISO against DK315 (vs BT $z=-5.481$, vs ISO $z=-4.399$, $p<0.001$ in both cases), between Benicia and DK315 ($z=-2.928$, $p=0.0182$) and

BT and Benicia (z=-2.622, p=0.0436) in 2005. In the following year, there were statistically significant differences for all pair wise comparisons of Thripidae densities except for ISO versus BT and Benicia versus DK315 (p<0.001 in all cases). For Phlaeothripidae, differences between ISO and both DK315 (z=-3.475, p=0.0029) and Benicia (z=-3.746, p=0.0011) were significant. In the last year, again, all pair wise comparisons were different except for the pairings DK315 and Benicia and MON88017 and ISO (p<0.047 for all comparisons).

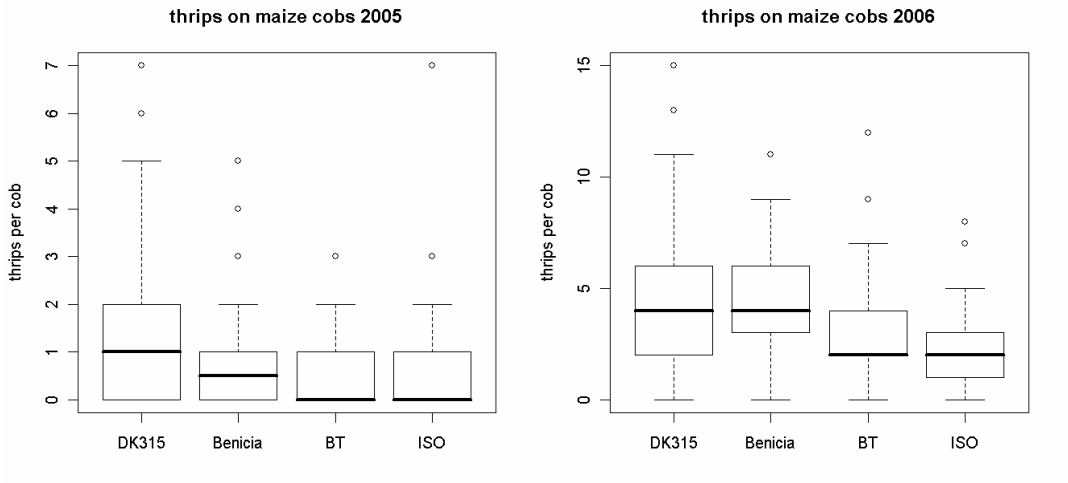


Figure 17: Mean number of thrips per cob on the four different maize lines in 2005 and 2006

4.4 Weather data

The average daily temperatures during the periods the maize was grown in the respective years were quite different. While there was in increase in the mean temperature from 2005 to 2006, the growing season 2007 was considerably cooler (Table 15). The main differences for the temperatures do not lie in the average values, however, but more in the temperature profile during the time from April/May to September/October. During the time of planting in May 2005 temperatures were fairly high, but then dropped towards the middle of June (Figure 18). From the middle of June (CW 24) to the beginning of September (CW 37) there was an extended period of time with temperatures ranging from 13° to 25° Celsius on average. The growing season 2006 started with lower temperatures, ranging down to below 10° Celsius at

the end of May. In the course of June they rose steadily, reaching a level at around 20° to 25° Celsius at average until the middle of August (CW 32).

Table 15: Minimum, maximum and average temperatures (° Celsius) in heights of 20 and 200 cm above ground and rain sum (in millimetres) averaged over the consecutive growing seasons (CW 19/21 through CW 39/41)

	20			200			
	min	avg	max	min	avg	max	rain sum
2005	10.1	17.6	25.8	11.1	17.4	23.6	226.6
2006	10.4	18.1	26.3	11.6	18.0	24.3	251.4
2007	10.0	16.5	23.9	11.1	16.6	22.3	435.1

This was considerably higher than in the same time of the previous year. Temperatures then dropped again and remained stable at a lower level until harvest at the end of September (CW 39). The beginning of the growing season in 2007 saw high temperatures, higher than in both preceding years. From June to the middle of July, however, they decreased steadily, being on the average 5° Celsius lower than in 2006 and somewhat lower than in 2005. The following weeks were characterized by a level of medium temperatures ranging from 13° to 26° Celsius, comparable to 2005.

Another major difference in the weather conditions of the three experimental years was the rainfall during the growing season: while in 2005 and 2006 a comparable total amount (in millimetres) of rain fell (Table 15), it was mostly during July in the former and mostly during May and June and after the middle of August in the latter year (Figure 19). In 2007, however, the amount of rain was double that of the previous years, with rainfalls from the middle of June to the middle of August. There were three single days with heavy rainfalls in May, June and August. In the seven days during the season with more than 20 millimetres of rain a total of 162 millimetres fell, only 60 to 90 millimetres short of the total rainfall for the entire 2005 and 2006 growing seasons, respectively. In conclusion, the first two years were comparable in the amount of rainfall, but not in terms of the temperature profile. The first and the last year were similar in temperature, but 2007 had much higher and heavier rainfalls than both preceding years.

4.5 Soil parameters

The data on soil parameters were compiled by the working group at the Institute for Applied Biotechnology in the Tropics at the Georg-August-University Göttingen. They can be found tabulated in the raw data section on CD-ROM (only with reviewer's copies).

