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Monitoring of the Environmental Effects of the Bt Gene

Research Project Sponsored by the Bavarian State Ministry for Environment, Health, and Consumer Protection (StMUGV)

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Monitoring of the Environmental Effects of the Bt Gene

- Final Report-

Research Project Sponsored by the Bavarian State Ministry for Environment, Health, and Consumer Protection (StMUGV)

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Overview of the Working Groups

Within the framework of the research program "Accompanying Research and Monitoring of Released Genetically Modified Organisms," sponsored by the Bavarian State Ministry for Environment, Health, and Consumer Protection (StMUGV), the Bavarian State Research Center for Agriculture (LfL) took on the subproject "Monitoring of the Environmental Effects of the Bt Gene".

During four years of field experiments (2000-2003), it was investigated whether the cultivation of Bt corn, protected from infestation with the European corn borer by an insecticidal protein (Cry1Ab) derived from the bacterium *Bacillus thuringiensis*, has effects on non-target species. Besides an investigation of possible effects on selected insect groups in the corn field and at its margin, soil microbial parameters and soil fauna groups were also considered since Bt toxins enter the soil through harvest residue and root exudates.
The research was performed on five state-owned experimental estates (Grub, Puch, Baumannshof, Neuhof, Schwarzenau) on permanent experimental plots through comparative cultivation of Bt corn (Bt176 ´Navares´ and MON810 ´Novelis´) and the conventional isogenic varieties (´Antares´ and ´Nobilis´). At each site, 1.5 acres of Bt corn were grown in continuous cultivation. In order to be able to compare possible environmental effects of Bt corn to those of commonly used spraying measures, half of each of these experimental setups were treated with insecticide (Baythroid) to combat the corn borer. Additionally, approximately 5 acres of further Bt corn fields were available for studies on alternating fields of the state-owned estates as well as on long-term soil observation fields on experimental farms.

Overall, the extensive data collected yielded the following results:

1. The soil microbial parameters (microbial biomass, enzymatic activity) showed no changes with Bt corn compared to conventional corn cultivation.
2. No significant influences of Bt corn on the indicator species (earthworms, springtails, nematodes) of the soil fauna studies could be proven.
3. For most of the non-target organisms investigated in the Bt corn and the control fields (aphids, cicadas, thrips, parasitizing wasps, lacewings, hoverfly larvae, lady beetles, assassin bugs, and spiders), no or only minor effects of Bt corn were observed. As opposed to that, after insecticide application, noticeable reductions in population density were observed in some of the animal groups.
4. Negative effects observed during feeding trials with Bt corn (Bt176) pollen could not be confirmed in the field. This risk as well as out-crossing of Bt corn to neighboring fields planted with conventional varieties could be eliminated through the planting of a [conventional] buffer zone.

Finally, it can be stated that according to the present state of knowledge, the cultivation of Bt corn (Cry1Ab), while making an effective defense against the European corn borer possible, does not constitute any additional risks and adverse effects compared to those resulting from the use of the *Bacillus thuringiensis* preparations that have been approved for many years for purposes of organic farming. However, the validity of such a direct comparison is limited from an ecotoxicological perspective.

According to good agricultural practice of crop protection measures, crop protection measures are to be used only in a targeted fashion, i.e. prompted by the surfacing of pests. Hence, Bt corn as an "insuring measure" should only be used in cases when there is

known infestation. Using Bt varieties, one basically applies insecticides preventively throughout the entire vegetation period. This may, eventually, foster the selection of resistance-breaking borers.

1 Introduction

The cultivation of genetically modified plants (GMPs) has increased continuously worldwide in recent years and now covers over 20M acres. While five countries (USA, Argentina, Canada, Brazil and China) hold 99% of the cultivated surface, in Europe, GMPs were planted predominantly for research and approval purposes, due to a 1998 approval moratorium issued by six EU member nations that prevented commercial cultivation, except in Spain, pending several studies about the safety of GMPs to be conducted by 2004.

The purpose of the research project “Monitoring of the Environmental Effects of the Bt Gene” at the LfL, sponsored by the Bavarian State Ministry for Regional Development and Environmental Affairs (StMLU), called State Ministry of the Environment, Health and Consumer Protection (StMUGV) since 10.14.2003, was not to prove assumed effects of Bt corn on the environment through modeling systems that are far from actual reality, but to gain insight in regular cultivation experiments within the framework of a wide-ranging experimental design. It was therefore planned to integrate so-called long-term observation fields on experimental farms into the state-wide monitoring. The generally low acceptance of GMPs understandably decreased farmers' interest in comparative cultivation of conventional and genetically modified corn varieties, and much less than the desired amounts were planted on long-term observation fields. Over the 4 year duration of the experiment, the number of initial sites went from initially five to zero in 2003. The research was therefore performed mainly on permanent long-term experimental fields, and on test fields on five state-owned experimental estates. Additionally, some of the goals set initially had to be modified and in some cases abandoned completely, in agreement with the sponsor. This applied mainly to the areas “bees” and “resistance in borers”. Both topics were also the subject of several year-long Federal Ministry of Education and Research (BMBF) projects (Effects of Bt Corn Pollen on the Honeybee, Project number 0312631J and Ecological Consequences of Insect-Resistant Bt Corn on Various Insects and the Corn Borer, Project number 031216), the results of which will not be published until after conclusion of this research project.

The present final report on Bt corn monitoring contains the main findings about the non-target organisms studied, separated according to the four working groups:

1. Insects and Spiders
2. Nematodes
3. Soil microbiology
4. Earthworms and Springtails.

The individual contributions were structured in accordance with the requirements listed under § 3 in the StMLU’s Letter of Approval dated 04.13.2000. They are preceded by some general statements on the issues, goals, and experimental design.

2 Issues, Goals, and Experimental Design

On March 12, 2001 the European Parliament passed Directive 2001/18/EG on the intentional release of genetically modified organisms (GMOs) into the environment. This Directive prescribes that before approval of a genetically modified organism, not only a risk assessment has to be conducted, but also, a monitoring plan through which possible effects on the environment and on human health after release are to be recorded, has to be prepared and implemented. The Directive distinguishes between two kinds of monitoring in this case: "Case specific" monitoring, on the one hand, "general surveillance", on the other. Any "case specific" monitoring, if needed, grows out of the pre-release risk assessment, i.e. any possible, specific problem is to be closely observed and monitored after release. The "general surveillance" monitoring plan has to be established for each GMO and is intended to detect unintended effects of GMOs (Wilhelm et al. 2002).

Since the year 2000, i.e. since before the issuance of the EU Directive, the then Bavarian State Ministry for Regional Development and Environmental Affairs had been financing an extensive research program, the content of which was work on relevant factors of risk assessment and monitoring. The Technical University Munich (Chair of Plant Cultivation and Plant Breeding and Chair of Plant Ecology), the Bavarian Environmental Protection Agency (LfU), and the State Research Center for Agriculture (LfL) were involved in this four year project. The LfL took on the subproject "Monitoring of the Environmental Effects of the Bt Gene".

Genetic engineering makes it possible to transfer genes for insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt) into corn. This genetically modified corn expresses Bt endotoxins (CryA1b) in a highly specific fashion protecting it from infestation by corn borers. The research at LfL dealt mainly with the question whether non-target organisms may be potentially affected by the use of the Bt gene. With a focus on a sustainable and environment-friendly agriculture, special attention was directed towards "species protection" "soil fertility", and "biological control". "Species protection" herein encompasses the possible endangerment of protected and/or rare species that live in the agro-ecosystem. With regards to "soil fertility", it is significant to what extent Bt toxins affect soil organisms through harvest residue or root exudates. Under the aspect of "biological control", it was investigated whether the cultivation of Bt corn might enhance any positive effects of beneficial organisms thereby enhancing natural protection against harmful ones.

Tab. 1 shows the selected "non-target organisms", which were further studied by four working groups. Their groups focused on "case specific" monitoring, meaning that possible environmental effects of Bt corn and of the respective isogenic conventional varieties were tested in field trials directly through comparative cultivation. Besides this focus, aspects of primary risk assessment and of "general surveillance" were also included. The research project encompassed the risk assessment of Bt cultivation relevant to the field situation as well as methodological questions that still need to be solved for purposes of future monitoring of genetically modified plants. The latter touches mainly upon aspects of procedures relevant to monitoring, to the selection of suitable monitoring parameters, as well as to the definition and selection of suitable reference systems in the context of GMP cultivation. In order to permit an objective evaluation of conceivable environmental effects of Bt corn, commonly used chemical measures to combat the corn

borers were also used within the experimental design. To achieve the goals of the project, it was therefore necessary to use an experimental design with variations.

Tab.1: Scope of the investigations within the project "Monitoring the Environmental Effects of the Bt Gene". The list shows the tested variables (indicators) with the respective experimental designs.

Indicators	Laboratory	Continuous cultivation of grain corn	"normal" cultivation (on experimental farms)	Long-term soil observation fields
Soil microbiology		X		X
Collembola		X		
Earthworms		X		X
Soil nematodes	X	X		
Aphids and Antagonists		X		
Butterflies	X	X	X	
Bees, resistance in borers*			X	
Fusarium toxins*		X	X	X

* these studies were abandoned

Experimental Design

Direct environmental effects of the Bt gene were tested through comparative studies. For this purpose, Bt corn of the respective isogenic non-Bt varieties was cultivated under identical site conditions. Mon810 by Monsanto ("Novelis") and Bt176 by Syngenta ("Navares") were chosen as Bt corn varieties since, qualitatively and quantitatively, they differ markedly in the expression of CryA1b. Besides the comparative cultivation on the long-term soil observation fields previously mentioned in the Introduction, with common crop rotations, most of the studies were performed on permanent experimental fields on state-owned estates under continuous cultivation of grain corn. Tab. 2 provides an overview of the five sites and Fig. 1 shows the uniform experimental design. Half of the respective experiments, with eight large plots of 1500 square meters each, were treated once a year with an insecticide (Baythroid 50) against the corn borer, in order to be able to compare interferences of different intensity as well as the environmental effects of Bt corn with conventionally practiced anti-borer measures. Large plots were chosen in order to avoid mutual influence (edge effects) of various parts of the experiment to the greatest extent possible. This *[elimination of the edge effect]* was achieved at the expense of more or less intense scattering of the data obtained on soil microbes and soil fauna, depending on the degree of soil inhomgeneity at the respective site.

Through the four years of continuous cultivation of grain corn, large amounts of harvest residue ended up in the soils; hence, the import of a high amount of Bt toxins as well as their possible accumulation could be expected. Besides this worst-case-scenario, additionally, on three of the state-owned estates, "normal" cultivation on 5 acres each of the same variety with and without the Bt gene (Bt176) occurred at a distance of about 0.6 miles from one another. These "normal" cultivation areas were used to study flying and other highly mobile insects.

Tab. 2: Sites for the permanent *[experimental fields]* used for the continuous corn cultivation experiments

State-owned estate	County	Gov.-Dist.	Weather Station No.	Elevation above sea level	Soil type	Quality of arable land Soil type	Annual average temp. °C	Precipitation mm Multiyear average
Baumannshof	PAF	Obb.	36	365	Brown earth	25 hS	7,8	636
Puch	FFB	Obb.	5	550	Rendzina	66 lS	8,0	920
Grub	EBE	Obb.	124	525	Parabrown earth	43 sL	7,4	967
Neuhof	DON	Schw.	99	516	Pseudogley	58 uL	7,6	764
Schwarzenau	KT	Ufr.	39	200	Pararendzina	74 uL	8,5	620

Bt 176 (Navares) without insecticide	conventional (Antares) without insecticide	Bt Mon810 (Novelis) without insecticide	conventional (Nobilis) without insecticide
Bt 176 (Navares) with insecticide	conventional (Antares) with insecticide	Bt Mon810 (Novelis) with insecticide	conventional (Nobilis) with insecticide

Fig.1 Experimental design for continuous corn cultivation on permanent *[experimental fields]*

For some of the indicators contained in Tab. 1, laboratory experiments were conducted in addition to the field experiments, in order to be able to prove cause and effect relationships under controlled conditions that would otherwise have been difficult to validate statistically in the field with a reasonable effort. Especially with regards to the future cultivation of further Bt corn varieties already approved in the US that express other Bt toxins (Cry3Bb1, e.g.) providing the plants with resistance to corn root worm, suitable bioindicators and standardized test procedures would be very helpful and a good complement to a monitoring. This is why within the framework of this project, accompanying research was carried out, which is also contained in the present report.

Regardless of the fact that the relevance of findings of laboratory tests and their transferability to the field are not universally accepted, the testing of beneficial organisms, for instance, in the approval process of chemical crop protection products is based on similar screening tests. Wilhelm, R., Beißner, L., Schiemann, J. (2002): Gestaltung des Monitoring der Auswirkungen gentechnisch veränderter Pflanzen im Agrarökosystem. Gesunde Pflanzen 54, 194-206.

3 Presentation of the Results by Working Groups

3.1 Insects and Spiders

3.1.1 Aphids and Antagonists

3.1.1.1 The Task at Hand

It was the goal of this subproject to investigate the potential risks of the cultivation of Bt corn on non-target organisms in the field. Besides its target, the European corn borer (*Ostrinia nubilalis*, Lepidoptera) the uptake of Bt toxin also has potential adverse effects on so-called non-target organisms like other invertebrates in the corn field. With regards to transgenic corn, the uptake of the Bt toxin by non-target organisms can occur via different mechanisms: 1.) The non-target organism consumes plant material of the corn plants, 2.) Pollen released by the plants and shed onto soil and corn plants is taken up by the organisms, 3.) Non-target organisms actively seek out the corn flowers, feeding on pollen there, 4.) Predatory non-target organisms feed on prey that previously ingested transgenic corn material. Furthermore, shifts on lower trophic levels, as for instance in sucking phytophages, may have indirect effects on populations of higher trophic levels (predators). Based on field studies (monitoring) in corn fields, it was now to be investigated whether the cultivation of Bt corn negatively affects invertebrates. Furthermore, the effects of conventional pest control with an insecticide, Baythroid, were studied for comparative purposes and as a control. The focus of the investigation was on the main sucking phytophages in corn, aphids, as well as their antagonists ("beneficial" organisms). By studying these antagonists, it was also investigated whether Bt corn and insecticide applications affect the biological control of aphids.

3.1.1.2 Extensive Documentation and Evaluation of the Literature Used

Research on field effects of Bt corn non-target organisms was published more frequently only in recent years, while this project was already underway (Tab. 1). With regards to the animal groups they have studied, duration of the experiments, corn varieties used, field sizes, sample size, testing methods used, geographic location, and many more, these studies are rather diverse, making direct comparisons between studies difficult (Orr & Landis 1997, Pilcher et al. 1997, Lozzia 1999, Manachini 2000, Wold et al. 2001, Bourguet et al. 2002, Hassell & Shepard 2002, Jasinski et al. 2003, Kiss et al. 2003, Musser & Shelton 2003, Dively & Rose 2003, Pons & Starý 2003, Volkmar & Freier 2003, Candolfi et al. 2004, Lumbierres et al. 2004). A large proportion of these studies was conducted in the US (47%), 13% each (= 2 publications) from France, Italy, and Spain, and only one each from Hungary and Germany (Volkmar & Freier 2003). Six studies (40%) covered only one year of research, eight were two-year studies (53%), and only one study from Spain lasted 3 years. Mainly aphid-eating predatory arthropods like lady beetles (Coccinellidae), e.g., lacewings (Neuroptera), parasitic wasps (Hymenoptera) and predatory heteropterans (Anthocoridae, Nabidae) were studied. Among herbivorous organisms, predominantly aphids (67%) and cicada (67%) were recorded, followed by thrips (40%).

Laboratory studies on the effects of Bt corn and Cry1A(b) toxin on aphids and antagonists were also published more frequently in recent years, sometimes combined with field studies (Pilcher et al. 1997, Hilbeck et al. 1998a,b, 1999, Lozzia et al. 1998, Zwahlen et al. 2000, Meier & Hilbeck 2001, Dutton et al. 2002, Lumbierres et al. 2004, Romeis et al. 2004). *Chrysoperla carnea* (Neuroptera) larvae were studied most frequently (in eight of the 11 references cited), further lady beetles, predatory heteropterans as well as aphids, and spider mites (Tab. 2).

3.1.1.3 Condition Under Which the Work Was Conducted

Our entomological studies on aphids and antagonists were carried out on continuous corn cultivation fields in five experimental estates from 2001 to 2003 (see chapter 2 "Issues at Hand and Goals, Experimental Design"). On each of the experimental estates, a randomized block design with 8 plots was implemented. The eight different plots and treatments resulted from the two corn varieties (Bt176 Navares, Mon810 Novelis), the respective isogenic control lines (Antares, Nobilis), an insecticide treatment with Baythroid 50 on half of the plots, (2 Bt varieties * 2 Control varieties * 2 Insecticide treatments = 8 plots). The work on the eight plots of a site including going there and coming back required, on average, one work day for four people. That means that five days each had to be projected for doing the work on the five experimental estates. Also, we had to wait for a good weather phase in order to ensure that there was not too much difference between the sampling dates for the individual experimental estates. Due to the short generation cycle of insects, samples become increasingly less comparable if more than two weeks pass between sampling dates. The subproject had to be carried out with fewer people than originally planned. The scheduled position of a biological lab assistant could only be used during the 2001 season due to one coworker leaving and subsequent elimination of the line. Due to a change in personnel, the planned participation of the head of the working area "Pests, Beneficial Organisms, Food Storage Protection" (IPS 2d) was not possible as planned. This loss of personnel could be only partially compensated through the hiring of seasonal help. In particular, seasonal help was not usable for the determination of animal groups in the field.

3.1.1.4 Planning and Progression of the Work

It had been planned to take 4 – 5 samples per season (2001 – 2003) on all experimental estates in each plot under continuous cultivation. In case there were any additional capacities available, experimental fields under continuous cultivation were to be worked on where possible. During the 2000 season, data gathering and methods were tested on the fields of the Grub experimental estate, validated, and finally optimized. It became apparent, there, that the validation for the various animal groups requires a great effort. On average, the validation of 10 corn plants took 1- 2 personnel hours (not counting going there and returning) so that 4 persons were busy for an entire day in one "continuous grain corn cultivation" field for each validation date. During 2001, only the continuous cultivation fields of three experimental estates (Grub, Neuhof, Baumannshof) were worked on. The fields at Puch were too badly damaged by hail; the Schwarzenau estate, which is very far away, could not be worked on due to our extremely tight personnel status during 2001. In 2002 and 2003, the continuous cultivation fields of all five experimental sites were sampled. Due to the personnel restrictions described in 3.3.1.3, the number of samplings performed was reduced to three per season. The validation dates were before insecticide application (and flowering), after insecticide application (and flowering), and

at full maturity (in 2001 only, the first sampling could not be performed until shortly after the insecticide application). Due to the strained personnel situation, no additional work on experimental fields under continuous cultivation could was possible.

