

EcoStrat GmbH



**Ecological Technology Assessment &
Environmental Consulting**

**REVIEW ON NON-TARGET ORGANISMS
AND BT-PLANTS**

Report to Greenpeace International, Amsterdam

April 2000

Published by: *EcoStrat GmbH*
Ecological Technology Assessment & Environmental Consulting
Feldblumenstrasse 10
CH-8048 Zurich, Switzerland

Authors: *Angelika Hilbeck*
Matthias S. Meier
Andrea Raps

CONTENT

Preface	1
Summary	3
1. Selectivity and mode of action of delta-endotoxins of <i>Bacillus thuringiensis</i> (Bt) var. <i>kurstaki</i> (Cry1 class) in microbially-produced insecticides and transgenic plants	10
2. Ecological availability and risk assessment.....	12
3. Laboratory studies	13
3.1. General comments on ecotoxicological studies	13
3.2 Comparison of insecticides versus transgenic Bt-plants.....	15
3.3. Unpublished studies	16
3.4. Published studies	25
4. Field studies	49
4.1. General comments on field ecological studies.....	49
4.2. Unpublished studies	51
4.3 Published studies	55
5. References	76

Preface

This is an independent report prepared by EcoStrat GmbH for Greenpeace International. The report was commissioned by Greenpeace International but EcoStrat GmbH is the sole author and Greenpeace International did not place any constraints on the substance of EcoStrat's analysis or recommendations. EcoStrat does not necessarily endorse or agree with Greenpeace's full position and recommendations on this issue.

We are very grateful to the following 8 scientists who reviewed all or parts of the report. The reviewers are listed in alphabetical order: David Andow (University of Minnesota, USA), Mike Cohen (International Rice Research Institute IRRI), Fred Gould (North Carolina State University, USA), Mike Hanson (Consumer Union, USA), Stefan Vidal (University of Göttingen, Germany), Jürg Zettel (University of Berne, Switzerland), and 2 scientists requesting anonymity.

The objective of the report was to review and analyze the methodologies used, results obtained and conclusions drawn from studies dealing with the ecological safety testing of transgenic plants expressing δ -endotoxins of *Bacillus thuringiensis*. EcoStrat GmbH wants to point out that this is not a 'risk' analysis since that would have to be done on a broader basis, including a comparison of costs and benefits of various other available options for pest management (including synthetic pesticides, mechanical and biological control), socio-economical and cultural considerations.

The initial basis for these analyses was a compilation of studies put together by Novartis. However, this report was extended to include similar studies dealing with the same issues but in other transgenic Bt-crops. Our report thereby does not address particular Bt-plants developed by a particular company but addresses all Bt-plants regardless of the companies involved in their development. The studies included both laboratory and field trials. All studies were divided into 'unpublished' studies, mainly but not exclusively carried out by industry for regulatory purposes demonstrating ecotoxicological safety for commercial registration, and 'published' studies mostly carried out by independent, public sector scientists, sometimes with industry sponsorship. Aside from the studies conducted for regulatory purposes on non-predaceous species, this report focuses on studies evaluating side effects on natural enemies. Further, no toxicological tests with birds, rodents or other small mammals were analyzed.

In the following table the reviewed studies are listed.

Table 1: List of reviewed studies

No.	Study	Page
1	48-hour static renewal toxicity of pollen from modified maize to water fleas (<i>Daphnia magna</i>)	15
2	Single dose test evaluating toxicity to earthworms (<i>Eisenia foetida</i>) using CryIAb enriched maize leaf protein	17
3	Effect of Bt maize pollen on larval honeybee (<i>Apis mellifera</i> L.) development	19
4	Effect of Bt maize pollen on larval honeybee (<i>Apis mellifera</i> L.) development	21
5	28-day survival and reproduction study in collembola (<i>Folsomia candida</i>) using CryIAb-enriched maize leaf protein	22
6	Pilcher, C.D.; Obrycki, J.J.; Rice, M.E.; Lewis, L.C. 1997. Preimaginal development, survival and field abundance of insect predators on transgenic <i>Bacillus thuringiensis</i> corn. <i>Environmental Entomology</i> 26(2): 446-454.	24
7	G.C. Lozzia, C. Furlanis, B. Manachini, I.E. Rigamonti. 1998. Effects of Bt corn on <i>Rhopalosiphum padi</i> L. (Rhynchota Aphididae) and on its predator <i>Chrysoperla carnea</i> Stephen (Neuroptera Chrysopidae). <i>Boll. Zool. Agr. Bachic. Ser. II</i> , 30(2): 153-164.	30
8	Hilbeck, A., Baumgartner, M., Fried, P.M., Bigler, F. 1998. Effects of transgenic <i>Bacillus thuringiensis</i> corn-fed prey on mortality and development time of immature <i>Chrysoperla carnea</i> (Neuroptera: Chrysopidae). <i>Environmental Entomology</i> 27(2): 480-487.	32
9	Hilbeck, A., Moar, W.J.; Pusztai-Carey, M.; Filippini, A.; Bigler, F. 1998. Toxicity of <i>Bacillus thuringiensis</i> CryIAb toxin to the predator <i>Chrysoperla carnea</i> (Neuroptera: Chrysopidae). <i>Environmental Entomology</i> 27 (5): 1255-1263.	34

10	Hilbeck, A., Moar, W.J.; Pusztai-Carey, M.; Filippini, A.; Bigler, F. 1999: Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator <i>Chrysoperla carnea</i> . <i>Entomologia Experimentalis et Applicata</i> 91: 305 – 316.	36
11	Sims SR 1995. <i>Bacillus thuringiensis</i> var. <i>kurstaki</i> (CryIA(c)) protein expressed in transgenic cotton: effects on beneficial and other non-target insects. <i>Southwestern Entomologist</i> 20(4): 493-500.	38
12	Dogan EB, Berry RE, Reed GL, Rossignol PA 1996. Biological parameters of convergent lady beetle (Coleoptera: Coccinellidae) feeding on aphids (Homoptera: Aphididae) on transgenic potato. <i>Journal of Economic Entomology</i> 89(5): 1105-1108.	41
13	Riddick EW & Barbosa P 1998. Impact of Cry3A-intoxicated <i>Leptinotarsa decemlineata</i> (Coleoptera: Chrysomelidae) and pollen on consumption, development, and fecundity of <i>Coleomegilla maculata</i> (Coleoptera: Coccinellidae). <i>Annals of the Entomological Society of America</i> 91 (3): 303-307.	43
14	Schuler TH, Potting RPJ, Denholm I, Poppy GM 1999. Parasitoid behavior and Bt-plants. <i>Nature</i> 400: 825.	46
15	Impact of transgenic maize expressing truncated CryIAb protein on several non-target insect populations: Diptera, Hymenoptera, and Coleoptera (Coccinellid family) as well as Homopterans:	50
16	Effects of CryIAb protein on several insect populations: Diptera, Hymenoptera, Coleoptera and Lepidoptera.	52
17	Candolfi M. et al. in prep. Effects of Bt-maize on non-target arthropods under field conditions.	53
18	Pilcher, C.D.; Obrycki, J.J.; Rice, M.E.; Lewis, L.C. 1997. Preimaginal development, survival and field abundance of insect predators on transgenic <i>Bacillus thuringiensis</i> corn. <i>Environmental Entomology</i> 26(2): 446-454.	54
19	Lozzia, G.C. and I.E. Rigamonti. 1998. Title: Prime osservazioni sull'artropodofauna presente in campi di mais transgenico. <i>ATTI Giornate Fitopatologiche</i> : 223 – 228.	56
20	Lozzia, G.C.; Rigamonti, I.E. and Agosti, M. 1998. Metodi di Valutazione degli Effetti di Mais Transgenico sugli Artropodi Non Bersaglio. <i>Notiziario sulla Protezione delle piante</i> 8: 27-39. (in Italian).	56
21	Lozzia, G.C. and I.E. Rigamonti 1998. Preliminary study on the effects of transgenic maize on non target species. <i>IOBC Bulletin</i> Vol. 21 (8): 171 – 180.	56
22	Orr, D.B. and Landis, D.L. 1997. Oviposition of European Corn Borer (Lepidoptera: Pyralidae) and impact of natural enemy populations in transgenic versus isogenic corn. <i>Journal of Economic Entomology</i> 90 (4): 905-909.	60
23	Hoffmann MP, Zalom FG, Wilson LT, Smilanick JM, Malyj LD, Kiser J, Hilder VA, Barnes WM 1992. Field evaluation of transgenic tobacco containing genes encoding <i>Bacillus thuringiensis</i> δ -endotoxin or cowpea trypsin inhibitor: efficacy against <i>Helicoverpa zea</i> (Lepidoptera: Noctuidae). <i>Journal of Economic Entomology</i> 85 (6): 2516-2522.	62
24	Wilson FD, Flint HM, Deaton WR, Fischhoff DA, Perlak FJ, Armstrong TA, Fuchs RL, Berberich SA, Parks NJ, Stapp BR 1992. Resistance of cotton lines containing a <i>Bacillus thuringiensis</i> toxin to pink bollworm (Lepidoptera: Gelechiidae) and other insects. <i>Journal of Economic Entomology</i> 85 (4): 1516-1521.	64
25	Fitt GP, Mares CL, Llewellyn DJ 1994. Field evaluation and potential ecological impact of transgenic cottons (<i>Gossypium hirsutum</i>) in Australia. <i>Biocontrol Science and Technology</i> 4: 535-548.	66
26	Hardee DD & Bryan WW 1997. Influence of <i>Bacillus thuringiensis</i> -transgenic and nectariless cotton on insect populations with emphasis on the tarnished plant bug (Heteroptera: Miridae). <i>Journal of Economic Entomology</i> 90 (2): 663-668.	68
27	Riddick EW, Dively G, Barbosa P 1998. Effect of a seed-mix deployment of Cry3A-transgenic and non-transgenic potato on the abundance of <i>Lebia grandis</i> (Coleoptera: Carabidae) and <i>Coleomegilla maculata</i> (Coleoptera: Coccinellidae). <i>Annals of the Entomological Society of America</i> 91 (5): 647-653.	70
28	Lozzia GC 1999. Biodiversity and structure of ground beetle assemblages (Coleoptera Carabidae) in Bt corn and its effects on target insects. <i>Boll. Zool. Agr. Bachic. Ser. II</i> , 31 (1): 37-58.	72

Summary

Laboratory studies

A total of 14 laboratory studies were evaluated. Of those 14 studies, 5 reported on unpublished, ecotoxicological tests carried out by industry mainly for regulatory purposes (in this case by Novartis, however, other companies submit very similar documentation on ecotoxicological safety) (studies 1, 2, 3, 4 and 5). One of these 5 studies is still on-going (study 4) and could therefore not be evaluated. The other 9 studies were all published in peer-reviewed journals (studies 6-14). Some of the data presented in one or two of these 9 published studies may also have been submitted as voluntary 'supplemental' documentation for regulatory purposes.

Application packages submitted for commercial approval of transgenic plants must also contain documentation on environmental safety assessment of transgenic plants. For such assessments, laboratory studies are conducted following standard ecotoxicological guidelines for synthetic pesticides (see 3.1.) testing for acute effects caused by the insecticidal protein genetically engineered into crop plants. These testing procedures are designed for pesticides and their mode of release – e.g. foliar application potentially causing drift into neighboring habitats, presence for a limited time period due to degradation, etc. – but these tests alone are insufficient for assessing ecological effects of transgenic, insecticidal plants on nontarget organisms. By making a plant produce an extra and novel protein (e.g. Bt), important characteristics of Bt's mode of release are changed: the temporal and spatial presence of the insecticidal toxin is extended due to constitutive expression and, thereby, the mode of exposure to herbivorous insects and, consequently, to predators and parasitoids is altered. Furthermore, the altered plant's metabolism (by virtue of producing a novel protein) may in itself affect interactions between the plants, their herbivores/pathogens and organisms of higher trophic levels. Therefore, it is recommended to adapt and modify the testing procedures for transgenic plants by including chronic lethal and sublethal toxicity parameters in addition to those testing for acute toxicity only.

Regulatory studies (unpublished). In three of the five ecotoxicological trials, 20 - 40 individuals were used per treatment always split among 2-4 within-experiment replications each consisting of 10 individuals per replication. In one of the two honeybee trials, 25 bee larvae were used per treatment and replication (4 within-experiment replications). This trial was repeated once over time but the experimental settings were different in the two repetitions. Small beehives with unknown numbers of individuals were used for the on-going trials. No information was provided for the on-going honeybee trial. Duration of the trials and exposure time to the toxin was variable. Duration of experiments with *Daphnia magna* (water fleas) (study 1) lasted 48 hours, with *Eisenia foetida* (earthworm or compost worm) (study 2) 14 days and with *Folsomia candida* (springtails) (study 5) 28 days. The test organisms were exposed to the toxin for the entire duration of the experiment. In contrast, exposure time of the honeybees was only 45 minutes and no information on total duration of the experiment was provided. The experiment ran until adults emerged. No information is provided regarding experiment duration for the on-going honeybee trial.

In the experiments, no fresh or dried plant foliage was tested but only pollen and a lyophilized corn-leaf protein preparation (a powder) was used as dietary test material. *E. foetida* was added to a mixture of soil and lyophilized leaf-protein powder, although, this species hardly eats soil and prefers decaying plant material. It needs to be demonstrated whether *E. foetida* actually ingested any Bt toxin when searching for food in the soil/powder mixture. *F. candida* feeds on saprophytic fungi growing on decaying plant material but was also offered lyophilized leaf-protein powder mixed into soil. In acknowledgment of the feeding preference of this species also

yeast was added. But it is unclear whether *F. candida* actually ingested any of the leaf-protein powder when ingesting the yeast cells. This was the only chronic exposure trial, and some adverse effects were observed suggesting some uptake of the toxin. *Apis mellifera* (honeybee) larvae were provided a mixture of pollen and water for 45 minutes and then returned to the hive. But, in particular, young larval bees cannot digest pure pollen. Worker bees need to pre-digest pollen for them. The degree to which older larval bees can digest pollen is still unclear. But generally, older insect larvae are typically less sensitive to Bt-toxins. The second, still on-going honeybee study accounts for this uncertainty and provides pollen via worker bees. Finally, pollen was added to water for testing toxicity to *D. magna*. Pollen does not or only insufficiently dissolve in water, therefore, it is unclear whether *D. magna* encountered toxins in such experiments. In addition, it is unclear whether the pollen particles offered were of appropriate size to be ingested by *D. magna*. This species usually digests particles in the size range of 1-5 μm in diameter. Corn pollen sizes range at around 70 μm . No information was provided whether pollen was cracked prior to the experiment.

In conclusion, except for the trial with *F. candida* and the on-going study with *A. mellifera*, all studies tested for acute toxicity, i.e. test animals were exposed to the Bt-protein only once for a relatively short time. No effects were observed. Adverse effects were noted in the chronic exposure trial using *F. candida* although suitability of the used dietary system was uncertain. None of the ecotoxicological tests considered multitrophic interactions between plants, herbivores or pathogens and natural enemies. Except perhaps for the on-going bee trial (only limited information available), for all dietary exposure systems reliable uptake of the Bt toxin into the gut of the test organisms (where the toxin is effective) must still be verified.

Published laboratory trials. A total of 9 published laboratory studies were reviewed. While numbers of individuals used for the trials varied (12-90), reasonable numbers were used for most studies with regard to the objectives. Three studies were not repeated over time (6, 11 and 13), one study was carried out twice (study 7), one was repeated 3 times (study 12), three studies were repeated 4 and 5 times over time (studies 8, 9, 10), and one was a behavioral study (study 14). Most studies were carried out for the entire immature life stage (studies 6-10 and 12). In two studies, experimental times were 7-11 days (study 11) and 20-21 days (study 13). In study 14, the behavior of parasitoids was observed for a 4-hour period. Of the 9 studies analyzed, in 6 studies tri-trophic systems were investigated (studies 7, 8, 10, 12, 13, 14), and in 3 studies (6, 9 and 11) direct feeding trials (bi-trophic) were carried out with the natural enemies. The parameters measured were predominantly developmental times and mortality of the natural enemy species (studies 6-10). Study 11 examined mortality and abnormal behavior. In study 12, several parameters were measured, developmental times, prey consumption, pupal weight, fecundity and longevity of the natural enemy adults. Also study 13 looked at consumption rates in addition to mortality and developmental times. Study 14 was particular in that it was the only study examining choice and no-choice behavior of parasitoids.

One of the three bi-trophic studies (6) tested transgenic and isogenic corn pollen using 3 different predator species. Pollen contains relatively low amounts of Bt toxin relative to other plant parts. Predators feed on pollen to supplement their carnivorous diet often for ovary maturation, hence as adults. Except for *Coleomegilla maculata*, the natural enemy larvae investigated in the analyzed study could not complete their immature development on pollen only. This is reflected in the fairly high control mortalities and the fact that occasionally some prey items had to be provided. Moreover, larvae of one species tested, *Chrysoperla carnea* (green lacewing) are not known to feed on pollen. Even though pollen was provided for 24 hours only during each of the

three larval stages (for the rest of the duration of the larval stage lasting approximately 2 weeks untreated meal moth eggs were provided) high control mortality was observed for both the Bt-pollen treatment and the isogenic control (51%; study 6a). No differences in mortality and developmental times due to the Bt-pollen were observed.

A second bi-trophic study (11) was primarily conducted according standard procedures for ecotoxicological testing of pesticides. Microbially produced Bt toxin was used and fed to honeybee larvae or adults, adult ladybeetles (*Hippodamia convergens*) or parasitoid adults either mixed into a honey solution or water. Since larval stages are typically most sensitive to Bt toxins, adults would not be expected to readily exhibit any effects. Further, *C. carnea* larvae were given meal moth eggs that had been coated with a Bt-solution. The forceps-like mouthparts of *C. carnea* larvae allow them only to suck out the liquefied contents of eggs or prey bodies. Unless it can be demonstrated that Bt toxin can cross the egg shells of the meal moth eggs and is present in significant amounts inside the egg, *C. carnea* larvae most likely did not ingest any or only very little Bt toxin in that experiment. All experiments were terminated after 7 to 11 days and no significant effects were observed.

The third bi-trophic study (9) used a Bt-incorporated artificial diet specifically developed for *C. carnea* larvae. This diet consisted of small paraffin spheres containing a liquid diet used for mass rearing of *C. carnea* larvae for biological control purposes. Bt toxin was incorporated into the liquid and, therefore, could be ingested by the *C. carnea* larvae. Significant adverse effects were observed compared to the control.

Two tri-trophic studies used aphids as the prey species (studies 7 and 12). While both studies investigated ecologically very relevant predator-prey-plant relationships, they probably did not test Bt-containing prey and Bt-toxicity. New studies strongly suggest that no Bt-toxin is present in the phloem. Hence, no differences in mortality or developmental times were observed. The experimental set-up in both studies was not optimal and the statistical analyses were inconclusive.

Study 8 and 13 were the only studies testing prey-mediated effects of Bt-plants on natural enemies. In study 8, two different Bt-containing (because foliage-feeding) prey species were provided to the natural enemy *C. carnea* in a tri-trophic set-up. Significantly higher mortality, and, for one of the two Bt-treatments, also significantly prolonged developmental times were observed. Results of study 9 confirmed direct toxicity of Bt toxin to *C. carnea* larvae but mortality was not as high as would have been expected considering the higher toxin concentration used in the bi-trophic trials (see above and detailed information on page 34). Study 10 is a follow-up trial to both studies 8 and 9, further exploring Bt toxin effects on *C. carnea* larvae again in a tri-trophic set-up. Artificial diet as food for the prey larvae was used that contained different concentrations of Bt toxin. Again significantly increased mortality of *C. carnea* larvae was observed for all concentrations exceeding those of the bi-trophic trials. The three studies 8, 9 and 10 did not only reveal a direct effect of Bt but also revealed ecologically very relevant plant x herbivore x natural enemy interactions that all contributed to the observed increased mortality of *C. carnea* larvae mediated by Bt-corn fed prey that was not lethally affected by the Bt corn.

In study 13, Bt-potato fed Colorado potato beetle larvae were provided to *C. maculata* larvae. Despite the partly inappropriate experimental set-up and the questionable evaluation of the data (details see page 43), in our opinion, this study did reveal some adverse effects of Bt-containing prey on consumption rate and mortality of *C. maculata*. Further investigations are necessary to determine whether this was due to the intoxicated prey or the Bt-toxin directly.