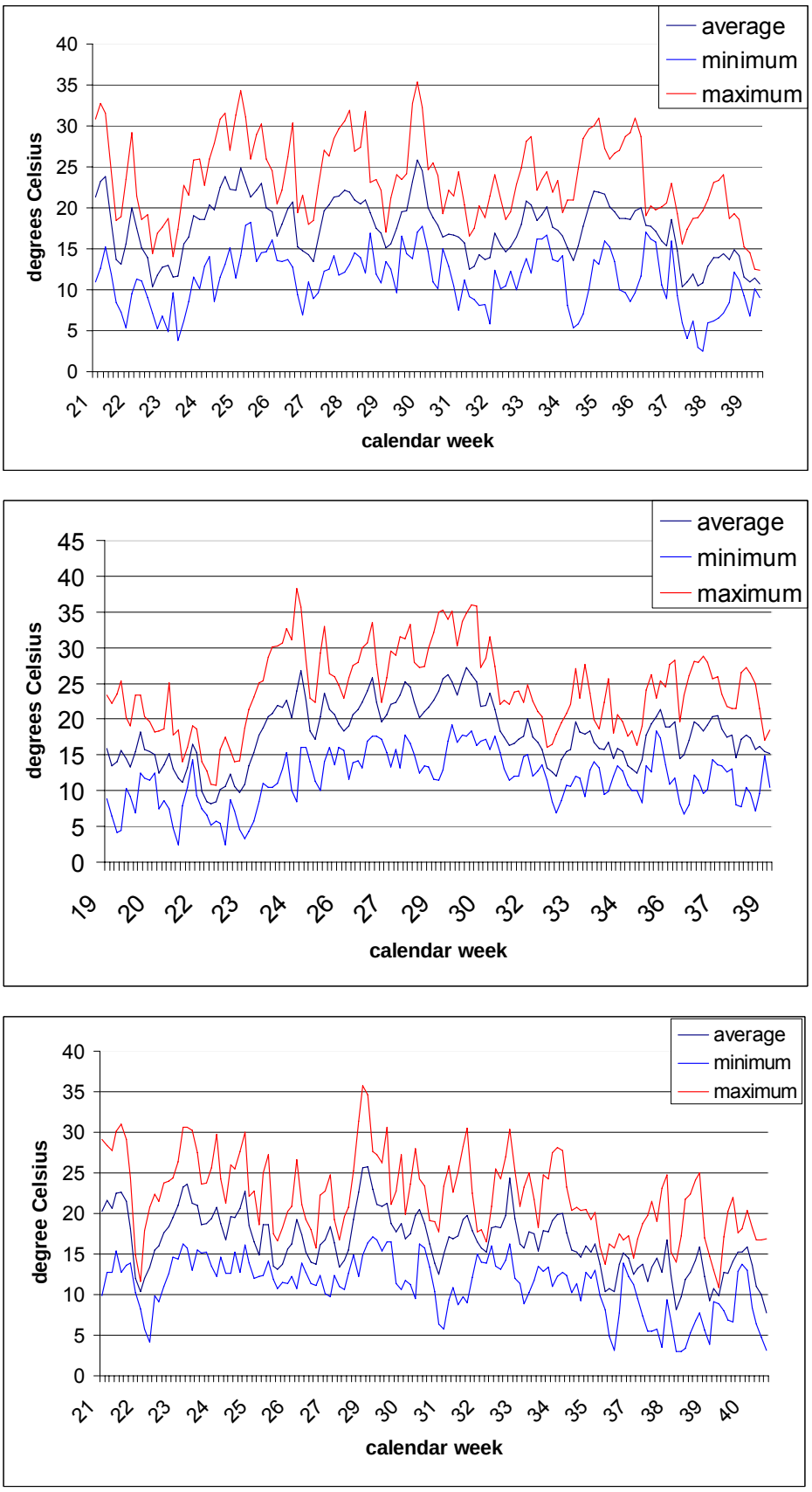


Figure 18: Temperature profiles of 2005 (upper), 2006 (middle) and 2007 (lower chart) growing seasons of maize (note the differences in the time-line)

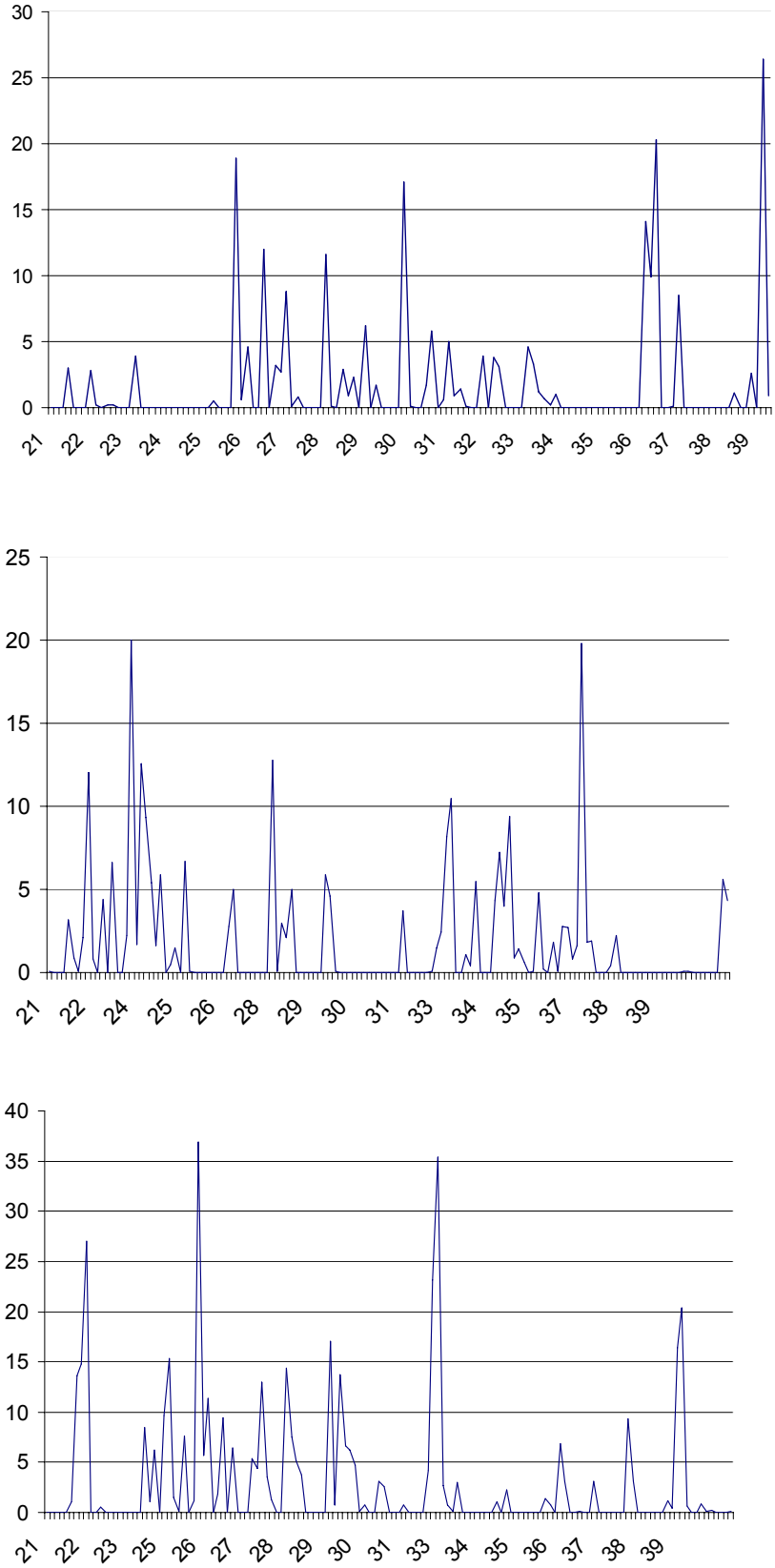


Figure 19: rainfall (sum per day in millimetres) during the growing seasons 2005 (upper), 2006 (middle) and 2007 (lower chart)

5. Discussion and Conclusions

Three major communities of arthropods were assessed with regard to potential impacts of Cry3Bb1 expressing MON88017 Bt-maize. The assemblage of arthropods living on and between the foliage of maize plants, i.e. the herb-layer, was the most complex. It comprised a number of organisms with special relevance for both Environmental Risk Assessment (problem formulation) and monitoring. The communities living on male inflorescences and on maize cobs were far more restricted in the number of species and featured only a handful of taxa occurring in high densities.

5.1 Community of foliage-dwelling arthropods in the herb layer in maize

The community of arthropods in the herb layer was highly diverse, both in systematic and in ecological (feeding behaviour, trophic layer, function within the ecosystem) terms. Most species or families, however, had only very small densities in the field and could not be sampled in numbers sufficient enough for a statistical analysis. This was especially true for the Chrysomelidae family of beetles, of which *D. virgifera virgifera* is also a member. Because of their close relationship to the target insect, all leaf beetles can be assumed to be affected when ingesting Cry3Bb1 with their food. This is true, for instance, for the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Galitsky et al., 2001; Meissle M., 2008). Leaf beetles are therefore of special interest in problem formulation, risk assessment and monitoring of Cry3Bb1 expressing maize lines (or others with resistance against coleopteran pests based on other Cry proteins with similar specificity).