3.1.1.5 Overview of the Overall Issues, and Currently Known Findings in the Area of the Task at Hand

The use of Bt sprays is generally considered to cause very little harm to beneficial organisms, since the various Bt endotoxins have very specific effects on the respective pests. While adverse effects on non-target species have been reported, they are usually restricted to members of the same systematic order as the targeted pests (Glare & O'Callaghan 2000). However, in Bt corn, the toxin is present in a (semi-)activated form (inactive in Bt spray); it is produced continually by the plant throughout the entire season, taken up by various herbivores and predators, and from there passed on to higher levels of the food web. These circumstances make it necessary to evaluate and test this novel plant protection measure and its effects on non-target organisms. The toxin formed in the Bt corn is derived from *Bt* var. *kurstaki* and therefore has a specific effect on the corn borer and other butterfly species. It is on lepidopterans that the largest number of adverse Bt effects have been reported (see 3.4 *[sic]* Butterflies).

There are relatively few toxicological laboratory studies on Bt corn and predatory invertebrates (Tab. 2); most focus on lacewing larvae *Chrysoperla carnea* (Neuroptera), with contradictory results. Hilbeck and colleagues found increased mortality and developmental delays in lacewing larvae after consumption of Cry1A(b) toxin isolate and lepidopteran larvae previously kept on Bt corn. (Hilbeck et al. 1998a,b, 1999). Romeis et al. (2004) found no effects of feeding Cry1A(b) toxin. Dutton et al. (2002) found an effect (mortality, duration of development) only when feeding *[predators]* caterpillars that previously consumed Bt corn, but not from feeding aphids and mites that had previously fed on Bt corn *[to predators]*. Romeis et al. (2004) interpret these results to mean that Bt toxin does not have an effect on the lacewing larvae, but that feeding on Bt corn weakens caterpillars, thereby lowering their food quality for their predators like *C. carnea*, which in turn has negative consequences for those predators. Since aphids apparently don't take up the Bt toxin (Head et al. 2001, Raps et al. 2001, Dutton et al. 2002), they also do not pass it on to their predators. Furthermore, lacewing larvae have a preference for aphids and caterpillars fed on Bt-free corn (Meier & Hilbeck 2001). Both circumstances should reduce their Bt exposure in the field. In terms of predatory arthropods, the only other studies were performed on lady beetles and predatory heteropterans, and no negative effects were found (Pilcher et al. 1997, Zwahlen et al. 2000). Since Bt toxin is not transported in phloem, no effect on the phloem-sucking aphids is to be expected (Head et al. 2001, Raps et al. 2001, Dutton et al. 2002). In spite of that, Lumbierres et al. (2004) report negative effects on the aphid *Rhopalosiphum padi*, but only on the wingless stages. The winged form reacted positively to Bt corn, with a shortened developmental period and a higher reproductive rate. Lumbierres et al. interpret this to result from "pleiotropic" effects, i.e. unexpected variety characteristics of the Bt event that positively affect the food quality for aphids of the variety compared to the isogenic line. The winged forms seem to adapt to this change differently than the wingless stages.

In recent years, a wide range of organisms, predators as well as herbivores, have been studied in Bt corn fields (Tab. 1). With the exception of the studies of Dively & Rose (2003) and Candolfi et al. (2004), no negative effects on non-target organisms (other than lepidopterans) were found. However, one has to take into consideration the limitations of

some of these studies, particularly with regards to field size, duration of the studies, and sample size (overall and per season). In view of these limitations, the absence of an effect can be excluded *[sic]* only with a certain probability that may, at times, be low (see also Bourguet et al. 2002, Perry et al. 2003, Lang 2004). There is demand here, particularly for future long-term studies on realistically-sized fields. While there are also reports about positive effects on some animal groups (Tab. 1), no explanations have been proposed for these findings. It is possible that these are again pleiotropic effects, as suspected in the case of the aphids (s. above, Lumbierres et al. 2004).

3.1.1.6 Scientific and Technical Methods Used

Ten corn plants each, in 10 plots, were marked with colored bands, and the animals on these marked plants recorded, each season, at the three sampling dates. Beginning at the Western margin of each plot, 2 plants each in rows 10, 15, 20, 25, and 30 were marked. The first plant in each row was 15 m away from the margin of the plot; the second plant was 30 m away. This resulted in 10 sampled corn plants per plot (for each treatment), i.e. 30 values (10 plants * 3 sampling dates). The ten individual values of each plot at a particular site were combined, resulting in five repeats per treatment and year (= 5 sites). The three sampling periods per year lay during calendar weeks (CW) 28 – 31, 35 – 36 and 38-40 in 2001, CW 26 – 27, 31 – 32 and 35 – 36 in 2002, and CW 26, 30 – 31 and 33 – 34 in 2003.

For purposes of sampling, each corn plant was visually inspected, beginning with the panicle, next the flag leaf, the rest of the leaves, and finally the cob. The top as well as the underside of the leaves were inspected, and, with regards to the cob, its inside as well. The following animal groups were recorded: Aphids (at the species level), cicadas, parasitizing wasps, (adults, mummies), lacewings (adults, larvae, eggs), hoverflies (larvae, eggs), lady beetles (adults, pupae, larvae, eggs), predatory heteropterans, and spiders. With the exception of the aphids, which can be determined directly in the field, species determination was omitted since that would have required the collection of too many organisms, and determining them in the lab. This omission was implemented in order to not disturb the system through such a destructive removal and in order to reduce the enormous work load that such practice would have entailed. For purposes of the evaluation, all aphid species and stages were combined. The specific aphid predators were also combined (adult lacewings, lacewing larvae, adult lady beetles, lady beetles larvae, hoverfly larvae) since the population densities of the individual groups were too low for individual evaluation. Representatives of the families *Anthocoridae*, *Reduviidae* and *Nabidae* were combined into “predatory heteropterans”.

Evaluation was performed separately for each year. For the statistical evaluation, the recorded values on each plot were combined into an annual mean and (Ln(x+1)) transformed. Then these values were tested with a multifactor ANOVA. The main factors were Bt status (yes/no), insecticide application (yes/no), and company (Syngenta, Monsanto); “Site” was treated as a random factor (5 different ones). “Company” was treated as a factor since the Bt varieties of the two companies differ, as for instance in their toxin content, in the toxin expression in different tissues, in the FAO number, and more. In an additional analysis, an ANOVA was performed for repeat measurements, in order to test whether any of the factors has a significant interaction with the date, i.e. has a different effect during different seasons of the year. The ANOVA factors for repeat measurements were “Bt”, “Insecticide application”, and “Site”. The values (Ln(x+1)) were

the means of each plot for a particular sampling date. The statistics program SPSS, version 11, was used for the calculations

3.1.1.7 Progress Made in Other Places That Became Known While This Study Was Conducted

The more recent studies that were published while our research project was underway have already been discussed under "3.3.1.5 Overview of the Overall Issues, and Currently Known Findings in the Area of the Task at Hand". In the Lang working group, however, an additional project on Bt corn, sponsored by the Federal Ministry of Education and Research (BMBF) (FKZ 0312631A), was carried out. Therein, Dipl.-Biol.[1] Claudia Ludy and Dipl.-Biol.[1] Michael Meissle were working on possible Bt corn effects on spider populations in corn fields and on corn field margins. In these studies, they not only looked at the abundances, but also at the species composition of the spider communities. The study showed that vacuuming up the spiders with a hand-held vacuum is the comparatively best method for a monitoring of spiders at the species level, (Meissle 2002, Ludy & Lang 2004a). While visual recording as performed in the present study yields comparable results regarding abundances, it does not provide information on species composition.

Negative effects on the population densities of spiders were found during the year 2001, in 2002, no difference was found between Bt fields and control fields, and during 2003, there were greater spider abundances in Bt fields (Meissle 2002, Ludy & Lang 2004b). With respect to species diversity, there was no difference in 2001 and 2002, and in 2003, more species of spiders were found in the Bt corn fields. In toxicological laboratory experiments, the consumption of Bt toxin, Bt corn pollen and /or of bees carrying Bt corn pollen had no adverse effects on the spiders *Mangora acalypha*, European garden spider (*Araneus diadematus*) and wasp spiders (*Argiope bruennichi*) (mortality, life span, weight gain, reaction to prey, and web parameters were studied) (Ludy & Lang 2004b). Within the framework of the mentioned BMBF research association, several additional studies were performed in other places. Their preliminary results can presently (June 21, 2004) be viewed on the internet at http://www.biosicherheit.de/forschungsergebnisse/295.doku.html

3.1.1.8 Presentation and Discussion of the Results

Overall, it can be stated that in all animal groups and throughout all years, site (experimental estate) always had a significant influence on abundances (ANOVA, $p < 0.05$). Furthermore, there was often an interaction between site and sampling date, meaning that the population dynamics differed at the various sites (repeated-measurement ANOVA, $p < 0.05$). Both factors increased the variance of the data sets, rendering a statistical proof of a possible effect of Bt corn on population densities more difficult.

Three aphid species were detected: *Rhopalosiphum padi* (oat aphid), *Metopolophium dirhodum* (rose-grain aphid) and *Sitobion avenae* (English grain aphid). Overall, *R. padi* clearly dominated, solely during the first evaluation dates *M. dirhodum* was found more commonly. Only individual specimens of *S. avenae* were found. In 2001 aphid counts in the Bt corn were significantly lower (Fig. 1, ANOVA, $p < 0.01$). The interaction Bt * Company pointed to a reduction only in Mon810 corn (Fig. 1A, ANOVA, $p < 0.05$). In 2002 as well, there was a tendentially lower aphid count on Bt corn (Fig. 1, ANOVA, p =

[1] German title of a biologist with a graduate degree, similar to Master's, not translatable

0.063). During the unusually hot and dry summer of 2003, there were practically no aphids on the corn, and therefore no testable effects (Fig. 1). In 2001, insecticide application led to increased aphid counts on the Monsanto corn (ANOVA, Insecticide * Company, $p < 0.05$), which was also noticed as a tendency in 2002 (repeated-measurement ANOVA, Insecticide * Company, $p = 0.092$). The lower counts on Bt corn are probably not due to a direct Bt effect, since aphids do not take up any Cry1A(b) while sucking (Head et al. 2001, Raps et al. 2001, Dutton et al. 2002). It is likely that here too, pleiotropic effects that may lower the food quality of Mon810 corn for aphids (cf. Lumbierres et al. 2004) are the cause (Saxena & Stotzky 2001). Insecticide application led to higher aphid counts since the insecticide also killed the antagonists. Due to their high immigration rate and short reproductive cycle, aphids compensate insecticide applications better than the predatory arthropods and allowing them to "escape" biological control since the remaining antagonists are no longer able to "get a handle" on the large numbers of aphids.

Bt corn had no effect on cicada (Fig. 2). During all three years, the application of Baythroid 50 led to a noticeable reduction in cicada counts (Fig. 2, repeated-measurement ANOVA, $p < 0.01$), however, the insecticide did not always have the same effect in each of the experimental estates (repeated-measurement ANOVA, interaction Site * Insecticide, $p < 0.05 - 0.10$).

In 2001, Bt corn had an effect on the aphid predators (Fig. 3, ANOVA, $p < 0.05$). the numbers were reduced on Bt corn, but only on Syngenta Bt176 corn (Fig. 3B, ANOVA, interaction Bt * Company, $p < 0.05$). In 2002, the Bt effect was date-dependent (repeated-measurement ANOVA, interaction Date * Bt, $p < 0.05$). In 2003, a Bt effect was also recognizable, but inverse, with higher counts on Bt corn (Fig. 3, ANOVA, $p = 0.05$). The insecticide's effect turned out to be highly variable. The reason is that in this case, negative lethal effects on the populations were overlaid by positive consequences like increased immigration of predators due to the high aphid counts (s. above), site-specific factors exerted additional influence. In 2001, the Baythroid effect seemed to depend on the site (interaction Insecticide * Site, $p = 0.09$) and company (Insecticide * Company, $p = 0.056$), in 2002, the effect of the insecticide differed between Monsanto- and Syngenta corn (Insecticide * Company, $p < 0.05$), and in 2003, all insecticide treated plots had lower aphid predator counts after the application (repeated-measurement ANOVA, Insecticide * Date, $p < 0.05$).

Bt corn had no significant impact on the number of "mummies" (= number of aphids parasitized by parasitic wasps). Insecticide application appeared to positively influence the number of "mummies" (Fig. 4, repeated-measurement ANOVA, $p < 0.05$ for all years), since aphid counts were higher in the insecticide-treated plots (Fig. 1), so that parasitic wasps could parasitize more aphids. It was not possible to conclusively prove a positive or negative effect on parasitation rates (= number of parasitized aphids), neither on Bt corn nor due to insecticide application.

Next to the spiders, the predatory heteropterans were the most common invertebrate predators on corn. In all three years, a tendency towards a Bt effect on the numbers of predatory heteropterans could be observed (repeated-measurement ANOVA, $p < 0.05$ for 2001 and 2002; ANOVA, Bt * Company, $p = 0.089$ for 2003). In 2001 and 2002 predatory heteropterans counts were reduced in Bt corn fields, while in 2003, the densities were higher in Bt corn than in conventional control fields (Fig. 5). During all three years, insecticide application led to a marked reduction in predatory heteropterans (repeated-measurement ANOVA, $p < 0.05$).

Next to predatory heteropterans, spiders were the most common invertebrate predators in corn. Spider abundances were lower on Bt corn in 2001 (repeated-measurement ANOVA, $p < 0.05$); this effect was more pronounced in Syngenta's Bt176 (Fig. 6B, ANOVA, Bt * Company, $p < 0.05$). During 2002 and 2003, no significant difference between Bt corn and control corn could be found (Fig. 6). Spider population densities were lower in the insecticide–treated than in untreated plots (repeated-measurement ANOVA, $p < 0.05$).

Aside from the aphids, insecticide application had a drastically negative effect on the abundances of all studied invertebrates, particularly on spiders and predatory heteropterans (Fig. 5 and 6). Negative insecticide effects were proven more frequently and were comparatively larger than Bt effects. It has to be emphasized, however, that contrary to results of prior field studies, we found reduced abundances of predatory arthropods in Bt corn. This applies to specific aphid predators in 2001, spiders in 2001, and predatory heteropterans in 2001 and 2002. It is a problem of relatively non-specific monitoring that it is often difficult to prove direct causes for the observed effects. In this case, there might be a direct or toxicological effect of Bt corn on the predatory invertebrates. According to the present state of knowledge, however, this argument appears questionable at least, since nobody has been able to prove toxic effects of Cry1A(b) from Bt corn on aphid predators, predatory heteropterans, or spiders. However, the number of available studies is still relatively low at present, especially as far as Mon810 corn is concerned (s. Tab. 2). Bt toxin concentrations in the corn plants might vary considerably between seasons, between years, and between sites (Nguyen Thu & Jehle, DLR Rheinpfalz, pers. comm.), with concurrent variations in the toxic effects on no-target organisms. A negative impact on predator populations can also be imagined to occur indirectly via the food quality of the prey animals (Romeis et al. 2004). In 2001, aphid counts were reduced particularly in Mon810 fields (s. above), which may have been responsible for the lower predator counts (at least in part). In a large scale cultivation of Bt corn, even such an indirect effect could lead to a regional or local reduction in population densities of beneficial organisms. For 2001, Meissle (2002) as well as Ludy & Lang (2004b) proved a reduction in spider numbers in Bt corn fields, on the same experimental estates, but with different detection methods, and in different fields.

In 2003, indications of a positive effect of Bt corn on aphid predators and predatory heteropterans were found. In 2003, Ludy & Lang (2004b) as well, found greater abundances of spiders in Bt corn fields compared to control fields. However, the year 2003 was unusually hot and dry, and definitely represents an exception. Possibly, again, in this case, pleiotropic effects on the corn plants were the determining factors for these results here (Saxena & Stotzky 2001). In the present study, too, differences between Bt corn plants and their isogenic control lines became apparent. For instance, during two years (2001, 2003), the Bt corn grew higher than the control plants, thereby providing more habitat to the animals living on the plant (repeated-measurement ANOVA, $p < 0.05$); other studies have reported similar findings (Magg et al. 2001, Hassell & Shepard 2002). In 2001, the Bt corn always developed slightly more slowly that than the control, it also died later, thereby providing a habitat for a longer time (repeated-measurement ANOVA, $p < 0.01$). Such developmental differences between Bt and isogenic corn are also known from other studies (Lumbierres et al. 2004). Drought stress can lead to lower flower biomass in corn plants (Traore et al. 2000), and during the 2003 season, some of the Bt corn had fewer dried leaves than the non-Bt varieties (repeated-measurement ANOVA, Bt * Date * Site, $p < 0.001$). Furthermore, Bt corn is not subject to additional stress through corn borer infestation. In 2003 that could have led to slightly more herbivorous insects on Bt corn since those plants stayed green longer and thereby continuing to provide food (the

cicadas in Mon810 corn, e.g., Fig. 2A). Eventually, the predators followed their prey, thereby also reaching greater abundances in Bt corn (the predatory heteropterans in Mon810, e.g. Fig. 5A). However, this interpretation has to remain speculative since a significant increase of herbivorous insects in Bt corn could not be proven for 2003 (we also did not record all herbivores).

The present results and interpretations underscore the necessity, in a monitoring, to record as many variables as possible that could potentially influence the monitoring parameters, in order to have some indications that would help to explain possibly detected effects. However, in order to prove a direct cause of an effect, if will often be unavoidable to conduct additional manipulative experiments in the field or toxicological studies. In this context it should also be pointed out that the lack of a positive proof of an effect does not automatically prove the absence of that effect. In order to test the reliability of the findings, or rather their p value, retrospective power analyses of the results are highly recommended (see also Hilbeck et al. 2000, Andow 2003, Perry et al. 2003, Andow & Hilbeck 2004, Lang 2004).

3.1.1.9 Summary

Through the transfer of DNA from the soil bacterium *Bacillus thuringiensis* var. *kurstaki* (Bt) into corn, the so-called Bt corn is able to form a *Lepidoptera*-specific toxin and thereby protect itself from being eaten by the caterpillars of the European corn borer. It was the goal of this subproject to investigate the effects of Bt corn cultivation on non-target organisms in the field, with the main focus on predatory "beneficial" species, as well as the main plant-sucking insect in corn (aphids, cicadas).

The studies were conducted from 2001 – 2003 on five experimental estates in Bavaria. At these sites, two Bt corn varieties (Mon810 by Monsanto and Bt176 by Syngenta) as well as their respective isogenic, conventional corn varieties were cultivated. Furthermore, conventional crop protection, treatment with insecticide, was conducted with Baythroid 50 and tested. The following animal groups were recorded and analyzed in the experimental fields during the growing season: aphids, cicadas, "mummies" (= parasitized aphids), aphid predators (lacewing larvae and adults, hoverfly larvae, lady beetle larvae and adults), predatory heteropterans (*Anthocoridae, Reduviidae, Nabidae*) and spiders.

Aphids were found in Bt corn fields in rather low numbers, particularly on Mon810-Bt corn, while Bt did not appear to affect cicadas. The abundances of aphid predators were reduced in Bt fields (2001) or increased (2003), while no Bt effects were found on "mummies" (= aphids parasitized by parasitic wasps). Predatory heteropterans showed a Bt effect each year: In 2001 and 2002 their population densities in Bt corn fields were significantly lower, while in 2003 their counts on Bt corn were higher. Spiders were less numerous on Bt corn in 2001, with this effect being most pronounced on Bt176 corn, and no effect was found during 2002 and 2003. The insecticide application with Baythroid had considerable negative effects on the population densities of nearly all animal groups studied, during several years: cicadas, aphid predators, predatory heteropterans and spiders were affected. Only the aphid numbers were increased by the insecticide application, probably because the insecticide reduced the numbers of their natural enemies. Hence, insecticide application negatively impacted biological control of aphids by antagonists. In the present study, the same could not be proven for Bt corn.