Study 14 was the only study investigating parasitoid behavior and addressed important aspects regarding potential changes in host-location and host-acceptance behavior of parasitoids that can be of great ecological relevance in Bt crop fields.

Control mortalities in studies 6 to 10 were 31-56%, 5%, 37%, 17% and 30%, and 26%, respectively. In study 11, except for *Apis mellifera* larvae, all control mortalities exceeded 20% after 5 to 11 days of exposure, reaching 30 and 33% for two species. In study 13, control mortalities ranged from 58 up to even 92%, a control mortality that raises doubts regarding the experimental setup and should normally lead to termination and disregarding of the trial. Multiple repetitions of the entire experiment would have been advisable. In study 14, control mortality was 37% and 44%.

In conclusion, protocols for ecotoxicological testing of transgenic plants on nontarget organisms, including higher trophic level organisms, for regulatory purposes must be improved, verified and adapted to the novel and extended route of exposure caused by constitutive expression of novel toxins in transgenic plants. Testing protocols for chronic lethal and sublethal effects should be included in addition to acute toxicity testing. However, although such regulatory trials provide valuable initial toxicity information, basing ecological assessments solely on bi-trophic feeding trials that provide the insecticidal protein in a highly processed form directly to the nontarget organism is not sufficient. Ecologically important interactions between plants and herbivores and natural enemies/nontarget organisms – potentially acting synergistically – may be missed. We propose to develop dual testing protocols where the direct feeding trials are complemented by multitrophic feeding trials. Routes of exposure should be used that represent a ‘best’ simulation of a natural situation where the toxin on hand is actually entering the food chain, i.e. is delivered to the higher trophic organism. Further, the selection of test organisms should include ecologically relevant species and consider multitrophic interactions.

Summary of field studies

A total of 14 studies were evaluated in this report (studies 15-28). The studies were carried out between 1992 and 1999 in the USA, Australia, Italy and France. Of these 14 studies, for one study the publication status is unclear (study 15) and 2 studies are unpublished (16 and 17). Two more studies (studies 19 and 20) were published in Italian and it was unclear to us whether these journals were peer-reviewed or not. One study (21) was an IOBC Bulletin, which often is not peer reviewed. Eight studies are clearly published (18, 22-28) in peer-reviewed journals.

The information provided in the two unpublished studies (16 and 17) regarding experimental setup (sizes of field plots and fields, cultivation practices, evaluation of data, sampling methods) is rudimentary and no data on arthropod fauna is provided. Therefore, only a limited evaluation was possible. Three other studies (23, 24 and 26) were designed to investigate the efficacy of transgenic plants on target and nontarget pest species and data on natural enemy abundance was only collected incidentally. Three other studies (19-21) reported about the same 2-year (1996 and 1997) field trials in northern Italy and, therefore, were analyzed jointly. Study 28 (same authors as in study 19-21) again reports about the 1997 field data but because they provided additional information about a third year (1998) data collection and focused more in detail on a specific insect group (carabids), this study was analyzed separately.

Evaluation and limitations of methods used. In 3 studies (16, 17, 25) no information regarding plot/field size was provided. In 4 of the 14 studies (15, 18, 23, 24,) small plots sizes of less than 0.005 ha (3 x 7m, 3 x 15m, 12 plants in a row, 4 rows x 9m) were used. In 5 of the 14 analyzed field studies (19-21, 26, 28) reasonable field sizes of 0.5 ha to 2.5 ha per treatment were used.

Except for two studies (27, 28) that were repeated twice at the same location, all studies were carried out for 1 field season in each location (not repeated over time). Some studies reported multiple year investigations but closer analysis revealed that either the experiment was carried out at different sites or different varieties of Bt-crops were used in the two seasons. Repe-

tition over time is important to consider inter-seasonal influences. One-season investigations limit the conclusions that can be drawn regarding long-term effects.

Various sampling methods were used, including yellow sticky traps, pitfall traps and D-Vac suction sampling. In studies 19-21 and 28 (same study over 3 years, see detailed description page 56 and 72), also area-wide sampling techniques such as pheromone and malaise traps were used.

Sampling frequencies for direct, visual field sampling or D-Vac sampling varied strongly. In 5 field experiments (16, 18, 19-21, 22, 23), field sampling was carried out only 3 or 4 times during the entire field season. In all other studies, sampling frequency varied between 5 to 10 times during the field season.

In study 22, also parasitism rates of the target pest (ECB *O. nubilalis*) were studied in addition to the sampling numbers of predators. Parasitism was also examined in study 17 but data has not been published yet. Only in study 25, population development curves of natural enemies were provided. All other studies established inventory lists of the insects sampled and compared abundances of those for Bt-crop fields and isogenic crop fields.

Evaluation and limitations of provided data. Identification level of sampled insects in most of the studies (15, 16, 17, 19 (except Noctuidae data), 20-21, 23, 25, 26, 28 (except Carabidae data)) did not go beyond order or family level. Furthermore, it was not differentiated between insect life stages (larval and adult stages). Thereby, important ecological information is missed. This level of taxonomic identification was rather crude and limits the conclusions that can be drawn regarding risk assessment of transgenic plants. The order of Hymenoptera for example contains also honeybees, wild bees and bumblebee species. Detailed information on these is desirable because of their importance for pollination. Similarly, for example, the orders of Diptera or Coleoptera contain both pest and natural enemy species. Detailed information on these is desirable because of their importance for biological control. Further, differentiating between life stages is crucial since for several natural enemy species only the larval stages are predaceous while the adults feed on nectar or pollen (e.g. Syrphidae and Chrysopidae), for others both immature and adult stages are predaceous. Unfortunately, although appropriate data in a number of studies was sampled, no detailed information was provided on population dynamics, predator-prey/host relationships (synchronies, ratios, etc.) that are essential for learning about existing biological control mechanisms and their effectiveness. More detailed information is important for an assessment of the ecological 'quality' of an existing insect community and potential observed changes.

Only in a few studies (18, 22 and 27), selected predator species were studied in detail. In study 18, insects of the family Chrysopidae, of the species *C. maculata*, *Hippodamia convergens*, *O. insidiosus*, *Nabis* spp. were sampled. In study 22, similar predator species (*O. insidiosus*, *C. maculata*, *C. carnea*) were recorded all of which are important predators in cornfields. In study 27, sampling focused on two predator species of the Colorado potato beetle, one prey-specific (*Lebia grandis*) and one generalist predator (*C. maculata*). Restricting investigations on selected relevant species (incl. their trophic relationships) in one-year, small plot trials may provide more appropriate data likely to detect effects and enhance our understanding on what is actually going on in the field.

In several studies (19-21, 23, 26, 27, 28), data were pooled across sampling sites, sampling dates and/or locations. In particular for the 2-year trials in northern Italy (19-21 and 28), this was done in various combinations in the different publications. However, it is generally recommended that pooling across locations, sample sites and dates should only be done after prior testing for insignificant differences. Otherwise valuable information may be lost regarding particular location effects, sample site effects etc.

Most authors reported no observed differences in abundances of insects between transgenic Bt-crop field and non-transgenic crop fields. Comparing one-year insect inventory lists and abundances at the taxonomic order and family level pooled over sites/locations without considering trophic relationships will only reveal rather drastic ‘acute’ and ‘instant’ effects that often occur immediately after the application of insecticides. Therefore, it is hardly surprising that insecticide treatments used as positive controls in some studies did in fact yield significant effects in these studies (see ‘Field ecological comments’ for further discussion of this issue). In contrast to insecticide treatments, potential adverse effects of Bt-plants on most beneficial insects are expected to be more subtle and on a long-term scale. Even if effects like those observed in studies 8, 9 and 10 would translate identically to the field, population effects in the field would probably manifest themselves after many years. The reason is that the effects observed in studies 8, 9 and 10 accumulated under laboratory conditions over a time period of one month, i.e. one generation. Hence, long-term effects will likely be inter-generational effects occurring over several generations when considering the life history of these insects. Once a lacewing larvae has completed its immature life stage, the adult usually leaves the field and moves to other habitats first in search of food for itself, then for mating partners and then last but not least for oviposition sites. All this occurs over many weeks, in various habitats that offer the necessary requisites for the quite differing needs and may include overwintering. One year, small field plot studies are not likely to detect such inter-generational and inter-seasonal effects. But when interpreted cautiously and within the given limitations, they can provide valuable initial information that can be used as baseline data for continuous investigations of such kind or long-term monitoring.

In study 27, the significant decline of a specialized predator was reported (*Lebia grandis*) because its primary prey species, the target pest Colorado potato beetle, was eliminated due to the Bt toxin expressed in transgenic potatoes. In the same study, the authors reported about a significant increase in leafhopper adult and nymph abundance (*Empoasca fabae*) in both the pure Bt-potato fields and the mixed field consisting of both transgenic and non-transgenic potatoes. Both findings point to important expected implications of large scale production of transgenic insecticidal plants: decline in specialized natural enemies and emergence of secondary pests trying to exploit the available abundant food source after the target pest is eliminated. It further shows that insecticide use had to continue in transgenic potatoes although perhaps at a lower frequency and targeted against another pest species, and that the remaining natural enemies then cannot necessarily be expected to control remaining secondary pest species. Similar developments seem to occur also in cotton in some areas of the South in the United States. As published in a report by the Economic Research Service of the USDA in summer 1999, pesticide use in 1997 could be reduced against the target pests in most (but not all) areas but were always higher, in one area almost double, against all other pests (IOBC Global Working Group 1999 Newsletter). Furthermore, the decline of a specialized natural enemy may seem ecologically irrelevant for the research or commercial field on hand since the target pest is eliminated. However, long-term implications of an area-wide decline of such a natural enemy for other cropping systems or natural habitats may still be multifold and should not be dismissed easily.

In study 28, in both years at one location, carabid diversity was higher in isogenic than in transgenic corn fields while at another location carabid diversity was higher in one year and lower in the other. The authors did not discuss the inconsistency of their results. In our opinion, they demonstrate the necessity for multiple year monitoring if community level parameters are to be assessed reliably and before any conclusions regarding long-term effects on biodiversity can be drawn.

As commonly observed in field studies, abundances of individual species were often low and associated with large variability. This is because these types of data are confounded with species-specific intra-field movement, inter-field emigration and immigration and predation behavior. These phenomena pose increasingly bigger problems as the size of field plots decreases. In order to handle the large variances, sample sizes and replications must be large often pressing logistical limits. Large variability also severely constraints statistical power because effects of 10 or 20% are seldom statistically significant while they can be of great ecological significance. Therefore, changes in biodiversity (decline or increase) of species over time or inter-generational effects of species should be studied in multiple year experiments. Because it is acknowledged that such extensive trials may not always be feasible, cautious interpretation of one or two year trials is advisable and the establishment of area-wide monitoring programs is strongly recommended.

1. Selectivity and mode of action of delta-endotoxins of *Bacillus thuringiensis* (Bt) var. *kurstaki* (Cry1 class) in microbially-produced insecticides and transgenic plants

Bacillus thuringiensis is a gram-positive bacteria that produces large amounts of insecticidal delta-endotoxins during sporulation. In these bacteria, the endotoxins are present in a crystalline, biologically inactive form. Microbial Bt-insecticides targeting lepidopteran pests typically contain Bt-proteins of the Cry1 class. In addition to Cry1 δ -endotoxin crystals, spores produced by *Bacillus* during the fermentation process also are present in these insecticides (Feitelson et al. 1992). Other potential bioactive substances occur in many Bt-insecticides, such as VIPs (vegetative insecticidal proteins), spores, phospholipases, zwittermycin, carrier and formulation ingredients, all of which exert an insecticidal effect in concert. The insects ingest this potpourri of biochemicals. The Bt crystals must undergo a complex cascade of biochemical reactions before they become active against susceptible insects. Firstly, the crystals must be dissolved in an alkaline midgut milieu and the presence of certain enzymes to yield a biologically inactive protoxin of 130-135kDa in molecular weight. Secondly, again in a stepwise chain of reactions, a 65 kDa toxic fragment is proteolytically cleaved from the protoxin. This fragment, commonly referred to as the activated toxin, binds to receptors located on the epithelium of the insect midgut. The receptor-bound toxin then induces pore formation leading to lysis of the midgut, which results in the death of the insect. For induction of death of a susceptible insect, binding and pore formation are essential. However, all steps preceding pore formation are equally crucial for determining the specificity of the Bt-proteins in killing insects (Goldburg and Tjaden 1990) (Fig. 1). In transgenic plants, the truncated semi-activated protein is expressed and will be ingested by the herbivorous insects feeding on it. Consequently, no crystal solubilization and relatively little protoxin-toxin conversion are necessary within the insect gut. It is conceivable that insects lacking the appropriate midgut enzyme composition or pH milieu may well have appropriate receptors. In past laboratory trials, increased activity could be observed in herbivorous insect larvae when fed the activated toxin instead of the protoxin. For example, *S. exigua* is 2 times more susceptible to Cry1Ac and Cry1C toxins, respectively, than to their respective protoxins (Moar et al., 1990, 1995).

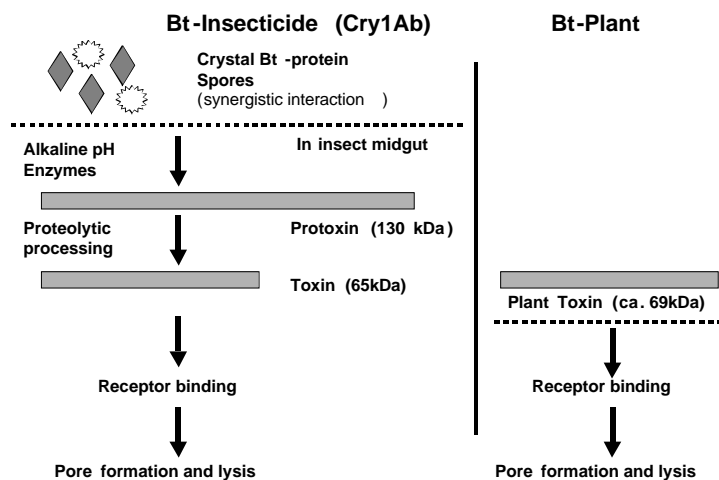


Figure 1. Differences Bt-insecticides and Bt-plants.

Limitations of our knowledge

There are still many unanswered questions regarding the mode of action of Bt-proteins, including the Cry1 toxins discussed in this report. For example, characterization of the receptors is still under investigation. Aminopeptidase N has been proven to bind Cry1 toxins. Also cadherin-like receptors have been suggested as well as glycolipids. Most likely there is more than one receptor competing for maximum binding and influencing efficacy of Bt-proteins, and hence, insect toxicity. Further, Bt-proteins occasionally can have an opportunistic biochemistry. Haider et al. (1986) reported that the insect specificity of *B. thuringiensis* var. *aizawai* IC1 could be altered depending on which digestive fluids processed the proteins. They showed that processing of *Bt* proteins by *Pieris brassicae* gut enzymes resulted in a 55-kDa protein that was only toxic to lepidopteran cell lines, whereas with *Aedes aegypti* gut extracts, the resulting 52-kDa protein was toxic only to mosquito cell lines and a *Spodoptera frugiperda* cell line. Also, the molecular structure and its implication for the mode of action still are not fully understood. For instance, researchers from Cambridge University published last year a paper demonstrating that the Cry1Ac Bt-molecule contains a site on the domain III (similar to fold of lectins) that can bind GalNAc (Galactosamine) (Burton et al. 1999). By GalNAc binding the specific receptor-Cry1Ac interaction is inhibited. Not only is inhibition by GalNAc so far unique among Cry toxins. Moreover, the location of the binding site is in a region that has a unique conformation compared to known Cry toxin structures. Additionally, its sequence is evolutionary distinct from that of other Bt Cry toxins. Although it is acknowledged that the above cited cases may be exceptional, they illustrate that the relatively large Bt-protein molecule is very complex and not well enough understood yet in its possible modes of action in particular in nontarget organisms. Our knowledge about Bt proteins stems mainly from research screening for susceptible target insects that potentially can be controlled by these proteins. For that matter, susceptibility is defined in terms of measurable lethal effects (LD/LC 50 or 99 etc.). Our knowledge further stems from research carried out to understand resistance mechanisms developing again in target insects. Both are important in an economic context. Only little information is available on fate and metabolism of Bt proteins in not or only sublethally susceptible insect herbivores and even less in higher trophic level organisms like insect natural enemies. All of this is important in an ecological context and has been of little interest or relevance prior to the utilization of transgenic Bt-plants expressing high concentrations of Bt-toxins constitutively.

2. Ecological availability and risk assessment

Ecologically even more important is the significantly extended temporal and spatial availability of Bt-toxins in the agroecosystem. In classic risk assessment, risk is the result of exposure x hazard, with 'exposure' being defined as a function of concentration, distribution and time.

Concentration. In compliance with the currently adopted pest resistance management strategy (high dose/refuge), transgenic technology strives to achieve the highest possible concentration of toxin expression in transgenic Bt-plants. The ultimate goal is to kill potential heterozygote resistant individuals. Most promising technology in that regard is chloroplast transformation increasing expression levels of Bt proteins in plants multifold over those currently expressed in transgenic Bt-plants (McBride et al. 1995).

Distribution. With Bt-insecticides, the Bt-proteins are only topically applied, whereas in transgenic plants, the Bt-proteins are expressed constitutively, i.e., in essentially all plant parts including, pith, kernels, roots and pollen albeit at different concentrations. Therefore, not only leaf-chewing, mandibulate insects are exposed to the toxin but also leaf-mining and cell-sucking insects such as diptera larvae, thrips, cicadellidae (leaf hoppers), spider mites.

Time. In contrast to Bt-insecticides, constitutive expression means that the Bt-toxins are expressed in transgenic Bt-plants throughout the entire field season from germination to plant senescence (Koziel et al. 1993, Perlak 1990) although the level of expression is dependant upon general plant vigor. Bt-insecticides degrade relatively quickly in the field due to UV-light and typically lose substantial activity within several days to two weeks after their application (Behle et al. 1997, Ignoffo and Garcia 1978).

This extended temporal and spatial expression results in considerably higher 'exposure' in the risk assessment scheme. Consequently, most, if not all, non-target herbivores colonizing transgenic Bt-plants in the field during the season will ingest plant tissue containing Bt proteins, which they may pass on to their natural enemies in a more or less processed form.

The ubiquitous and continuous availability of Bt-proteins in the agroecosystem, in addition to its modified form and mode of release, prohibits a simple deduction of the safety of transgenic Bt-plants from the past record of Bt-insecticide use. Hence, more rigorous and appropriate assessment techniques need to be developed that account for these differences (Jepson et al. 1994). As suggested by Jepson et al. (1994), extended dietary exposure bioassays of natural enemies would be one logical approach toward this goal.

3. Laboratory studies

3.1. General comments on ecotoxicological studies

Test designs as used in studies 1-3 and 5 are conducted according to standardized laboratory test protocols for ecotoxicological risk and damage assessment. The basis of most of these test protocols is the OECD guidelines for testing of chemicals. Depending on the substance tested and on the regulatory requirements of the specific country, other guidelines such as EC-guidelines, USEPA-, DIN-, ISO-, etc. are also applied. Ecotoxicological laboratory tests are designed to determine the potential adverse effects of synthetic chemicals such as pesticides and industrial pollutants on natural ecosystems. The mode of action of still widely used, older synthetic pesticides aim at disrupting vital life functions such as, for example, transmission of nerve signals or respiration in insects, nematodes or mollusks (organophosphates, carbamates, pyrethroids, etc.). Death induced by such compounds occurs often almost instantly or within minutes. Therefore, most of the ecotoxicological testing during the development of new insecticides assess acute toxicity (see below), some also assess chronic toxicity of a test substance often later during the development of a new pesticidal product. Toxicity of such synthetic compounds depends on the dose and duration of exposure. With increasing dose and exposure time, toxicity increases as well. But it is acknowledged that newly developed pesticides often have a 'softer' mode of action and do not necessarily kill instantly.