Five genera of chrysomelids were caught at the experimental site, of which *Phyllotreta*, *Chaetocnema* and *Oulema* were present in all three years. These three generally seem to be the most common genera of leaf beetles in maize, albeit that there is not much published literature on the occurrence and density of these arthropods in this crop (Kiss et al., 2002, undated; Jörg Leopold, personal communication). At the field site near Würzburg, they played only a minor role as members of the biocoenosis, judged by their overall low total numbers. In Hungary, however, they can reach high densities and even cause visible symptoms on maize plants through feeding (Kiss et al., 2002, undated). An assessment of a potential

hazard caused to these species by Cry3Bb1 expressing maize has to take into account their low prevalence in the receiving environment (area under maize cultivation) and their status as secondary pests. In this light, even an identified hazard may be acceptable from a regulatory standpoint, since management goals and the anthropocentric values of species are fundamental features driving the selection of species to test in ERA and to monitor during General Surveillance, and their relevance in the overall risk assessment (Johnson et al., 2006; Romeis et al., 2008; Todd et al., 2008).

Of the approximately 500 species of leaf beetles in Germany, about 37 species might have a more or less likelihood of being negatively affected by the cultivation of Cry3Bb1 expressing Bt-maize (Leopold & Felke, 2008). This estimate is based on species the host plants of which occur on and near maize cropping areas. The majority would be affected by the deposition of pollen on the plants they are feeding on, rather than feeding on maize itself. In this scenario, there are a variety of factors that influence the actual exposure of the respective species: 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 flowering of maize; 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 characterizations for Lepidoptera and Cry1Ab expressing Bt-maize (Sears et al., 2001; Gatehouse et al., 2002). To fully assess the relevance of this scenario, at least the major components – i.e. exposure and hazard – need to be characterised (see Johnson et al., 2006).

For three species of chrysomelids tested by Leopold & Felke (2008) in acute toxicity setups in the laboratory at the Julius Kühn-Institute for Biological Control (the former Federal Biological Research Center BBA, Darmstadt, Germany), a potential hazard was identified (i.e. higher mortality), but only with unrealistically high exposure scenarios, i.e. higher pollen deposition than would occur on the host plants under natural conditions. Two of these species had a low to moderate probability of exposure to the Cry3Bb1 protein in their natural environments. One of these was the Colorado potato beetle *Leptinotarsa decemlineata* (Say), a severe pest of potatoes in

Europe and the US. Thus, a high probability for an overall negligible risk for chrysomelid beetles was determined, although there remains some uncertainty for most leaf beetles.

Besides species closely related to the target organism, all arthropods exposed to the novel protein and occurring in high enough densities to warrant their ecological relevance are of potential interest for and relevance in ERA and monitoring. Two species that match these criteria were found at the experimental site: The first is the plant bug *T. caelestialium* which will be dealt with in the following. The other, the maize leafhopper *Z. scutellaris*, will be discussed later in the section about the Auchenorrhyncha assemblage in maize.

Density patterns of the rice leaf bug (T. caelestialium) in maize

The different maize lines exhibited different density patterns for adults and nymphs of the rice leaf bug, which changed over the growing seasons but were consistent over the three years. The differences between the two conventional hybrids DK315 and Benicia were statistically significant in almost all cases, while there was only one significant difference between Bt-maize MON88017 and the near-isogenic line. In general, lowest and highest abundances were recorded in DK315 and Benicia, respectively, while the values for BT and ISO mostly lay in between these two. The reasons for the observed differences and changes in distribution are unclear. Since *T. caelestialium* takes olfactory cues from its host plants (Niiyama et al., 2007), differences in plant volatile composition between the maize lines may be one factor in causing the density variations. Other factors may include plant phenology and physiology, leaf surface microstructure or differences in the microclimatic conditions of the maize stands. The specific reasons for the observed differences fall outside the scope of this work. The marked differences in the densities of *T. caelestialium* between the two conventional varieties DK315 and Benicia were consistent over the years. This indicates the existence of a variety effect, some difference in the characteristics of the conventional maize lines that exerts an influence on the occurrence of this herbivorous arthropod. There were no differences between MON88017 and the near-isogenic line.

There was no consistent influence of individual or specific sets of soil parameters on the densities of the rice leaf bug. Generally, varying combinations of parameters

explained a variable portion of the variance in the data. Since it is unclear in what way and extend the different maize lines react to local differences of certain soil parameters, the soil parameters cannot be easily used to explain the differences in densities of *T. caelestialium*. A much better understanding of the influence of certain soil parameters on the performance of different maize lines is needed to assess the possible workings of these influences on the next trophic level. However, the reduction of variance in field count data is a valuable asset in itself, enhancing the statistical analyses.

Exposure of T. caelestialium to the Cry3Bb1 protein

The results of the ELISA tests demonstrate that individuals of *T. caelestialium* are exposed to Cry3Bb1 through all stages of their life: Nymphs contained around 8 ng Cry3Bb14 corresponding to 4 µg Cry3Bb1/g. Since insects in nymphal stages have no completely developed wings, their sole mode of dispersal is walking or jumping. They may not cover large distances and will therefore likely remain at more or less the same vicinity, thus being exposed to the genetically modified protein during their whole development. The actual exposure in this case is closely related to the concentration of the genetically modified protein in the plant parts that are fed on. Expression of genetically modified proteins might vary considerably, as has recently been reported by Nguyen & Jehle (2007) for Cry1Ab in MON810. Similar differences between plant organs and with regard to different growth stages can be observed for Cry3Bb1 from MON88017 (Rauschen et al., 2008b; Hang Thu Nguyen, personal communication). An analysis of the tissue-specific expression of the genetically modified protein over time can therefore be a valuable asset in a non-target risk assessment.

Adult insects were tested for varying amounts of Cry3Bb1, ranging from a few to over 60 ng, corresponding to 2 – 10 µg Cry3Bb1/g. They do have the ability to fly, however they do not seem to maintain longer flights, but only cover rather small distances between plants (personal observations; see also Goto & Higuchi, 2004). This observation is corroborated by the absence of Cry3Bb1 positive samples from non-Bt plots on the experimental field and only very small amounts in the bulk sample from 2005. The large amount of protein that a single individual may contain might be the cause for the positive detection of Cry3Bb1 in the latter sample. A few insects with an average payload of Cry3Bb1 that had recently moved into a non-Bt plot and

were caught there would have easily caused the values obtained with ELISA. In conclusion, the lack of long range dispersal may lead to prolonged exposure of rice leaf bug adults to Cry3Bb1 during their whole life span.

Experimental work done during a diploma thesis (Schultheis, 2008) has shown that Cry3Bb1 is rapidly lost from *T. caelestialium* adults after a very short period of feeding on non-genetically modified maize or of food deprivation. This means that insects, after moving from a Bt-maize plot into a plot with conventional maize, will have a very low internal concentration of Cry3Bb1, due to not having fed on Bt-maize for some time and due to feeding on non- genetically modified maize. This may be an additional explanation for the lack of Bt-positive test individuals from plots with non-transgenic maize. The ELISA test used would have been able to quantitate down to 1 ng per individual bug tested (limit of detection at 0.5 ng Cry3Bb1/ml sample extract).

The exposure of *Trigonotylus spp.* to transgenic protein has been investigated for Cry1Ab expressing maize Bt176. Obrist et al. (2006a) found around 1.5 µg Cry1Ab/g in insects collected from Bt-maize fields before and during anthesis. This value is considerably lower than the ones reported in this work. But when taking the respective expression levels into account, the internal concentrations are similar: Cry1Ab in Bt176 was measured by Obrist et al. (2006a) at a concentration of 6 µg Cry1Ab/g in leaves. In *Trigonotylus*, they found 1.5 µg Cry1Ab/g. This means that a quarter of the amount found in the maize leaves could be detected in the insects. Leaves of MON88017 express Cry3Bb1 with around 35 µg/g (Nguyen, personal communication). In *T. caelestialium* we measured around 7 µg/g, which is one fifth of the original concentration. Based on these calculations, the relative concentrations of the different Bt-proteins found in *Trigonotylus* sampled from different genetically modified maize varieties are very similar.