Overall, fewer negative effects on non-target organisms and fewer effects on their population densities were observed with Bt varieties than with a crop protection measure

with a pyrethroid insecticide. As opposed to previous studies, however, Bt corn effects on populations of beneficial organisms could be proven; the effects were negative or positive depending on the year. This seems to be significant particularly for the affected predatory heteropterans and spiders, since these two groups represent the most numerous predatory arthropods on corn as well as on other agricultural plants. A substantial reduction in predatory heteropterans and spiders could have far-reaching consequences for the agro-ecosystem and biological pest control. However, in this study we were not able to prove that the observed effect on heteropterans and spiders was a Bt effect, or whether the populations of these predatory arthropods are influenced indirectly, for instance through the population densities of their prey species or through habitat availability. In view of the proven Bt effects and of the importance of predatory heteropterans and spiders on the agro-ecosystem it appears crucial to solve this question. This would ideally be achieved through long-term field experiments, supported by laboratory studies, carried out on the population as well as on the species level, as well as with as many varieties of Bt and conventional corn as possible.

3.1.1.10 Appendix with Tables, Figures, References

List of Tables and Figures

Table 1: Overview (06.22.04) of published field studies with Bt corn and non-target organisms (except. Lepidoptera & Hymenoptera).

Authors	**Bt event**	**Field / plot size**	**N per treatment**	**Study period**	**Samples per season**	**Country**	**Animals**	**Abundance in *Bt* corn compared to control**
Bourguet et al. 2002	Mon810	200 – 900m^2	4	1yr	15	France	Aphids, Anthocoridae, syrphid larvae, ladybird beetle, lacewings (Neuroptera), parasitoids	No effects
Candolfi et al. 2004	Bt176	1.2 – 1.7 ha	2 – 3	1yr	6 – 8	France	Soil-dwelling arthropods, plant-dwelling arthropods, flying-arthropods	Some Diptera including a syrphid species (-) Cicadina (+) Neuroptera (+)
Dively & Rose 2003	Cry1A(b)	342m^2	4	2yrs	6 – 8	USA	Foliage-dwelling arthropods, soil-surface dwelling arthropods	Sap beetles (Nitidulidae) (-) Predators in maize[2] litter (+)
Hassell & Shepard 2002	Cry1A(b)	29m^2	6	1yr	2	USA	Chrysomelidae, predacious Heteroptera, Coccinellidae, Thripidae, Cicadellidae, aphids, spiders, parasitoids	No effects
Jasinski et al. 2003	Cry1A(b)	4 – 20 ha	5	1yr	3 – 7 (?)	USA	Spiders, mites, parasitoids, ladybirds, Anthocoridae, Staphylinidae, Carabidae, lacewings (Neuroptera), syrphids	No effects
Kiss et al. 2003	MON810	900m^2	6	2yrs	weekly	Hungary	Aphids, Thrips, Cicadina, Chrysomelidae, Coccinellidae, Carabidae, Staphylinidae, Anthocoridae, lacewings (Neuroptera), syrphids, aphids, spiders	Aphids (+)
Lozzia 1999	Bt176	10 ha	2 (- 8)	2 yrs	every 14 d	Italy	Carabidae, spiders, aphids, Cicadellidae, and others	No effect
Lumbierres et al. 2004	Bt176	0.4 – 1 ha	4	3yrs	5	Spain	Aphids	Winged aphids (+) Unwinged aphids (-)
Manachini 2000	Bt176	10 ha	1	2 yrs	?	Italy	Carabidae, Diptera, Lepidoptera, Thrips, Hymenoptera, Cicadellidae, spiders	No effect
Musser & Shelton 2003	Cry1A(b)	32m^2	3 – 4	2yrs	2	USA	Coccinellidae, Anthocoridae, Aphids	Coccinellidae (+) Athocoridae (+)
Orr & Landis 1997	Cry1A(b)	0.4 ha	3	1yr	3	USA	Coccinellidae, Anthocoridae, lacewings (Neuroptera)	Neuroptera (+)
Pilcher et al. 1997	MON810	22 – 45m^2	3	2yrs	3	USA	Coccinellidae, Anthocoridae, lacewings (Neuroptera)	Overall predators (+)
Pons & Starý 2003	Bt176	?	?	1yr	2	Spain	Aphids, parasitoids	No effects
Volkmar & Freier 2003	Bt176	7 – 29 ha	2	2yrs	6	BRD	Spiders (on ground)	No effects
Wold et al. 2001	Cry1A(b)	21 – 543m^2	4	2yrs	3	USA	Coccinellidae, Anthocoridae, lacewings (Neuroptera)	No effects

[2] Translator's Note: The German "Mais" will be translated according to US practice as "corn" throughout these sections. "corn" and "maize" are used interchangeably in the references and original English figures to mean *Zea mays*.

Table 2: Overview of the literature (status: 06.22.04) of published laboratory studies with Bt corn and non-target organisms (except Lepidoptera & Hymenoptera).

Authors	**Bt event**	**Toxin source**	**Organism(s) tested**	**Exposure to toxin**	**Parameters tested**	**Effects of *Bt* on study organism(s)**
Dutton et al. 2002	Bt176	Green plant material	Aphids	Aphids on whole plant	Intrinsic rate of increase	No effect
			Mites	Mites on whole plant	Intrinsic rate of increase	No effect
			Lepidoptera	Larvae on whole plant	Mortality, development	Higher mortality, delayed development
			C. carnea (Neuroptera)	aphids fed with *Bt* maize	Mortality, Development	No effect
			C. carnea (Neuroptera)	Mites fed with *Bt* maize	Mortality, development	No effect
			C. carnea (Neuroptera)	Lepidoptera larvae fed with *Bt* maize	Mortality, development	Higher mortality, delayed development
Hilbeck et al. 1998a	Cry1A(b)	Isolated toxin	*C. carnea* (Neuroptera)	Toxin in artificial diet	Mortality, development	Higher mortality
Hilbeck et al. 1998b	Bt176	Maize plant	*C. carnea* (Neuroptera)	Lepidoptera larvae fed with *Bt* maize	Mortality, development	Higher mortality, delayed development
Hilbeck et al. 1999	Cry1A(b)	Isolated toxin	*C. carnea* (Neuroptera)	Lepidoptera larvae fed with toxin	Mortality, development	Higher mortality
Lozzia et al. 1998	Bt176	Maize plant	Aphids, *Chrysoperla* (Neuroptera)	Aphids on plant leaves, *C. carnea* fed with aphids	Mortality & fecundity of aphids, mortality of *C. carnea*	No effects
Lozzia et al. 2000	Bt176	Maize plant	Mites	Plant leaves	Mortality, development, reproduction	Longer development in some stages
Lumbierres et al. 2004	Bt176	Maize plants	*R. padi*, winged aphids	Aphids on whole plants	Mortality, development, reproduction	Short development, high reproduction
			R. padi, unwinged aphids			Long development, low reproduction
Meier & Hilbeck 2001	Bt176	Maize plant	*C. carnea* (Neuroptera)	Aphid fed with *Bt*	Prey preferences	Preference for lepidopteran larvae fed with non-*Bt* maize, preference for aphids over lepidopteran larvae
				Lepidoptera fed with *Bt*		
Pilcher et al. 1997	Bt176	Pollen	Coccinellid beetle	Pollen	Mortality, development	No effects
			Anthocorid bug			
			C. carnea (Neuroptera)			
Romeis et al. 2004	Cry1A(b	Isolated toxin	*C. carnea* (Neuroptera)	Toxin in artificial diet	Mortality, development	No effects
Zwahlen et al. 2000	Bt11	Maize plant	Anthocorid bug	Thrips fed with *Bt* maize	Mortality, development	No effects

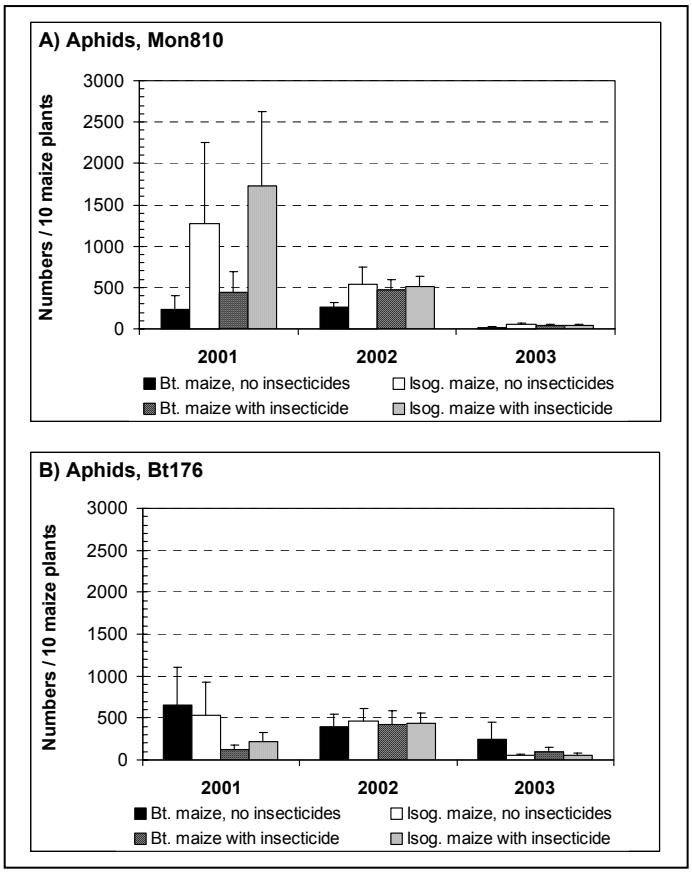


Fig. 1: Abundances of aphids in Bt corn fields with Mon810 by Monsanto (A) and Bt176 (B) by Syngenta during the years 2001 – 2003 compared to conventional control fields and one insecticide application (Baythroid 50). Shown are annual means of 10 plants each (+ SD) from three dates and from three (2001) and five sites, respectively (2002, 2003).

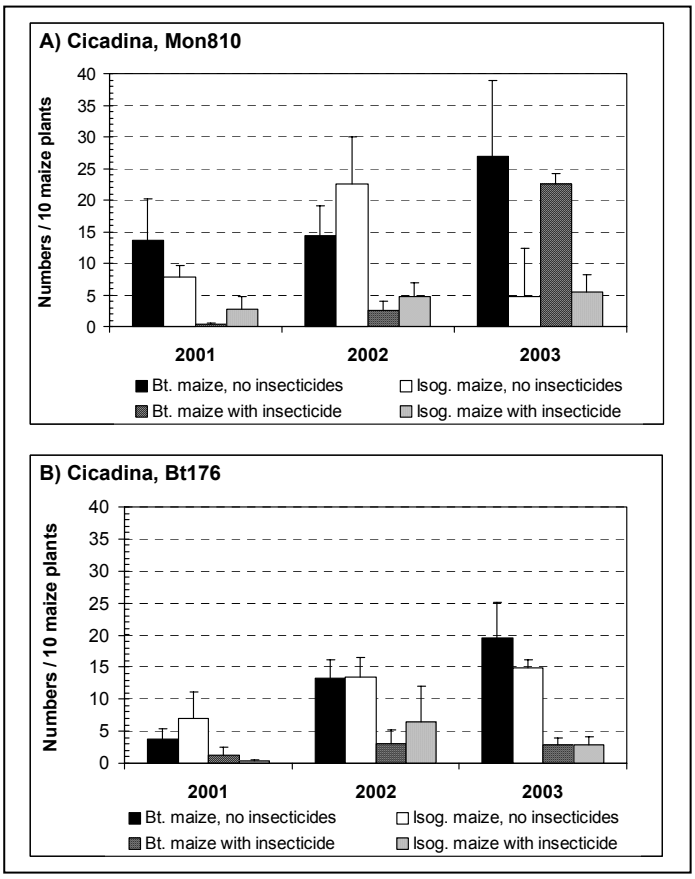
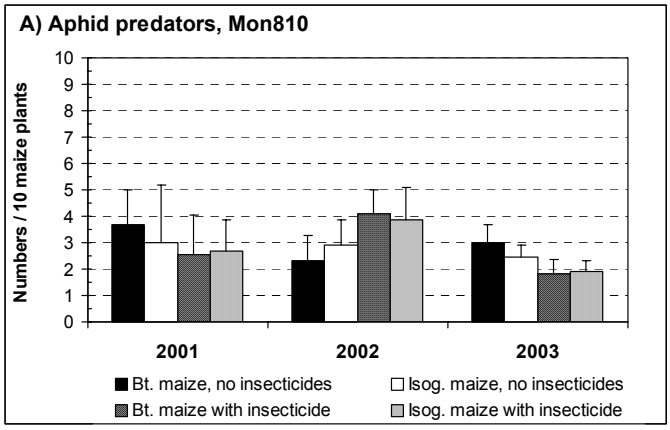


Fig. 2: Abundances of cicadas in Bt corn fields with Mon810 by Monsanto (A) and Bt176 (B) by Syngenta during the years 2001 – 2003 compared to conventional control fields and one insecticide application (Baythroid 50). Shown are annual means of 10 plants each (+ SD) from three dates and from three (2001) and five sites, respectively (2002, 2003).



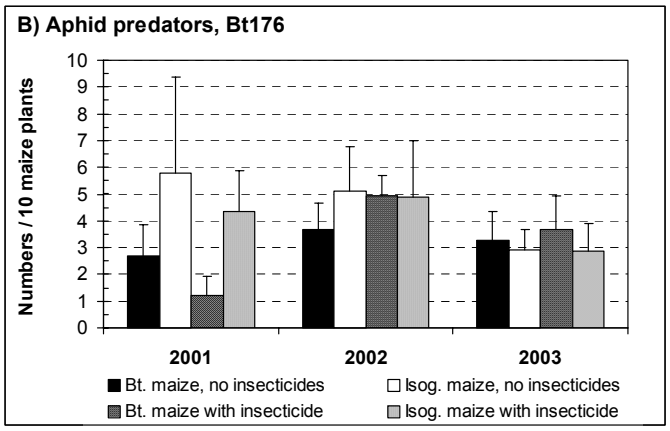
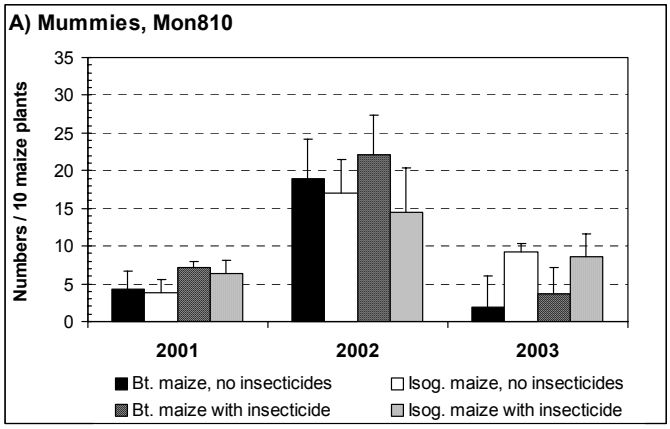


Fig. 3: Abundances of aphid predators in Bt corn fields with Mon810 by Monsanto (A) and Bt176 (B) by Syngenta during the years 2001 – 2003 compared to conventional control fields and one insecticide application (Baythroid 50). This combines adult hoverflies, hoverfly larvae, adult lady bugs, lady bug larvae, and lacewing larvae. Shown are annual means of 10 plants each (+ SD) from three dates and from three (2001) and five sites, respectively (2002, 2003).



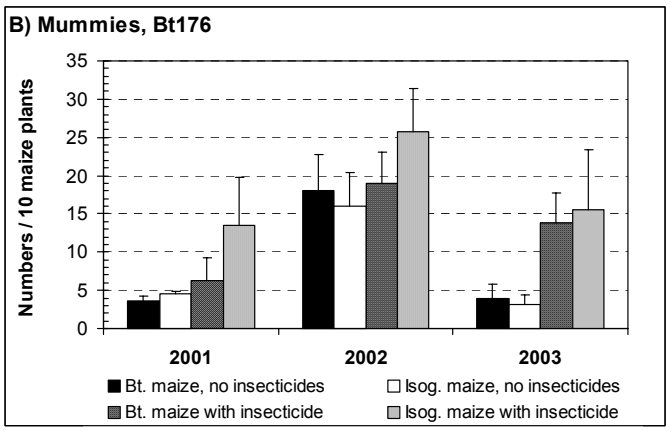
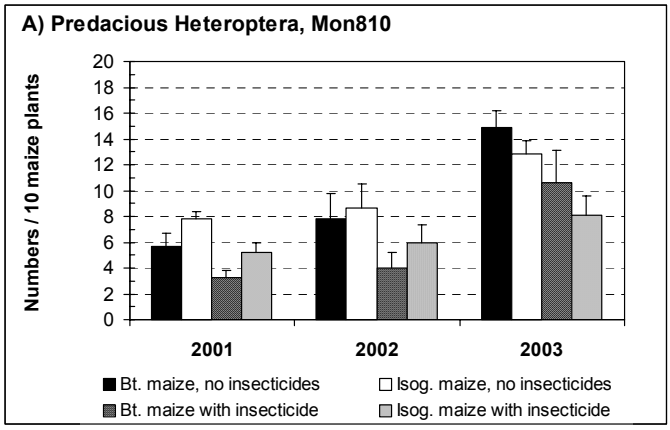


Fig. 4: Number of „mummies“ (aphids parasitized by predatory wasps) in Bt corn fields with Mon810 by Monsanto (A) and Bt176 (B) by Syngenta during the years 2001 – 2003 compared to conventional control fields and one insecticide application (Baythroid 50). Shown are annual means of 10 plants each (+ SD) from three dates and from three (2001) and five sites, respectively (2002, 2003).



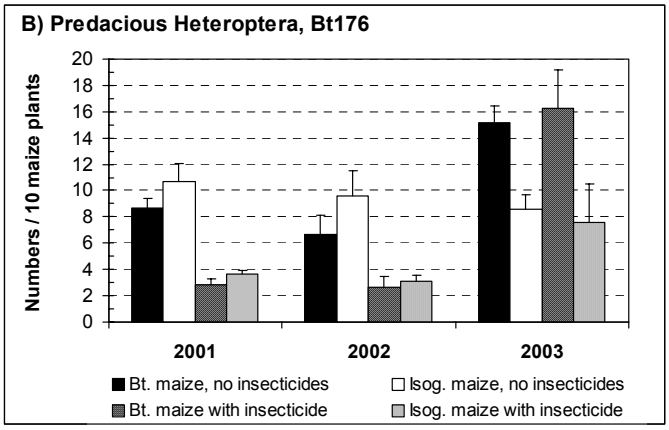
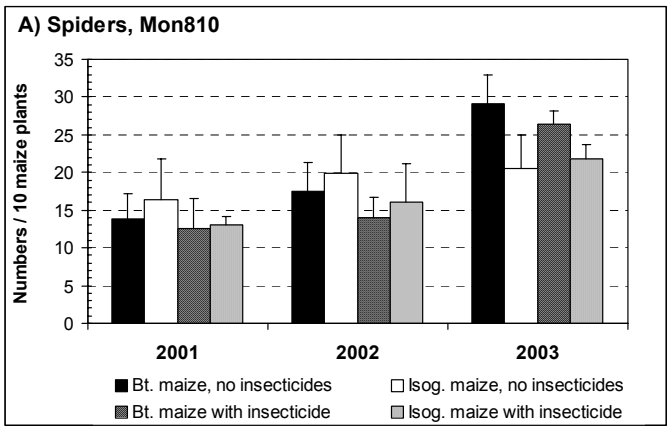


Fig. 5: Abundances of predatory heteropterans in Bt corn fields with Mon810 by Monsanto (A) and Bt176 (B) by Syngenta during the years 2001 – 2003 compared to conventional control fields and one insecticide application (Baythroid 50). Shown are annual means of 10 plants each (+ SD) from three dates and from three (2001) and five sites, respectively (2002, 2003).