Acute toxicity tests. Acute toxicity tests determine the concentration of a chemical that produces harmful responses on the test animals after a single application or after a relatively short term exposure (Reinecke 1992). Usually, acute toxicity occurs after application of a high dose of a substance. Therefore, the exposure time is not exactly defined but differs for different animals and their life cycles. For fish, signs of acute toxicity may occur after an exposure time of 48 to 96 hours, for daphnids after 24 to 48 hours (Fent 1998). Usually, acute toxicity tests are lethal tests determining LD₅₀ (lethal dose required to kill 50% of the population) or LC₅₀ (lethal concentration required to kill 50% of the population) parameters. The LC₅₀ is defined as the mean lethal concentration of a substance found in the environment (air, water, or soil) that kills 50% of the test animals during the time of exposure. LC₅₀-values are specific for the animal tested, the particular exposure time, and the mode of entry (Fent 1998). LC₅₀-values can vary between different species, different life stages and sex of a single species. Finally, LC₅₀-values do not assess chronic toxicity and, therefore, are not appropriate to assess long-term effects. From the results of acute lethal tests, no conclusion can be drawn regarding sublethal effects of a substance under conditions of chronic exposure (Reinecke 1992). Besides LC₅₀-values, acute toxicity tests may also determine parameters such as the body weight of a test animal in order to provide some additional information about sublethal effects.

Chronic toxicity tests. Chronic toxicity tests determine adverse effects of chemicals on test animals under long term exposure. Chronic toxicity is defined as the slowly progressing adverse health effects that occur after permanent or repeated exposure over a long time period, and often during the entire life of an organism. The exposure time leading to chronic toxic effects varies for different life stages of different organisms. Signs of chronic toxicity may begin to develop after several days to years. Sometimes measurable effects may occur only after exposure of several generations of the test animals. Chronic toxicity tests assess physiological parameters (e.g. growth and development), biochemical parameters (e.g. enzyme activity), cell structural parameters (e.g. histology), behavioral parameters (e.g. mobility), and reproduction parameters (e.g. fertility). The test value of chronic toxicity tests mostly used is the NOEC-value (no observed effect

concentration) that is defined as the concentration of a substance causing no adverse effects on the test animals.

3.2 Comparison of insecticides versus transgenic Bt-plants.

There are several differences between synthetic insecticides and Bt-plants that severely restrict a short-term, direct comparison of both pest control methods. Synthetic pesticides are often effective immediately after application, because they induce instant, acute toxic effects. Hence, effects are rapidly and easily visible (dead insects in field furrows). They can affect a broad range of organisms and are often applied repeatedly in the same field. Foliar application makes the use of pesticides temporally and spatially restricted and often drift into other habitats by air or water occurs where it can affect many non-target organisms.

In transgenic Bt-corn, the Bt-protein is produced during the entire life span and in almost every part of the plant in high concentrations. The persistent presence of the active insecticidal compound in transgenic Bt-corn (several weeks to months) can induce chronic toxicity effects (see above). Further, Bt-proteins do not induce instant death as many synthetic insecticides do. It usually takes even the most susceptible, neonate insect larva a minimum of 1 to 2 days before it dies, often more. Hence, effects in general are far less visible for target and non-target pests alike. All herbivores that feed on Bt-plant tissue and are not killed by the Bt-toxin will continuously ingest Bt-proteins produced by the plant. Thereby, the Bt-protein enters into the food chain throughout the entire field season and, thus, may exert various sublethal and lethal effects on different trophic levels in the ecosystem. Whereas modern synthetic pesticides typically have a known, relatively short half-life after application, degradation of Bt-protein in dead plant material after harvest of the plants above ground and below ground is still unclear. Various researchers reported conflicting results (Donegan et al. 1995, Koskella & Stotzky 1997, Sims & Holden 1996). 'Drift' into other ecosystems of transgenic Bt-toxin by air is limited to the transport of transgenic pollen or through outcrossing and introgression of the transgenic, insecticidal trait into wild/weedy relatives.

This results in very different routes of exposure and exposure times than for pesticides, thus, inducing different effects in an agroecosystem than pesticides that require different methods for assessment. For example, through foliar application, herbivores and plants are externally contaminated with pesticides, including Bt-insecticides (as spore/crystal mixture). Only externally feeding herbivores ingest the pesticides. Cell-sucking, phloem-feeding, or mining insects, like thrips, mites, aphids, dipteran larvae do not ingest the Bt-insecticide. Consequently, hemipteran, chrysopid, arachnid or parasitoid natural enemies that parasitize or feed on these herbivores by sucking out their liquefied body content (without ingesting the potentially contaminated external cuticula) do not ingest the toxin. For transgenic Bt-plants, which express the toxin internally, cell sucking or mining insects will also ingest the toxin. Consequently, also their predators and parasitoids are likely to ingest the toxins.

Through the temporally and spatially extended presence of Bt-proteins in the agroecosystem, the range of potentially affected non-target organisms within a field is also extended. This extended exposure in addition to its 'non-instant' mode of action is not likely to have short term, readily visible effects like synthetic insecticides. Consequently, standard laboratory tests done according to the risk assessment protocols for pesticides are insufficient as the sole data basis for risk assessment of transgenic plants. Laboratory tests must take into account realistic routes of exposure of transgenic Bt-toxin. For example, spraying a prey item with a Bt-protein solution and feeding it to a sucking neuropteran (such as the green lacewing larvae) or hemipteran predator is an inadequate route of exposure. Clearly, for Bt-plants, the methodological focus should be expanded to test for acute AND chronic lethal and sublethal toxicity.

3.3. Unpublished studies

Study 1: for regulatory purposes

48-hour static renewal toxicity of pollen from modified maize to water fleas (*Daphnia magna*).

Species name: *Daphnia magna* (water fleas)

Methods

Number of individuals used: 20 daphnids per concentration treatment (<24 hours old); (n=2 groups of ten)

Food sources = treatments:

- 1) Bt-176 corn pollen at five different concentrations: 19, 32, 54, 90, 150 mg pollen/liter water
- 2) Isogenic corn pollen at five different concentrations: 19, 32, 54, 90, 150 mg pollen/liter water
- 3) No pollen (negative control group)

Duration of experiment: 48 hours under static conditions with 24-h renewal period

Parameters measured: Survival and mobility of *D. magna*, concentration of dissolved oxygen

Repetition of the experiment: The entire experiment was not repeated over time.

Type of experiment: acute toxicity test also described in the OECD guidelines for testing of chemicals no. 202, bi-trophic

Our comments

- Since *Daphnia magna* is extremely sensitive to metal ions like copper and zinc, pesticides, detergents, bleaches, and other dissolved toxins, it is a widely used species in ecotoxicological tests to assess water quality.
- The toxicity test with *D. magna* is designed for substances soluble in water. However, pollen and Bt-protein do not or only insufficiently dissolve in water. In order to exert any activity, Bt-proteins have to be ingested. *Daphnia*'s natural food source are various groups of bacteria, yeast, micro algae, detritus, and dissolved organic matter. All these food groups are of the size of 1 to 5 μm in diameter. Corn pollen on the other hand has a size of around 70 μm in diameter. From daphnids of the family *Cladocera* it is known that by filtering their food particles of inadequate size are excreted unprocessed via the abdomen (J. Zettel University of Berne, Switzerland, personal communication). Therefore, it needs to be shown that *D. magna* can actually ingest insufficiently dissolved pollen or pollen fragments before conclusions based on this testing procedure can be drawn.
- An exposure time of 48 hours is designed to test for acute toxicity. But even in susceptible insects, adverse effects of Bt-toxins are reliably measurable only after time spans of 24 to 48 hours. According to the OECD guidelines for testing of chemicals, a chronic toxicity study on *D. magna* requires an exposure time of at least 14 days (Guideline no. 202, Reproduction Test). This was not done.

Results and conclusions drawn by authors

100% survival for all concentrations of the Bt-corn pollen and the isogenic corn pollen treatment and the negative control group. No immobilization or sublethal signs of toxicity were observed. EC_{50} after 48 hours based on immobilization was >150 mg pollen/L for Bt- as well as for isogenic pollen, thus the NOEC was 150 mg genetically modified or isogenic pollen/L.

Our comments

Control(s) survival: 100%

Treatment(s) survival: 100%

- One might argue that pollen from transgenic Bt-corn cannot be toxic to *D. magna* since it may not be able to ingest it. In the case of corn pollen, toxicity tests with *D. magna* seem to be of minor relevance considering the fact that only daphnids in water bodies near corn fields will be exposed.
- NOEC value is given, which is a value typically provided for chronic exposure tests. However, this was an acute toxicity test.
- Even though the study indicated that no sublethal signs of toxicity were observed, it did not mention on what measured parameters this conclusion was based.

Study 2: for regulatory purposes

Single dose test evaluating toxicity to earthworms (*Eisenia foetida*) using CryIAb enriched maize leaf protein.

Species name: *Eisenia foetida* (earthworm, compost worm)

Methods

Number of individuals used: 40 *E. foetida* per treatment (n=4 groups of ten)

Food sources = treatments:

- 1) Lyophilized protein preparation from Bt-176 corn leaves (powder) at four different concentrations: 21.2, 90.9, 455, 500 mg protein/kg soil
- 2) Lyophilized protein preparation from isogenic corn leaves (powder) at four different concentrations: 21.2, 90.9, 455, 500 mg protein/kg soil
- 3) no corn protein (negative control group)
- 4) chloroacetamide (reference test)

Duration of experiment: 14 days

Parameters measured: mortality, toxicity, behavior: observed on day 7 and 14;
body weight: observed on day 0 and 14

Repetition of the experiment: The entire experiment was not repeated over time.

Type of experiment: artificial soil test according to OECD guideline for testing of chemicals no. 207 ("Earthworm, Acute Toxicity Tests"), bi-trophic

Our comments

- *Eisenia foetida* is a standard test organism for ecotoxicity testing of industrial pollutants and synthetic pesticides.
- *E. foetida* prefers habitats that contain high amounts of decomposing organic matter. Hence, they are widely commercially sold as 'compost worms' to enhance compost decomposition. Because of the relatively low content in organic matter, *E. foetida* is not able to survive over a long time in most field soils (J. Zettel University of Berne, Switzerland, personal communication). Therefore, *E. foetida* is of minor ecological relevance in corn fields.
- Aside of the fact that it is a marginally relevant species for most agricultural settings, it needs to be demonstrated first whether *E. foetida* ingests any Bt-protein in this test. *E. foetida*, a typical epedaphic species, does not feed through soil but ingests concentrated organic debris (J. Zettel University of Berne, Switzerland, personal communication). By mixing corn leaf protein powder into a test soil substrate (consisting of peat, clay and industrial sand, OECD guideline no. 207), the study tests primarily for contact toxicity but it needs to be proven that it is an adequate system to test for adverse effects due to ingestion of corn plant residues.

Results and conclusions drawn by authors

No effects on survival were noted for all treatments after day 7 and 14. No adverse effects on body weight were recorded. LC₅₀ for genetically modified corn protein was found to be >500 mg protein/kg soil, the NOEC was 500 mg protein/kg soil.

Our comments

Control(s) survival: 100%

Treatment(s) survival: 100%

- No data and statistical analyses of the body weight is given.
- The above method is an acute toxicity test designed to determine the concentrations of a chemical that causes harmful responses in *E. foetida* during relatively short term exposure. However, acute toxicity tests alone are not sufficient to assess long-term effects of transgenic plants that might also be on a sublethal scale.
- Chronic effects of Bt-protein in the soil should be determined by long term survival and reproduction tests using natural corn plant material and ideally earthworm species naturally found in or on soils of corn-fields.

Study 3: for regulatory purposes

Effect of Bt maize pollen on larval honeybee (*Apis mellifera* L.) development.

Species name: *Apis mellifera* L. (honeybee)

Methods

Number of individuals used: 100 larval honeybees per treatment (n=4 groups of 25), three to five days old (fourth instar)

Food sources = treatments:

- 1) 1 mg of Bt-176 corn pollen per larvae mixed with a drop of water (2 mg per larvae in the second study)
- 2) 1 mg of isogenic corn pollen mixed with a drop of water (2 mg per larvae in the second study)
- 3) no treatment (negative control)
- 4) non transgenic pollen mixed with a carbaryl insecticide (first study) and potassium arsenate (second study)

Duration of experiment: single dose application during 45 minutes

Parameters measured: emergence frequency and development time

Repetition of the experiment: 2 (study was conducted twice, however, different set-ups were used)

Type of experiment: acute toxicity test, bi-trophic

Our comments

- The test is designed as an acute oral toxicity test analogous to toxicity testing of synthetic pesticides to larval honeybees.
- A 45 minutes exposure period seems short considering the foraging behavior of bees (repeated visits of the same pollen source by worker bees).
- Tests should be carried out exposing honeybees to transgenic Bt-toxin during their entire life cycle.
- Pollen has to be pre-digested by nurse bees in order to be digestible for larval honeybees (Wittmann 1982). In their hypopharyngeal glands, nurse bees produce the protein-rich jelly also called brood food, which is fed to the larvae (Crailsheim 1992). The pollen contained in this jelly has been broken down in the nurse bee's midgut (Wittmann 1982). Larvae older than 3 days (3rd to 4th instars) receive brood food containing some unprocessed pollen (Haydak 1970) but the significance of this pollen is not known and it is not an essential constituent of the food of worker bee larvae (Haydak 1970). Therefore, it is questionable whether the pollen is digested and the Bt-toxin released in the larval gut.
- A more appropriate way to administer pollen to honeybee larvae should consider that pollen is provided through the worker bees (it seems that this was taken into account in study 4).

Results and conclusions drawn by authors

The studies were performed twice.

In the first trial there was no effect between the bee larvae fed Bt-pollen and the negative control (water). However, emergence frequency of the larvae fed isogenic pollen was significantly lower than of the larvae fed Bt-pollen and no pollen. Possible cause of the reduced emergence frequency of the larvae fed isogenic pollen is differences in hive vigor or genetic variability. Lowest emergence frequency was found in the larvae fed with carbaryl treated pollen.

The second trial produced no statistical difference in emergence frequencies for all groups except for the one treated with insecticide-contaminated pollen, which was significantly less than the others.

Based on these results, there are no measurable detrimental effects of ingestion of Bt-protein containing pollen on larval honeybee development.

Our comments

Control(s) emergence frequency:

First study: 96% in the untreated group, 65% in the group feeding on isogenic pollen, 4% for the carbaryl treated group.

Second study: 95% in the untreated group, 92.5% in the group feeding on isogenic pollen, 6.25% for the group treated with potassium arsenate.

Treatment(s) emergence frequency:

First study: 95% in group treated with Bt-pollen.

Second study: 92.5% in group treated with Bt-pollen.

- An acute toxicity test of 45 minutes exposure is not sufficient to support the conclusion that ingestion of Bt-protein containing pollen has no measurable detrimental effects on larval honeybee development.
- Longer time exposure studies considering the appropriate routes of exposure are recommended to study potential adverse effects of Bt-corn pollen on *A. mellifera*.

Study 4: ongoing

Effect of Bt maize pollen on larval honeybee (*Apis mellifera* L.) development.

Species name: *Apis mellifera* L. (honeybee)

Methods

Number of individuals used: small bee colonies

Food sources = treatments: 1) bee colony held over plots of transgenic Bt-corn during pollination

2) bee colony held over plots of isogenic corn during pollination

Duration of experiment: no information given

Parameters measured: mortality, foraging activity, brood development

Repetition of the experiment no information given

Type of experiment: semi-field study

Our comments

From the information provided it seems that this is a chronic toxicity test. However, since the study is still ongoing and no detailed data on the test procedure is available, no further comments are possible.

Study 5: for regulatory purposes

28-day survival and reproduction study in collembola (*Folsomia candida*) using Cry1Ab-enriched maize leaf protein.

Species name: *Folsomia candida* (springtails)

Methods

Number of individuals used: 40 collembola per treatment (10-12 days old), (n=4 groups of 10)
Food sources = treatments: 1) Lyophilized Bt-176 corn leaf protein at 125 mg protein/kg soil
2) Lyophilized Bt-176 corn leaf protein at 250 mg protein/kg soil
3) Lyophilized Bt-176 corn leaf protein at 500 mg protein/kg soil
4) Lyophilized isogenic corn leaf protein at 500 mg protein/kg soil (negative control)
5) untreated soil

Additionally, all treatments were provided with 2 mg of yeast as food on day 0 and 14.

Duration of experiment: 28 days

Parameters measured: survival and reproduction

Repetition of the experiment: The entire experiment was not repeated over time.

Type of experiment: chronic toxicity and reproduction test, bi-trophic

Our comments

- This kind of test has been used to assess chronic toxic effects of pesticides.
- As stated by the authors, the proper food for *F. candida* is saprophytic fungi found on decaying plant matter. Consequently, the laboratory experiment is a poor simulation of actual field environments. An appropriate experiment would have to involve corn plant matter.

Results and conclusions drawn by authors

Survival and reproduction with the two highest Bt-corn leaf protein concentrations (250 and 500 mg protein/kg soil) were significantly reduced compared to the controls. Thus, the NOEC was found to be at 125 mg Bt-leaf protein/kg soil.

The author comments that the scenario of pre-harvest soil incorporation of genetically modified maize is most unlikely, and therefore, collembolans would not be likely to ingest significant quantities of fresh maize plant tissue but rather ingest partially digested plant tissue colonized by fungi. The toxic potential of Bt-protein may then be less because the fungi are likely to degrade some of the Bt-toxin. Therefore, the actual safety margins will likely be greater.

Our comments

Control(s) survival: 100% in the untreated as well as in the negative control group.

Control(s) reproduction: 147 offspring/replicate in the untreated and 155 offspring/replicate in the negative control group.

Treatment(s) survival: 95%, 38%, and 13% in the 125, 250, and 500 mg Bt-leaf protein/kg soil treatment.

Treatment(s) reproduction: 96, 27, and 4 offspring/replicate in the 125, 250, and 500 mg Bt-leaf protein/kg soil treatment.

- Even though the test substance used was a Bt-protein preparation and the test was designed as a bi-trophic study, adverse effects were observed.
- The conclusion drawn by the author that the actual safety margin will likely be greater due to degradation of the Bt-protein in the plant tissue by fungi is speculative. No data are provided

that fungi are likely to degrade Bt-toxin. With the same justification also the opposite may be speculated.

- Even if an incorporation of plant material prior to harvest may be unlikely, after harvest there are corn stalks and roots left in the soil as post-harvest plant tissues. Sims and Holden (1996) have shown that Bt-proteins contained in post-harvest plant material from transgenic Bt-corn were active for 2 to 120 days.
- In order to investigate the toxic potential of Bt-corn on *F. candida*, first, tests should be carried out verifying whether the saprophytic fungi on transgenic plant material contains Bt-toxin and whether it serves as a mediator of the toxin to *F. candida*.

3.4. Published studies

Study 6a

Pilcher, C.D.; Obrycki, J.J.; Rice, M.E.; Lewis, L.C. 1997. Preimaginal development, survival and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology 26(2): 446-454.

Species name: *Chrysoperla carnea*

Methods

<u>Number of individuals used:</u>	90 larvae per treatment.
<u>Food sources = treatments:</u>	1) optimal diet of <i>Sitotroga cerealella</i> (meal moth) eggs or non-Bt pollen only or Bt-pollen only (Ciba Seeds hybrid) (Ciba 3906X, Event 176) 2) Bt-pollen (Ciba 3906X, Event 176) or non-Bt pollen (Ciba Seeds hybrid) offered for 24 hours during each larval stage before <i>Sitotroga cerealella</i> eggs (meal moth eggs) were added (pollen only as a food source in total 72 hours = 3 days).
<u>Duration of experiment:</u>	total immature development.
<u>Parameters measured:</u>	Survival and developmental time (stage specific, first instar to adult eclosion)
<u>Repetition of the experiment:</u>	The entire experiment was not repeated over time.
<u>Type of experiment:</u>	bi-trophic; predators kept individually in container.
<u>Statistical analyses:</u>	Developmental time: analysis of variance on individual data. Survival: proportion of surviving <i>C. carnea</i> was arcsine transformed and an ANOVA was carried out. Means and standard errors were determined.

Our comments

- *C. carnea* larvae have forceps-like mouthparts. Therefore, *C. carnea* larvae pierce through the skins and shells of prey items (insects or insect eggs), inject enzymes into the prey body to liquefy and pre-digest the content and then suck out the liquefied content. To the best of our knowledge, *C. carnea* larvae have not been reported to feed on pollen. Their mouthparts only allow them to feed on liquid food sources. Therefore, it can be concluded *C. carnea* larvae did not ingest the toxin.
- Repetition of the experiment is recommended.