Bt- and other insecticidally active proteins can be carried over from exposed herbivorous arthropods to their predators (Zwahlen et al., 2000; Burgess et al., 2002; Ferry et al., 2003; Álvarez et al., 2005; Obrist et al., 2005; 2006a, b, c; Bai et al., 2006; Torres & Ruberson, 2007; Álvarez-Alfageme et al., 2008). Depending on the feeding rate, the actual internal concentration of the Bt-protein in the consumed prey, the stability of the Bt-protein during and after feeding, and the rate of digestion and excretion, a predator will be exposed to a certain amount of protein via its prey. By consuming a single *T. caelestialium* individual a predator might take up a considerable amount of Bt-protein. The role of *T. caelestialium* as a mediator of these

proteins in the food-web is not clear, but can be assumed to be of some importance. Whether or not this carry-over is of ecotoxicological relevance has not been examined in detail however, since Bt-proteins are generally regarded as being specific to narrow spectra of arthropods (Schnepf et al., 1998). A non-target action is neither to be suspected nor has there been any convincing evidence for it in the field. Another aspect that has to be kept in mind is the fact that current ELISA methods do also detect protein fragments containing the antibody-specific epitope, and not only the full-length proteins (Lutz et al., 2005b). This means that a positive ELISA results does not necessarily indicate the presence of bioactive Cry-protein.

There are a number of coleopteran predators in the maize biocoenosis that could be affected by Cry3Bb1. Most belong to either the Coccinellidae or the Carabidae families. Experimental work done in the laboratory and the field has shown that for selected species from both groups there was no harmful effect of Cry3Bb1 (Ahmad et al., 2006). The same was true for the heteropteran predator *Orius insidiosus* – and might also hold true for other predacious bugs, notably species from the *Nabis* genus, based on their phylogenetic relationship. For carabids, some work has also been done with MON88017, again demonstrating no acute toxicity of Cry3Bb1 to selected species from this arthropod group (Priesnitz et al, 2007). On the other hand, there is no published data available in the peer-reviewed scientific literature from thorough toxicity studies with this protein and predatory insects. Work on another coleopteran-specific Cry-protein, Cry3A from genetically modified potatoes directed against *L. decemlineata*, has shown that neither the ladybird beetle *Harmonia axyridis* (Pallas) nor the carabid beetle *Nebria brevicollis* (Fabricius) were negatively affected (Ferry et al., 2007). This again can be taken as evidence for the high specificity of Bt-crops to their respective target organisms. It can be concluded, that even in the same insect order as that of the target pest effects on an NTO might be absent.

Despite the exposure to Cry3Bb1, there were no indications of a negative impact of the Bt-maize on *T. caelestialium*. Densities of the rice leaf bug were always similar in MON88017 and the near-isogenic line. At one occasion, there were even more adult bugs in the BT plots. From these field-data it can be concluded, that there were no major impacts on life-table and fitness parameters of this species. Generally, Cry-proteins from *B. thuringiensis* are regarded to be highly specific against certain

groups of insects, due to their mode of action (Schnepf et al., 1998; Crickmore et al., 1998). Therefore, a negative impact on *T. caelestialium* seems very unlikely.

Auchenorrhyncha assemblage in maize

The Auchenorrhyncha assemblage found in maize can be categorised into two groups: the first group encompasses species that have an association with maize, i.e. which feed extensively on maize and complete at least part of their lifecycle on this host plant. The most notable representative of this group is the maize leafhopper *Z. scutellaris*, which has been reported from maize fields in different parts of Europe (Schmitz & Bartsch, 2001; Kiss et al., 2002, Rauschen et al., 2004; Candolfi et al., 2004; Pons et al., 2005). Moreover, exposure of *Z. scutellaris* to proteins from genetically modified maize has been documented (Dutton et al., 2004; Obrist et al., 2006a). Other members of this group are the two phloem feeding, non-exposed *J. pellucida* and *L. striatella*; and *E. atropunctata*, for which exposure to the Cry3Bb1 protein from MON88017 has been demonstrated (Meissle M., 2007). *P. alienus*, *M. laevis* and *E. pteridis* also belong to this group and have also already been found in maize fields (Kiss et al., 2002; Rauschen et al., 2008a). Problem formulation leads to the conclusion that a non-target risk assessment is valuable for these plant- and leafhoppers and especially *Z. scutellaris*, based on their affiliation to host plant and exposure to the Bt-protein.

The second group consists of vagrants from off-crop furrows and ridges or the surrounding cultivated land. These insects probably spend only little time in maize stands, while searching for a suitable host. Feeding on maize may occur, but is limited and does not support the development or maintenance of larger populations. The 10 species that together accounted for 0.7% of individuals all belong to this group. At least some of these species may have been hosted by a hedgerow of shrubs, trees and herbaceous undergrowth beyond the perimeter of conventional maize around the experimental field. Others may have come from grassy field margins. Exposure of species in this group is low to nil: the only two species that are exposed based on their feeding on xylem are *P. spumarius and N. campestris*. All other species are phloem feeders and will therefore not get into contact with transgenic proteins, since these are regularly not found in the phloem sap of maize

(Head et al., 2001; Raps et al., 2001). No toxic effect of the Bt-protein on these arthropods and their specialised antagonists can therefore be suspected.

Density patterns of the maize leafhopper (Z. scutellaris)

A more in depth analysis of the density data could only be carried out for the most abundant leafhopper *Z. scutellaris*. There were no consistent differences in the densities of this species between plots with MON88017 and plots planted with the near-isogenic line. At two times, there were slightly more leafhoppers present in the Bt-maize, at one time there were more in the near-isogenic maize. The differences were smaller than those between the two conventional hybrids DK315 and Benicia. There was, however, no consistent pattern with respect to the density of the maize leafhopper in these two hybrids: at times, there were more *Z. scutellaris* in DK315, at others this species was more abundant in Benicia. Whether these fluctuations are based on physiological differences between these varieties and can be interpreted as a variety-effect, possibly modulated by other factors, remains unclear.

The two sampling methods reflected the population density of *Z. scutellaris* differently (Schultheis, 2008): with the sweep nets large numbers of the maize leafhopper were caught later in the season, while with the sticky traps most individuals were caught in early summer and considerably lower counts were obtained in August and September. A potential explanation is given by observations in the field: in the first part of the growing season, maize leafhoppers could be observed actively flying between the rows of maize plants, covering distances of some meters during a single flight. This is consistent with leafhoppers colonising the maize field. Insects got caught on sticky traps with their wings spread and could easily be recognised based on this. Toward the end of the season, the predominant mode of locomotion was jumping over short distances. Single maize leafhoppers could be observed landing on the sticky traps with their tarsi first, walking around on the glued surfaces and then jumping off again. Therefore, only very few insects were caught, although their density in the field was very high. Thus, a different behaviour of *Z. scutellaris* may be the cause for the differences between the two methods.