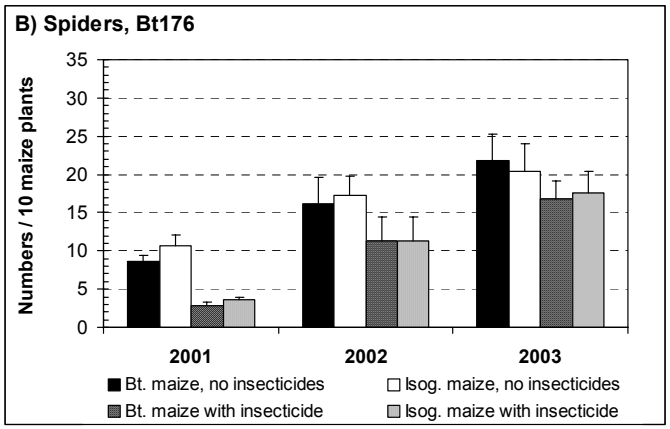


Fig. 6: Abundances of spiders in Bt corn in Bt corn fields with Mon810 by Monsanto (A) and Bt176 (B) by Syngenta during the years 2001 – 2003 compared to conventional control fields and one insecticide application (Baythroid 50). Shown are annual means of 10 plants each (+ SD) from three dates and from three (2001) and five sites, respectively (2002, 2003).

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3.1.2 Butterflies

3.1.2.1 The Task at Hand

This subproject was to investigate potential dangers to the butterfly fauna arising from the pollen of Bt corn. During the flowering of corn, corn pollen can be shed into adjacent and neighboring fields, where it may be consumed by non-target organisms like caterpillars feeding on leaf material of their food plants contaminated with corn pollen, thereby ingesting the toxin contained in the pollen. The potential negative effect of the corn pollen is likely to be correlated to its Bt toxin content, so that comparatively, a stronger effect would be exerted by the variety "Navares" (Bt176 by Syngenta) then from the variety "Novelis" (Mon810 by Monsanto). Various approaches and issues were studied within this research project on the lepidopteran fauna: Studies on the flowering dates of corn, determinations of toxin contents in the pollen of Bt corn, quantification of deposits of Bt pollen at the field margins, assessment of the relevant butterfly community, selective evaluation of the conservation-related species mapping of Bavarian Environmental Protection Agency and the cases of the species *Papilio machaon* (swallowtail) and *Arctia caja* (woolly bear), which were studied more intensively as case examples.

3.1.2.2 Extensive Documentation and Evaluation of the Literature Used

More frequent published laboratory trials on the effects of Bt corn on non-target butterflies did not appear until this research project was already underway. Overall, eleven studies were researched and/or considered (Pilcher et al. 1997a, Losey et al. 1999, Hansen Jesse & Obrycki 2000, Wraight et al. 2000, Felke & Langenbruch 2001, Hellmich et al. 2001, Zangerl et al. 2001, Felke et al. 2002, Binning & Rice 2002, Hansen Jesse & Obrycki 2002, Felke & Langenbruch 2003). As with the aphids and antagonists (s. above) most of the studies came from the US (Tab. 1). Of the species dealt with there, about half are also relevant in Europe, which is proven mainly by three studies of the Federal Biological Research Centre for Agriculture and Forestry (BBA), Darmstadt (Felke & Langenbruch 2001, 2003, Felke et al. 2002). The US studies (laboratory and field) focus very strongly on the monarch, *Danaus plexippus* (Losey et al. 1999, Hansen Jesse & Obrycki 2000, Hellmich et al. 2001, Stanley-Horn et al. 2001, Zangerl et al. 2001). Most of the laboratory experiments were pollen feeding experiments (Tab. 1), but also green corn plant material in the case of secondary lepidopteran pests (Pilcher et al. 1997a, Binning & Rice 2002). Among the various Bt events, Bt176 was tested most frequently (82% of the eleven studies), followed by Bt11 (45%), then Mon810 (18%) and a few additional events (some studies tested several events at a time). The first larval stage (L1), which is sensitive to Bt toxin, was tested most often, the usually less sensitive older stages (L2 – L4) were tested only in the European species. Most of the time, larval sample sizes tested were sufficient. It should be considered however, that the larval numbers presented in Tab. 1 often have to be divided up between the various Bt events as well as the respective controls.

As far as we know, only four published field studies on the effects of Bt corn on non-target butterflies exist, all of them from the US (Pilcher et al. 1997a, Wraight et al. 2000, Stanley-Horn et al. 2001, Zangerl et al. 2001). One studies deals with secondary harmful lepidopterans in corn (Pilcher et al. 1997a), and the other three with the effects of Bt corn pollen on two butterflies, *Danaus plexippus* and *Papilio polyxenes* (Tab. 2). The three most commonly used Bt events dealt with were: Bt176, Mon810, and Bt11. With the exception of Pilcher et al. (1997a) the experiments were conducted only during one season and during the flowering period of corn.

3.1.2.3 Conditions Under Which the Work Was Conducted

In order to carry out the work of the "Butterflies" subproject, all experimental fields as well as all lands of the experimental estates were used (see chapter 2 "Issues, Goals, and Experimental Design"). To determine the toxins in Bt corn pollen, the fields of the long-term experiments as well as the test fields were sampled. This way, pollen samples of both of the relevant Bt events (Bt176, Mon810), pollen from different years, sites, and cultivation settings were included, making the results more representative. In order to assess pollen deposition in field margins we had the fortune to be able to use fields of realistic size on the approximately 5 acres of the "normal" cultivation fields *[on the experimental farms]*, and thereby study a situation that was relevant to normal agricultural practice. The counting of the butterflies in the margins of corn fields was performed by mapping (visual observation) of adult individuals, a common assessment method, which is less time-consuming than the counting of larval stages. The mapping was carried out over four years at a total of seven sites from Southern (Schwaiganger) to Northern Bavaria (Schwarzenau), making the collected data relevant with regards to the assessment period and geographical distribution. From the Bavarian Environmental Protection Agency we obtained species conservation mapping data, which can be analyzed regarding habitat use and phenology of the various butterfly species. We chose two species as case examples, the swallowtail (Papilionidae, *Papilio machaon*) and the woolly bear (Arctiidae, *Arctia caja*). Habitat use (i.e. agricultural or adjacent lands), overlap of the phenology with the flowering period of corn, ability to breed them in the laboratory, and endangered status (listing in the Bavarian or German Federal "Red List"[3] were used as selection criteria for the two species. The field margins of the 5 acres of test fields at Grub and Neuhof (Bt176 corn "Navares") were also used for field experiments with swallowtail larvae. As explained previously in the "Aphids and Antagonists" chapter under 3.3.1.3 (see there), due to personnel constraints, the subject "Pests, Beneficial Organisms, Protection of Stored Crops" work area IPS 2d of the subproject could not be realized to the extent it had been planned.

3.1.2.4 Planning and Progression of the Work

Flowering Time. In order to determine the flowering times of various corn varieties, we used the LfL data base containing the studies of corn varieties they had conducted. We used data from the years 1995 – 2000 since it was only since the 2001 season that flowering times were no longer recorded in the corn variety studies. Overall, we evaluated "only" the data from 6 years since all of those data were still stored in mainframe format and conversion into PC formats was very time-consuming. In order to obtain data about the flowering time of Bt corn, the flowering data for "Navares" (Bt176 by Syngenta) in the "normal" cultivation test fields and in the permanent experimental plots from 2000 to 2003 were recorded and mined. "Novelis" (Mon810 by Monsanto) was not evaluated; however its flowering time should be similar since both isogenic varieties, Nobilis by Monsanto (K270) as well as Antares by Syngenta (K220), flower relatively early.

Determination of Toxins. Toxins in Bt corn pollen were determined by serological ELISA. For this, we tested mainly Bt176 pollen since it has a higher Cry1A(b) load, while the toxin concentrations of Mon810 pollen are often below the lower detection limit of the test kits.

Pollen Deposition. Distribution of corn pollen was assessed in and near the test fields, using microscopic slides (see "3.1.2.6. Scientific and Technical Methods Used") in and on the

[3] List of Endangered Species

test fields with Navares corn (Bt176). Since the microscopic slides were placed at distances of up to 10 m from the corn field (s. Fig. 2), there had to be corresponding pollen-free areas at the field margins. Due to the predominant topography and the structure of the spaces around the fields, the North side of corn fields ended up being overrepresented while no data could be recorded from the South side (based on predominant wind directions, the deposition is expected to be lowest on the South side). The wild carrot (*Daucus carota*) was chosen to study corn pollen density on the leaves of the butterflies' food plants, since it is a preferred food plant of swallowtail caterpillars, the caterpillars of the species that was studied for this project (see below).

Butterfly Mapping. Visual observation of adult butterflies was used to record butterfly species occurring in the margin of corn fields. Recording the larvae would have yielded the important proof of autochtonicity, however, recording of larvae is much more time consuming and would not have been possible within the framework of this project. Occasionally, however, autochtonicity could be proven by accidental observation of oviposition, caterpillars or pupae (see also Tab. 3). Moths, another important group that would have been interesting within the framework of the project, were not recorded, since this would have exceeded the time and personnel limits of the working group. Later, the data of the butterfly mapping were used to calculate the required sample size for a future monitoring.

Mapping for Species Conservation. The Species Conservation Maps of the LfU can be used to analyze seasonal appearance, as well as habitat use of any butterfly species in Bavaria. These parameters are of eminent importance in order to be able to determine the overlap in time and space of these species with the Bt corn flowering period. Such an analysis was performed for the two species, swallowtail (*Papilio machaon*) and woolly bear (*Arctia caja*). We used the data for adult butterflies since the sample size of the larval records were too small. In principle, such an evaluation would be useful and advisable for all butterfly species, but it would have exceeded the time and personnel limits of the subproject.

Laboratory Experiments. The laboratory experiments were carried out with caterpillars of the swallowtail (*P. machaon*) and the woolly bear (*A. caja*) that were fed Bt176 corn pollen. Pollen of Bt176 corn was used since it has a higher Bt toxin concentration than Mon810 pollen. The swallowtail experiments were conducted with first stage larvae (L1). The woolly bear caterpillars were to be used to study whether feeding on Bt corn pollen influences the various larval stages to a different degree, which is why larval stages L1 – L3 were used. Unfortunately, the delicate L1 larvae in all attempted experimental setups passed away so that we have results only for woolly bear larvae of the stages L2 and L3.

Field Experiments. The field experiments were conducted 2001 with L1 swallowtail caterpillars in the margins of the Bt176 test fields in Grub and Neuhof (Vojtech 2002). Due to the local topographic conditions and possibilities, the experiment took place in the Northern field margin in Grub, and in the Eastern field margin in Neuhof. The caterpillars were in the field during the corn flowering period and thereafter transferred into the lab, where they were to complete their development into adults. Due to a microbial infection, a large number of the animals died and sublethal parameters like duration of development e. g., could not be recorded. During the years 2000 (Grub) and 2002 (Schwarzenau), field experiments with swallowtail larvae in Bt corn fields were also carried out. However, in both of those years, the data had to be discarded because of excessive rain during the experimental period (no or low pollen deposition as well as increased larval mortality due to intense rain). Such experiments are very work-intensive, among others since it is necessary to breed the larvae so that they are ready at the exact flowering time of the corn, daily attendance of the experiment on site, as well as aftercare for the animals (including the cultivation of food plants). Furthermore, an extremely high number of caterpillars have to be raised and cared for in order to achieve

sufficient sample sizes, and to be able to compensate for the high larval mortality during the field phase. This is why the field experiments were not repeated during the last season, 2003, since the (uncertain) gain of knowledge from the experiment was not commensurate with the efforts required for it.

3.1.2.5 Overview of the Overall Issues, and Currently Known Findings in the Area of the Task at Hand

Bt toxins var. *kurstaki* (Cry1Ab) have toxic effects on caterpillars and have been used as biological sprays against lepidopteran pests in agriculture for a long time (Glare & O'Callaghan 2000). Lethal and sublethal effects of Bt preparations on non-target lepidopteran larvae have been proven in the laboratory as well as in the field (e.g. Miller 1990, Johnson et al. 1995, Wagner et al. 1996, Peacock et al. 1998). Losey et al. (1999) pointed out that, due to the wind, Bt corn pollen drifts onto food plants at the field margin where it can be consumed by lepidopteran larvae living there. Since pollen of Bt corn also contains Bt toxin, this could lead to damage to non-target butterfly species. The extent of this negative effect on larvae depends on the toxin concentrations in the pollen, the degree of exposure (flowering time, pollen distribution, and amounts), and the toxic effects of Bt pollen consumption on the butterfly larvae (Sears et al. 2001).

It is known that the Bt toxin (Cry1Ab) content in pollen differs between different events and varieties. For instance, the pollen of Bt176 contains several times the amount of Bt toxin (1.1 to 2.9 µg Cry1A(b) per g pollen) compared to Mon810 pollen, the content of which ranges from 0.002 to 0.09 µg Cry1A(b) per g pollen (Pilcher et al. 1997b, Hansen Jesse & Obrycki 2000, 2002, Wraight et al. 2000, Sears et al. 2001, Wolt 2003).

In Central Europe, the flowering time of corn fields is usually in July, and corn fields flower 5 to 8 days on average (Zscheischler et al. 1990, Treu & Emberlin 2000, Feil & Schmid 2001, Oberhauser et al. 2001, Wolt 2003). There are many publications on corn pollen shedding (amount and reach) (Fleischmann 1942, Raynor et al. 1972, Emberlin et al. 1999, Hansen Jesse & Obrycki 2000, Treu & Emberlin 2000, Wraight et al. 2000, Pleasants et al. 2001, Stanley-Horn et al. 2001, Zangerl et al. 2001, Eastham & Sweet 2002). However, the results of these studies are often not comparable, due to different field sizes, different methods, different durations of recording and more. Corn pollen can drift very far (up to 2000m, Fleischmann 1942), but the majority of the corn pollen probably does not fly further than 60 m (Raynor et al. 1972). Eastham & Sweet (2002) estimate that approximately 90% of the corn pollen is deposited within a 10 m radius around the flowering corn field.

There are practically no studies on the extent to which pollen deposited in field margins ends up on the food plants of butterfly larvae. Pleasants et al. (2001) report that at the field margin, about 40% of the pollen that drifts into it settles on the leaves of milkweed, the food plant of the monarch butterfly. All other studies that measure pollen concentration on food plants originate in the US and are not transferable to European conditions since they focus on the monarch butterfly (*Danaus plexippus*) and milkweed (*Asclepias syriaca*) (Hansen Jesse & Obrycki 2000, Stanley-Horn et al. 2001, Zangerl et al. 2001). Only the von Zangerl et al. (2001) study reports about pollen deposition on the leaves of wild parsnip (*Pastinaca sativa*) which is also a food plant of the larvae of the European swallowtail (*P. Machaon*). The highest average amount of corn pollen on wild parsnip leaves directly at the field margin was 320 pollen grains per cm^2 leaf area, and 2 m away from the corn field 180 pollen grains per cm^2 (Zangerl et al. 2001).

Laboratory studies on the effects of Bt corn pollen consumption are summarized in Tab. 1 . According to our present knowledge, the consumption of Bt176 pollen causes increased

mortality in butterflies as well as sublethal effects like reduced weight gain and delayed development (Felke & Langenbruch 2001, 2003, Hansen Jesse & Obrycki 2000, Wraight et al. 2000, Felke et al. 2001, Hellmich et al. 2001, Zangerl et al. 2001). The toxic effect of Mon810 and Bt11 pollen seems to be comparatively mild (Hansen Jesse & Obrycki 2000, 2002, Wraight et al. 2000, Hellmich et al. 2001). Older larval stages of several butterfly species appear to be less sensitive to Bt toxin than the L1 larvae (Binning & Rice 2002, Felke & Langenbruch 2001, 2003, Felke et al. 2002, Hellmich et al. 2001). In several studies, the respective LD50 values were calculated (Felke & Langenbruch 2001, 2003, Hellmich et al. 2001, Felke et al. 2002). It should be noted that in laboratory studies, Bt corn pollen is usually offered to the larvae only short-term (1 – 2 days), whereas pollen deposition under field conditions lasts longer (average 5 – 8 days, maximum up to 14 days). It is also apparent that only a few studies considered the entire development period from egg to adult. The reproductive rate of adult females would be a particularly important parameter since it plays a key role in determining changes in population density. It should also be noted that the standardized laboratory conditions represent ideal conditions for the larvae. Butterfly larvae under stress would react more severely to toxins, and in the field, caterpillars are exposed to numerous additional stress factors, adverse climatic conditions, food shortages, diseases and predators. Food shortages as well as infections, for instance, increase the caterpillar's sensitivity to Bt toxin (Pierce et al. 2001, Ben-Dov et al. 2003). The intake of Bt corn pollen can also lead to lower activity levels of the larvae, which may spend more time on the leaf surface, making them more susceptible to predation (Felke & Langenbruch 2001).

Only three (!) field studies on the effect of Bt corn pollen on caterpillars have been published to date (Tab. 2), which is not surprising, considering how involved such studies are. There are no published field studies on European butterfly species, here we depend totally on published results of laboratory studies (but see also “3.1.2.7 Progress Made in Other Places That Became Known While This Study Was Conducted”). In principle, the published field studies reflect the results of available laboratory studies: Bt176 corn can cause sublethal as well as lethal damage to butterflies at the field margin, whereas Mon810 and Bt11 corn seem to have fewer negative effects (Tab. 2). However, the sample sizes of some of the studies appear too low (due also to the high natural mortality of the larvae), to permit statements with a high level of probability and validity.

3.1.2.6 Scientific and Technical Methods Used

Flowering Time. The experiments on varieties conducted at the LfL during the years 1995 to 2000 were researched in order to determine the flowering time of corn in Bavaria. Overall, 307 different corn varieties grown on 1680 fields at 22 different sites were considered. To determine the flowering time of Bt176 corn “Navares”, flowering times in the 5 acre “normal” cultivation test fields on the experimental estates between 2000 and 2003 were recorded.