Results and conclusions drawn by authors

No differences between the treatments were observed. No Bt-pollen effect on *C. carnea* larval development was detected. Authors acknowledge the low survival of *C. carnea* and noted that *C. carnea* larvae have been reported to be unable to survive longer than 24 hours without food. The authors attributed the low survival to a missing dietary requirement in the corn pollen.

Our comments

Control mortality and developmental time (*S. cerealella* eggs plus 72 hours isogenic pollen):

- First instar: 37%; duration: 5.6 days
- Total immature development: 51%; duration: 22 days

Treatment mortality and developmental time (*S. cerealella* eggs plus 72 hours Bt-pollen):

- First instar: 38%; duration: 5.6 days
 - Total immature development: 51%; duration: 22 days
-
- A statistical analysis of mortality is unclear. Although the whole experiment was not repeated and, therefore, only one value for “proportion survival”, i.e. inverse mortality, was obtained an ANOVA was carried out and means and SE were determined. Inspection of statistical parameters (degrees of freedom = 10) suggests that the 90 *C. carnea* larvae may have been placed in subgroups, thus, indicating the creation of pseudo-replications for statistical purposes.
 - We suggest, that the likely cause for the observed high mortality of *C. carnea* larvae in both the control and Bt-treatment was due to starvation of *C. carnea* for 24 hours during each of their 3 larval stages.
 - Before conclusions regarding Bt-toxicity can be drawn, it should be proven first, whether *C. carnea* feeds on pollen and whether Bt-toxin contained in pollen is ingested.

Study 6b

Pilcher, C.D.; Obrycki, J.J.; Rice, M.E.; Lewis, L.C. 1997. Preimaginal development, survival and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. *Environmental Entomology* 26(2): 446-454.

Species name: *Coleomegilla maculata*

Methods

Number of individuals used: 45 larvae per treatment.

Food sources = treatments: 1) Bt-corn pollen (Ciba 3906X, Event 176) plus 3 pea aphids (*Acyrtosiphon pisum*); pollen was dried for 24 h at app. 23°C, additional water was provided by placing a soaked 1-cm piece of dental wick in the vials. Food was renewed each time the larva molts to a new instar.
2) Isogenic-corn pollen (Ciba Seeds hybrid) plus 3 aphids (*A. pisum*); pollen was dried for 24 h at app. 23°C, additional water was provided by placing a soaked 1-cm piece of dental wick in the vials. Food was renewed each time the larva molts to a new instar.
3) *A. pisum* only

Duration of experiment: total immature development.

Parameters measured: Survival and developmental time (stage specific, first instar to adult eclosion)

Repetition of the experiment: The entire experiment was not repeated over time.

Type of experiment: predominantly bi-trophic/short-term tri-trophic; predators were kept individually in container.

Statistical analyses: Developmental time: analysis of variance on individual data.
Survival: proportion of surviving *C. carnea* was arcsine transformed and an ANOVA was carried out. Means and standard errors were determined.

Our comments

- *C. maculata* can complete development only on pollen, and in some cases may actually do so in the field (D. Andow, University of Minnesota, USA, personal communication). However, generally larvae are predaceous. Therefore, in addition to bitrophic studies, feeding studies with a tri-trophic set-up (Bt-plant – herbivorous prey – *C. maculata*) are recommended before conclusions about Bt-ecotoxicity can be drawn.
- Repetition of the feeding experiment is recommended.

Results and conclusions by authors

No differences in developmental times and survival were observed between the Bt-pollen and isogenic pollen treatments. Aphid-fed predator larvae developed significantly slower to adulthood than both pollen-treatments.

Our comments

Control(s) mortality and developmental time: 31% (isogenic pollen plus 3 aphids); duration: 21 days
36% (aphids only); duration: 24 days

Treatment(s) mortality and developmental time: 11% (Bt-pollen plus 3 aphids); duration: 21 days

- A statistical analysis for mortality is unclear. Although the whole experiment was not repeated and, therefore, only one value for “proportion survival”, i.e. inverse mortality, was obtained an ANOVA was carried out and means and SE were determined. Inspection of statistical parameters (degrees of freedom = 6) suggests that the 45 *C. maculata* larvae may have been placed in subgroups, thus, indicating the creation of pseudo-replications for statistical purposes.
- Although survival/mortality was recorded, the quite varied mortality rates between both control groups and the Bt-treatment go unmentioned and unexplained. Only time until death is discussed.
- Based on the results of this study, it seems that B-corn pollen is not toxic to *C. maculata*. However, for a complete safety conclusion tri-trophic feeding studies are recommended in particular when considering the higher Bt-toxin concentrations of leaves.

Study 6c

Pilcher, C.D.; Obrycki, J.J.; Rice, M.E.; Lewis, L.C. 1997. Preimaginal development, survival and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. *Environmental Entomology* 26(2): 446-454.

Species name: *Orius insidiosus*

Methods

<u>Number of individuals used:</u>	45 nymphs per treatment.
<u>Food sources = treatments:</u>	1) Bt-corn pollen (Ciba 3906X, Event 176); pollen was dried for 24 h at app. 23°C, additional water was provided by placing a soaked 1-cm piece of dental wick in the vials. Food was renewed every 2-4 days 2) Isogenic-corn pollen (Ciba Seeds hybrid); pollen was dried for 24 h at app. 23°C, additional water was provided by placing a soaked 1-cm piece of dental wick in the vials. Food was renewed every 2-4 days 3) <i>Ostrinia nubilalis</i> eggs; food was renewed every 3-4 days.
<u>Duration of experiment:</u>	total immature development.
<u>Parameters measured:</u>	Survival and developmental time (first instar to adult eclosion)
<u>Repetition of the experiment:</u>	The entire experiment was not repeated over time.
<u>Type of experiment:</u>	bi-trophic; predators kept individually in container.
<u>Statistical analyses:</u>	Developmental time: analysis of variance on individual data. Survival: proportion of surviving <i>C. carnea</i> was arcsine transformed and an ANOVA was carried out. Means and standard errors were determined.

Our comments

- *Orius insidiosus* is a predaceous insect feeding occasionally on plant material to supplement its predominantly carnivorous diet. Therefore, the experimental setup does not consider the typical natural feeding behavior.
- Repetition of the experiment is recommended.

Results and conclusions drawn by authors

No differences in development time and mortality/survival between the Bt- and isogenic pollen treatments were observed. Nymphs reared on *O. nubilalis* eggs always developed significantly faster than the pollen-fed nymphs. The authors suggested that plant tissue may not be the optimal food source.

Our comments

Control(s) mortality and developmental time:	56% (isogenic pollen); duration: 22 days 43% (<i>O. nubilalis</i> eggs); duration: 18 days
Treatment(s) mortality and developmental time:	37% (Bt-pollen); duration: 22 days

- A statistical analysis of mortality is unclear. Although the whole experiment was not repeated and, therefore, only one value for “proportion survival”, i.e. inverse mortality, was obtained an ANOVA was carried out and means and SE were determined. Inspection of statistical parameters (degrees of freedom = 6) suggests that the 45 *O. insidiosus* nymphs may have been

placed in subgroups consisting, thus, indicating the creation of pseudo-replications for statistical purposes.

- Although survival/mortality was recorded, the quite different mortality rates between the isogenic pollen control versus the Bt-treatment and *O. nubilalis* egg control go unmentioned and unexplained. Repetition of the study would have been advisable.
- *O. insidiosus* nymphs are predaceous and prefer carnivorous prey. Pollen typically serves as supplementary food source. As the authors speculated, plant tissue alone is suboptimal for a predaceous insect. Therefore, in addition to bitrophic studies, feeding studies using a tri-trophic set-up (Bt-plant – herbivorous prey – *O. insidiosus*) are recommended before conclusions about Bt-ecotoxicity can be drawn.

Study 7

G.C. Lozzia, C. Furlanis, B. Manachini, I.E. Rigamonti. 1998. Effects of Bt corn on *Rhopalosiphum padi* L. (Rhynchota Aphididae) and on its predator *Chrysoperla carnea* Stephen (Neuroptera Chryopidae). Boll. Zool. Agr. Bachic. Ser. II, 30(2): 153-164.

Species name: *Chrysoperla carnea*

Methods

<u>Number of individuals used:</u>	Numbers of individuals unknown (n=?). First trial used second instars, second trial used neonates.
<u>Food sources = treatments:</u>	1) <i>R. padi</i> (bird cherry aphid) reared on transgenic Bt-corn (Event 176); amount of aphids fed is not given 2) <i>R. padi</i> reared on isogenic corn; amount of aphids fed is not given
<u>Duration of experiment:</u>	Entire immature life stage (first instar until hatch of adult).
<u>Parameters measured:</u>	Developmental time and mortality
<u>Repetition of the experiment:</u>	The experiment was partly repeated 2 times over time.
<u>Type of experiment/Evaluation:</u>	The complete statistical methods were: 'The percentages of <i>C. carnea</i> mortality recorded in 1997 were analyzed with the χ^2 test. During the 1998 test, fewer deaths of <i>C. carnea</i> larvae occurred, and the data available were insufficient for a statistical analysis.'

Our comments

- Experimental set-up is unclear. It is not possible to determine whether the *C. carnea* larvae were kept individually or all in one container. From the description of the methods, either could be assumed. Experimental set-up and arrangement is unclear. Rearing methods for aphids is not specified.
- No stage-specific measurements of mortality and development times were carried out.
- As a dietary supplement for the aphid prey cut leaves were used for 5 days. Within that period of time, phloem translocation of all compounds is highly disrupted and the leaves undergo degradation regardless of nutrient additives. The authors acknowledged that it was unclear from the beginning whether or not any Bt-toxin would be present in the phloem at all. It would have been advisable to exchange the leaves and prey daily, or better, to use intact, living plants.

Results and conclusions drawn by authors

No significant differences in mortality and developmental times were found when aphids either fed on transgenic Bt-corn or on isogenic corn. The authors acknowledge that this may have been due to the absence of Bt toxin in the phloem.

Our comments

Control(s) mortality and developmental time: 5%; duration: 18 days
Treatment(s) mortality and developmental time: 11%; duration: 18 days

- The results on mortality are unclear. From Table 4 of the article, it appears that the authors used 74 individuals for the control test and 70 individuals for the Bt-treatment. No sample size is given explicitly at any point. The authors stated earlier that for the 1998 experiment,

‘the number of larval deaths was lower, and not sufficient for statistical analysis.’ No reasons are mentioned for this conclusion. The data provided in table 4 is confusing and allows for the conclusion that pooling may have been done over both year’s data sets. However, pooling of data should not be done without prior statistical justification.

- When reviewing the data provided in table 4 of the original publication, the expected values are very different from the observed values (observed: 4 dead and 70 alive on isogenic plants; 8 dead and 62 alive on Bt-plants; expected: 3.08 dead and 33.91 on isogenic plants; 2.91 dead and 32.08 alive on Bt-plants). A χ^2 -value of 0.854 was determined, P -value was 0.355. When we repeated the χ^2 test using the provided observed mortality values of 4 dead in the isogenic control and 8 dead in the Bt-treatment and the provided numbers of total individuals per treatment we obtained the following χ^2 test statistics: expected mortality-values of *C. carnea* reared on *R. padi* feeding isogenic plants: 6.17 dead and 67.83 alive; expected mortality-values of *C. carnea* reared on *R. padi* feeding Bt-plants: 5.83 dead and 64.17 alive. Our χ^2 -value was determined at 1.708 with a P -value of 0.1912. The reason of the difference between the calculation in the paper and our calculation remains unexplained.
- Mortality in the Bt-treatment was twice as high than in the isogenic control. However, the second run seemed to give a different result but data were not shown. In our opinion, this would have made it necessary to conduct at least one if not two more experimental runs to see what result would be confirmed.
- During the first trial, the most susceptible first larval stage was not included. Reasons for this and details of how the larvae were handled up to the time they were used in the experiment were not given.
- The authors pointed out that toxicity of Bt-protein was probably not tested with this experiment since Bt protein may not be translocated into the phloem sap (confirmed by Raps et al. in review, unpublished data) and hence may not be present in aphids.
- Aphids are a relevant prey for *C. carnea* that is preferred where present and therefore investigations on the effect of Bt-fed aphids on *C. carnea* are of high ecological relevance.
- We recommend to repeat the experiment using intact living plants as food source for *R. padi* before final conclusions can be drawn.

Study 8

Hilbeck, A., Baumgartner, M., Fried, P.M., Bigler, F. 1998. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology* 27(2): 480-487.

Species name: *Chrysoperla carnea*

Methods:

<u>Number of individuals used:</u>	50 per treatment in each experiment
<u>Food sources = treatments:</u>	4
	1) <i>O. nubilalis</i> larvae (small instars) fed transgenic Bt-corn (Event 176) for 24 hours
	2) <i>Spodoptera littoralis</i> (small instars) larvae fed transgenic Bt-corn (Event 176) for 24 hours
	3) <i>O. nubilalis</i> larvae (small instars) fed isogenic corn for 24 hours
	4) <i>Spodoptera littoralis</i> (small instars) fed isogenic corn for 24 hours
<u>Duration of experiment:</u>	Entire immature life stage (hatch of first instar to eclosion of adult)
<u>Parameters measured:</u>	Developmental time and mortality
<u>Repetition of the experiment:</u>	The entire experiment was repeated 4 times over time.
<u>Type of experiment:</u>	tri-trophic, predators kept individually in containers, plant and prey exchanged daily. All herbivorous prey was allowed to feed on Bt-plant material for 12 to 24 hours prior to use in the experiment
<u>Statistical analysis:</u>	stage specific mortality analyzed using a logistic regression (accounts for binomial distribution of mortality data). Model tested for repetition, treatment main effects (Bt-treatment and prey type) and their interaction effect. In addition, a means comparison procedure (Student-Newman-Keuls test) was used. Similarly, stage-specific development times were analyzed by ANOVA and means comparison procedure using the same model as for mortality. In addition, total mortality and developmental time from first instar to adult was analyzed accordingly.

Our comments

The goal was to test potential effects of Bt-containing prey on *C. carnea* larvae in a tri-trophic set-up and to explore whether these effects were due to intoxication of prey or could also be observed when prey was not or only sublethally affected suggesting a more direct Bt effect. Therefore, two species of prey were used, the target pest *O. nubilalis* and the non-target pest *Spodoptera littoralis*. Both herbivores feed on plant foliage and, therefore, ingest the Bt toxin.

Results and conclusions drawn by authors

In both Bt-free control treatments (*O. nubilalis* and *S. littoralis* fed isogenic corn), total mortality during entire immature life stages did not exceed 37%. Mean total mortality for *C. carnea* larvae raised on Bt-fed *O. nubilalis* was 65% and on Bt-fed *S. littoralis* was 59%. While the effect of

the prey species was not statistically significant, the effect of the Bt treatment was. Development time was significantly prolonged when Bt-fed *O. nubilalis* were provided to the predators but not when Bt-fed *S. littoralis* were provided. Although some unnoticed adverse effects of *S. littoralis* may have occurred due to feeding on Bt-corn (herbivores fed Bt-corn for a maximum of 24 hours), the results suggest that the reduced fitness of chrysopid larvae was associated with Bt. With respect to field implications the authors acknowledge that no conclusions can be drawn at this point as to how results from our laboratory trials might translate in the field. They state, that further experimentation under field conditions is necessary.

Our comments

Control(s) mortality and developmental time: 37% for both controls; duration: 29 days
Treatment(s) mortality and developmental time: 59% (Bt-fed *S. littoralis*); duration: 31.5 days
65% (Bt-fed *O. nubilalis*); duration: 32 days

- Equal control mortality and developmental times showed that both prey species were similarly suitable regarding these both parameters.
- The experiment demonstrated adverse effects of Bt-corn fed prey on *C. carnea*. The effect of Bt-toxin could not be completely separated from possible effects of altered host physiology. Therefore, follow-up studies were carried out (study 9 and 10).

Study 9

Hilbeck, A., Moar, W.J.; Pusztai-Carey, M.; Filippini, A.; Bigler, F. 1998. Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology* 27 (5): 1255-1263.

Species name: *Chrysoperla carnea*

Methods

<u>Number of individuals used:</u>	30 per treatment in each experiment
<u>Food sources = treatments:</u>	two types of food sources: a special artificial diet for <i>C. carnea</i> larvae and <i>Ephesia kuehniella</i> (meal moth) eggs. 5 treatments: 1) artificial diet with Bt toxin (100 µg/ml diet) during all larval stages 2) artificial diet without Bt-toxin during all larval stages 3) <i>E. kuehniella</i> eggs during first instar and artificial diet with Bt-toxin (100 µg/ml diet) during second and third instars 4) <i>E. kuehniella</i> eggs during first instar and artificial diet without Bt-toxin during second and third instars 5) <i>E. kuehniella</i> eggs only during all larval stages.
<u>Duration of experiment:</u>	Entire immature life stage (first instar until adult eclosion)
<u>Parameters measured:</u>	Stage-specific developmental times and mortality
<u>Repetition of the experiment:</u>	The entire experiment was repeated 5 times over time.
<u>Type of experiment:</u>	bi-trophic, predators were kept individually
<u>Statistical analysis:</u>	stage specific mortality analyzed with logistic regression accounting for binomial distribution of mortality data. Model tested for repetition, treatment main effects (Bt-treatment and diet type) and their interaction effect. In addition, a means comparison procedure (Student-Newman-Keuls test) was used. Similarly, stage-specific developmental times were analyzed by ANOVA and means comparison procedure using the same model as for mortality. In addition, total mortality and developmental time from first instar to adult was analyzed accordingly.

Our comments

By using an appropriate artificial diet for *C. carnea* larvae, the activated toxin could be fed directly to *C. carnea* larvae, hence, toxicity through ingestion could be tested and compared to the results observed when feeding the toxin via a lepidopterous prey.

Results and conclusions drawn by authors

When reared only on artificial diet containing Cry1Ab toxin, total immature mortality was significantly higher than in the respective control (treatment 1 and 2). Also, significantly more chrysopid larvae died when receiving the Cry1Ab toxin later during their larval development compared to the respective control (treatment 3 and 4). Lowest mortality was observed when *C. carnea* received *E. kuehniella* eggs only, which represented an optimum diet. No or only small differences in developmental times were observed between the various treatments. Therefore, Cry1Ab is toxic to *C. carnea* at 100 µg/ml of diet. However, when considering the multifold higher concentration used in these trials compared to those present in transgenic plants of the

previous trials (see study 8) (approximately ranging at 5 µg/g fresh weight) the mortality was lower than one would expect. This suggested that unknown interactions between the herbivore and the Bt toxin occur that increase the toxicity of Bt-plant fed prey to *C. carnea* larvae. This could be due to secondary effects or a processing of the Bt toxin to a perhaps more toxic stage. To explore these interactions further a third series of trials was conducted (see study 10 described below).

Our comments

Mortality and developmental time data.

Treatment	mortality	developmental time
artificial diet with Bt-toxin	57%	37 days
<i>E. kuehniella</i> eggs and artificial diet with Bt-toxin	29%	28 days
artificial diet without Bt-toxin	30%	37 days
<i>E. kuehniella</i> eggs and artificial diet without Bt-toxin	17%	27 days
<i>E. kuehniella</i> eggs only	8%	23 days

- The experiment demonstrated adverse effects of Cry1Ab on the development of *C. carnea* larvae.

Study 10

Hilbeck, A., Moar, W.J.; Pusztai-Carey, M.; Filippini, A.; Bigler, F. (1999): Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator *Chrysoperla carnea*. *Entomologia Experimentalis et Applicata* 91: 305-316.

Species name: *Chrysoperla carnea*

Methods

<u>Number of individuals used:</u>	30 neonate larvae per treatment in each experiment
<u>Food sources = treatments:</u>	Artificial diet for lepidopterous prey (<i>Spodoptera littoralis</i>) incorporated with: 1) Cry1Ab protoxin at 200, 100 and 50 µg/g diet 2) Cry1Ab toxin at 100, 50 and 25 µg/g diet 3) Cry2A protoxin at 100 µg/g diet 4) control diet without Bt toxin
<u>Duration of experiment:</u>	Entire immature life stage (first instar until adult eclosion)
<u>Parameters measured:</u>	Stage-specific mortality and developmental stage
<u>Repetition of the experiment:</u>	The entire experiment was repeated 4 times over time.
<u>Type of experiment:</u>	tri-trophic
<u>Statistical analysis:</u>	stage specific mortality analyzed using logistic regression (accounts for binomial distribution of mortality data; excluding Cry2A data to provide for a balanced design). Model tested for repetition, treatment main effects (Bt type and concentration) and their interaction effect. In addition, a means comparison procedure (LSD) was carried out (including the Cry2A data). Proportion of individuals that had molted to the next life stage (second and third instar, pupae, adult). A regular ANOVA (excluding Cry2A data to provide for a balanced design) and mean comparison procedure (including the Cry2A data) was carried out using the same model as for mortality.