Beside the difference in reflecting the population density of *Z. scutellaris*, the two methods also caught different ratios of male to female insects. The percentage of males was lower in sweep nets than on the sticky traps. The cause of this is unclear, but may be related to a different locomotive behaviour as discussed previously. More

research would be needed to assess the possible reasons for the results, the above-mentioned observations and their influence on the number of individuals of insects caught with either method.

The two sampling methods were also conflictive in their detection of differences in the abundance of the maize leafhopper in the four maize varieties. With the sweep nets, significant differences were detected between DK315 and Benicia in five cases over the three years, and in three cases between MON88017 and the near-isogenic line. The sticky traps only showed one significant difference at one sampling date. Thus, the two methods are not consistent in the representation of field-populations of *Z. scutellaris* and of distribution patterns of this species in different maize cultivars.

In conclusion, both sweep net catches and sticky traps can be used to assess the presence and density of plant- and leafhoppers in maize, but only with certain limitations: with sweep netting, the development of the field population of *Z. scutellaris* can be monitored closely, while the sticky traps fail to detect the increase in density over the growing season. Instead, large numbers of *Z. scutellaris* are caught in early summer, when they are comparatively less abundant in the field based on observations and sweep net catches. For a reliable quantitation of leafhopper abundance that is consistent with observations in the field, the sweep netting should be used. For the monitoring of presence and absence of plant- and leafhoppers, the sticky traps are most valuable, since they can be used over long periods of time without great expenses and effort. Flight activity can be estimated based on caught numbers of leafhoppers, but the field densities of *Z. scutellaris* are underestimated. The methods may complement each other, but sticky traps should not be used exclusively. This is generally in line with an earlier report on these two methods (Rauschen et al., 2008a), but sharpens the view for possible methodological issues and thus leads to a more refined choice of field sampling techniques.

5.2 Communities of insects occurring on the male inflorescences and on maize cobs

The assemblages of arthropods on panicles and maize cobs are similar systematically: In both cases, thrips (Thysanoptera), aphids (Aphidae) and flower bugs (Anthocoridae) of the *Orius* genus are the most abundant groups. Panicle samples are characterized by the presence of other foliage-dwelling insects, like

plant bugs (Miridae), plant- and leafhoppers (Auchenorrhyncha) and spiders, while on maize cobs the small detritivorous beetle species *Cortinicara gibbosa* (Lathridiidae) is typical (Eckert et al., 2006).

Both communities are characterised by trophic connectivity: the herbivorous thrips (mainly from the Thripidae family) are preyed upon by *Orius* bugs, which take up Bt-protein in the process (Torres & Ruberson, 2007). The same holds true for the predatory thrips from the Aeolothripidae and Phlaeothripidae families. The presence of secondary prey, like the non-exposed aphids, might reduce the actual exposure of predators to the Bt-protein in the field. Also, for *Orius albidipennis* it has been shown that neither of Cry1Ac, Cry1Ab and Cry2Ab proteins have a toxic effect (González-Zamora et al., 2007). This is likely to be true for other Cry proteins and other Orius species as well, calling into question the ecotoxicological significance of the exposure. This can lead to a more streamlined problem formulation.

Nonetheless, the trophically linked assemblages are of general ecological interest and give the opportunity to study potential long-term Bt- and variety effects on closely tied arthropod communities within monitoring. In the following, therefore, the major findings for these arthropod groups are discussed.

For panicle samples, there were a number of statistically significant differences in the densities of certain arthropod groups between the four maize lines. These differences were inconsistent, however, and not found in all three years: In 2005 and 2007, there were more *Orius* bugs in ISO plots in comparison to plots planted to DK315. In the 2006 growing season, there were no statistically significant differences. There were differences in single years for Thripidae, Aeolothripidae, aphids and the mirid *T. caelestialium*. In all cases but the last, the differences were only between the conventional hybrids, including the near-isogenic line. The only statistically significant difference between MON88017 and the near-isogenic line was for *T. caelestialium* in the 2nd sampling during the 2006 season, with the density of this plant bug being higher in the transgenic line. In conclusion, there are variations in the densities of certain insect taxa between the conventional lines. Differences between selected pairs of these lines are greater in magnitude than differences between MON88017 and the near-isogenic line. The densities of non-target organisms observed in the transgenic line are always well within the range of those in the conventional maize lines. Thus, the panicle samples give no indication of a negative impact of the

transgenic maize line on the major arthropod groups occurring on the male inflorescences.

The timing of sampling was difficult, due to the maize lines having different flowering phenologies. DK315 and Benicia flowered earlier than MON88017 and DKC5143. Depending on the weather conditions in a given year, the first two maize lines started flowering between the end of July and the beginning of August, with the other two maize lines following approximately a week later. The time taken for completion of anthesis, from a few days up to 2 weeks, again largely depended on the weather during that period. This circumstance led to the panicles being sampled in an asynchronous way during the three years. Also in the last year it was decided to sample the panicles only once during the peak of anthesis, since the insects seemed to be more abundant when pollen and anthers were present in large quantities.

In conclusion, differences observed between DK315 and Benicia on the one hand, and MON88017 and its near-isogenic line on the other, can be based on the different attractiveness of the flowers to arthropods, but also on different environmental conditions during the time of sampling and on different population developments of the arthropods over time. This has to be taken into account when interpreting these data.

In cob samples, there were again no consistent differences between the four maize lines for most of the arthropod groups, except for aphids that were more abundant in MON88017 and the near-isogenic line during all three years. This difference can be interpreted as a cultivar effect. In only one case, the density of a non-target organism was reduced in MON88017 when compared to ISO: in 2007 there were fewer *C. gibbosa* on cobs from the transgenic maize. The density in MON88017 was still considerably higher, however, than in the conventional hybrid Benicia during the same year. Therefore, it appears questionable to assume an effect of the Cry3Bb1 protein. Differences of this kind may occur by chance, especially with species occurring in such low instances and densities.

The composition of the arthropod community on the maize cobs and the densities of some groups or species were similar to the data of Eckert et al (2006). This means that this community is rather stable in its overall make-up and that for the interpretation of the field data already published data can be fully taken into account and serve as a comparator.

There are, however, two factors that can be problematic with this sampling method: in years with high infestations with *Ustilago maydis* (Basidiomycota: Ustilaginales), the causal agent of corn smut, finding uninfected and normally developed cobs can be difficult. Sampling representatively then can be almost impossible, especially if different maize lines exhibit different degrees of susceptibility to infection or the infection occurs in a patchy distribution. Another confounding factor is the incidence of high aphid densities, as they occurred during the 2005 and 2007 growing seasons. Large numbers of aphids make the sampling of arthropods from the cobs and the sorting of specimen under the binocular microscope very labour intensive. Since aphids are not exposed to the Cry protein expressed in Bt-maize (Head et al., 2001; Raps et al., 2001; Dutton et al., 2004), this work is to little avail with respect to the environmental risk assessment and problem formulation in general. While cob samples are in general a good tool to assess arthropod communities in maize, these drawbacks have to be taken into account.

5.3 Non-target organism selection for Environmental Risk Assessment including problem formulation and General Surveillance

There is a multitude of criteria to define the relevance of specific organisms or groups of arthropods for Environmental Risk Assessment and General Surveillance (Romeis et al., 2008; Todd et al., 2008). For species closely related to the target organism of a given Bt-maize line, a risk assessment is reasonable since it can be assumed with certainty that the related non-target organism will be affected by the Bt-protein. The significance of that impact for the overall risk assessment, however, will largely depend on the importance of the species at risk, its ecological function and relevance, and its anthropocentric value. If, as for the chrysomelids in this study, their ecological relevance seems to be small based on their low densities in the field and their status as secondary pests, it could be decided that a risk of some magnitude can be acceptable.