Determination of Toxins. The determination of toxins was performed for pollen of Mon810 (“Novelis” by Monsanto) and Bt176 (“Navares” by Syngenta), as well as on the controls with pollen of the isogenic varieties (“Nobilis” by Monsanto, “Antares” by Syngenta). Pollen was collected between 2000 and 2003 on the experimental fields of the state-owned estates. The pollen was passed through a 1mm sieve and then stored at –20°C. For determination of toxin content the pollen was thawed for 12 hours at 28°C and sieved once more before analysis (0,1 mm mesh). An ELISA test kit EnviroLogix QuantiPlate™ for Cry1Ab/Cry1Ac by Adgen© was used for the determination. This kit has a detection limit of 250ppb. Of each pollen sample, 2 to 3 aliquots were taken and determined and the mean of the Bt content of the pollen samples calculated.

Pollen Deposition. The corn pollen deposition in field margins was analyzed at the margin of the test fields during the years 2000 to 2002. The number of pollen grains was determined using microscopic slides (76 * 26 mm) coated with a thin layer of Vaseline. These microscopic slides were placed at a height of 6 – 20 cm and at different distances from the margin of the corn field (-5 m into the corn field proper, directly at the margin at 0 m distance, and different distances of up to 10 m). The microscopic slides were exposed for 24 hours, and then the pollen count per area counted manually in the lab or by using image analysis (Leica Qwin). Overall, 300 microscopic slides were exposed during these tests. To analyze which variables most strongly determine pollen counts, multiple regression analysis was performed with the dependent variable "Pollen density" and the independent variables "Air temperature", "Precipitation", "Relative humidity" "Wind speed" "Distance from corn field" "Height of the microscopic slide" "Direction of the field edge", "Developmental stage of the corn" (i.e. flowering stage of the corn, BBCH scale according to Meier 1977), and "Site".

To determine how much pollen gets deposited on food plants, experiments were carried out at Neuhof in 2002. Right at the margin of the Bt176 test field, during flowering of the corn, wild carrot (*Daucus carota*) leaves were exposed. The average length of the leaves was 15 cm, and they were placed at a height of 9 cm for 24 h. Overall, 36 leaves were exposed on July 22, 23, and 26 (BBCH state of the corn = 65). At the end of the exposition period, transparent tape was pressed onto the upper side of the leaves, the leaves collected, and the pollen count on the upper side of the leaves determined manually. The leaf area was determined by image analysis (Leica Qwin) and the number of corn pollen grains per cm^2 calculated. The pollen deposition on the microscopic slides (see above) was measured at the same time.

Butterfly Mapping. During the years 2000 to 2003, mapping walks occurred from May to September on seven sites of the state-owned experimental estates to capture the occurrence of adult butterflies (Grub, Finsing, Schwaiganger, Puch, Baumannshof, Neuhof, Schwarzenau). The species observed as well as frequencies in field margins along the corn fields were recorded (Bt as well as conventional fields). Overall, 24 field margins were sampled with differing intensity. There was no systematic recording of proofs of reproduction of adult butterflies, however, accidental findings of eggs, larvae, or pupae were recorded (for methods see also Lang 2004). Based on the butterfly numbers found and their simple variances, the required sample size for a monitoring that should possibly accompany the cultivation of Bt corn in the future was determined. The number of field margins required to be sampled for statistical validation of differences between edges of Bt corn vs. conventional corn fields was calculated. The software nQuery, version 4.0, was used for this purpose. The calculations were based on the following assumptions: normal distribution of the data, equal distribution of samples between the two types of edges (Bt vs. conventional) a t test for independent samples is used, the test is one-sided, the significance level $\alpha = 0.05$, and the power of the test is = 80% (i.e. the probability of discerning a difference if α is a given).

Species Conservation Mapping. The observation sites and data of the Species Conservation Mapping (ASK) of the Bavarian Environmental Protection Agency were studied for both butterfly species, the swallowtail (*Papilio machaon*) and the woolly bear (*Arctia caja*) (status: 04.19.2004). We used the data for adults regarding habitat use (occurrence in their habitat) and flight times. We used only data for up to 800 m above sea level since, in Bavaria, corn is not grown above that (information obtained from Agricultural Services Kaufbeuren, Weilheim, Bad Tölz-Wolfratshausen, Passau-Rotthalmünster and others). The program MS-Access 2000 was used to analyze the data. Observation of a butterfly was considered as "Occurs on agricultural land" if the habitat description of the ASK matched one of the following criteria: unmanaged grassland, meadows and pastures, grassland, rich

meadow/rich pasture, field, arable land, special cultures, vineyard (unmanaged), vine cultures, vineyard (managed), extensive grassland, agricultural lands, mesophilic grassland. A mention of the following in the vegetation description of the ASK also led to the assignment to "Occurs on agricultural land": field, agricultural weed community, agricultural land, rich meadow/rich pasture, vineyard, grain field, barley field, clover field, corn field, rape field, oat field. For purposes of graphic representation (Fig. 5, 7), the categories were classified as belonging to one of the following four agricultural habitats "Grassland", "Fields", "Vineyards" and "Areas in unspecified agricultural use." In order to categorize observation sites that were not on areas in agricultural use, but adjacent to them we used the information contained under surrounding vegetation and surrounding uses of the ASK analogously.

Laboratory Experiments. First stage caterpillars (L1 larvae) of *P. machaon* were fed the pollen of Bt176 corn (variety "Navares") in the laboratory, controls were not fed pollen. For this purpose, varying amounts of corn pollen were applied to standardized food platelets made from wild parsnip (*Pastinaca sativa*) leaves, one L1 caterpillar placed on each of the platelets, and the amount of pollen consumed recorded after 48 h. The L1 larvae used for this experiment were between 12 and 24 hours old and were not fed for approximately 4 – 5 hours before the experiment in order to induce a standardized hunger state. The experiments were conducted at a temperature of 25°C, 50% rel. humidity and 16 h days. Mortality of the larvae, sublethal effects like feeding performance, duration of the development, weight gain, pupa weight, as well as size and weight of the adult butterflies were recorded and evaluated. In analogy to the laboratory experiments described above, woolly bear caterpillars (*Arctia caja*) of developmental stages 2 and 3 were fed Bt176 corn pollen (variety "Navares"). The controls received no pollen. Again, different amounts of corn pollen were applied to standardized food plant platelets (dandelion), one caterpillar placed on each of the platelets, and the amount of pollen consumed recorded after 24 h. The L2 larvae used for this experiment were on average 10, the L3 larvae 12 days old and were not fed for approximately 4 – 5 hours before the experiment in order to induce a standardized hunger state. The experiments were conducted at a temperature of 25°C, 50% rel. humidity and 16 h days. Mortality of the larvae, sublethal effects like feeding performance, duration of the development, weight gain, pupa weight, as well as size and weight of the adult butterflies, and their lifespan were recorded and evaluated.

Field Experiments. The field experiments were conducted with newly hatched L1 larvae of the swallowtail (*P. machaon*) (Vojtech 2002). The experiments were conducted at Grub (07.20. – 07.24.2001) and Neuhof (07.25.– 07.30.2001). When the corn was flowering, potted food plants (wild carrot) were placed at the margin of the Bt176 corn field ("Navares") at different distances (0 – 10m) and thereby exposed to different pollen concentrations (see also Fig. 2). The distances used at Grub were 0m, 1m, 5m, and 10m. At 0m distance, at Grub, 4 carrot plants were placed, and three plants each at the other distances. The distances tested at Neuhof were 0m, 1m, 3m, 5m, and 10m. At Neuhof, 6 carrot plants were placed at each of the distances. Both, at Grub and at Neuhof, 10 caterpillars each were placed on each carrot plant. The larvae were left to remain in the field 5 (Grub) to 6 days (Neuhof) and the survival rates recorded.

3.1.2.7 Progress Made in Other Places That Became Known While This Study Was Conducted

M.H. Pham-Delègue, L. Kaiser, R. Ramirez-Romero, J. Chantaux and R. Delorme of the French Institut National de la Recherche Agronomique (INRA) have conducted laboratory experiments on the effects of Bt corn pollen on butterfly larvae (not yet published). L1 larvae were fed Mon810 corn pollen, and the LD50 determined. They tested the following European species: *Pieris brassicae*, *Colias crocea*, *Nymphalis urticae*, *Maniola jurtina*, *Papilio*

machaon, *Lycaena phlaeas*, *Laothoe populi* and *Smerinthus ocellata*. For all species, the respective LD50 was found to be above the dosages that can realistically be expected in the field. Nothing has been published about the exact extent of the lethal and sublethal damage.

The working group of I. Schuphan and A. Gathmann at the RWTH Aachen, Chair of Biology V, sampled seeded strips of wild mustard (*Sinapis arvensis*) along corn fields during pollen shedding. They sampled and evaluated caterpillars of the butterfly species *Plutella xylostella* and *Pieris rapae* next to Bt corn, conventional corn, and corn with insecticide treatment. The results (unpublished) show a reduction of caterpillar abundances due to the insecticide application, and no difference in the mortality between Bt corn and controls during the flowering phase of the corn.

Besides their laboratory experiments, M. Felke and G.A. Langenbruch (s. Tab.1) also conducted field experiments with larvae of the peacock (*Inachis io*) and Bt176 corn. These unpublished studies show that under field conditions as well, the pollen shedding of Bt176 corn has negative effects on the peacock caterpillars.

B. Darvas of the Plant Protection Institute in Budapest, Hungary, researched the effects of Bt corn pollen of Mon810 on L1 larvae of the peacock (*I. io*) and of the comma (*Polygonia c-album*) in laboratory experiments. Upon administration of naturally occurring amounts of pollen, a reduced weight gain was observed in the caterpillars after feeding on Bt pollen. However, this effect disappeared during subsequent larval development to the adult. That study has not yet been published either.

For results that were published while this research project was on its way, see "3.1.2.5 Overview of the Overall Issues, and Currently Known Findings in the Area of the Task at Hand".

3.1.2.8 Presentation and Discussion of the Results

Flowering Time. Most of the corn (91%) flowered around mid-July 07.18. ± 9 days (mean ± SD) (Fig. 1A). The earliest date was June 27, and the latest was August 8. The Bt variety "Navares", on average, flowered on July 12, with the earliest date being June 30, and the latest July 26 (Fig. 1B). Since corn fields can shed pollen for up to 14 days, (Oberhauser et al. 2001, Treu & Emberlin 2002), one can expect flowering corn fields in Bavaria from the end of June to the third week of August.

Toxin Determination. The pollen of Bt176 corn "Navares" contained 2.59 ± 0.40µg Cry1Ab / g pollen (n = 10, mean ± SD). The pollen of Mon810 corn "Novelis" contained on average 0.25 ± 0.09 µg Cry1Ab and 0.13 ± 0.16 µg Cry1Ab per gram pollen, depending on whether one calculates the value of the lower detection limit as zero or as 250 ppb (= detection limit) (n = 6, mean ± SD). The samples of the isogenic, conventional comparative varieties (n = 14) did not contain any Bt toxin. The toxin contents in pollen found in this study are within the previously reported range, however, in the upper third thereof. (Pilcher et al. 1997, Hansen Jesse & Obrycki 2000, Wraight et al. 2000, EPA 2001, Sears et al. 2001; Hansen Jesse & Obrycki 2002, Wolt 2003).

Pollen Deposition. Pollen deposition at the field margin was highly variable, ranging from 0 to 429 pollen grains per cm^2 and 24 hours (Fig. 2). Pollen density decreased with increasing distance from the field, but could still reach up to 93 pollen grains per cm^2 at a distance of 10 m (Fig. 2). Pollen density was most intensely influenced by the relative humidity of the air, the distance from the margin of the corn field, and the flowering stage of the corn, in accordance with the multiple regression equation: Pollen density (n/cm^2) = 17,11 – 3,37 * Rel. humidity (%) – 4,00 * Distance from the field (m) – 3,26 * Flowering stage

(BBCH) (R^2 = 0,48; p < 0,001; values for Pollen density Ln(x+1) transformed, Rel. humidity arcsine(x)-transformed, Distance from the field margin square root (Ln(x+10)) transformed, and of the Flowering stage arcsine (x/67) transformed). The other tested variables (s. 3.3.2.6) had comparatively less influence on pollen density. Compared to the few other studies published to date, it should be noticed the pollen densities at the field margin recorded here are somewhat higher than previously reported (Raynor et al. 1972, Hansen Jesse & Obrycki 2000, Wraight et al. 2000, Stanley-Horn et al. 2001, Zangerl at al. 2001). In this project as well, a steady decrease of pollen density as a function of the distance to the margin of the corn field was observed, however, the decrease was less rapid then previously reported so that relatively high pollen counts could still be found at a 10 m distance.

The more pollen was shed at the field margin, the more was found on the wild carrot leaves (Fig. 3). On average, there were 33,5 ± 24,9 pollen grains per cm^2 leaf area and 24 h (mean ± SD; minimum = 7,1 pollen/cm^2; maximum = 93,0 pollen/cm^2). Hence, 31,6%, on average, of the shed pollen ended up on the leaves (minimum = 12,4%; maximum = 63,5%). It is known about pollen densities in this range that they can have adverse effects on caterpillars (Hansen Jesse & Obrycki 2000; Stanley-Horn et al. 2001; Zangerl et al. 2001; Felke & Langenbruch 2003). It should further be considered in this context, that the values presented here are based on 24 h measurements, while pollen can accumulate on the plants at the field margin over several days while the corn flowers. This becomes apparent from several other studies, for instance, that describe higher pollen densities on caterpillar food plants, resulting from several days of accumulation. On the other hand, intense rain or wind can also wash or blow the pollen off the plants (Hansen Jesse & Obrycki 2000, Pleasants et al. 2001, Stanley-Horn et al. 2001, Zangerl et al. 2001).

Butterfly Mapping. Overall, 949 individuals were observed at the margin of corn fields, belonging to a total 36 butterfly species (Tab. 3). The most common species were the cabbage white (*Pieris rapae*), the green-veined white (*P. nape*), the small tortoiseshell (*Nymphalis urticae*) and the small heath (*Coenonympha pamphilus*), while the remaining species made up less than 5% each of the sample. 22% of the species found are listed in the Bavarian "Red List" *[of endangered species]* (Golz & Geyer 2003) and 28% in the "Red List" *[of endangered species]* for Germany (Pretscher 1998). This mapping describes a realistic butterfly species community in Bavarian field margins. It should be noted, though, that the observed field margins are located on intensively used agricultural lands and that additional, including more rare species are likely to be found in more extensively used area, more natural habitats, or other geographic regions. On the other hand, some of the species observed are definitely not endangered by Bt corn, since their larvae are hardly exposed to corn pollen at the margin of fields (*Apatura ilia,* e.g.). Table 4 shows the number of field margins that would have to be sampled in order to prove a Bt effect on butterflies within the framework of a field monitoring (see also Lang 2004). Depending on the prevailing circumstances, the number of field margins may become quite large. For example, in order to prove a 5% reduction in species numbers one would have to sample 2156 field margins, i.e. 1078 field margins next to Bt corn fields plus 1078 next to conventional fields. Or, sampling 2 x 12 field margins would be sufficient to statistically validate only a change that is equal to or greater than 50%. When studying individual species, the cost and time involved in carrying out these studies may increase considerably, depending on the butterfly species (Tab. 4). To be able to statistically validate a 10% reduction in swallowtail populations would necessitate the sampling of 1728 field margins. The exact number of field margins to be sampled does not only depend on the variance of the different variables, but also on the desired accuracy of the monitoring, i.e. which effect is to be shown with which probability at which level of significance (see also Fig. 4). Therefore, the present calculations on the sample sizes should not be considered as absolute values, but rather as an approximate estimate of the sample sizes required for a

monitoring. Standardizing of the mapping, a particular sampling design, the way the original data are structured and consideration of covariants might possibly reduce variance and thereby the required sample sizes (Perry et al. 2003).

Species Conservation Mapping. Of a total 2700 ASK records evaluated, 27% of the observation sites of *Papilio machaon* were on lean grasslands, 22% in bogs, wetland meadows and shores, and another 22% from lands under agricultural management; all other habitat types were represented at levels below 10% (Fig. 5). For a total of 682 (= 25%) observation sites not under agricultural management, adjacent agricultural use was reported. If one adds the latter observation sites to the observations on the agricultural lands it turns out that 47% of the Bavarian sightings of the swallowtail were made on lands under agricultural influence. The share of butterflies and swallowtails flying around on agricultural lands is probably underestimated since the a) data entered into the ASK mainly come from habitats that have special value from a conservation point of view, among which agricultural lands are therefore underrepresented, and b) since the people recording for ASK often do not note the use of the adjacent land. In Bavaria, *P. machaon* has two generations: The first generation flies between mid- and end-May, and the second generation, which is about three times as numerous as the first one, form approximately mid-July to mid-August (Fig. 6). Hence the flying period of the second generation overlaps with the corn's flowering period (see also Fig. 1).

Of a total 212 mined ASK entries, 27% of the sightings of *Arctia caja* were made in woods, and 15% in wetland meadows and bogs (Fig. 7). The second most common habitat was agricultural lands (15%). For a total of 38 (= 18%) sighting sites not under agricultural management, lands under agricultural management were listed as adjacent (Fig. 7). If one adds the latter observation sites to the observations on the agricultural lands it turns out that 33% of the Bavarian sightings of the woolly bears were made on lands under agricultural influence. In Bavaria, the woolly bear has a single generation during which it flies from mid-July to mid-August (Fig. 8). Hence the flying period overlaps with the flowering period of corn (see also Fig. 1).

Laboratory Experiments. The more Bt corn pollen L1 swallowtail caterpillars consumed, the lower were their survival rates (Fig. 9). The survival curve differed significantly from the control at dosages of 5mg/10ml and above. Tab. 5 shows that the pollen density on the leaf at a dose of 5mg/10ml may well correspond to naturally occurring pollen densities (24 hour value) (see also Fig. 3), so that a lethal effect of Bt176 corn pollen on swallowtail larvae in the field cannot be excluded. If one bases the calculation on the measurements of accumulated pollen densities on wild parsnip conducted by von Zangerl et al. (2001) even the treatment with 20mg/10ml in Table 5 is still within the range of naturally occurring pollen exposure. The more pollen there was on the wild parsnip leaves, the more of it the caterpillars consumed (Fig. 10A). The consumption of Bt corn pollen caused subsequent reduced feeding performance in swallowtail larvae (Fig. 10B) and furthermore, reduced body weight (Fig. 11A). Swallowtail larvae with a lower second day body weight pupated later (Fig. 11B). The length of chrysalis stage and chrysalis weight did not differ significantly between the treatments (ANOVA, $p > 0,05$, data not shown). The consumption of Bt corn pollen by the larvae had an effect neither on wing size of adult *P. machaon* nor on male body weight (Fig. 12A-C). However, Bt pollen led to lower body weight of the adult females (Fig. 12A). Overall, the experiments show that the consumption of Bt176 corn pollen can have lethal and sublethal consequences for swallowtail caterpillars and adults at pollen concentrations that naturally arise in the field. It should be considered here that corn fields flower for several days and that pollen can accumulate on the food plants, whereas the caterpillars of the laboratory experiment were exposed to a single dose of Bt pollen for only two days. Not just increased

mortality, but sublethal effects as well would have negative consequences for the populations. A delayed development means increased risk of capture by predators, and pushes the reproductive phase later into the season. Since female body weight correlates positively with egg production, lower body weight means fewer offspring.