Our comments

This study was the third of 3 series trials investigating possible food chain effects of transgenic Bt-plants on a natural enemy, *C. carnea* (green lacewing) observed in previous experiments (study 8 and 9). The goal was to explore effects caused by herbivore x Bt interactions on this ubiquitous predator.

Results and conclusions drawn by authors

Mean total immature mortality for chrysopid larvae reared on Bt-fed prey was always significantly higher than in the Bt-free control. Total immature mortality of *C. carnea* reared on Cry1Ab toxin 100-fed prey was highest and declined with decreasing toxin concentration. Cry1Ab protoxin-exposed *C. carnea* larvae did not exhibit a dose response. Prey-mediated, total mortality of Cry1Ab protoxin-exposed chrysopid larvae was intermediate to Cry1Ab toxin exposed and Cry2A protoxin exposed *C. carnea*. Total developmental time of *C. carnea* was not significantly affected by the Bt-treatments except at the highest Cry1Ab toxin concentration (100 µg/g diet). At this concentration *S. littoralis* exhibited increased mortality but at a much lower level than the predator feeding on it. Mortality of *C. carnea* may have been confounded by this increased intoxication of *S. littoralis*. At all other Bt-toxin and -protoxin concentrations and all

Bt-prototoxins, *S. littoralis* was not lethally affected. Comparative analysis with the two previous trials (studies 8 and 9) revealed that in addition to confirmed Bt x herbivore interactions, also triple Bt x herbivore x plant interactions exist that all contribute to the observed toxicity of Bt-prey fed *C. carnea* larvae.

Our comments

Mortality data.

Treatment	mortality
Cry1Ab toxin 100-fed prey	78%
Cry1Ab toxin 50-fed prey	67%
Cry1Ab toxin 25-fed prey	55%
Cry1Ab protoxin 200-fed prey	62%
Cry1Ab protoxin 100-fed prey	46%
Cry1Ab protoxin 50-fed prey	56%
Cry2A protoxin 100-fed prey	47%
Bt-toxin free prey (control)	26%

- This study demonstrated adverse effects of Cry1Ab toxin- and protoxin- and Cry2A protoxin-fed prey on the development of *C. carnea* larvae.
- The three studies 8, 9, and 10 showed effects of Bt-toxin from transgenic corn on *C. carnea* larvae additionally revealing that herbivore x toxin and plant x herbivore x toxin interactions contribute to the toxicity of Bt-toxin from transgenic corn.
- Based on the results of this study evaluations of the data for field implications are necessary. Field ecological parameters such as the main prey species for *C. carnea* in corn fields of the area of interest should be considered. Additionally, a risk-benefit analysis has to be carried out dealing with questions like: “What mortality of beneficial insects are we willing to accept?”. This is probably the most difficult decision society has to decide on.

Study 11

Sims SR 1995. *Bacillus thuringiensis* var. *kurstaki* (CryIA(c)) protein expressed in transgenic cotton: effects on beneficial and other non-target insects. *Southwestern Entomologist* 20(4): 493-500.

Species names:

In the following only the natural enemies and honeybee data are discussed, i.e. *Chrysoperla carnea*, *Nasonia vitripennis* (a pupal parasitoid of house flies), *Hippodamia convergens*, and *Apis melliferae*.

Methods

- Number of individuals used:
- 1) *N. vitripennis*: 2 groups of 25 adult wasps per treatment
 - 2) *H. convergens*: 2 groups of 25 adult beetles per treatment
 - 3) *C. carnea*: 30 larvae per treatment (no age given)
 - 4) *A. melliferae* - larvae: 4 brood frames per treatment, one brood frame with a minimum 50 larvae (1 to 3 days old)
 - 5) *A. melliferae* - adults: 3 containers per treatment, one container with approximately 40 bees (1 to 3 days old)
- Food sources = treatments:
- optimal diet with full length Cry1Ac produced in fermentation procedures using recombinant *E. coli* (Cry1Ac equivalent to protein expressed in transgenic cotton). For all beneficials an attenuated Cry1Ac_{inact} protein (= heat inactivated protein control) was tested in addition to the test diet control
- 1) *N. vitripennis*: 25% honey solution; 20 µg toxin (Cry1Ac, Cry1Ac_{inact}) / ml solution;
 - 2) *H. convergens*: 47% honey solution; 20 µg toxin (Cry1Ac, Cry1Ac_{inact}) / ml solution
 - 3) *C. carnea*: *Sitotroga* eggs; 17 µg toxin (Cry1Ac, Cry1Ac_{inact}) incorporated in distilled water/g eggs
 - 4) *A. melliferae* - larvae: 5 µl test substance in the wells with the larvae; test substance: 20 mg Cry1Ab toxin (Cry1Ac, Cry1Ac_{inact}) / ml distilled water
 - 5) *A. melliferae* - adults: 50:50 honey : water mixture containing 20 µg toxin (Cry1Ac, Cry1Ac_{inact}) /ml
- Duration of experiment:
- 1) *N. vitripennis*: 9 days (then control mortality exceeded 20%)
 - 2) *H. convergens*: 10 days (then control mortality exceeded 20%)
 - 3) *C. carnea*: 9 days (then control mortality exceeded 20%)
 - 4) *A. melliferae* - larvae: 11 days (until emergence of adult bees)
 - 5) *A. melliferae* - adults: 7 days (then control mortality exceeded 20%)
- Parameters measured:
- 1) *N. vitripennis*: mortality and/or abnormal behavior
 - 2) *H. convergens*: mortality and/or abnormal behavior
 - 3) *C. carnea*: mortality and/or abnormal behavior
 - 4) *A. melliferae* - larvae: Percentage larval surviving from dosing to capping and from capping to adult emergence
 - 5) *A. melliferae* - adults: mortality and signs of toxicity
- Numbers of surviving insects were compared using ANOVA.
- Repetition of the experiment: none of the experiments were repeated over time

Type of experiment: bi-trophic studies
Statistical analysis: Number of surviving insects, within treatment and control groups of each species, was compared using analysis of variance (ANOVA). Percentage mortality data were arcsine transformed prior to analysis.

Our comments

- The methods applied were standard procedures for ecotoxicological testing of pesticides. Because of the short experimental periods, they are designed to detect acute, short-term toxicity. However, they are not sufficient for investigating non-target effects of transgenic plants, which are likely to occur over a long-term period and a sublethal scale (for further discussion see chapter 2).
- As a pupal parasitoid of house flies, *N. vitripennis* is unlikely to be exposed to Cry1Ac in Bt-cotton. Therefore, it is of minor agroecological relevance. Additional testing of other species (e.g. those listed in the SETAC, Society of Environmental Toxicology and Chemistry – Europe) is recommended.
- Only adults of *N. vitripennis* and *H. convergens* were analyzed. However, for testing of susceptibility to Cry proteins the immature and adult stages of insects should be investigated.
- Exposure of *C. carnea* larvae to Cry1Ac will likely be negligible if the toxin is applied externally to insect eggs. *C. carnea* larvae have forceps-like mouthparts with which they pierce through the skins and shells of prey items (insects or insect eggs), inject enzymes into the prey body to liquefy and pre-digest the content and then suck out the liquefied contents (see also our comments study 6a).
- The age of the *C. carnea* larvae tested is not given.
- Repetition of the entire experiment is recommended.
- An ANOVA with n=2 per treatment is unlikely to reveal significant results. With such a low sample size, the differences have to be very large to result in statistically significant effects.

Results and conclusions drawn by authors

Cry1Ac protein produced no toxic effects on the four species of beneficial insects. All surviving insects were normal in appearance and behavior during the tests. Overall the data presented in the study support the conclusion that Cry1Ac proteins expressed in the tissues of transgenic cotton have no activity against beneficial or non-target insects other than those in the order Lepidoptera.

Our comments

Test duration and mortality data.

Species name	Test duration	mortality		
		Cry1Ac	Cry1Ac _{inact.}	control
<i>N. vitripennis</i>	9 days	2%	16%	30%
<i>H. convergens</i>	10 days	21%	12%	22%
<i>C. carnea</i>	9 days	3%	10%	23%
<i>A. melliferae</i> adults	7 days	24%	22%	25%
<i>A. melliferae</i> larvae ¹	11 days	20%	23%	17%
<i>A. melliferae</i> larvae ²	10 days	0%	23%	5%
<i>A. melliferae</i> larvae ³	5 days	42%	27%	33%

¹dosage to capping, ²capping to adult emerging, ³adults

- Except for *A. mellifera* larvae, all control mortalities exceeded 20% reaching 30% and 33% in two cases after only 5 to 10 days of exposure. These are high levels of mortality and may

imply that the rearing of the insects was not appropriate. Thus, significant effects of Bt could be masked by the high background mortality.

- With the method used, it is unlikely that *C. carnea* is exposed to Cry1Ac.
- The results at best gave an indication about the acute toxicity of Cry1Ac toxin as a substance.
- The results do not support the conclusions drawn by the authors, neither do they prove “no activity”. In order to draw such conclusions, additionally long-term tests with improved methodologies have to be conducted.

Study 12

Dogan EB, Berry RE, Reed GL, Rossignol PA 1996. Biological parameters of convergent lady beetle (Coleoptera: Coccinellidae) feeding on aphids (Homoptera: Aphididae) on transgenic potato. *Journal of Economic Entomology* 89(5): 1105-1108.

Species name: *Hippodamia convergens* Convergent lady beetle (Guérin-Ménéville) (Coleoptera: Coccinellidae), *Myzus persicae* green peach aphid (Sulzer) (Homoptera: Aphididae), potato (Russet Burbanks) expressing the δ -endotoxin specific for Coleoptera

Methods

<u>Number of individuals used:</u>	12 individuals per treatment in each experiment
<u>Food sources = treatments:</u>	<i>M. persicae</i> that had fed on transgenic or non-transgenic potatoes. One experimental unit contained: one leaflet (changed once a week), one lady beetle larva, aphids daily supplied (10 aphids for the 1st, 20 for the 2nd, 30 for the 3rd, 40 for the 4th instar).
<u>Duration of experiment:</u>	entire immature life stage (first instar to adult)
<u>Parameters measured:</u>	number of aphids consumed, developmental time for each stage, pupal weight, fecundity (number of eggs laid) and longevity of adults.
<u>Repetition of the experiment:</u>	The entire experiment was repeated 3 times over time
<u>Type of experiment:</u>	tri-trophic study
<u>Statistical analysis:</u>	Statistical analysis was done using multifactor analysis of variance (ANOVA). The response parameters were aphid consumption, time between molts, pupal weight, number of eggs laid, and longevity. The source of variation were: trial (3 repetitions), treatment, trial * treatment, and residual.

Our comments

- The experimental design (whole developmental period, 3 repetitions) and the parameters measured are appropriate.
- The sample size of 12 individuals per treatment seems to be rather low.
- The 12 leaflets per treatment came from 6 plants, hence they do not represent true replicates.
- The cut leaflets were used for one week. Within that period of time, phloem translocation of all compounds is highly disrupted and the leaves undergo degradation regardless of nutrient additives. The presence of endotoxin in the cut leaflets after one week was not verified.
- At the time the study was carried out, it was unclear whether aphids ingest Bt-protein when feeding on phloem and, thus, would pass the toxin to the natural enemy. Our own research indicates that Bt-protein does not occur in the phloem (Raps, unpublished data).
- For biosafety testing of aphids on transgenic plants feeding trials should be conducted on whole plants to reassure intact phloem transportation and potential presence of the toxin in aphids.

Results and conclusions drawn by authors

No significant differences were found in either parameter measured. However, aphid consumption in the 2nd and 4th instar revealed less consumption on transgenic potatoes with small p values (0.002, 0.0005) which 'are due to blocking effects', that is due to differences between the 3 experiments. The same effect occurred for the developmental time of the 4th instar being longer on transgenic potatoes (p=0.0001) and which 'was also a result of the blocking effect'. Differ-

ences in fecundity (slightly more eggs on the transgenic treatment) and longevity (slightly longer on the transgenic treatment) were not significant, but may be biologically important. The experiments demonstrate that the effect of transgenic potatoes on the convergent lady beetle is not significant. As it is not known whether aphids are exposed to the toxin and whether they are susceptible, the results are not easy to interpret. Field tests and further studies on the exposure of aphids and the susceptibility of aphids and the lady beetles are recommended.

Our comments

- The interpretation of significant p values with "blocking effects" is unclear. Based on the description of the statistical analysis given, differences between the 3 experiments (=blocks) should be analyzed as an independent effect. Thus, the significant p values may indicate differences due to treatment effects suggesting an impact on developmental time.
- Data on the susceptibility of *H. convergens* to Cry3A would be helpful to interpret the results.
- Investigations on effects of Bt-fed aphids on beneficial insects are of high ecological relevance because aphids are widely spread insect pests and serve as prey for beneficial insects. The crucial question is whether the Bt-toxins are translocated and transported in the phloem sap because only there aphids may acquire the toxins in reasonable amounts. Our own investigations on Bt-corn showed that Cry1Ab is not transported in the phloem sap (Raps et al., unpublished data).

Study 13

Riddick EW & Barbosa P 1998. Impact of Cry3A-intoxicated *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and pollen on consumption, development, and fecundity of *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Annals of the Entomological Society of America* 91 (3): 303-307.

Species names: *Coleomegilla maculata* (Coleoptera: Coccinellidae), *Leptinotarsa decemlineata* Colorado potato beetle (CPB) (Coleoptera: Chrysomelidae), transgenic potato expressing the Cry3A endotoxin

Methods

- Number of individuals used:
- a) Toxicity to CPB: n = 11 petri dishes (10 first instar CPB per petri dish), no control
 - b) Consumption of *C. maculata*: 4 different experiments:
 - 1) n = 6 per treatment (male adult *C. maculata*)
 - 2) n = 10 per treatment (primarily fourth instar of *C. maculata*)
 - 3) transgenic n = 15, non-transgenic n = 14 (primarily third instar of *C. maculata*)
 - 4) transgenic n = 14, non-transgenic n = 15 (fourth instar of *C. maculata*)
 - c) Developmental time of *C. maculata*: n = 40 second instar per treatment
- Food sources = treatments:
- a) Toxicity to CPB: 2 disks (10 mm average) of transgenic potato foliage
 - b) Consumption of *C. maculata*:
 - 1) - 3) 10 first instar of CPB per treatment previously exposed to transgenic or non-transgenic potato foliage for 24 hours
 - 4) 8 first instar of CPB previously exposed to non-transgenic potato foliage / 12 first instar previously exposed to transgenic potato foliage
 - c) Developmental time of *C. maculata*:
 - 1) intoxicated prey versus healthy prey (one neonate larvae each day or every other day, see above for intoxication)
 - 2) intoxicated prey + pollen versus pollen alone
- Duration of experiment:
- a) Toxicity to CPB: 48 hours
 - b) Consumption of *C. maculata*: 24 hours
 - c) Developmental time of *C. maculata*: 20 and 21 days
- Parameters measured:
- a) Toxicity to CPB: percentage survivors after 24 hours and 48 hours
 - b) Consumption of *C. maculata*: dry mass of prey consumed, proportion of dry mass consumed
 - c) Developmental time of *C. maculata*: proportion of *C. maculata* reaching adult / pupal stage, life weight of adults
- Repetition of the experiment: The entire experiment was not repeated over time.
- Type of experiment: bi- and tri-trophic experiment

Our comments

- *Toxicity to CPB*. No control treatment was used.
- *Consumption of C. maculata*. According to the study, larvae of CPB show temporary paralysis after 24 hours exposure to Cry3A. After 48 hours, 98% of the larvae of CPB were killed (This seems extraordinary fast). Most larvae died after 24 to 48 hours (see below), which is the time period the authors measured the consumption rate of *C. maculata*. Whether and how lethal effects on CPB may contribute to toxicity of Cry3A to *C. maculata* was not discussed.
- In addition to the dry weight of prey consumed, the numbers of prey larvae eaten would be useful to determine, because this is an indicator for the biocontrol capacity of *C. maculata*.
- *Developmental time of C. maculata*. It is not the developmental time that was measured but the survival rate until adulthood and adult weight. From the description it is not clear whether the neonate prey larvae that are added daily to the dishes are previously exposed to potato foliage for 24 hours.
- The experiment started with the second instar of *C. maculata*. The most susceptible first larval stage was not included. How the larvae were handled up to the time they were used in the experiment is not mentioned (note, that in the toxicity trial with CPB the first instar is used).
- In the second trial, pollen is provided together with intoxicated prey (it is not described which pollen) and the control treatment consisted of pollen only. Usually, carnivorous insects cannot develop on pollen alone. However, *C. maculata* has the potential to do well on pollen (D. Andow, pers. comm.). The high mortality in the experiment (see below) is suspect and might indicate suboptimal experimental methodologies. Using pollen alone as the control for the ‘pollen and prey’ treatment does not represent the appropriate control for the question investigated.
- None of the experiments were repeated over time.

Results and conclusions drawn by authors

Toxicity to CPB. After 24 hours 84%, after 48 hours only 2% of the larvae were still alive,. *Consumption of C. maculata*. In the first three experiments, significant lower amount of prey biomass was consumed when the prey was reared on transgenic potatoes. In the 4th experiment, where number of prey neonates was manipulated to account for the greater body weights of the healthy neonates, no significant differences were found. In all experiments, the actual proportion of fed prey was not significantly different. *Developmental time of C. maculata*. No significant differences were detected in the proportion developing into pupae or adults. Also, the fresh weight of teneral adults was not significantly different. In the second experiment, a greater proportion of adults emerged after consuming transgenic fed prey with pollen versus pollen alone.

As the proportion of prey consumed was not different, no real differences in consumption existed. *C. maculata* was capable of completing its development on transgenic fed prey, thus the uptake of Cry3A will have no chronic effects on *C. maculata*. A diet of transgenic fed prey with pollen resulted in a significantly greater proportion metamorphosing to adults than when reared on pollen alone. This suggests that a diet containing animal and plant material is more suitable for *C. maculata* development. The lack of any significant impact of Cry3A-intoxicated CPB on the consumption and development suggests, that *C. maculata* will not be deterred from feeding on CPB in transgenic potato fields.

Our comments

Development of *C. maculata*.

	Experiment 1		Experiment 2	
	pupae	adult	pupae	adult
Control(s) mortality:	58%	68%	80%	92%
Treatment(s) mortality:	73%	83%	68%	74%

- In contrast to the opinion of the authors, to our understanding, this study did reveal effects of Cry3A on *C. maculata*. First, the consumption rate was significantly lower on Bt-potato fed prey in experiments 1 to 3. Second, control mortality in the study was very high, and Cry3A further increased mortality of *C. maculata*. Considering that most of the prey larvae were severely affected by feeding on the transgenic potatoes, this seems not surprising. However, further experimentation needs to verify this. In general, experiments with high control mortality must be interpreted with caution because it might be an indication for inappropriate experimental conditions. Results and interpretation of experiment 2 do not allow conclusions on Bt-toxicity to *C. maculata* because of the inappropriate control used.
- Repetition of the experiments and improvement of the experimental design are strongly recommended.
- From an ecological point of view, in addition to these experiments, feeding studies using non-target herbivores as prey (for instance leaf hoppers) are recommended. These herbivores are expected to be not or only slightly susceptible to Cry3A and will therefore represent the major prey for beneficial insects in a Bt-potato field. Furthermore, complementary data on susceptibility of *C. maculata* to Cry3A (bi-trophic trials) would be desirable for a complete safety assessment.

Study 14: published

Schuler TH, Potting RPJ, Denholm I, Poppy GM 1999. Parasitoid behaviour and Bt-plants. Nature 400: 825.

Species name: *Cotesia plutellae* (Hymenoptera: Braconidae), *Plutella xylostella* (Lepidoptera: Plutellidae), oilseed rape expressing Cry1Ac (cv. Oscar, line O52).