Besides the close relatives of the target organism, species with high densities in the field, and thus probably high ecological relevance, are also potential candidates for risk assessment and monitoring. This is especially true, when these species are exposed to high levels of the transgenic protein due to their mode of feeding. They might be directly affected by the toxic action of these proteins, although this is highly

unlikely, which might in turn cause effects on higher trophic levels based on reduced prey quality or abundance (Dutton et al., 2002; Meissle et al., 2005, Romeis et al, 2004). They will also directly carry the transgenic protein over to higher trophic levels, potentially causing toxic effects there (Groot & Dicke, 2002; Meissle et al., 2005; Obrist et al., 2005; 2006a, b; Torres & Ruberson, 2007; Romeis et al., 2006). Not much is known about trophic links in the maize biocoenosis (Scholte & Dicke, 2005). High tier field release experiments can therefore yield more ecological data that are of high relevance for ERA and monitoring, since they can inform risk assessors and regulators about what species are of long-term relevance in the environment receiving a transgenic crop.

Two species found at the field site were of particular interest. Both are herbivores occurring in high densities throughout the growing season and exposed to considerable amounts of the novel protein. Based on the abundance data gathered in this study, a toxic effect of Cry3Bb1 on these two species is not to be expected. However there is a lack of long-term data on possible hazards, so there some arguments for monitoring studies on these two key species as listed below.

Indicator potential of T. caelestialium

The rice leaf bug is a pest of small grains, including agronomically important crop plants, notably rice (Wheeler & Henry, 1985). In maize, it can be regarded as a minor secondary pest. The bug has a global distribution (Wheeler & Henry, 1992). It is exposed to considerable amounts of transgenic protein from the genetically modified Bt-maize, especially during the early stages of development (see also Obrist et al., 2006a). During the growing season, it reaches high densities in the field, evidence for its importance as prey for generalist predators in the maize biocoenosis (Árpás et al., 2005). With the sweep netting method employed in this work, it is possible to survey and sample the field population of *T. caelestialium* during the whole growing season of maize. Different life stages can be enumerated. The method yields robust data that can be statistically analysed.

Indicator potential of Z. scutellaris

The maize leafhopper is bound to maize as host plant and has a broad geographic distribution that covers central Europe (Nickel, 2003; Pons et al., 2005; Rauschen et al., 2004). It is exposed to considerable amounts of transgenic protein from the

genetically modified Bt-maize (Dutton et al., 2004; Obrist et al., 2006a). During the growing season, it reaches high densities in the field (Pons et al., 2005; Rauschen et al, 2008a), evidence for its importance as prey for generalist predators in the maize biocoenosis (Árpás et al., 2005). With the two sampling methods employed in this work, it is possible to survey and sample the field population of *Z. scutellaris* during the whole growing season of maize. The methods showed different patterns and trends, a fact that has to be minded when deciding upon the use of these methods at certain times of the growing season. Nonetheless, the data obtained are well suited for statistical analyses.

In conclusion, both the rice leaf bug and the maize leafhopper are interesting candidates as indicator organisms in the monitoring of the environmental impact of genetically modified Bt-maize.

5.4 The relevance of field experiments for Environmental Risk Assessment and monitoring approaches

The legislation of the European Community stipulates an Environmental Risk Assessment of genetically modified plants before and a monitoring of possible adverse environmental effects after these are placed on the market (EC, 2001).

The ERA is to be performed on a case-by-case and step-by-step basis. This means that each genetically modified plant event has to be assessed individually, taking into account assessments of already regulated plant events and other existing knowledge. The review of existing knowledge and the identification of areas of concern or uncertainty are done during Problem Formulation, in the first phase of the ERA (Romeis et al., 2008). Here, the familiarity and substantial equivalence of a GM crop are assessed and the further data needed for the approval are identified. This procedure streamlines the following ERA substantially. This is especially true with regard to the so-called “stacked” events that combine different transgenic traits in a single plant (EFSA, 2007; Schrijver et al., 2007). The decision for or against approval of such plants might not warrant much new information, if these are substantially equivalent to other already authorised GM plants (Romeis et al., 2008). The assessment of possible impacts is then performed in a tiered approach, beginning with experiments under controlled conditions in the laboratory and then proceeding, if

deemed necessary, to more complex and realistic scenarios under greenhouse, semi-field or field conditions (Romeis et al., 2008).

Monitoring comprises two separate and very different components, the Case Specific Monitoring and the General Surveillance (Sanvido et al., 2005). The former is mandated only as a follow-up of the ERA, while the latter has to be performed always as matter of the precautionary principle.

A number of clarifications, guidelines and proposals have been put forward to more clearly outline the methodological approaches for ERA, in terms of its scope, problem formulation and hypothesis testing, the organisms to be tested and the proceeding within the tiered assessment approach (EC, 2002; Andow & Hilbeck, 2004; Sanvido et al., 2005; EFSA 2006a, b; Raybould, 2006; Romeis et al., 2008; Todd et al., 2008). While currently a consensus on ERA emerges between different stakeholders (Romeis et al., 2008), there is still considerable need for discussion and regulation of monitoring approaches, especially for General Surveillance (Graef et al., 2005a, 2007; Züghart et al., 2007; Schmidt et al., 2007; Sanvido et al., 2008). The Case Specific Monitoring comes into effect when the overall risk assessment calls for certain issues associated with a genetically modified plant to be further investigated. This means that areas of uncertainty or potential risk have been identified after testing of clearly defined scientific hypothesis. In CSM, these uncertainties or risks are then specifically addressed. General Surveillance, on the other hand, is not based on any hypothesis on how an adverse effect is caused by the cultivation of genetically modified plants, or on any specific assumptions, mechanistic causes or specific risks associated with that cultivation. On the contrary, General Surveillance is a general overseeing of the biogeographical regions where GM plants are grown and is aimed at finding effects of the cultivation of GM plants or of changes of agricultural practices accompanying that cultivation on the human environment (Graef et al., 2005a; EFSA, 2006b). It should be linked to clearly defined environmental protection goals (Bartsch, 2008) and take all factors active in the receiving environment into account (Sweet, 2008). Moreover, it should complement and not replace general environmental monitoring conducted by Member States (Graef et al., 2005a; EFSA, 2006b). The European Safety Authority proposes as a definition of effect “an alteration that results in values that are outside the normal variation range given the constant change and flux of the agriculture, the agricultural practices, the rural environment and the associated biota in the European Union” (EFSA, 2006b). In

addition to that, EU Directive 2004/35/EC on environmental liability gives evidence for how “damage” might be defined, quantitated and its significance assessed (EC 2004).

The EFSA definition of “effect” premises deeper and wider knowledge of the natural variation within the agroecosystem. Baselines, threshold values, corridors and multiple comparators are necessary to draw scientifically sound conclusions on whether an observed value, e.g. the density of an herbivorous arthropod in maize, falls outside the normal range of the current agricultural practice and thus indicates the presence of an effect. There are, however, no detailed quantitative baselines available, especially not for different geographical scales (Graef et al., 2005a; Sanvido et al., 2007). Furthermore, the constant change of agricultural practice (e.g. crop rotations, ploughing and spraying techniques, structure of the arable land) and its impact on biodiversity (Matson et al., 1997; Cortet et al., 2002; Hails, 2002; Holland, 2004; Butler et al., 2007; Krogh et al., 2007), the deployment of new cultivars and possibly new crop species and even the possibility of climate change would necessitate the continuous updating of such baseline data (Schorling & Freier, 2006; Sanvido et al., 2007). The sheer amount of information needed to form such baselines and the need for harmonisation and standardisation of data gathered within different monitoring frameworks (Graef et al., 2007) casts the possibility that General Surveillance could be performed with low intensity (and thus low monetary input) on a large regional scale (see Graef et al., 2005b as an example for Brandenburg only) into doubt (Sanvido et al., 2007).