No effect of Bt corn pollen on the mortality of L2 and L3 larvae of the woolly bear (*A. caja*) were found (Fig. 13). The amount of dandelion consumed as well as weight gain differed between the two larval stages (ANOVA, $p < 0.05$), but not between the Bt corn pollen treatments (Fig. 14, 15). We also found no significant effects of consumption of Bt pollen on the parameters duration of larval development, weight at pupation, and duration of the pupa stage, or size, weight, and life span of the adult butterflies (data not shown). As opposed to swallowtail caterpillars, woolly bear larvae therefore showed no adverse effects of Bt176 pollen. This may be due to the fact that older larval stages (or maybe this species?) react to the Bt toxin with a lesser sensitivity (Binning & Rice 2002, Felke & Langenbruch 2001, 2003, Felke et al. 2002).

Field Experiments. Within the scope of these field experiments, no effect of Bt176 corn on mortality of L1 caterpillars of the swallowtail (*Papilio machaon*) could be proven (Fig. 16). Neither at Grub nor at Neuhof did the caterpillars placed directly at the field margin, where they were exposed to the highest amount of pollen shedding, differ from those placed further away. However, their natural mortality was already very high, especially at Neuhof (Fig. 16B), which might have concealed a possible Bt effect. As far as the caterpillars at Neuhof are concerned, it can also not be excluded with certainty that they might not already have suffered from a bacterial infection. In principle, at least sublethal effects of Bt176 pollen on swallowtail larvae are also to be expected under field conditions. On the one hand, the caterpillars in the laboratory were sensitive to the administration of Bt corn pollen (s. above). On the other hand, it is known from field experiments with another species of the same genus *Papilio polyxenes*, that pollen shedding of Bt176 corn damages caterpillars of that species under field conditions (Wraight et al. 2000, Zangerl et al. 2001).

3.1.2.9 Summary

The genetically modified Bt corn varieties approved in Europe contain DNA of the soil bacterium *Bacillus thuringiensis* var. *kurstaki* and are thereby able to produce a toxin (Cry1Ab) that protects them against caterpillars of the European corn borer, a lepidopteran pest. While this toxin forms in all tissues of Bt corn, its concentrations in different tissues vary depending on tissue and Bt corn variety. In this subproject, we took a closer look at the potential endangerment of the butterfly fauna through the pollen of Bt corn. Corn is wind-pollinated, and during its flowering period, the corn pollen can be shed on adjacent and nearby areas where it may be taken up by non-target organisms. This could have a particularly negative effect on butterfly caterpillars at the field margin, since the Bt toxin is specific to lepidopterans. Caterpillars make contact with the toxin when Bt corn pollen is shed onto their food plants and the caterpillars then feed on the leaves contaminated with corn pollen, thereby ingesting the toxin contained therein.

Within the framework of these studies of the butterfly fauna, various approaches and issues were investigated while the project was ongoing (2000 – 2004): The flowering time of corn, toxin determination of pollen in Bt corn varieties, quantitative analysis of the shedding of Bt corn pollen into field margins, assessment of the butterfly societies found at the field margins, selected evaluation of the species conservation mapping of the Bavarian Environmental Protection Agency were researched, as well as case studies of the species *Papilio machaon* (swallowtail) and *Arctia caja* (woolly bear), which were investigated more

deeply as examples. The effects of a consumption of Bt corn pollen were studied in the laboratory as well as in the field.

In Bavaria, most of the corn by far (91%) flowers in July. The earliest onset of the corn flowering period was June 27, the latest was August 8. The Bt corn variety "Navares" (Event Bt176) flowered predominantly in July. The pollen of "Navares" (Bt176) contained on average 2590 ppb Cry1Ab toxin, the pollen of "Novelis" (Event Mon810) 90 – 250 ppb Cry1Ab toxin. Corn pollen deposition in field margins was highly variable, ranging from 0 to 429 pollen grains per cm^2 area and 24 hours. The pollen density decreased with increasing distance from the field margin, but was found to still be up to 93 pollen grains per cm^2 at a distance of 10 m. The more pollen was deposited at the field margin, the more pollen was found on the leaves of the wild carrot, a food plant of swallowtail caterpillars, after we planted them there. On average, 31% of the deposited pollen was found on the carrot leaves (mean 33.5 pollen grains per cm^2 leaf area and 24 h). A total 36 butterfly species were found at the field margins, 28% of which are listed as endangered in either the Bavarian or German "Red List" *[of endangered species]*. In Bavaria, the swallowtail (*Papilio machaon*) is found mainly on poor swards, but agricultural lands are another important habitat of this species: 47% of the sightings of this butterfly were made either on areas under agricultural management or in neighboring habitats. The woolly bear (*Arctia caja*) occurs mainly in wood- and wetlands, its second most important habitat are agricultural areas: 33% of the sightings of the woolly bear were made on areas under agricultural management or in neighboring habitats. The majority of the populations of both butterfly species flies in July, at the time of corn flowering. The consumption of Bt176 corn pollen had negative effects on L1 larvae of the swallowtail in the laboratory. The more Bt corn pollen the caterpillars consumed, the lower were their survival rates, the lower their body weight, the longer was the delay of their development, and the lower the body weight of adult females. These lethal and sublethal effects occurred at pollen concentrations low enough to occur naturally under field conditions. The consumption of Bt176 corn pollen had no effect on L2 and L3 larvae of the woolly bear. In two field experiments, no effect of Bt corn on caterpillars of the swallowtail could be found at the field margin. However, natural mortality was already very high in the field experiment (up to 95%), which may have concealed a BT effect. A sample size estimate showed that in a monitoring, a large number of field margins would have to be sampled in order to statistically validate a Bt effect on butterflies; for instance, proving a 10% reduction in swallowtail populations would require the sampling of 1728 field margins.

Due to the flowering period of corn and to the extent of the pollen deposition in field margins, exposure of non-target butterfly caterpillars appears highly probable. This exposure is, among others, determined by the flying period of the butterflies, and it is to be expected that in July, many butterflies are found at the field margins. Particularly in case of large scale cultivation of Bt176 corn, negative effects on the butterfly fauna at the field margin cannot be categorically excluded. In order to protect the butterfly fauna, Bt corn varieties with a lower toxin content in pollen (Mon810, Bt11) should therefore be planted. Alternatively, a 10 m wide buffer zone of conventional corn should be seeded around Bt corn fields. Because of the sparse present knowledge about field effects of Mon810 and Bt11 corn on indigenous butterflies, extensive field experiments and studies with these varieties are highly recommended. The species conservation mapping of the *[Bavarian]* Environmental Protection Agency provides the necessary data for the selection of the butterfly species to be considered therein if examined regarding habitats and flying times of the respective species.

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3.1.2.10 Appendix with Tables, Figures, References

List of Tables Figures

Tab. 1: Overview (Status: 06.22.04) of published laboratory studies with Bt corn and non-target butterflies (i.e. excluding "pests").

Study	Event, toxin	Diet	Organism(s) tested	Origin of species	Instar of larvae	Number of larvae tested	Parameters tested	Effects of *Bt* on study organism(s)
Binning & Rice 2002	Bt11 Cry 9C	Maize plants and green plant material	*Papaipema nebris* (Noctuidae), secondary pest	USA	L1 – L4	not known	Larval mortality & body weight	Higher mortality, lower weight, L3 – L4 less susceptible, no difference between Bt events
Felke & Langenbruch 2001	Bt176	Pollen	*Pieris brassicae* (Pieridae) *Pieris rapae* (Pieridae) *Plutella xylostella* (Yponomeutidae)	Europe	L2 – L3 L2 – L3 L4	ca. 440 ca. 670 ca. 550	Larval mortality & body weight	Higher mortality, lower weight, LD50 values differ among species, older larvae less susceptible
Felke et al. 2002	Bt 176	Pollen	*Pieris brassicae* (Pieridae) *Pieris rapae* (Pieridae) *Plutella xylostella* (Yponomeutidae)	Europe	L2 L2 L4	174 359 206	Larval mortality & body weight, consumption rate	Higher mortality, lower consumptio lower weight, LD50 values differ a species, older larvae less susceptible
Felke & Langenbruch 2003	Bt176	Pollen	*Nymphalis io* (Nymphalidae)	Europe	L2 – L4	3153	Larval mortality & body weight	Higher mortality, lower weight, L3 – L4 larvae less susceptible, LD5
Hansen Jesse & Obrycki 2000	Bt176 Bt11	Pollen	*Danaus plexippus* (Danaidae)	USA	L1	455	Larval mortality & body weight, pupal weight, adult wing length, adult lipid content	Higher larval mortality, higher eff Bt176, no effect on adults
Hansen Jesse & Obrycki 2002	Bt176, Bt11	Pollen, anthers	*Euchatias egle* (Arctiidae)	USA	L1 – L2	45	Larval mortality	No effects
Hellmich et al. 2001	Bt176,Bt11,Mon810, Cry9C, Cry1Ac,Cry1F	Artificial diet, pollen	*Danaus plexippus* (Danaidae)	USA	L1 – L3 L1	> 1000	Larval mortality & body weight, consumption rate	Higher mortality & lower weight consumption of Bt176, L3 – L4 susceptible, contaminants in pollen n (anthers) increase adverse effects, LC
Losey et al. 1999	Bt11	Pollen	*Danaus plexippus* (Danaidae)	USA	L2 – L3	50	Larval mortality & body weight, consumption rate	Higher mortality & lower weight, consumption rate
Pilcher et al. 1997a	Bt176	Maize: green plant material	*Agrotis ipsilon* *Papaipema nebris* *Pseudaletia unipuncta* *Helicoverpa zea* (all Noctuidae and secondary pests)	USA, Europe USA USA, Europe USA	L1 L1 (?) L1 L1	240 160 240 240	Larval mortality & development, pupal weights (male, female)	*P. unipuncta* larvae had higher mor longer development & less pupal w *H. zea* had higher mortality & l development
Wraight et al. 2000	Mon810, Bt176	Pollen	*Papilio polyxenes* (Papilionidae)	USA	L1	96	Larval mortality	No effect of Mon810, increased mo with Bt176
Zangerl et al. 2001	Bt176	Pollen	*Papilio polyxenes* (Papilionidae)	USA	L1	?	Larval mortality	Higher mortality, LD50 value

Table 2: Literature overview (Status: 06.22.04) of published field studies with Bt corn and non-target butterflies.

Study	Event	Field / plot size	N per treatment	Seasons	Country	Species	Instar of larvae (n = total number)	Parameters tested	Effects of *Bt* maize
Pilcher et al. 1997a	Bt176	22.8m^2	80 Bt plants, 80 control plants	2	USA	*Agrotis ipsilon* *Agrotis ipsilon* *Papaipema nebris* *Pseudaletia unipuncta* *Helicoverpa zea* (all species are Noctuidae and secondary pests)	L1 (n = 800 per yr) L4 (n = 320 per yr) L2 – L3 (n = 160 in 1 yr) L1 (n ca. 3000 per yr) L1 (n = 800 – 4000)	Consumption, consumption, consumption, consumption, consumption	No effect, no effect, less damage in Bt corn, less damage in Bt corn, less damage in Bt corn
Stanley-Horn et al. 2001	Bt176 Bt11 Mon810	21m^2, 5 – 17 ha 0.2 ha	3 – 10	1	USA	*Danaus plexippus* (Danaidae)	L1 (n = 96), Bt176 L1 (n = 40), Bt176 L1 – L3 (n = 480), Bt11 L1 (n = 540), Bt11 L1 (n = 150), Mon810	Larval survival & weight; for Bt11 corn only: developmental time, pupal and adult weight, and wing length.	Less survival and less weight with Bt176 corn, insecticide application increased mortality
Wraight et al. 2000	Mon810	1.2 ha	5	1	USA	*Papilio polyxenes* (Papilionidae)	L1 (n = 100)	Larval mortality, larval weight	No effects
Zangerl et al. 2001	Bt176	90m^2,	4 – 5	1	USA	*Danaus plexippus* (Danaidae) *Papilio polyxenes* (Papilionidae)	*D. plexippus* L1 (n = 600), *Papilio polyxenes* L1 (n = 300)	Larval mortality, larval weight	Less weight of *P. polyxenes* larvae, only 22 larvae of *D. plexippus* survived in total (i.e., statistical power is very low)

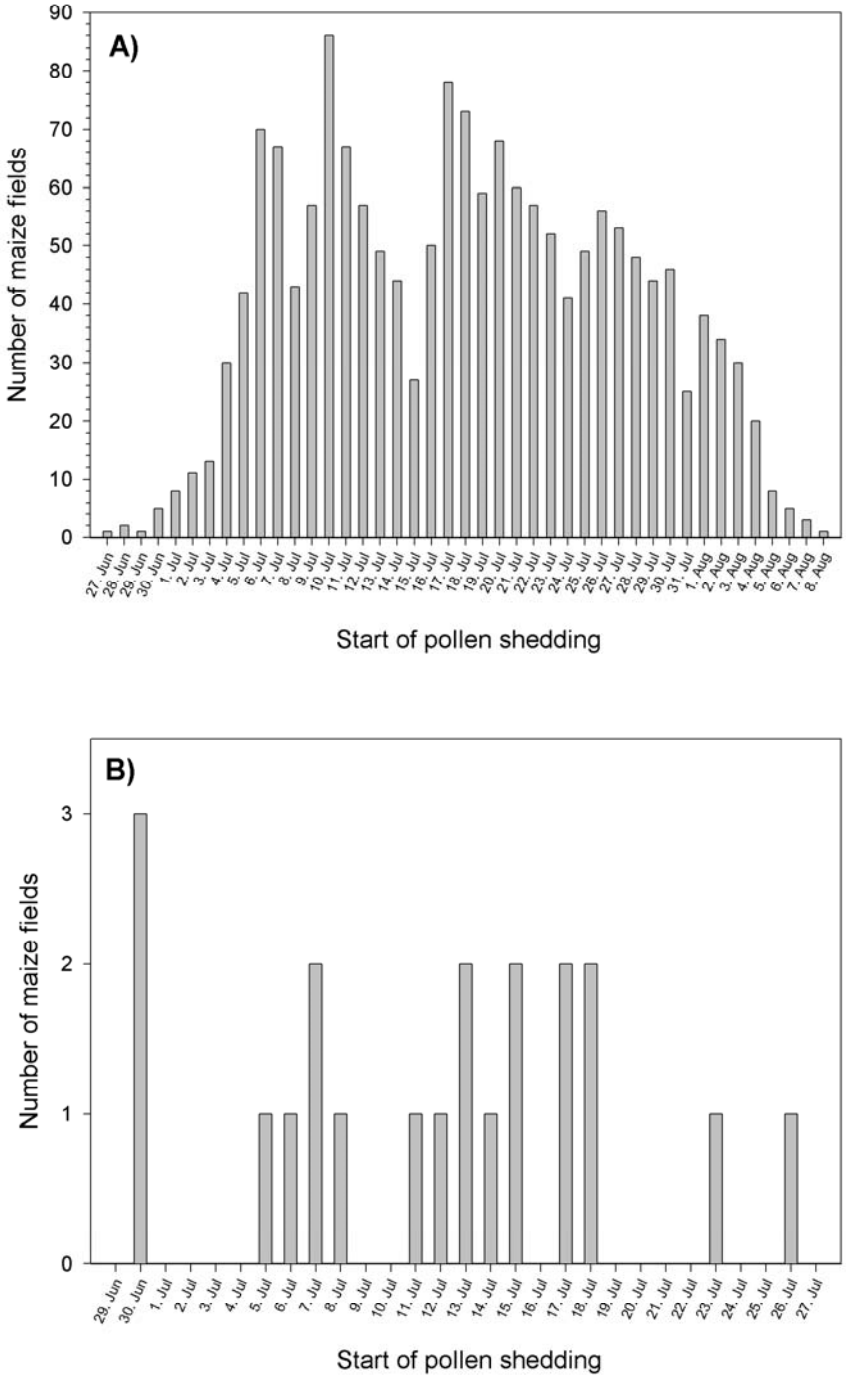

Fig. 1: (A) Onset of corn flowering in Bavaria 1995 – 2000 (N = 307 different corn varieties in 1680 fields at 22 sites). (B) Onset of the flowering of the Bt176 corn "Navares" on the Bavarian experimental estates 2000 – 2003 (N = 21 fields).

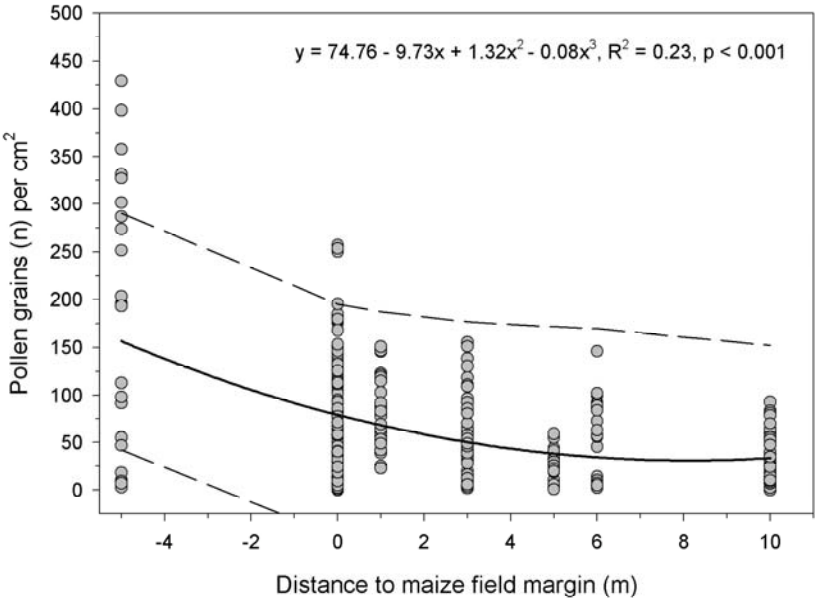

Fig. 2: Corn pollen deposition at the field margin as a function of distance from the corn field in (number of corn pollen grains on microscopic slides covered with Vaseline, n = 300, 24 hour values). Regression line, 95% confidence interval and regression equation shown in the diagram.

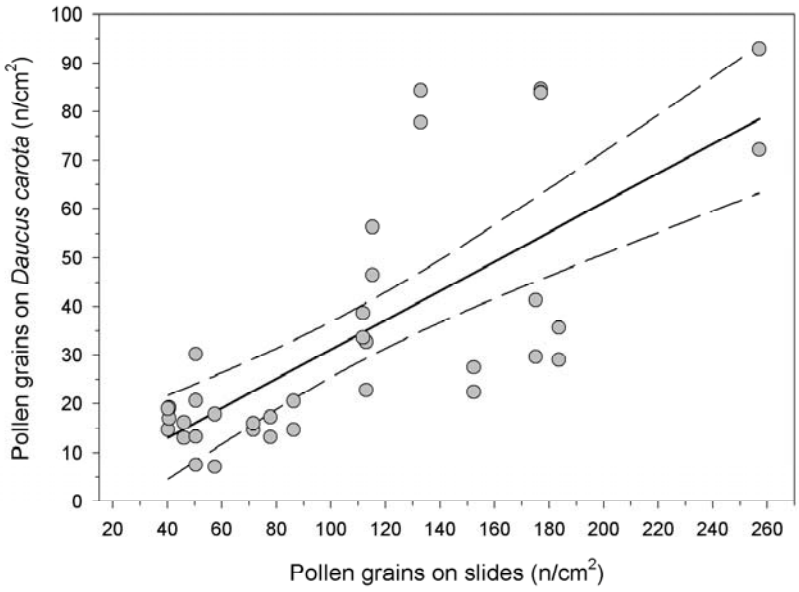

Fig. 3: Corn pollen density on wild carrot (*Daucus carota*) leaves as a function of pollen deposition. The diagram shows 24 hour measurements of images taken at the margin of a Bt176 corn field in Neuhof. Pollen deposition was measured with microscopic slides (see also Fig. 2). The linear regression (95% confidence interval shown) is: $y = 0.99 + 0.30x$, $R^2 = 0.54$, $p < 0.001$, $n = 36$.