Methods

- Number of individuals used: no choice test: no numbers given; choice experiments in the wind tunnel: approximately 40 females per bioassay
- Food sources = treatments: a) no choice test: Bt-susceptible and Bt-resistant larvae of *P. xylostella*, both feeding on leaves of wild type or Bt-oilseed rape, respectively;
b) choice test: leaves of Bt- and wild-type oilseed rape damaged by Bt-susceptible and Bt-resistant larvae of *P. xylostella* in various combinations
- Duration of experiment: a) no choice test (petri dish): 4 hours exposure of susceptible and resistant *P. xylostella* larvae to female of *C. plutellae*; afterwards larval developmental period until emergence of adults,
b) choice tests (wind tunnel): 5 min exposure of *C. plutellae* females to oilseed rape leaves
- Parameters measured: a) no choice test: performance (no. of adult parasitoids emerged)
b) choice test: feeding damage of *P. xylostella*, no. of female parasitoids landing on a leaf
- Repetition of the experiment: The entire experiment was not repeated over time.
- Type of experiment: tri-trophic study

Our comments

- To our knowledge this is the first study where the impact of transgenic Bt-plants on mortality and behavior of parasitoids was investigated. Parasitoids show a complex host location and host acceptance behavior where often volatile cues released from the plants play an important role. Therefore, these kinds of investigations in combination with toxicity studies are crucial in risk assessment of transgenic plants.
- Repetition of the experiments, mainly the investigation of the performance of the parasitoids, would be advisable.

Results and conclusions drawn by authors

No choice test: On Bt-resistant hosts, no significant differences in survival between Bt or wild type oilseed rape were found. *C. plutellae* larvae forced to develop in Bt-treated, susceptible hosts inevitably died with their hosts. -choice test: For Bt-resistant host larvae, no difference in feeding damage and parasitoid landing rate between Bt- and wild-type oilseed rape were found. Bt-susceptible host larvae feed significantly less on Bt-oilseed rape and parasitoids distinguish between the two treatments. When Bt-leaves damaged by either Bt-resistant hosts or by Bt-susceptible hosts were compared, parasitoid females preferred the ones damaged by the resistant hosts. In conclusion, behavioral factors are likely to limit the scale of the detrimental effect of Bt-oilseed rape under field conditions. The apparent lack of effect on survival or host-seeking ability of the pests enemy indicates that Bt plants may have an environmental advantage over broad spectrum insecticides. In this case, *C. plutellae* might even help to constrain the spread of genes

for Bt-resistance. The study highlights the need to consider behavioral as well as toxicological aspects when looking at side effects on non-target organisms.

Our comments

Control(s) mortality: Bt-susceptible host: 37%; Bt-resistant hosts: 44%

Treatment(s) mortality: Bt-susceptible host: 100%; Bt-resistant hosts: 46%

As long as *P. xylostella* is susceptible to Bt-oilseed rape, *C. plutellae* as a specialized antagonist is likely to disappear from these fields. The data presented mainly become relevant once *P. xylostella* has developed resistance against Cry1Ac or when refugia with non-Bt-plants are available, where *P. xylostella* can develop normally.

Nevertheless, behavioral studies like these in combination with studies of chronic, sublethal effects on non-target insects are very important to assess the complex situation in agroecosystems, like interactions between transgenic insecticidal plants, herbivores and beneficial insects.

4. Field studies

4.1. General comments on field ecological studies

Measuring field abundance of natural enemies is difficult and complex. Often different life stages of natural enemies are present with very different nutritional requirements. For many species, only the larval stages are predaceous or parasitic and the adult stages feed on pollen or nectar (examples are green lacewing adults or syrphid flies). Hence, their searching and feeding behavior is very different. Further, the adult stages of natural enemies are often highly mobile and only visit a field based on certain clues, such as presence of prey/hosts for their offspring if they are in oviposition mode. The presence of appropriate prey/hosts can be signaled by the prey/host species itself but also by the wounded host plants. If the adult natural enemy is still fairly young and not yet ready to oviposit, it may visit the field in search for food for itself such as pollen for ovary maturation or nectar, or they may be in search for appropriate mating partners. These different life stages are all associated with very different, species-specific behavioral patterns. Further, many prey/host species have several natural enemies and many natural enemies are polyphagous and feed on a number of different prey/host species while others can be very prey/host – specific and only feed on one or a few prey/host species (mono- or oligophagous). To complicate matters further, predator abundance can be driven significantly by landscape components or factors outside of the field of interest. For example, abundance of adult *Coleomegilla maculata* an important coccinellid predator (ladybird beetle) in North America, is strongly influenced by presence of tasseling corn plants and landscape structures such as hedges or trees that serve as overwintering sites.

Whether or not these intricate natural enemy – prey/host relationships work to the benefit of a farmer, i.e. exert a biological control impact, largely depends on two major factors, a qualitative and a quantitative one: 1) the spatial and temporal synchrony of the appropriate prey/host with the respective natural enemy species community (in terms of both age and size); and 2) the quantitatively appropriate ratios of natural enemies vs. prey/host densities. Slight divergences from the fine-tuned quantitative and qualitative natural enemy – prey/host synchronies can result in complete inefficiency of the system through disruption.

To investigate these intricate relationships and learn about the factors influencing their efficacy, researchers measure the species- and stage-specific abundance of natural enemies together with that of the herbivores present in the field. This is being done by sampling as often as possible in as short as feasible time intervals whatever insects occur on, near or below the plant. This can be done destructively by catching the natural enemies and herbivores in traps or non-destructively by inspecting whole plants (marked or randomly selected) and recording everything present, natural enemies and herbivores alike. The latter methods provide a momentary record on what happened to be present at that particular moment. All methods have drawbacks which is why many researchers use various methods, destructive and non-destructive sampling. Sampling methods are also chosen according to the scope of the planned study. For area wide qualitative detection of presence of species, for example, malaise traps, pheromone traps and light traps are suitable. For smaller, within field studies, individual plant sampling or D-Vac sampling methods are appropriate.

The natural enemies of the prominent and, therefore, often problematic pest species are then analyzed jointly for their seasonal population dynamics, the synchrony of appropriate predaceous life stages with appropriate prey life stages. The data are then presented as joint population curves (natural enemy and host/prey) and analyzed by regression analyses, repeated measures analyses, covariance analysis, or as natural enemy : prey/host ratios (also plotted over time. On the multi-trophic, multi-species community level, food web analyses are carried out by evaluat-

ing characteristics such as food chain lengths and species composition using various diversity indices all of which fluctuate within and over years.

However, regardless which method of sampling and analysis chosen, the abundance per plant or ratios are naturally always low and associated with high variability. This is because these types of momentary data are confounded with species-specific intra-field movement, inter-field emigration and immigration and predation behavior. These phenomena pose increasingly bigger problems as size of field plots decreases. In order to handle the large variances, sample sizes and replications must be large often pressing logistical limits. This also severely constraints statistical power because effects of 10 or 20% are seldom statistically significant while they can be of great ecological significance. Rarely, effects double or half of that in the control can be detected. Depending on the questions asked, like for example, changes in biodiversity (decline or increase) of species over time or inter-generational effects of species multiple year experiments in similar or the same location(s) are recommended as are large and multiple field plots. And even such extensive experimental set-ups may only result in reliable trends.

4.2. Unpublished studies

Study 15: state unclear (published?)

Impact of transgenic maize expressing truncated CryIAb protein on several non-target insect populations: Diptera, Hymenoptera, and Coleoptera (Coccinellid family) as well as Homopterans:

Methods

<u>Field locations:</u>	Bloomington, IL, USA
<u>No. field plots:</u>	3 replicate field plots per treatment
<u>Size field plots:</u>	one field plot: 7 m long x 3 m wide
<u>Experimental design:</u>	Monitoring of insect populations with yellow sticky traps. Two traps per plot.
<u>Cultivation practices:</u>	no information given
<u>No. of seasons investigated:</u>	1 (1993)
<u>Treatments applied:</u>	transgenic hybrid maize, isogenic hybrid maize, wild type maize, permethrin treated maize
<u>Sampling methods:</u>	not given
<u>Parameters measured:</u>	Number of total insects and numbers in the specific orders Diptera, Hymenoptera and Coleoptera (Coccinellid family) with focus on beneficial predators and parasites as well as Homopterans as an important food source.
<u>Frequencies of field checks:</u>	weekly over a 10 week period from mid-June through early September
<u>Evaluation of data:</u>	no information given

Our comments

- The plots were very small.
- Only mobile stages of insects (actively or passively flying) were caught by yellow sticky traps, thus the non-flying larval stages of predator species were not monitored.
- With this method only large differences in insect populations will be detected.
- Only one season was investigated.

Results and conclusions of the authors

Results indicated no difference in the number of total insects or the numbers of specific orders between the transgenic maize plots and either the isogenic or wild type control maize plots. There was no shift in the taxonomic distribution of insects. In contrast, treatment with permethrin had significant effects on the total numbers of insects and on the numbers within the specific groups. The beneficial lady beetles were particularly susceptible to permethrin. Coccinellids, dipterans, and hymenopterans represent the majority of beneficial insects associated with maize. The results suggest that expression of CryIAb in maize should not adversely affect insects in these groups.

Our comments

- Based on the data given it is difficult to assess the experimental design and the data analysis. It seemed that the authors have monitored insect populations on order and family level and have not distinguished between the different developmental stages.

- As expected, treatment with the conventional chemical insecticide permethrin had dramatic effects on insect populations due to its documented, high acute toxicity and wide spectrum of efficacy.
- Bt plant effects that are likely to be on a long-term, often sublethal scale, and will likely differ between individual insect species, are probably not detected by this field experiment.
- The data provided do not allow to exclude potential adverse effects of Bt-corn on beneficial insects.

Study 16

Effects of CryIAb protein on several insect populations: Diptera, Hymenoptera, Coleoptera and Lepidoptera.

Methods

<u>Field locations:</u>	Two locations in the Po valley, Italy
<u>No. field plots:</u>	3 replicate plots per treatment
<u>Size field plots:</u>	no information given
<u>Experimental design:</u>	no information given
<u>Cultivation practices:</u>	no information given
<u>No. of seasons investigated:</u>	1 (1994)
<u>Treatments applied:</u>	transgenic hybrid maize, isogenic control hybrid
<u>Sampling methods:</u>	no information given
<u>Parameters measured:</u>	Observation of insects belonging to the orders Coleoptera, Diptera, non-target Lepidoptera and Hymenoptera.
<u>Frequencies of field checks:</u>	4 times during the growing season
<u>Evaluation of data:</u>	no information given

Our comments

- The description of the methods is too rudimentary to be reviewed.
- The number of sampling times per season was low.
- Only one season was investigated.

Results and conclusions of the authors

No significant differences were observed between the plots with the genetically modified maize and the control hybrid. Only the aphid *Rhopalosiphum maidis* could not be detected later in the season probably due to presence of aphid predators. The results of the monitoring study suggest that expression of CryIAb in maize does not affect insects in these groups except for the expected impact on the target organism.

Our comments

- Based on the given facts, it is hardly possible to review the experimental design, the sampling methods used or the data analysis.
- The authors seem to have monitored insect populations on order level, which is crude for ecological investigations, and they had not distinguished between different developmental stages. This kind of insect sampling could reveal acute, instant impacts e.g. wide spectrum mortality. However, Bt plant effects, which are likely to be on a long-term, often sublethal scale, and which will likely differ between single insect species, are probably not recorded by this field experiment.
- The conclusions drawn by the authors are not supported by the information provided in the study.

Study 17

Candolfi M. et al. in prep. Effects of Bt-maize on non-target arthropods under field conditions.

Methods

<u>Field locations:</u>	Burgundy, France
<u>No. field plots:</u>	no information given
<u>Size field plots:</u>	“normal sized” agricultural maize plots (no measures given)
<u>Experimental design:</u>	Soil living animals: traps; plant inhabiting insect: direct plant counts, knock-off assays; flying arthropods: yellow traps; parasitism of European corn borer
<u>Cultivation practices:</u>	no information given
<u>No. of seasons investigated:</u>	no information given
<u>Treatments applied:</u>	Bt-176 maize, isogenic non-transgenic control hybrid, Bt-sprays, synthetic insecticides.
<u>Sampling methods:</u>	no information given
<u>Parameters measured:</u>	Arthropod fauna (Lycosidae, Linyphiidae, Opiliones, Staphylinidae, Carabidae, Cicadellidae, Thysanoptera, Anthocoridae, Nabidae, Coccinellidae, Chrysopidae and Chalcidoidae)
<u>Frequencies of field checks:</u>	no information given
<u>Evaluation of data:</u>	no information given

Our comments

- The study is not published yet and description of methods given is insufficient to be reviewed.

Results and conclusions of the authors

Bt-maize efficiently protects itself against the European corn borer. Neither reduction in quantity, nor any changes in population development could be detected in the soil and plant dwelling fauna. The analysis of the flying fauna has to be completed.

Our comments

- Based on the information given, we assume that again insects were analyzed on family level, without considering trophic relationships, which is crude for ecological investigations (including a risk assessment of Bt-maize).
- Based on the given facts it is not possible to review the experimental design, the sampling methods used or the data analysis. Statements on results of this field trial can only be made after the evaluation is completed and published.

4.3 Published studies

Study 18: Field part of study 6a-c.

Pilcher, C.D.; Obrycki, J.J.; Rice, M.E.; Lewis, L.C. 1997. Preimaginal development, survival and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology 26(2): 446-454.

Methods

<u>Field locations:</u>	2 different locations. 1994: Iowa State University, Ames, USA (Insectary field) 1995: Iowa State University, Woodruff farms, 2 km southwest of Ames, USA
<u>No. field plots:</u>	3 replicate field plots per treatment
<u>Size field plots:</u>	1994: 3 x 7.6 m; 1995: 3 x 15.2 m
<u>Experimental design:</u>	Randomized complete block design
<u>Cultivation practices:</u>	no information given
<u>No. of seasons investigated:</u>	1 (two one-season studies using different varieties)
<u>Treatments applied:</u>	1) 1994: Bt-corn from formerly Ciba Seeds (event 176), 1995: Bt-corn from Monsanto (MON 810) 2) 1994: Isogenic control from formerly Ciba Seeds; 1995: Isogenic control from Monsanto
<u>Sampling methods:</u>	6 plants/plot were marked and whole plants were visually inspected for above parameters.
<u>Parameters measured:</u>	All life stages of predatory species, numbers of <i>Ostrinia nubilalis</i> egg masses
<u>Frequencies of field checks:</u>	3 during total season (before, during and after tasseling)
<u>Evaluation of data:</u>	Mean comparison between counts on a total of 3 sampling dates; ANOVA and t-tests.

Results and conclusions of authors

1994: No differences were observed in the number of predators colonizing either isogenic control corn or transgenic Bt-corn.

1995: More predators were observed on transgenic Bt-corn than on isogenic control corn.

The authors concluded that there were no indications that the presence of transgenic Bt-corn pollen affected predator abundance. However, they also concluded that the absence of significant differences may have resulted from plot size. Due to the small plot sizes separated by only one buffer row, pollen from Bt-corn and isogenic corn may have been mixed by wind. Corn pollen is predominantly a wind-dispersed pollen. They concluded that the inconsistent results between the two years in the field indicate that additional studies on a larger scale are needed.

Our comments

- The size of the field plots was very small.
- The sample size (6 plants per plot) was very small.
- Each trial was carried out in a different location, hence, do not represent a true repetition but are two individual studies
- Number of checks (3 sampling dates during the entire season) are low .

- No information on the population dynamics, natural enemy-prey relationships, synchronies between natural enemies and their prey, etc. were obtained. This information would be useful for an environmental assessment (see chapter 4.1 for detailed commentary on field evaluation).
- In the types of study presented here, the momentary abundance (numbers per plant) of an individual, stage-specific natural enemy species can be measured, which is typically low and varies quite drastically (see also standard errors in table 4 of the original study). This is because the data are confounded with species-specific intra-field movement, inter-field emigration and immigration, and predation behavior.
- With the experimental set-up reported here, only acute, instant toxic effects may be detectable, right after the application of the toxic substance. (This is often done for efficacy tests of new synthetic insecticides with an acute mode of action.) However, Bt plant effects that are likely to be on a long-term, often sublethal scale, and will likely differ between individual insect species, are probably not detected by this type of field experiment. (Additionally, even in the most susceptible organisms, Bt toxin induces lethal toxic effects only after a number of days rather than within seconds or minutes as many synthetic insecticides mostly.)

Study 19, 20, and 21

19. Lozzia, G.C. and I.E. Rigamonti. 1998. Title: Prime osservazioni sull'artropodofauna presente in campi di mais transgenico. ATTI Giornate Fitopatologiche: 223 – 228.

20. Lozzia, G.C.; Rigamonti, I.E. and Agosti, M. 1998. Metodi di Valutazione degli Effetti di Mais Transgenico sugli Artropodi Non Bersaglio. Notiziario sulla Protezione delle piante 8: 27-39. (in Italian).

21. Lozzia, G.C. and I.E. Rigamonti 1998. Preliminary study on the effects of transgenic maize on non target species. IOBC Bulletin Vol. 21 (8): 171 – 180. (non-peer-reviewed; language: English)

Study 19 and 20 both report on the same data from 1996 and 1997 field trials.

Study 21 reports on the data from the 1996 field trials only.

Methods

<u>Field locations:</u>	4 different locations during 2 years 1996: 1) Maleo (Lodi) in Lombardy; 2) Oppeano (Verona) Veneto 1997: 3) San Genesio (Pavia); 4) Mogliano Veneto (Treviso)
<u>No. field plots:</u>	2 per location, a total of 8 fields/location (4 fields at 2 locations per year).
<u>Size field plots:</u>	10 ha each field
<u>Experimental design:</u>	Unclear; probably completely randomized design. It seems that each 10 ha field was subdivided into 4 plots of 2.5 ha. This is supported by a drawing of the field layout of the experimental plots in study 20 and 21.
<u>Cultivation practices:</u>	normal cultivation practices
<u>No. of seasons investigated:</u>	1 (each trial was carried out once at each location).
<u>Treatments applied:</u>	1) Bt-176 corn provided by formerly Ciba Seeds 2) isogenic control corn also provided by formerly Ciba Seeds
<u>Sampling methods:</u>	4 methods were used 1) pheromone traps: monitoring flight of 4 lepidopteran adults whose larvae are common lepidopteran herbivores on corn in these areas (<i>Agrotis ipsilon</i> , <i>Agrotis segetum</i> , <i>Mythimna unipuncta</i> , <i>Peridoma saucia</i>) 2) pit-fall traps: to collect epigeic insects, predominantly carabid beetles and spiders 3) D-Vac (motorized suction sampling method): to monitor foliage dwelling arthropods with emphasis on non-target pests species like aphids and leaf hoppers and beneficial insects, such as spiders, coccinellids and hymenopterans. 4) in 1997 only, additionally, malaise traps were established.
<u>Parameters measured:</u>	numbers of insects caught in each type of trap.
<u>Frequencies of field checks:</u>	during season (lasting from June until September): Pheromone traps: 1996, checked 10 times (7 to 16 days) in both locations 1997, checked 8 times in Mogliano and 7 times in S. Genesio.

Pitfall traps: 1996: 10 times in Maleo and 4 times in Oppeano
(checked every 10 – 14days)

D-Vac: 1996, a total of 4 sampling dates

1997, a total of 5 sampling dates

Evaluation of data:

Inventory of organisms collected

Our comments

In all 3 articles, the same data sets were presented in different ways and combinations. The statistics presented in all 3 papers are not systematically justified. The methods and evaluation procedures are poorly presented and confusing. While in one paper total numbers per season are given followed by a statistical analysis differing from the data set presented (for example on the seasonal, pooled data while the corresponding table listed the total numbers date-specifically), the same data set is presented in another paper in form of statistical parameters of another test only. In addition to the poor presentation of the methods, the inconsistent data presentation makes it difficult for a reader to understand what was actually done and gain a clearer own picture on the results. The following is a more detailed analysis:

- Unclear statistics and set-up of traps. Unclear what was analyzed. While it seemed that several traps per 2.5 ha plot had been established, the statistics did not report on replication effects and the data presented were pooled data per site, often across sampling dates and locations. We gather from the tables, that probably treatment main effects were tested in an ANOVA for each sampling date and a non-parametric mean comparison test was carried out to test for differences between the two treatments (Bt- and isogenic control corn).