The work on hand shows that forming baseline corridors can be difficult. For all sampling methods, there were large between-year differences in the abundances of the caught arthropods. This led in some cases to a given species or even family not being observed altogether, especially when that species usually occurred with only small densities. In the case of the major arthropod taxa that occurred in high densities throughout the growing season, differences in population densities by factors in excess of 30 were observed between years. These differences make between-year comparisons very difficult and decrease their explanatory power. Because of these large natural variations it seems problematic to define sensible thresholds, baselines or corridors for what some call the “good ecological state” (Schorling & Freier, 2006) or “favourable conservation status” (Bartsch, 2008). Based on the presented data, such baselines would have large ranges, calling into question

their usefulness in evaluating the possible (small) impacts of genetically modified plants on non-target organism field densities. The matter is even more complicated by the fact that, as shown for the rice leaf bug *T. caelestialium*, an arthropod species might exhibit different densities already in different conventional maize lines.

Until now, most field experiments have focussed solely on one or two genetically modified crop line/s and its or their near-isogenic counterpart/s (Bourguet et al., 2002; Dively & Rose, 2003; Lumbierres et al., 2004; Árpás et al., 2005; Bitzer et al., 2005; Pilcher et al., 2005; Pons et al., 2005; Poza et al., 2005; Ahmad et al., 2006; Daly & Buntin, 2006; Schorling & Freier, 2006), sometimes combined with different conventional insecticide treatments (Al-Deeb et al., 2003; Candolfi et al., 2004; Bitzer et al., 2005; Dively, 2005; McManus et al., 2005; Eckert et al., 2006; Ludy & Lang, 2006; Floate et al., 2007; Rose & Dively, 2007; Sharma et al., 2007; Toschki et al., 2007; Rauschen et al., 2008a) (see Marvier et al., 2007 for a review of 42 field studies on Bt-maize and Bt-cotton and their impact on non-target arthropods). Conventional crop lines have only recently been taken into consideration in field and laboratory experiments to assess baseline data on the natural variability between different varieties (Wandeler et al., 2002; Cortet et al., 2007; Griffiths et al., 2007; Rauschen et al., 2007). Developing a sensible baseline corridor or reflecting a “good ecological state” for an arthropod species or group that shows different field densities in different maize lines would require assessing the occurrence of that taxon in multiple maize lines over many consecutive years, using the same method and sampling schemes in very different local environments. This work would have to be done by specialists or at least specially trained personnel. It remains doubtful, whether the stakeholders in the regulation and monitoring of genetically modified plants would be willing to impose or finance such measures over long periods of time. Generally, it is proposed to enhance or widen the scope of already existing national environmental monitoring networks, integrating them to also cover the General Surveillance of genetically modified crops. In Germany, this could apply to the Ecological Area Sampling (EAS, Middelhoff et al., 2006) combined with other efforts to survey and monitor certain arthropod or floral groups, and even farm questionnaires (Schmidt et al., 2007). This is thought to meet the requirements laid down in the EC regulations and also be efficient in terms of resources spent in relation to the amount and scientific reliability and robustness of the data gathered.

Whether these efforts will succeed in reaching their aims and whether they are actually suitable for their purposes in the first place, remains arguable.

In summary, there are currently the following main problem areas in the implementation of schemes for the monitoring of genetically modified plants: 1) the lack of agreed protection goals and hence uncertainty about what to monitor in the receiving environment; 2) the lack of quantitative baseline data that allow the comprehensive ecological assessment of potential deviances from the natural variability and the differentiation between GM-related and other effects. On the other hand, there are qualitative baseline data available in terms of farmers' experience concerning their land use or of conservation specialists regarding endangered and red list species; 3) the lack of an uniform, EU-wide framework in terms of methodological and analytical standards, choice of monitoring sites, data collating and reporting agencies, defined thresholds that indicate when action has to be taken to prevent (further) damage. Considerable time and effort will be needed to reach agreements that will be acceptable to all stakeholders involved and also serve the purpose of protecting the environment from unintended harm.

6. Summary and Outlook

"It's tough to make predictions, especially about the future."
Lawrence Peter "Yogi" Berra

The ecological impact of the cultivation of Bt-maize MON88017 was studied in a field-release experiment during three study years. This genetically modified maize line has an insect pest resistance against the Western corn rootworm *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) which has been recently introduced to Europe, as well as tolerance against the herbicidal agent glyphosate. The arthropod communities in the herb layer, on maize panicles and on maize cobs were assessed with regard to their species composition, diversity and density based on the results from different field sampling methods. Multiple pair-wise comparisons were made between Bt-maize MON88017, its near-isogenic line and the two conventional maize lines DK315 and Benicia, to range potential differences in the overall natural variability existing between maize lines with different characteristics. Non-target organisms suitable for Environmental Risk Assessment including problem formulation and the monitoring of adverse effects of the cultivation of genetically modified maize were identified. The methods employed were evaluated in their applicability for monitoring unanticipated long-term effects.

The arthropod communities investigated were systematically and trophically highly diverse. Two herbivorous arthropod species were identified in the herb-layer community that occurred in consistently high densities throughout the three consecutive growing seasons. These were the maize leafhopper *Zyginidia scutellaris* (Herrich-Schäffer) (Auchenorrhyncha: Cicadellidae) and the rice leaf bug *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae). Both are exposed to considerable amounts of Cry3Bb1 from MON88017 as shown by the results of ELISA tests. Direct relatives of the target organisms, i.e. members of the Chrysomelidae family (the leaf beetles), were detected inconsistently and in overall very low densities. Their role in the maize agroecosystem, at least in central Europe, appears to be limited. Therefore, it appears that the cultivation of MON88017 will likely pose a negligible risk to non-target leaf beetles.

The herb-layer community in general featured the most taxa, while the communities on maize panicles and cobs were far more restricted in their composition. In the latter two assemblages, herbivorous and predatory thrips (Thysanoptera), flower bugs (Heteroptera: Anthocoridae) and aphids (Homoptera: Aphidae) were the most prevalent arthropods. Of these groups, thrips and flower bugs are of special interest,

since both are exposed to proteins from genetically modified maize and also serve important biological (control) functions.

The statistical analyses of the data gathered on the different communities yielded no evidence for a negative impact of MON88017 on any of the investigated non-target taxa. The densities of selected arthropods in the genetically modified and the near-isogenic line were similar and fell within the range of the variability and differences between the two conventional maize varieties. Differences between the two conventional maize lines were always more pronounced than those between MON88107 and its near-isogenic varieties.

For *T. caelestialium* significant and consistent differences in the densities could be detected between the two conventional varieties. This proves that a non-target organism might be differently affected by conventional maize varieties already. Such effects necessitate the consideration of multiple comparators in Environmental Risk Assessment and ecological field-release experiments to assess the biological relevance of these findings. The natural variability in the densities of non-target organisms in different conventional maize varieties needs to be assessed to draw scientifically sound conclusions on possible differences between transgenic varieties and their near-isogenic parents. The experimental setup and methods utilised in this work were suitable for detecting and also sensitive enough to quantitate such differences. In conclusion, MON88017 and its near-isogenic varieties can be regarded as equivalent for the investigated non-target organisms, while this is not totally true for the two conventional varieties DK315 and Benicia.