Table 3: Species list of butterflies found at the corn field margin. Observations of adult butterflies during the years 2000 to 2003 on six experimental estates in Bavaria (total observation period = 2235 min).

No.	Species	**Endangerment**		**Observations**	**Rel. share**
		BY[2]	FRG[3]	(n)	(%)
1	*Papilio machaon* L.[1]		V[4]	16	1.69
2	*Leptidea sinapis* L. / *reali* Reiss.	D[5]	V[4]	2	0.21
3	*Colias hyale* L. / *alfacariensis* Ribbe			2	0.21
4	*Gonepteryx rhamni* L.			10	1.05
5	*Aporia crataegi* L.	3[6]	V[4]	1	0.11
6	*Pieris brassicae* L.			27	2.85
7	*Pieris rapae* L.[1]			318	33.51
8	*Pieris napi* L.[1]			89	9.38
9	*Anthocharis cardamines* L.			10	1.05
10	*Apatura iris* L.	V[4]	V[4]	1	0.11
11	*Apatura ilia* Denis & Schifferm.	V[4]	3[6]	3	0.32
12	*Limenitis camilla* L.	V[4]	3[6]	1	0.11
13	*Nymphalis io* L.[1]			33	3.48
14	*Nymphalis c-album* L.			3	0.32
15	*Nymphalis urticae* L.[1]			72	7.59
16	*Vanessa atalanta* L.[1]			19	2.35
17	*Vanessa cardui* L.[1]			34	3.58
18	*Araschnia levana* L.[1]			15	1.58
19	*Melitaea diamina* Lang	3[6]	3[6]	1	0.11
20	*Argynnis paphia* L.			11	1.16
21	*Issoria lathonia* L.			20	2.11
22	*Brenthis ino* Rottemburg	3[6]	V[4]	10	1.05
23	*Boloria selene* Denis & Schifferm.	3[6]	V[4]	3	0.32
24	*Melanargia galathea* L.			33	3.48
25	*Maniola jurtina* L.			41	4.32
26	*Aphantopus hyperantus* L.			39	4.11
27	*Coenonympha pamphilus* L.			57	6.01
28	*Pararge aegeria* L.			5	0.53
29	*Lasiommata megera* L.			2	0.21
30	*Neozephyrus quercus* L.			1	0.11
31	*Lycaena phlaeas* L.			4	0.42
32	*Polyommatus icarus* Rottemburg			25	2.63
33	*Carterocephalus palaemon* Pallas		V[4]	3	0.32
34	*Thymelicus sylvestris* Poda			14	1.48
35	*Thymelicus lineola* Ochsenheimer			19	2.00
36	*Ochlodes sylvanus* Esper			5	0.53

[1] Proof of reproduction: Observation of oviposition, caterpillars or pupae

[2] Bolz & Geyer (2003), Red list *[of endangered species]* Bavaria

[3] Pretscher (1998), Red List *[of endangered species]* Germany

[4] V = Species on the early warning list

[5] D = Data incomplete

[6] 3 = Endangered

Tab. 4: Random sample estimation for a butterfly monitoring. The numbers indicate the number field margins that would have to be sampled, in order to prove a Bt effect on the number of species and overall abundance of 11 selected species. The number of field margins has been calculated for several scenarios (5 – 50% reduction), and numbers would have to doubled to render the total number of the overall random samples (i.e. field margins adjacent to Bt corn fields plus field margins adjacent to conventional field margins *[sic]*). The calculations are based on the means (± 1SD) of the 2000 – 2002 butterfly mappings of 15 field margins (for additional preconditions see 3.3.2.6 and Lang 2004).

Variable	Number per 60min and field margin (± 1SD)	Number of random sample to prove a reduction of							
		5%	10%	15%	20%	25%	30%	40%	50%
Number of species	5.95 ± 2.78	1078	271	121	69	44	31	18	12
Overall abundance	23.29 ± 8.88	720	181	81	46	30	21	12	8
P. machaon	1.51 ± 1.26	3406	864	383	215	139	97	55	36
P. rapae	10.78 ± 4.70	940	236	106	60	39	27	16	11
P. napi	2.89 ± 1.31	1005	254	113	64	42	29	17	11
I. io	1.24 ± 0.59	1114	279	153	71	46	32	19	12
V. atalanta	1.30 ± 1.36	5451	1364	607	342	219	153	86	56
C. cardui	1.08 ± 1.11	5266	1317	586	330	212	147	83	54
A. urticae	1.30 ± 0.95	2621	656	292	165	105	74	42	27
I. lathonia	1.75 ± 1.51	3642	922	409	231	148	103	59	38
M. jurtina	1.79 ± 1.08	1814	449	201	114	73	51	29	19
C. pamphilus	1.61 ± 1.10	2288	580	257	145	94	65	37	24
P. icarus	1.34 ± 0.90	2216	555	250	141	90	63	36	23

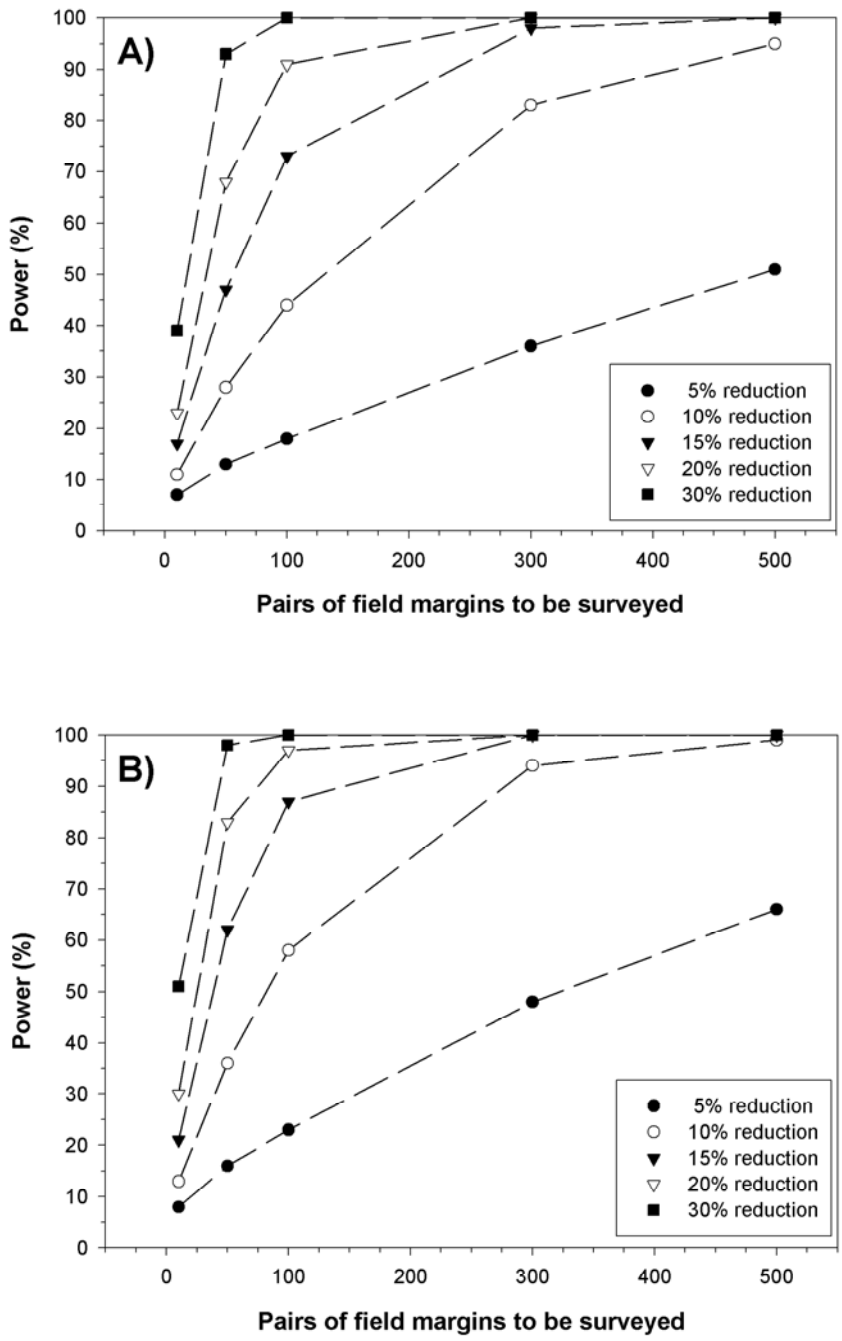

Fig. 4: Power analysis of the monitoring of a Bt corn effect on the number of butterfly species (A) and overall butterfly abundance (B) at the field margin. The probability of finding an effect (= power) is shown for various case examples that assume a 5% to 30% reduction in the number of species and individuals, respectively. The sample sizes are given as pairs from field margins, meaning that to show the entire sampling effort, the numbers on the x axis would have to be multiplied by 2 (= margin of Bt fields plus margins of non-Bt fields). For the preconditions of the calculations see paragraph 3.3.2.6 and Lang (2004).

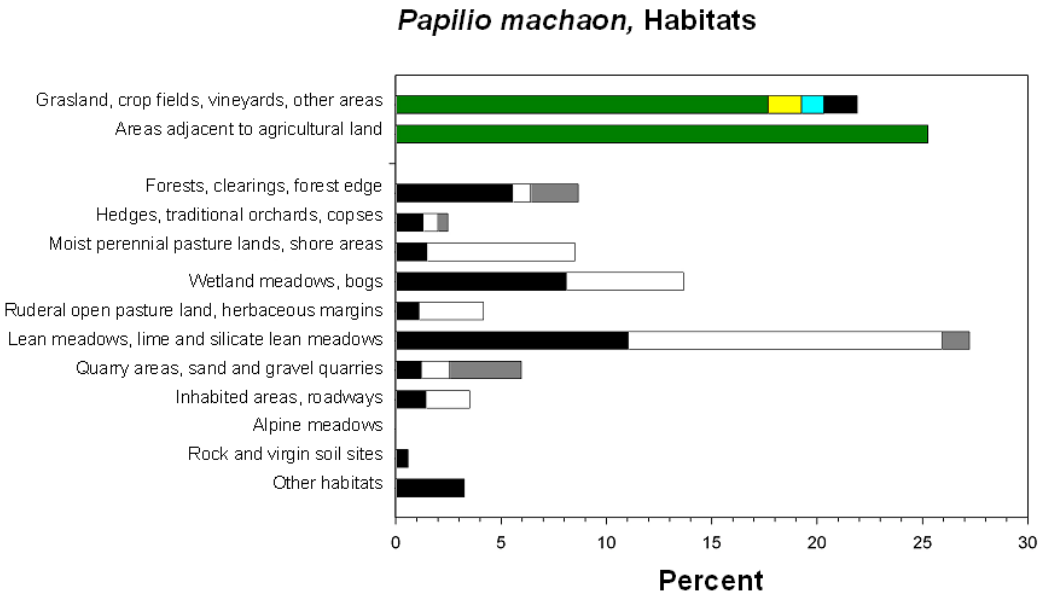

Fig. 5: Habitats of the swallowtail (*Papilio machaon*) in Bavaria. Proof of adults according to the endangered species maps of the Bavarian LfU (n = 2700 sighting sites, status 04.19.2004). "Areas adjacent to agricultural land" designates the percentage of sightings on areas that are not under agricultural management, but are adjacent to areas under agricultural management.

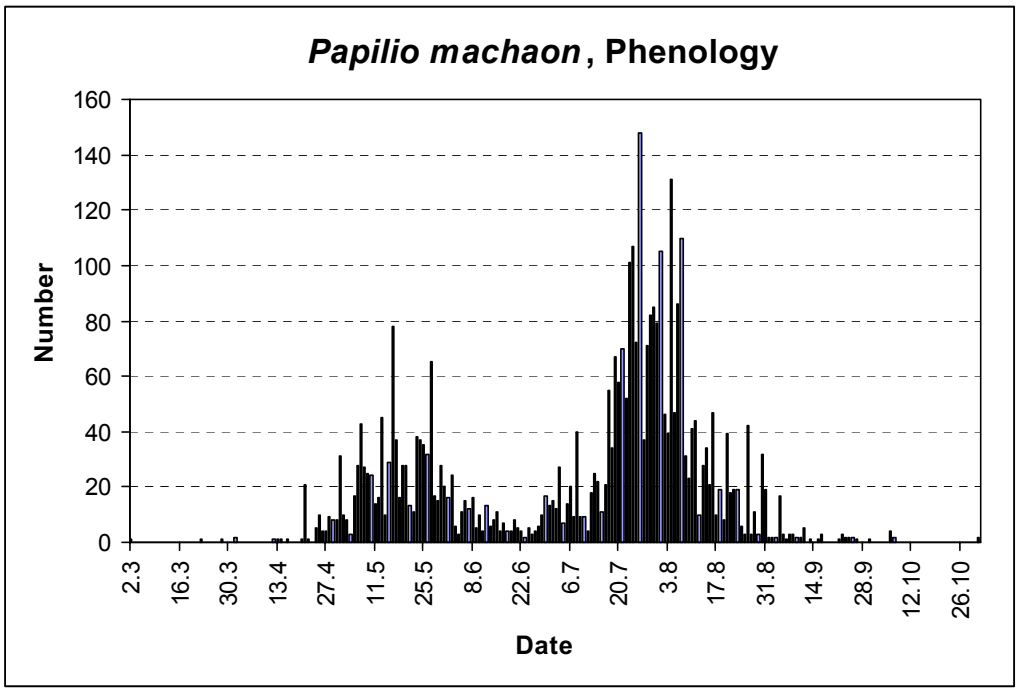

Fig. 6: Seasonal occurrence (phenology) of the swallowtail (*Papilio machaon*) in Bavaria. Proof of adults according to the endangered species maps of the Bavarian LfU (n = 3706 observations, status 04.19.2004).

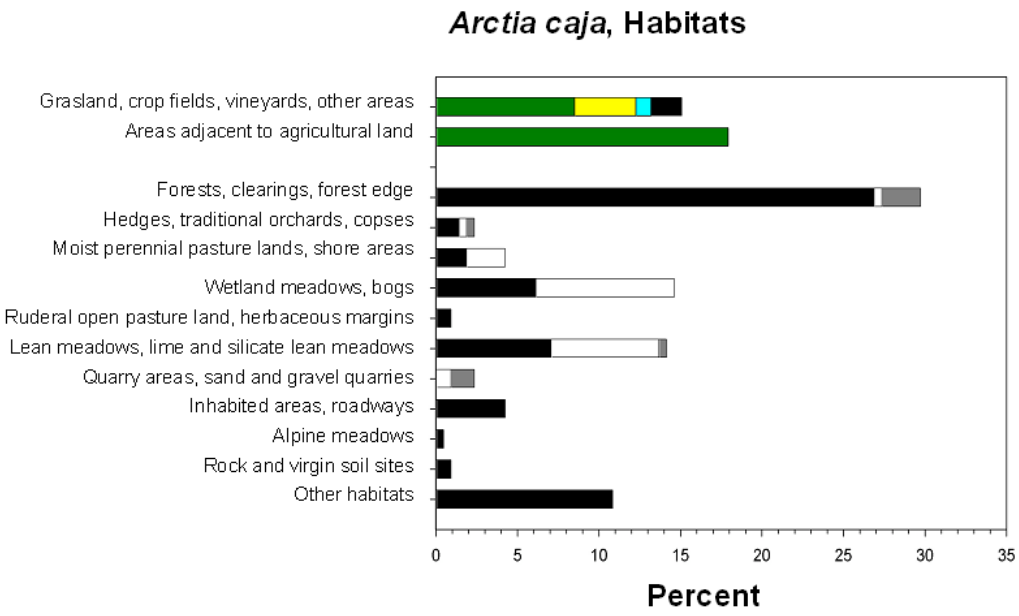

Fig. 7: Habitats of the woolly bear (*Arctia caja*) in Bavaria. Proof of adults according to the endangered species maps of the Bavarian LfU (n = 212 sighting sites, status 04.19.2004). "Areas adjacent to agricultural land" designates the percentage of sightings on areas that are not under agricultural management, but are adjacent to areas under agricultural management.

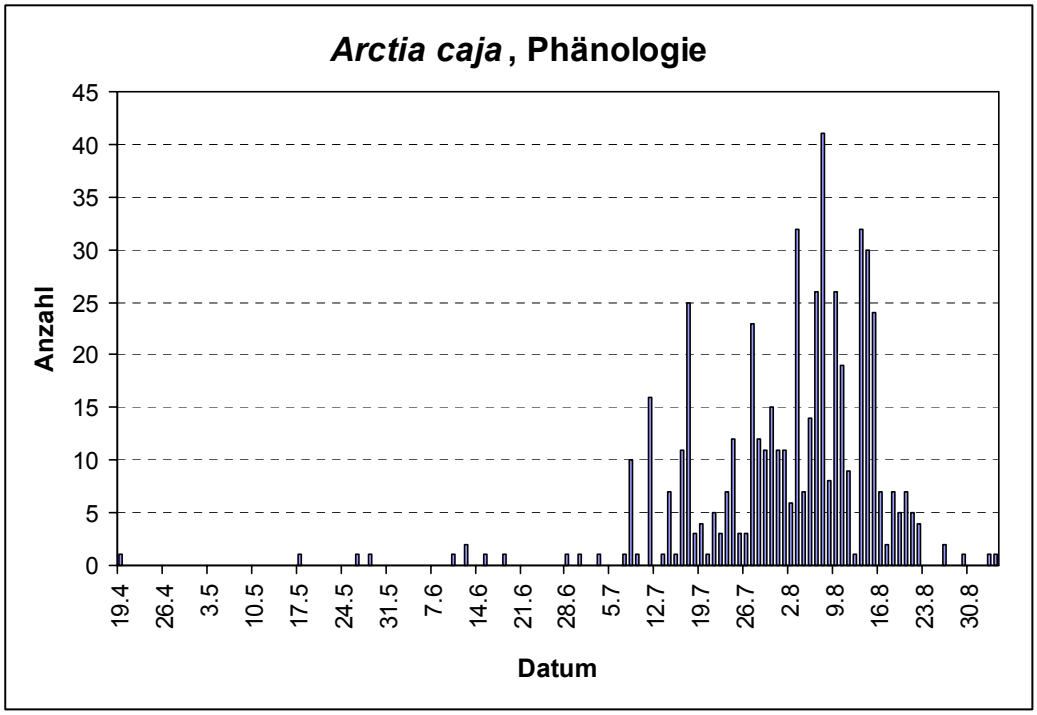

Fig. 8: Seasonal occurrence (phenology) of the woolly bear (*Arctia caja*) in Bavaria. Proof of adults according to the endangered species maps of the Bavarian LfU (n = 526 observations, status 04.19.2004).