- *Pheromone traps*. Study 19: Total number of the 4 lepidopteran species collected at 4 sites and at all sampling dates (7 to 10). Statistics (Mann-Whitney parameters, mean comparison between catches in transgenic and non-transgenic fields) on total numbers probably across entire season.

Study 20: Total numbers of the 4 lepidopteran species collected at all 4 sites across entire season. No statistics.

Study 21: Statistical parameters of ANOVA test (degrees of freedom, correlation coefficient R, F-values, p-values, Variance) carried out probably on total numbers of the 4 lepidopteran species collected at the two locations across entire season. Non-parametric mean comparison parameters (Mann-Whitney test) provided for one location only (Oppeano). Probably across entire season, comparing ‘observation posts’.

Pitfall traps. Study 19: total numbers of carabid species caught per site across entire season are given. No statistics, no detailed data on population dynamics.

Study 20: total number of carabids caught in one site (S. Genesisio) across one season (1997) are given. No statistics, no detailed data on population dynamics.

Study 21: total numbers of carabids per species, site and checking date (10 in Maleo and 4 in Oppeano) are given for 1996. P-values are given per species, site and checking date. An ANOVA testing for treatment effects between catches in transgenic and non-transgenic fields per site and date was carried out.

- *D-Vac*. Study 19: Total numbers of individuals caught at all 4 locations per sampling date in both years (1996 and 1997) are provided. Taxonomic level: order and family. Statistics (Mann-Whitney parameters) were provided for differing numbers of orders and families per location. The analyses were probably carried out on the total numbers of arthropods caught per taxa during entire season.

Study 20: Total numbers of individuals caught for one site (Mogliano, Veneto) per sampling date in one year (1997) are provided. Taxonomic level: order and family. The analyses were probably carried out on the total numbers of arthropods caught per taxa during entire season.

Study 21: Total numbers of individuals caught at 2 locations per sampling date in one year (1996) are provided. Taxonomic level: order and family. Statistics (p-values of ANOVA test) were provided for 9 orders and families per location and sampling date.

- *Malaise traps*. Study 19: Total numbers of individuals caught at 3 sampling dates in one year (1997) are provided. Taxonomic level: order and family. One location only (Mogliano). Statistics (Mann-Whitney parameters, mean comparison between catches in transgenic and non-transgenic fields) on total numbers of individuals out of 4 orders and families probably across entire season. No significances detected.

Study 20: Total numbers of individuals caught at 3 sampling dates in one year (1997) are provided. Taxonomic level: order and family. One location only (Mogliano). Statistics (Mann-Whitney parameters, mean comparison between catches in transgenic and non-transgenic fields) on total numbers of individuals out of 6 orders and families probably across entire season.

Results and conclusions of authors

No significant differences between insects caught in transgenic and non transgenic fields were found.

The authors concluded that on the basis of the results, it is important to continue the research in the same fields.

Study 21: The authors acknowledge that the data was only taken during 1 year and that the observed variability in the data makes it necessary to carry out further experiments in more depth and detail before reaching sound conclusions.

Our comments

- Studies 19-21 and 28 report on data of field trials carried out over a three year period (study 19 describes the 1996 data only, studies 20 and 21 report both about the 1996 and 1997 data and study 28 describes the 1997 and 1998 data).
- *Detail of data and taxonomic level*. Only the data on the pheromone catches of 4 adult noctuid species (study 21) and the carabid species of all pitfall catches (study 19 and 20) are presented in somewhat more detail. All other data regarding the insects caught on the foliage in the fields were presented on a rather crude taxonomic level, i.e. mostly order, occasionally family level (study 19: tables 7-13; study 20: tables 3-6; study 21: tables 10-13). The order Heteroptera and Diptera, for example, comprise both pest and natural enemy species that have not been differentiated. Also 'Hymenoptera parasitoids' is a very large group including highly specific parasitoids that are very meaningful for an agroecosystem. 'Other Hymenoptera' comprises for example honeybees and bumble bees, more detailed information on these species would also be very desirable. Further, the authors did not report data differentiated by life stage, which is crucial for an ecological assessment. The adult stages of Dipteran natural enemy species are often non-predaceous while their larvae are important predators (for example syrphid flies). All of the more detailed data is important for an assessment of the ecological 'quality' of the fauna present in an agroecosystem. Sheer numbers alone on a order and family level have a limited meaning in population and community ecology.
- *Sampling frequencies*. 4 and 5 sampling dates (1 sampling per month) during 1996 field season and 6 sampling dates during the 1997 field season both lasting from May through September (4-6 months) is insufficient to carry out population dynamic analyses (which was consequently not done in these studies). But the major parameters of interest regarding a new pest management tactic involving the large scale release of a toxin into the agroecosystem is how natural regulation by natural enemies may be affected. However, it was one of the objectives of the study to test for negative effects on beneficial insects that could 'question the va-

lidity of the possibility of a practical application on a large scale' (Introduction study 21). However, for an evaluation on predator/parasite – prey/host dynamics and the degree of disruptedness or synchrony of natural regulation processes, a joint analysis of BOTH in-field, relevant prey and predator abundance is necessary (covariance analyses of prey-predator ratios, density dependent or independent population fluctuations, analyses of population curves, etc. see 'general comments' above). These abundances have to be measured on a as frequent schedule as is logistically possible but at least weekly or twice a week.

- *Precision of sampling technique.* In general, the more frequent and detailed a sampling technique is the more intricate relationships can be monitored. Pheromone, light or malaise traps are suitable techniques for area-wide, multiple year monitoring of certain insect species attracted by the volatile substances or light over a large area used repeatedly in the same locations. However, for one-year studies aimed at detecting differences on a plot or field level, they lack precision.

Conclusion

Based on sampling frequency, precision of sampling techniques chosen, data detail presented and lack of multi-seasonal investigations, no conclusions can be drawn yet on the long-term impact of insecticidal transgenic Bt-corn on non-target insects and natural regulation mechanisms. No detailed natural enemy–prey interactions or specific non-target herbivore population developments were monitored.

Study 22

Orr, D.B. and Landis, D.L. 1997. Oviposition of European Corn Borer (Lepidoptera: Pyralidae) and impact of natural enemy populations in transgenic versus isogenic corn. *Journal of Economic Entomology* 90 (4): 905-909.

Methods

<u>Field locations:</u>	1 = Hensell farm near Constantine, Michigan, USA. In a center-pivot irrigated soybean field at least 201.3 m away from the nearest commercial corn field.
<u>No. field plots:</u>	6 plots total; 3 plots per treatment (3 replications). Arranged in a 2 x 3 pattern. Each plot surrounded by a 4.58 m border of annual rye grass and entire experiment surrounded by a 4.58 m strip of isogenic control corn.
<u>Size field plots:</u>	0.405 ha (64.05 x 62.83 m); 84 rows wide.
<u>Experimental design:</u>	Completely randomized design (CRD) with 3 replications
<u>Cultivation practices:</u>	conventional agronomic practices, recommended application of herbicide and fertilizer
<u>No. of seasons investigated:</u>	1 (1994)
<u>Treatments applied:</u>	1) Cry1Ab corn by formerly Ciba Seeds (Event 176) 2) Isogenic control hybrids
<u>Sampling methods:</u>	Whole-plant visual counts
<u>Parameters measured:</u>	Within each plot, 2 sample sites spaced 21 m apart were marked in 5 rows (row 21, 32, 43, 54 and 65). One sample site consisted of 5 consecutive corn plants. This resulted in a total of 10 5-plant sites per field and 30 5-plant sites per treatment. Whole plants were inspected and number of <i>O. nubilalis</i> egg masses and egg per egg mass were counted. Plant sizes and leaf surface area were measured. Predator sampling: '2 scouts, 1 on each side of a sampled row, counted potential predator numbers. <i>Orius insidiosus</i> , coccinellids (almost exclusively <i>Coleomegilla maculata</i>) and <i>C. carnea</i> larvae were sampled. Parasitism: Since <i>O. nubilalis</i> larvae will die soon in a transgenic field, isogenic plants were grown within a transgenic plot in so called 'microplots' (= 4 adjacent plants across 5 rows). 3 microplots per plot. Laboratory reared <i>O. nubilalis</i> egg masses in the 'blackhead' stage were brought out on August 19 (i.e. they would hatch within the next 24 hours on Aug. 20) and allowed to hatch on these plants. All larvae were removed from 1 plant in each row of the microplot on two sampling dates August 26 and September 15. Larvae were reared to adulthood or until parasite emerged. Percentage of parasitism was calculated from that data.
<u>Frequencies of field checks:</u>	during one field season: 3 dates (chosen to fall near the beginning, peak and end of <i>O. nubilalis</i> ovipositional period (measured at another field station nearby).
<u>Evaluation of data:</u>	Means for each plot for oviposition, egg fate, predator counts and larval parasitism.

ANOVAs were carried out on the plot means to compare the plant types. Distribution of *O. nubilalis* oviposition was analyzed by analysis of covariance (ANCOVA). Plant sizes were analyzed with 2-tailed Student t-test.

Results and conclusions of authors

No significant differences in *O. nubilalis* egg populations, or its predators or parasitoids were observed. Mortality factors (i.e. exerted by predators) were consistent in all plots, therefore, corn type had no impact on these factors. The authors suggest that all measured predator species are attracted to the corn pollen regardless of which variety and, therefore, density independence is suggested. Also larval parasitism was not significantly different and therefore probably density-independent.

Authors acknowledge that plot sizes may not be large enough to account for landscape-scale population effects but could still be used to assist in development of strategies for managing *B. thuringiensis* resistance and conserving natural enemies.

Our comments

- This was a one-year and one-field experiment with sampling carried out on 3 dates during the entire season. Although, sampling was designed to explore the prey–predator dynamics around the peak oviposition period of *O. nubilalis*, the sampling dates were still spaced temporally so distantly apart that a joint population dynamics analysis of both the observed prey and predators is not possible. Therefore, the individual species were analyzed independently.
- The sampling methods focused on a reasonable number of parameters and were designed to detect within-plot differences and did not attempt to measure “everything”. Also field plot sizes were reasonably large.
- In Table 3 of the original publication, the authors list the fate in terms of parasitism of 2nd generation *O. nubilalis* larvae collected from isogenic and Bt-transgenic plots. While in all isogenic plots, apparently, two parasitoid species were detected, *E. terebrans* and *M. grandii*, in transgenic Bt-plots only one is observed, *E. terebrans*. This observation goes unmentioned and unexplained.
- Field trials were not repeated for another year. From this one year field trial no long-term population developments of non-target insect populations including both herbivores and natural enemies can be concluded.

Study 23

Hoffmann MP, Zalom FG, Wilson LT, Smilanick JM, Malyj LD, Kiser J, Hilder VA, Barnes WM 1992. Field evaluation of transgenic tobacco containing genes encoding *Bacillus thuringiensis* δ -endotoxin or cowpea trypsin inhibitor: efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* 85 (6): 2516-2522.

<u>Field locations:</u>	near Woodland, California, USA
<u>No. of field plots:</u>	1 field, divided into 4 blocks
<u>Size of field plots:</u>	1 block approximately 41 m length x 12 m wide, 1 plot = 12 plants in a row, spaced 55 cm apart
<u>Experimental design:</u>	randomized complete block design, 4 replicates; 48 plots per block (6 plots x 8 rows) = 48 treatments (see below); plants were infested with eggs of <i>H. zea</i> (100 and 200 per plant) on 4 different dates. Thereby, predators (mainly Nabidae) that were present at the uppermost parts of the plants were removed and the <i>H. zea</i> eggs were protected with a plastic bag for the first 7 to 10 days.
<u>Cultivation practices:</u>	insecticides applications: carbaryl (24.8), diazinon (29.8.) for control of flea beetle
<u>No. of seasons investigated:</u>	1 (1989)
<u>Treatments applied:</u>	a) genotype: 4 different genotypes of tobacco (Samsun / Samsun + CptI; Xanthia / Xanthia + Bt); (Bt: Cry protein from Bt-strain HD 73) b) types of infestation (with eggs of <i>H. zea</i>): "seasonal" (weekly samples); "harvest" (undisturbed until harvest); "none" (no infestation) c) times of infestation: 4 dates (15. Sept.; 22.Sept.; 2.Oct.; 9.Oct.)
<u>Sampling methods:</u>	destructive plant sampling of 3 randomly selected plants per plot (plants were cut at soil level, put into bags and analyzed in the lab).
<u>Parameters measured:</u>	"seasonal"/"none": no. of insects collected (<i>H. zea</i> , Nabidae, Aphididae, flea beetle (Chrysomelidae); "harvest": no. of <i>H. zea</i> larvae found on 8 plants per plot (15.11.) and leaf damage of <i>H. zea</i> on 3 uppermost leaves of each of 4 plants per treatment (31.10.); "none": leaf damage of <i>H. zea</i> on 3 uppermost leaves of 36 to 48 leaves
<u>Frequencies of field checks:</u>	weekly, after infestation <ul style="list-style-type: none">• <i>H. zea</i>: "seasonal": 4 times; uninfested plots fewer (not described)• other insects: "seasonal", "none": first 2 observations following infestation; pooled (n=8);• "harvest": 15. Nov. and 31.Oct.
<u>Evaluation of data:</u>	repeated measurement ANOVA for genotype, date of infestation, block, sample, week and interactions. Data subsequently were analyzed within the sample week (average across infestation date) and by each combination of sample week and infestation date. Data from "harvest" evaluation were subjected to ANOVA.

Results and conclusions of authors

Under "Data summary and Statistical Analysis" the authors mentioned that "...Potato virus Y infection was apparent throughout the field but was most common in one block. ... It was assumed that the indirect effect (if any) of the virus on insect abundance would be accounted for by the blocked experimental design" (This infection was not considered when results were discussed.). The results demonstrated that transgenic tobacco was efficacious against artificial infestations of *H. zea*, the level of control obtained with Xanthi + Bt was excellent. Neither Xanthi+Bt nor Samsun + CpTI had an apparent effect on the number of naturally occurring Nabidae, Aphididae and Chrysomelidae. In conclusion, transgenic crop plants have many advantages, but resistance development should be considered.

Our comments

This early field experiment with tobacco as a model plant was designed and analyzed for efficacy of transgenic plants expressing promising insecticidal proteins against *H. zea*. Hence, emphasis was put on the abundance of and damage caused by *H. zea*. In contrast, abundance of non-target insects was investigated incidentally. Only mean numbers (+ S.E.M.) on transgenic and control genotypes were given and the results were not discussed.

From this experiment, no conclusions can be drawn about non-target effects on beneficial insects because:

- the plots were very small
- the treatment with insecticides at the beginning of the experiment may have had an impact on insect populations, especially naturally enemies, but was not considered in the discussion of the results.
- the effect of the transgenic genotypes on predators of the family Nabidae was investigated during the first two weeks after infestation with *H. zea*. However, all Nabidae were initially removed from the plants and plants were protected with bags during the first week. Both manipulations may have influenced the abundance and distribution of this predators but are not considered in interpretation of the results.
- only mean numbers over time and space (blocks) of Nabidae, Aphididae and Chrysomelidae were given. Species and developmental stages were not differentiated and predator/prey-relationships, time curves, statistical analysis and discussion of the results were missing.
- a virus infection may have significant impact on the abundance of insects and should be considered in interpretation of the results.
- only one season was investigated

Study 24

Wilson FD, Flint HM, Deaton WR, Fischhoff DA, Perlak FJ, Armstrong TA, Fuchs RL, Berberich SA, Parks NJ, Stapp BR 1992. Resistance of cotton lines containing a *Bacillus thuringiensis* toxin to pink bollworm (Lepidoptera: Gelechiidae) and other insects. *Journal of Economic Entomology* 85 (4): 1516-1521.

Methods

<u>Field locations:</u>	University of Arizona Maricopa Agricultural Center, Pinal County, Arizona, USA
<u>No. of field plots:</u>	1
<u>Size of field plots:</u>	Subplot size was 4 rows, each 9.1 m long, spaced 1.01 m apart
<u>Experimental design:</u>	Split plot design with 6 replicates; insecticide treatment and no insecticide treatment for lepidopterous insects as main plots and cotton lines as subplots.
<u>Cultivation practices:</u>	The entire experiment was treated once with acephate (against <i>Lygus hesperus</i>)
<u>No. of seasons investigated:</u>	1 (1990)
<u>Treatments applied:</u>	cotton lines: <ul style="list-style-type: none">• three experimental lines '62', '65', and '82' of Monsanto, each expressing Cry1Ab endotoxin.• control: Coker 312, MD51ne The whole field plot was surrounded by a 30.5 m wide belt of non-transgenic cotton. insecticide treatments: at the beginning application of Methyl parathion, 4 weeks later bifenthrin, then in weekly intervals bifenthrin and lambda-cyhalothrin A total of 22000 pink bollworm moths were released into the plots during the first 4 weeks of the experiment to establish an early-season population.
<u>Sampling methods:</u>	Sweep net sampling (40 sweeps per plot)
<u>Parameters measured:</u>	Number of beneficial insects caught in sweep nets (beside other parameters)
<u>Frequencies of field checks:</u>	weekly, 5 to 6 times per season
<u>Evaluation of data:</u>	ANOVA for a split-plot design with insecticide treatment versus no treatment as main plots and cotton lines as subplots

Results and conclusions of the authors

Beneficial insect populations were low (< 6 per 40 sweeps) on all the cotton lines for the entire sampling period. An exception was the sample of July the 5th, where fewer *Geocoris* spp. were found on MD51ne than on Coker 312 or on the three transgenic lines. Number of beneficial insects were very low (< 6 per 40 sweeps) for the entire sampling period. The transgenic lines apparently had little effect on populations of beneficial predator insects.

Our comments

This field experiment was designed and analyzed for efficacy of transgenic Bt-cotton against the pink bollworm *Pectinophora gossypiella* and other lepidopterous pests. Non-target effects on

beneficial insects were investigated incidentally. From this experiment, no conclusions can be drawn about non-target effects on beneficial insects because:

- The experiment was not designed to study non-target effects.
- Numbers of beneficial insects were generally low, and no data and statistical analyses are given. Instead, the species found were listed (*Chrysoperla carnea*, *Collops vittatus*, *Hippodamia convergens*, *Nabis* spp., *Orius tristicolor*). Potential reasons of the low abundance of beneficial insects are not discussed.
- Only one season was investigated.

Study 25

Fitt GP, Mares CL, Llewellyn DJ 1994. Field evaluation and potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia. *Biocontrol Science and Technology* 4: 535-548.

Methods

<u>Field locations:</u>	Narrabri Agricultural Research Station, New South Wales, Australia
<u>No. of field plots:</u>	200 transgenic plants comprising 6 Bt-cotton varieties in the center of an 8 ha unsprayed field of conventionally planted non-transgenic cotton
<u>Size field plots:</u>	200 Bt-plants (6 lines) / 200 non-transgenic plants (the respective 6 control lines)
<u>Experimental design:</u>	Two replicate rows of each variety, row spacing 1 m; within each row the respective Bt+/Bt- varieties were planted at random at a density of 2.5 plants per meter (commercial planting 10-12 plants per meter). Plants were individually tagged and numbered for identification throughout the season.
<u>Cultivation practices:</u>	Fertilized and irrigated as for commercial cotton. Two late season applications of the organophosphate dimethoate (aphid control, <i>Aphis gossypii</i>); pesticide drift from nearby commercial fields during January/February, which influenced insect abundance and disrupted some aspects of the study.
<u>No. of seasons investigated:</u>	1 (1992/93 growing season)
<u>Treatments applied:</u>	6 varieties transformed with the Cry1Ab and their respective control lines
<u>Sampling methods:</u>	Whole-plant visual counts
<u>Parameters measured:</u>	abundance of <i>Helicoverpa</i> , other pests and beneficial insects was recorded of 20 transgenic and 20 non-transgenic (240 in total) plants.
<u>Frequencies of field checks:</u>	weekly
<u>Evaluation of data:</u>	time curves of abundance of the major predatory groups (spiders, predatory bugs, predatory beetles).

Results and conclusions of the authors

Numbers of beneficial insects were similar on control and transgenic plants; the impact of spray drift disrupted beneficials clearly. As expected from the known toxicity spectrum of Bt, there was little impact on abundance of beneficial insects. However, studies with larger areas are needed to fully evaluate such effects.