The two arthropod species *Z. scutellaris* and *T. caelestialium* are interesting candidates for monitoring of long-term effects and could also be used in future Environmental Risk Assessment approaches regarding other Cry-proteins. They are important members of the maize biocoenosis, based on their high densities and their function as herbivores/primary consumers, and are therefore reasonable surrogate species for this guild and their respective taxonomic groups. They are exposed to considerable amounts of the Bt-proteins from genetically modified maize and could therefore be directly affected themselves or pass the entomotoxic activity of these proteins over to higher trophic levels. Ecotoxicological baseline data on potential effects of new Bt-proteins on these species are lacking, however.

The four different methods employed, transect-wise catching with a sweep net, sticky traps, panicle and maize cob samples can all be used to assess potential impacts of

genetically modified maize on the arthropod communities. For the herb-layer, a combination of sweep net catches and sticky traps is recommended, since these two methods reflect certain parts of the community differently. Both methods are cost and time efficient. Sampling panicles and maize cobs on the other hand both have some serious limitations under certain circumstances, which call into question their general feasibility for longer term monitoring. For medium-term studies during field-release experiments, these methods can yield valuable data, however.

Field experiments with an ecological scope such as the one on hand are of considerable importance. They can help to refine and inform Environmental Risk Assessment approaches including problem formulation by providing fundamental ecological data on the occurrence of non-target organisms in different geographic regions and cropping systems, and on the trophic interconnectedness of the arthropod communities on the cultivated land itself, on off-crop areas and between these two. Moreover, they alone are able to confirm or refute conclusions made during Environmental Risk Assessment on a larger scale and under realistic environmental conditions. In ERA, field-release experiments are the highest tier and only necessary when previous tiers have shown evidence for potential negative impacts that warrant a further, more complex step of the assessment process. From an ecological standpoint, field-release experiments are important to continuously refine assessment approaches, since these can only be as good as the current state of scientific knowledge and methodology. Field experiments are needed to further develop and optimise methods and to gather new ecological data that paint a clearer picture of the complex and changing interactions of the living and non-living environment into which genetically modified plants are and will be released.
Environmental Risk Assessment is stipulated as a case-by-case, step-by-step, hypothesis driven scientific process that is based on clearly defined risk hypothesis for selected assessment endpoints. Hypothesis are tested under suitable conditions with experimental setups sensitive enough to detect effects transgressing previously defined thresholds or being in excess of natural variation assessed concurrently during the experiment, taking duly into account already existing knowledge. Higher tier tests within the assessment process are called for when lower tiers show considerable risks that warrant further research or when unacceptable uncertainties remain. This ensures that sufficient work is done to comprehensively assess potential

risks and at the same time prevents unnecessary or duplicate work being done. After commercialisation of a genetically modified crop, a Case Specific monitoring is implemented only if potential risks to the environment have been identified in the preceding ERA and some degree of uncertainty remains. A General Surveillance of the environmental impact of the cultivation of genetically modified crops has to be carried out in all cases. A focussing of efforts on specific goals will allow a cost-effective implementation.

While there is growing consent among various stakeholders on how ERA is to be performed, there is still no full agreement on the scope and implementation of General Surveillance. It is demanded to be general for all GMO grown and to be hypothesis-driven, but at the same time aimed to detect effects not foreseen in the preceding ERA, that probably are very hard to nearly impossible to detect and which in the end have to be traced back to a certain GM crop. Also, it should take advantage of existing environmental monitoring schemes and networks so as not to cause too much costs. These existing monitoring efforts are in part very general and not necessarily appropriate for the assessment of effects associated with the cultivation of a certain genetically modified crop, of effects brought about by the cultivation of such crops in general and changes in the agricultural practices accompanying that cultivation but unrelated to the crops themselves. Although some more detailed and specific recommendations on the implementation of General Surveillance approaches have been put forward, there is still a considerable need for legislation, regulation and developing an agreement between all stakeholders involved, especially the general public that has major concerns and growing reservations against the use of genetically modified crops for food and feed.

Public sector research can help steering the discussion on genetically modified plants into calmer waters. Data gathered by experienced (public sector) scientists and advertised to the general public in an understandable and accessible manner can improve public perception of the usefulness, environmental compatibility and sustainability of current and future GM plants. Few other technologies hold so much promise, but are met with so much fear, uncertainty and doubt. It is the responsibility of the stakeholders from the public sector (i.e. public sector scientists, risk assessors and regulators, institutions) to inform the general public comprehensively on the potential risks, but also on the advantages and chances, and thus the risks of not adopting this technology. Confidence in the motivations and trust in the

independence of these stakeholders is a prerequisite for achieving that goal. Trust can only be built on communication, by all out addressing reservations and perceived risks, by treating the opponents of this technology with understanding and respect, by taking their concerns seriously.

This all requires 1) the independent, continuous and ample funding of public sector research, 2) the possibility to conduct biosafety related research, especially field-release experiments over prolonged periods of time, with a reasonable scope, and without undue legal restrictions and unhindered by active opponents of GM technology and 3) the clear and comprehensible communication of scientific results to the general public.

Ecological field experiments are more tangible and thus probably more persuasive than laboratory experiments. And the acceptability of scientific results to the general public should be taken into account in an area as contested as plant biotechnology.

7. Appendices

I. References

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II. List of abbreviations

AG – stock corporation
AIC – Akaike information criterion
BBA – Federal Biological Research Center (Biologische Bundesanstalt), now Julius Kühn-Institute
BMBF – Federal Ministry for Education and Research (Bundesministerium für Bildung und Forschung)
Bt – Bacillus thuringiensis
BT – denoting the Bt-variety MON88017
BVL – Federal Office for Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit)
C – carbon content
CA – California
CD-ROM – compact disc read-only memory
CEC – cation exchange capacity
Co. – Company
CP4 – *Agrobacterium* strain CP4
CSM – case-specific monitoring
CW – calendar week
DAS-ELISA – double antibody sandwich enzyme linked immunosorbent assay
DIN A4 – paper size according to german industry norm A4 (Deutsche Industrienorm A4)
EAS – ecological area sampling
EC – European Commission
EFSA – European Food Safety Authority
EPPO – European and Mediterranean Plant Protection Organization
EPSPS – 5-Enol-pyruvylshikimate-3-phosphate synthase
ERA – Environmental Risk Assessment
EU – European Union
GLM – generalized linear model
GmbH – limited company (Gesellschaft mit beschränkter Haftung)
GM – genetically modified
GMO – genetically modified organism
IN - Indiana
Inc. – Incorporated
ISO – denoting the near-isogenic line of MON88017 (DKC5143)
LFL – State Research Center for Agriculture (Landesanstalt für Landwirtschaft)
MI – Michigan
N – nitrogen content
N_{min} – mineralized nitrogen
NTO – non-target organism
URL – uniform resource locator
USA – United States of America
USDA-APHIS – United States Department of Agriculture, Animal and Plant Health Inspection Service
WCR – Western corn rootworm

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8. Raw data

The reviewer's copies of this work contain CD-ROMs with the raw data stored as the orginal .XLS files. The online .PDF version of this work does not contain raw data. They can be obtained however, from the author. Current email adress is:

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