Our comments

From this experiment, no conclusions can be drawn about non-target effects on beneficial insects because:

- The field plots were very small (approximately 30 plants per variety).
- The statistical analysis is missing.
- The analysis of beneficial abundance is very crude - on group levels, where it is not clear to which families the 'spiders, bugs and beetles' belong. No discrimination of developmental stages was made.

- Considering the mobility of the insects investigated, it is questionable, whether it will be possible to find any effects at all in seed mixtures that are surrounded by non-transgenic plants.
- According to the data presented in the paper, it looks like fewer beneficial insects were found on transgenic plants. However, since data are not statistically analyzed no statements can be made.
- As the authors also concluded, field experiments on a larger scale are necessary to draw conclusions. Fewer numbers of beneficials on the transgenic plants should motivate a more careful examination into non-target effects.
- Only one season was investigated

Study 26

Hardee DD & Bryan WW 1997. Influence of *Bacillus thuringiensis*-transgenic and nectariless cotton on insect populations with emphasis on the tarnished plant bug (Heteroptera: Miridae). Journal of Economic Entomology 90 (2): 663-668.

Methods

<u>Field locations:</u>	1 km (field plots 1 and 2) and 3 km (field plots 3 and 4) east of USDA-ARS Jamie Whitten Delta States Research Center at Stoneville, Mississippi, USA		
<u>No. of field plots:</u>	4 sites		
<u>Size of field plots:</u>	0.5 ha - 1.5 ha per variety at each site		
<u>Experimental design:</u>	randomized complete block design, with 2 replications of 3 (1994) and 4 (1995) cotton cultivars at each site; outside rows or ends of plots of Bt-cotton were bordered with 24.4 m of the non-transgenic nectariless variety MD51ne.		
<u>Cultivation practices:</u>	multiple insecticide applications each year, beginning with the start of the experiment and then throughout the season with acetate, oxamyl, and dicotophos in varying intervals (ranging from weekly to monthly applications)		
<u>No. of seasons investigated:</u>	1; two one year field studies (1994, 1995) using different Bt varieties		
<u>Treatments applied:</u>		<u>1994</u>	<u>1995</u>
	1) nectariless/ high fibred variety	MD51ne	MD51ne
	2) Bt	line 757	NuCotn33
	3) control	DES 119 or SureGrow 501	DP5415 (background for NuCotn33) Coker 312 (background for line 757)
<u>Sampling methods:</u>	Sweep net sampling (4 sets of 25 sweeps per plot).		
<u>Parameters measured:</u>	Abundance of beneficial insects, and others (fruiting characteristics, abundance of tarnished plant bug, bollworms and budworms). In both years, approximately 10 times during the growing season, beneficial insects were collected with a sweep net. All beneficial insects (predators) and spiders were counted as one group with no attempt to separate them by species. Adults and immatures were counted but not recorded separately by life stage.		
<u>Frequencies of field checks:</u>	weekly		
<u>Evaluation of data:</u>	During the first 3 weeks of observation, many data points were zero and were excluded from the analysis. For each sampling date and observation made an ANOVA was computed, means were compared by least significant differences (LSD) at the 0.05% level.		

Results and conclusions of the authors

It appeared that in 1994 fewer beneficials were recorded in line 757 than in grower varieties (not significant) and significantly fewer in MD51ne in 1995 than in other varieties. Analyses for 'location by treatment interaction' each year showed that the interaction was seldom significant,

indicating the validity of using plots at different locations as replications. Transgenic cotton have a promising future in management of bollworm, budworm and cabbage loopers, the transgenic character itself did not cause an increase of any insect pest population.

Our comments

This study was designed and analyzed to investigate the efficacy of Bt-cotton against its main pests and the potential of development of secondary pests. Emphasis was placed on the abundance of the tarnished plant bug and the boll weevil, which may become more important when fewer or no sprays against bollworm and budworms are used in Bt-cotton. In contrast, abundance of non-target insects was investigated incidentally. From this experiment, no conclusions can be drawn about non-target effects on beneficial insects because:

- As a result only the total mean for all beneficial insects (BENF) found on the different varieties per year is given.
- The statistical analysis is missing.
- The beneficial insects were not discriminated by species or by developmental stages.
- The high insecticide input is likely to have had an impact on abundance of beneficial insects but is not considered in interpretation of the results.
- Although two growing seasons have been investigated, only one season was investigated per variety.

Study 27

Riddick EW, Dively G, Barbosa P 1998. Effect of a seed-mix deployment of Cry3A-transgenic and non-transgenic potato on the abundance of *Lebia grandis* (Coleoptera: Carabidae) and *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Annals of the Entomological Society of America* 91 (5): 647-653.

Methods

<u>Field locations:</u>	3 experimental farms of the Central Maryland Research Education Center: Upper Marlboro (Prince Georges County), Beltsville (Prince Georges County), Ellicott City (Howard County), USA
<u>No. of field plots:</u>	4 isolated fields on each farm, approximately 500 m apart
<u>Size of field plots:</u>	one field approximately 0.05 ha (22 x 22 m); 24 rows, 23 m long
<u>Experimental design:</u>	one field comprises a seed mixture of different proportions of transgenic and non-transgenic plants (see treatments)
<u>Cultivation practices:</u>	in both years insecticide applications; in the control fields application of Esfenvalerate twice per season (against Colorado potato beetle (CPB)), all fields once with Dimethoate (against the potato leafhopper), which is toxic to Coleoptera.
<u>No. of seasons investigated:</u>	2 (1994, 1995)
<u>Treatments applied:</u>	Bt-potatoes expressing the Cry3A endotoxin and the respective control line, planted in different proportions per field: 100% non-transgenic, 50% non-transgenic/ 50% Bt-potatoes, 30% non-transgenic / 70% Bt-potatoes, 100% Bt-potatoes. Abundance of CPB was manipulated by releases in 1992, 1993 (1 adult/plant in the non-transgenic fields) and by translocations from non-transgenic fields to the other ones in 1994/95 (in 1995 CPB release to seed mixtures only because of 100% mortality in the pure Bt field).
<u>Sampling methods:</u>	Sweep net sampling and visual counts (1995 only) (predators), pitfall traps (Carabid beetles)
<u>Parameters measured:</u>	numbers of predators and carabid beetles
<u>Frequencies of field checks:</u>	sweep nets at approximately weekly intervals; visual counts 11 (Upper Marlboro, Beltsville) or 8 (Clarksville) times; pitfall traps (exposed for 48 hours in 1994, 24 hours in 1995) at intervals between 7 days (1994) and 10 days (1995) during the season.
<u>Evaluation of data:</u>	Data were pooled and means computed for each season because of low abundance of both predators on many of the sampling dates. Kruskal-Wallis test was applied to test for significance and the Dunn test (non parametric multicomparison test) to detect any differences between treatment means. The Spearman Rank Correlation coefficient was used to identify any trends between species abundances.

Results and conclusions of the authors

L. grandis adults were significantly more abundant in the pure non-transgenic fields than in the 50:50% mixture and was practically absent in the 30:70% mixture or the pure Bt field. In contrast, *C. maculata* was equally distributed over the treatments in both years. A positive correlation occurred between *L. grandis* and *L. decemlineata* for both years. Adult and nymphal leaf-

hoppers, primarily *Empoasca fabae*, caught in the sweep net were significantly more abundant on foliage in the seed mixtures and in the pure Bt fields. This indicates that *E. fabae* has to be controlled in transgenic potato fields. In conclusion, *L. grandis* will not persist in 100% potato fields and is likely to disperse from these fields rapidly. However, *C. maculata* will likely thrive and flourish in fields with Bt potato.

Our comments

- This field study was designed and analyzed for abundance of two predatory beetle species differing in specification for the Colorado potato beetle (CPB). Two growing seasons were investigated and statistical analysis was given. The predators were determined to species levels but they were not discriminated for developmental stages. No time curve of abundances were given.
- Bt-potatoes are designed to protect themselves from its main insect pest, the CPB *L. decemlineata*. It is highly susceptible to the Cry3A toxin expressed in the transgenic plants and usually 100% of the larvae are killed. With the elimination of the target pest, specialized antagonists of CPB will also be eliminated because they can no longer find suitable hosts. Thus, the results of this study is not surprising: abundance of *L. grandis* as a specific predator of *L. decemlineata* decreases when no hosts are available, the polyphagous predator *C. maculata* may switch to another host and will persist.
- However, based on these data it cannot be concluded that *C. maculata* will "flourish and thrive". Abundance of *C. maculata* in general was very low in both years and analysis of the data is too crude. Pooling over 3 sites ignores differences in environmental conditions and by averaging over the sampling time a lot of information is lost. No data are given about the susceptibility of *C. maculata* against Cry3A (and other Cry proteins expressed in transgenic crops) although this information is crucial to assess potential long-term sublethal effects on this polyphagous predator. In general, fitness and abundance of general predators like *C. maculata* depends on their susceptibility to the Cry toxins passed on to them via their prey, and on the availability of suitable prey. Therefore, susceptibility of non-target herbivores is an important parameter in risk assessment of transgenic plants, and time curves on population development and analysis of predator-prey dynamics are desirable.

Study 28

Lozzia GC 1999. Biodiversity and structure of ground beetle assemblages (Coleoptera Carabidae) in Bt corn and its effects on target insects. *Boll. Zool. Agr. Bachic. Ser. II*, 31 (1): 37-58.

Methods

<u>Field locations:</u>	North Italy, near Pavia and Treviso
<u>No. of field plots:</u>	4 (2 fields at each site)
<u>Size of field plots:</u>	10 ha per field
<u>Experimental design:</u>	a field was divided into 4 plots of 2.5 ha = 4 replicates
<u>Cultivation practices:</u>	not described
<u>No. of seasons investigated:</u>	2 (1997, 1998)
<u>Treatments applied:</u>	1) Bt-corn (Event 176) 2) non-transgenic corn (isogenic line)
<u>Sampling methods:</u>	Carabidae: pitfall traps (one trap per subplot) aerial fauna: <i>blower vac-aspirator</i> (2 minutes per block); <i>malaise traps</i> (one per field); <i>visual counts</i> (10 randomly sampled plants / replicate) During the first trial, the most susceptible first larval stage was not included. Reasons for this and details of how the larvae were handled up to the time they were used in the experiment were not given.
<u>Parameters measured:</u>	Carabidae: number of insects caught (determined to species level) aerial fauna: number of insects caught (determined to order/family level)
<u>Frequencies of field checks:</u>	approximately every 14 days from sowing to harvest
<u>Evaluation of data:</u>	General linear models (ANOVA) with corn hybrid (transgenic/non-transgenic), locality (Pavia, Treviso) and year (1997, 1998) as factors. Diversity of Carabidae was determined using biodiversity indices (Shannon-Weaver, Hill diversity numbers, Simpsons dominance index, Margalefs species-richness, Sørensen index). Aerial fauna was analyzed with Mann-Whitney test (pooled over time, locality, year).

Our comments

- Description of methods, analysis of data and presentation of results is confusing and not clear.
- A description of the environment of the different fields and the distance between them or the locations would be helpful because surroundings may be important for diversity of Carabidae. Also, cultivation practices are not described.
- Sampling of only 10 plants at 4 locations in a 10 ha field is very low.

Results and conclusions of the authors (original citation)

Carabid numbers. The number of carabids in transgenic corn was higher than that collected in isogenic corn, except for Treviso 1998. Carabids were more abundant in Pavia than Treviso. Although the number of captures was quite different, no statistical difference was evident considering the total number of carabids and the hybrid type. The ground beetle assemblages in transgenic and isogenic corn crops in Pavia in 1997 were quite similar. In 1998, the specific spectrum was rather restricted, especially in Treviso, probably due to a rather dry season. In both corn crops, the abundance of many species was less than 3% of the total catch, and several species

were caught in one hybrid field only. Although the number of catches and ground beetle assemblages were quite different, this demonstrates the similarity of the isogenic and transgenic corn. The data for both years show that *Pseudophonus rufipes* was more abundant in transgenic corn than in isogenic corn. -*Species richness/Diversity*. Carabidae diversity was relatively low. The Shannon-Weaver index for both years was higher for isogenic corn than for transgenic corn, being more prominent in Treviso. Also, the Hills index N2 was generally higher for isogenic corn than for transgenic corn. There was no decreasing trend in the biodiversity indices from the first to the second year and considering the data as a whole, the two years appear comparable. The difference in biodiversity recorded for some indices is not due to the presence of transgenic corn. -*Aerial fauna. Blower-vac aspirator*. The arthropofauna as a whole (calculated as a whole for both years and both localities) was not different. Similarly, abundance of aphids, leaf hoppers, other Homoptera, thrips, leaf beetles, spiders, lady bird beetles, Hymenoptera parasitoids, other Hymenoptera, and Diptera were not different (calculated for both years and for both localities). *Malaise traps*. The number of arthropods was higher in the transgenic corn, though not significant. *Visual counts*. The abundance of coccinellid larvae (only *Adalia bipunctata*) was not statistically different.

All the sampling methods and the statistical analysis, supported by visual checking, show that there was no significant difference in abundance, composition or biodiversity of non target arthropods in isogenic and transgenic corn crops.

The results show that there is (original citation) “no potential for plant antibiosis, neither to influence phytophagous species nor for predatory species over the 3 or 4 trophic levels. ... From the results it appears that the transgenic character itself does not lead to an increase or decrease of any insect populations. ... This means that Cry1Ab proteins do not directly affect the phytophagous species nor does it have any indirect influence on other trophic levels or activities such as behavior, oviposition, predators-prey.”

Our comments

- Studies 19-21 and 28 report on data of field trials carried out over a three year period (study 19 describes the 1996 data only, studies 20 and 21 report both about the 1996 and 1997 data and study 28 describes the 1997 and 1998 data).
- By pooling data over years, localities and sampling times a lot of information is lost. Moreover, pooling of data should not be done without statistical justification that is when no significant difference between the data sets to be pooled has been demonstrated. But even then it is better not to pool data. No analysis on differences between the sites and years are given.
- *Carabid beetles*. Regarding interpretation of the data, the author's arguments are inconsistent: while high levels of captures of *P. rufipes* in transgenic fields demonstrate the absence of negative effects, differences in biodiversity recorded for some indices is not due to the presence of transgenic corn. Both statements were made without further explanations.
- Generally, the data for Treviso in 1998 should be treated with caution because very few individuals were found during the whole growing season (a total of 29 in isogenic corn and 43 in transgenic corn). The abundance of *P. rufipes* only seemed to be higher in the Bt-field in 1998 in Pavia (1 out of 3). The diversity indices for Pavia for both years gave inconsistent results, for Treviso in 1997, the biodiversity indices seemed to be lower in transgenic crops.
- -*Aerial fauna*. The provided results do not allow to draw conclusions regarding plant antibiosis, population in- or decreases, behavior and oviposition of beneficial insects or predator-prey dynamics. None of these parameters have been investigated.
- From this experiment, no conclusions can be drawn about non-target effects on beneficial insects because:

- description of arthropod fauna is too crude, insect groups are insufficiently specified - some only on order level.
- the beneficial insects were not differentiated by species or developmental stage.
- statistical analyses of pooled data over years, locations and sampling dates is too crude to provide meaningful results.

5. References

- Behle, R. W., M. R. McGuire, & B. S. Shasha. 1997. Effects of sunlight and simulated rain on residual activity of *Bacillus thuringiensis* formulations. *Journal of Economic Entomology* 90: 1560-1566.
- Burton, S. L., D. J. Ellar, J. Li and D. J. Derbyshire. 1999. *N*-Acetylgalactosamine on the Putative Insect Receptor Aminopeptidase N is Recognised by a Site on the Domain III Lectin-like Fold of a *Bacillus thuringiensis* Insecticidal Toxin. *Journal of Molecular Biology* 287: 1011-1022.
- Crailsheim, K. 1992. The flow of jelly within a honeybee colony. *J. Comp. Physiol. B.* 162: 681-689.
- Donegan, K. K., C. J. Palm, V. J. Fieland, L. A. Porteus, L. M. Ganio, D. L. Schaller, L. Q. Bucao & R. J. Seidler. 1995. Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var *kurstaki* endotoxin. *Applied Soil Ecology* 2: 111-124.
- Feitelson, J. S., J. Payne, and L. Kim. 1992. *Bacillus thuringiensis*: insects and beyond. *Biotechnology* 10: 271-275.
- Fent, K. 1998. Chapter 3 Allgemeine Prinzipien der Ökotoxikologie. In: Ökotoxikologie. Umweltchemie Toxikologie Ökologie. Georg Thieme Verlag. Stuttgart, New York: 51-76.
- Goldburg, R. J., and G. Tjaden. 1990. Are B.T.K. plants really safe to eat? *Bio/Technology* 8: 1011-1015.
- Haider, M. Z., B. H. Knowles, and D. J. Ellar. 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal delta-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *Eur. J. Biochem.* 156: 531-540.
- Haydak, M. H. 1970. Honeybee Nutrition. *Ann. Rev. Entomol.* 15: 143-156.
- Ignoffo, C. M. & C. Garcia, 1978. UV-photoinactivation of cells and spores of *Bacillus thuringiensis* and effects of peroxidase on inactivation. *Environmental Entomology* 7: 270-272.
- IOBC Global Working Group Newsletter 1. 1999. Global Working Group on 'Transgenic Organisms in Integrated Pest Management and Biological Control', eds. A. Hilbeck and A. Raps, EcoStrat GmbH, Zurich (formerly Swiss Federal Research Station, Zurich), Switzerland.
- Jepson, P. C., B. A. Croft, and G. E. Pratt. 1994. Test systems to determine the ecological risks posed by toxin release from *Bacillus thuringiensis* genes in crop plants. *Molecular Ecology* 3: 81-89.
- Koskella J. & G. Stotzky. 1997. Microbial utilization of free and clay-bound insecticidal toxins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. *Applied and Environmental Microbiology* 63 (9): 3561-3568.
- Koziel, M. G., G. L. Beland, C. Bowman, N. B. Carozzi, R. Crenshaw, L. Crossland, J. Dawson, N. Desai, M. Hill, S. Kadwell, K. Launis, K. Lewis, D. Maddox, K. McPherson, M. R. Meghji, E. Merlin, R. Rhodes, G. W. Warren, M. Wright, and S. V. Evola. 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Biotechnology* 11: 194-200.
- McBride, K.E., Z. Svab, D. J. Schaaf, P. S. Hogan, D. M. Stalker & P. Maliga. 1995. Amplification of chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *Bio. Technology* 13: 362-365.
- MacIntosh S. C., T. B. Stone, S. R. Sims, P. L. Hunst, J. T. Greenplate, P. G. Marrone, F. J. Perlak, D. A. Fischhoff & R. L. Fuchs, 1990. Specificity and efficacy of purified *Bacillus*

- thuringiensis* proteins against agronomically important insects. *Journal of Invertebrate Pathology* 56: 258-266.
- Moar, W. J., L. Masson, J. T. Trumble & R. Brousseau, 1990. Toxicity to *Spodoptera exigua* and *Trichoplusia ni* of individual P1 protoxins and sporulated cultures of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and NRD-12. *Appl. Environ. Microbiol.* 56: 2480--2483.
- Moar, W. J., M. Puzsai-Carey, & T. P. Mack. 1995. Toxicity of purified proteins and the HD-1 strain from *Bacillus thuringiensis* against lesser cornstalk borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 88: 606--609.
- Perlak, F. J., R. W. Deaton, T. A. Armstrong, R. L. Fuchs, S. R. Sims, J. T. Greenplate, and D. A. Fischhoff. 1990. Insect resistant cotton plants. *Biotechnology* 8: 939-943.
- Reinecke, A. J. 1992. A review of ecotoxicological test methods using earthworms. In: Greig-Smith, P. W., H. Becker, P. J. Edwards & F. Heimbach (eds.). *Ecotoxicology of Earthworms*. Intercept Ltd, UK: 7-19.
- Sims, S. R. & L. R. Holden. 1996. Insect bioassay for determining soil degradation of *Bacillus thuringiensis* subsp *kurstaki* CryIAb protein in corn tissue. *Environ. Entomol.* 25: 659-664.
- Wittmann, D. 1982. Entwicklung von Testverfahren und Experimente zur Beurteilung von Insektizid-Wirkungen auf Bienenlarven. Dissertation Universität Tübingen.