Tab. 5: Laboratory feeding experiment with Bt176 corn pollen and L1 larvae of the swallowtail (*P. machaon*): Experimental exposure to pollen as well as leaf and pollen consumption of the different treatments (treatment = pollen solution applied to the leaf (mg pollen per 10ml water); applied pollen = absolute number of pollen grains on the leaf; pollen per leaf area = pollen density; leaf eaten = amount of leaf eaten by the larvae in 48h; pollen eaten = number of pollen grains, eaten by the larvae in 48h). The variable "Leaf eaten" was recorded in all four classes (0% - 25%, - 50%, - 75%, - 100% eaten) and converted. Standardized leaves of wild parsnip (*Pastinaca sativa*) were given as leaves. The values shown are means ± SD. Stars designate significant differences between treatments within the same column ($p < 0.05$, Kruskal-Wallis test). Values within a column with different letters differ significantly ($p < 0.05$, Tamhane test); n = respective number of caterpillars.

Treatment (pollen per 10ml)	Pollen applied* (n)	Pollen per leaf area* (n / cm^2)	Leaf eaten* (cm^2)	Pollen eaten* (n)
Control (n = 133)	0 a	0 a	1.64 ± 0.49 a	0 a
1.0mg (n = 31)	4.71 ± 2.36 b	18.78 ± 9.39 b	1.39 ± 0.49 a	4.42 ± 2.17 b
2.5mg (n = 44)	9.86 ± 1.83 c	39.34 ± 7.28 c	0.90 ± 0.51 b	8.86 ± 2.14 c
5.0mg (n = 44)	18.61 ± 2.83 d	74.24 ± 11.29 d	0.86 ± 0.49 bc	16.02 ± 4.10 d
7.5mg (n = 47)	30.77 ± 5.45 e	122.71 ± 21.75 e	0.61 ± 0.31 cd	23.62 ± 7.23 e
10mg (n = 38)	39.89 ± 6.68 f	159.12 ± 26.65 f	0.49 ± 0.27 de	27.61 ± 11.47 e
20mg (n = 35)	68.63 ± 14.44 g	273.72 ± 57.61 g	0.31 ± 0.16 f	34.71 ± 19.96 e
30mg (n = 32)	107.84 ± 28.44 h	430.14 ± 113.41 h	0.37 ± 0.24 ef	65.91 ± 37.41 f

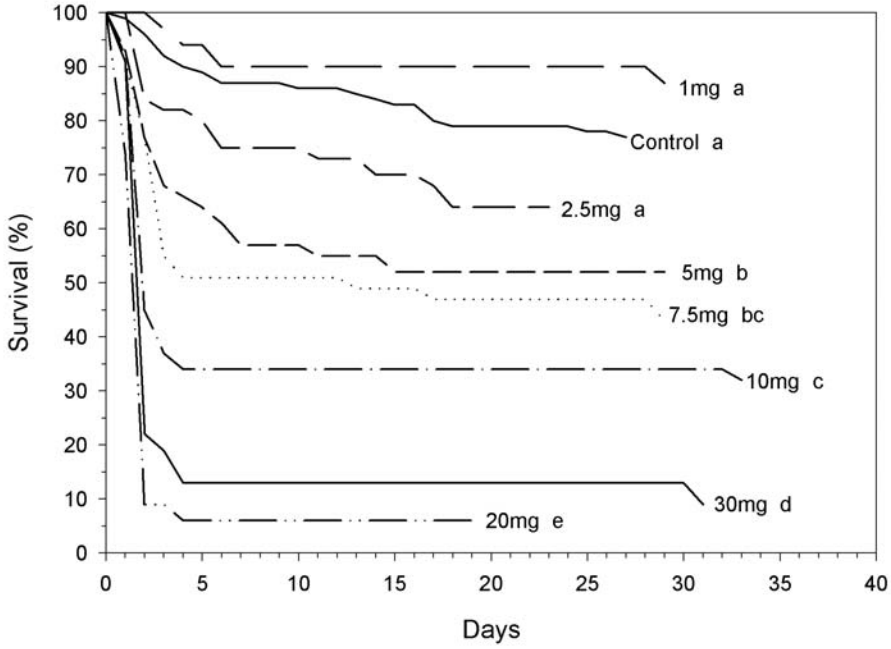

Fig. 9: Survival curves of the swallowtail (*P. machaon*) after feeding on Bt176 corn pollen. Treatment = pollen solution applied (mg pollen per 10ml water), regarding amount of pollen eaten see Tab. 5. Curves with different letters differ significantly ($p < 0.05$; Kaplan-Meier analysis).

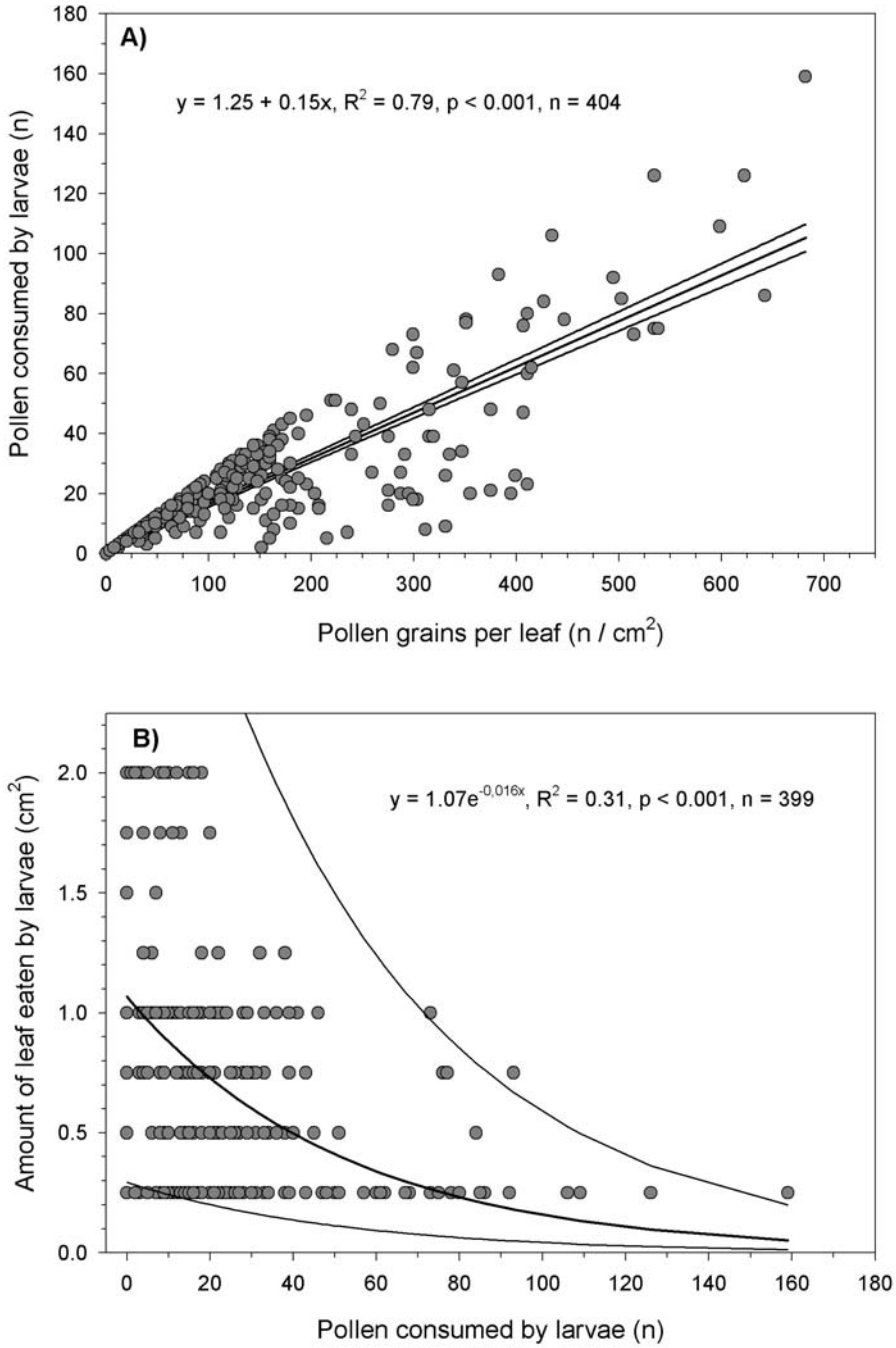

Fig. 10: (A) Pollen consumed as a function of the pollen density on the leaf surface; (B) Relationship between leaf material eaten and consumed Bt corn pollen. Laboratory experiments with L1 caterpillars of the swallowtail (*P. machaon*) and Bt176 corn pollen; all feeding values refer to a 48 hour period. The diagram shows the respective regression lines with 95% confidence intervals, regression equations, R^2 and p values shown (n = Number of caterpillars).

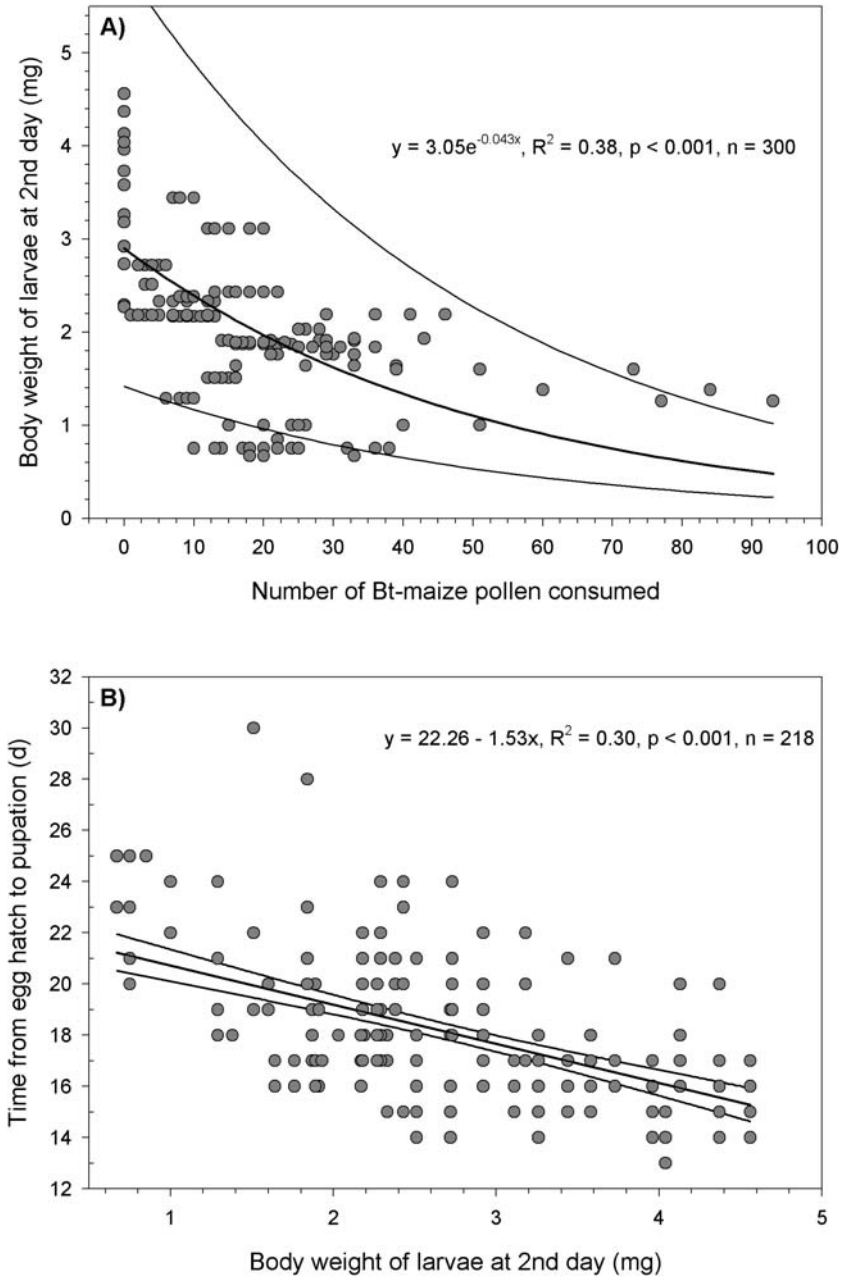

Fig. 11: (A) Body weight of two day old caterpillars as a function of the number of Bt corn pollen grains consumed (in 48h); (B) Development time of the caterpillars as a function of body weight on day two. Laboratory experiments with L1 caterpillars of the swallowtail (*P. machaon*) and Bt176 corn pollen. The diagram shows the respective regression lines with 95% confidence intervals, regression equations, R^2 and p values shown (n = number of caterpillars).

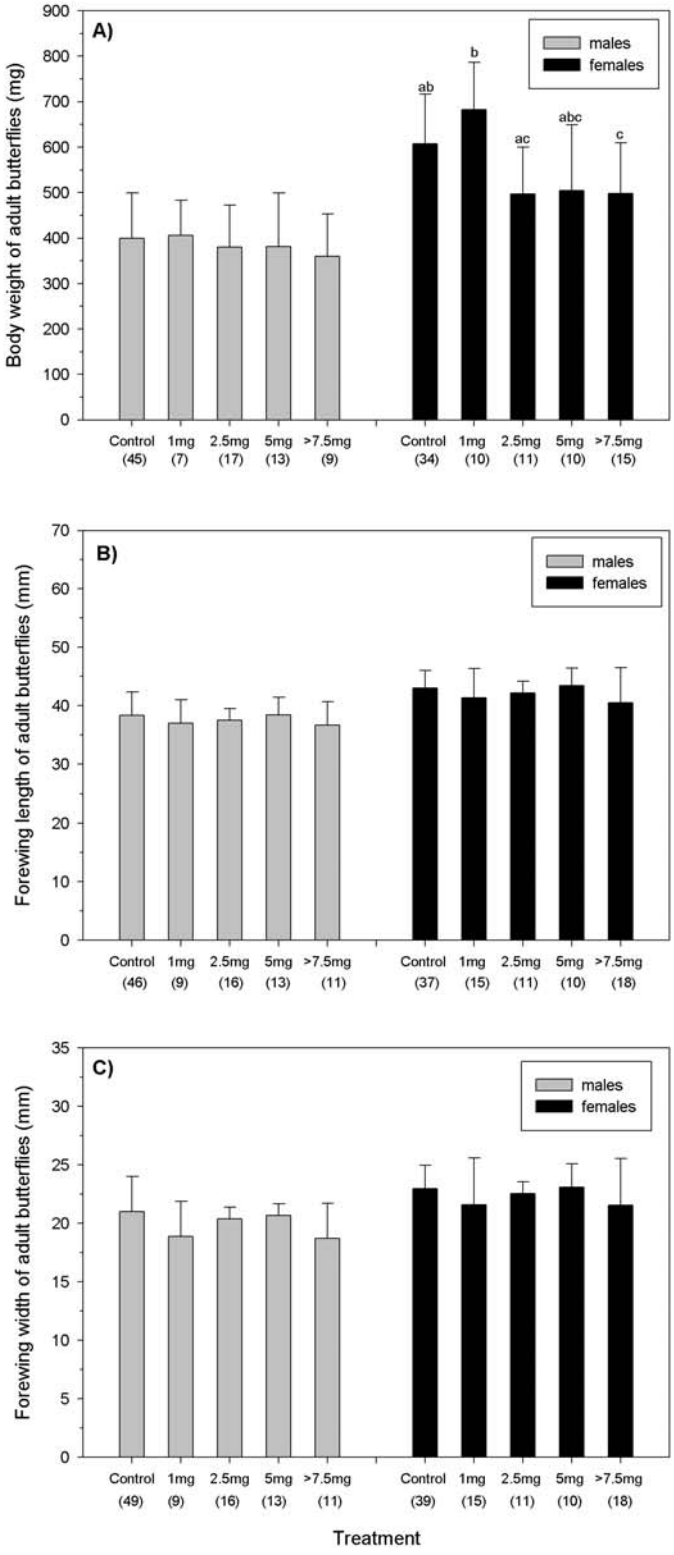

Fig. 12: Body weight (A), front wing length (B) and front wing width (C) of adult swallowtails (*P. machaon*), which ate Bt corn pollen as L1 larvae. The different treatments designate the ratio of the different pollen dosages (mg Bt176 corn pollen per 10ml water), to the respective amount of pollen consumed Tab. 5, and the figures in parentheses indicate the respective number of butterflies. The diagram shows means with SD. Columns in (A) with different letter differ significantly ($p < 0.05$, Tamhane test)

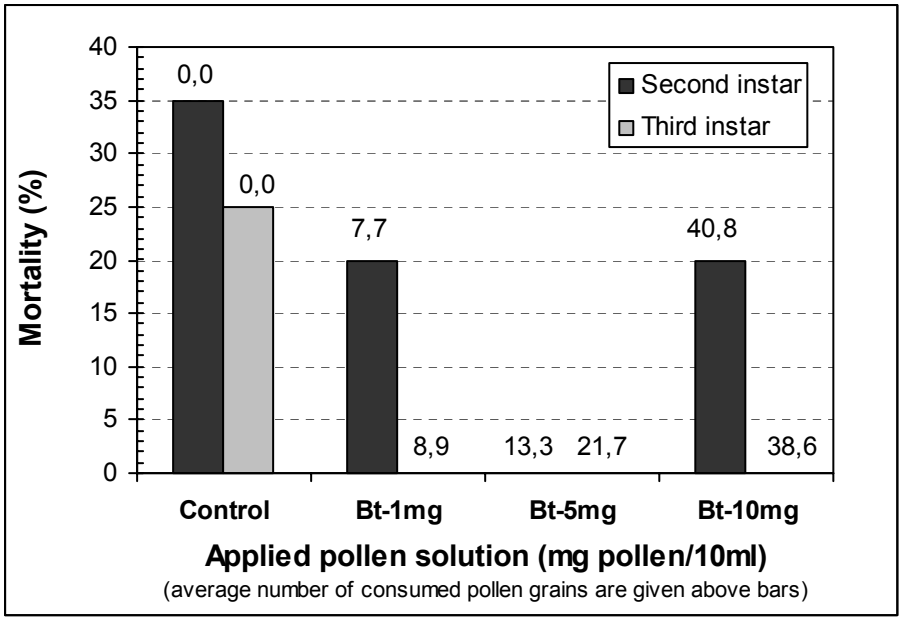

Fig. 13: Mortality (%) of L2 and L3 caterpillars of the woolly bear (*A. caja*) 48 hours after eating Bt176 corn pollen (laboratory experiment). The diagram shows the mean + SD for different treatments (mg Bt corn pollen per 10ml aqueous solution applied to a dandelion leaf) separately, for the second and third larval stage. Values above the column indicate the average number of Bt pollen grains eaten per larva for the different treatments. Sample size is 10 – 20 caterpillars per treatment and larval stage.

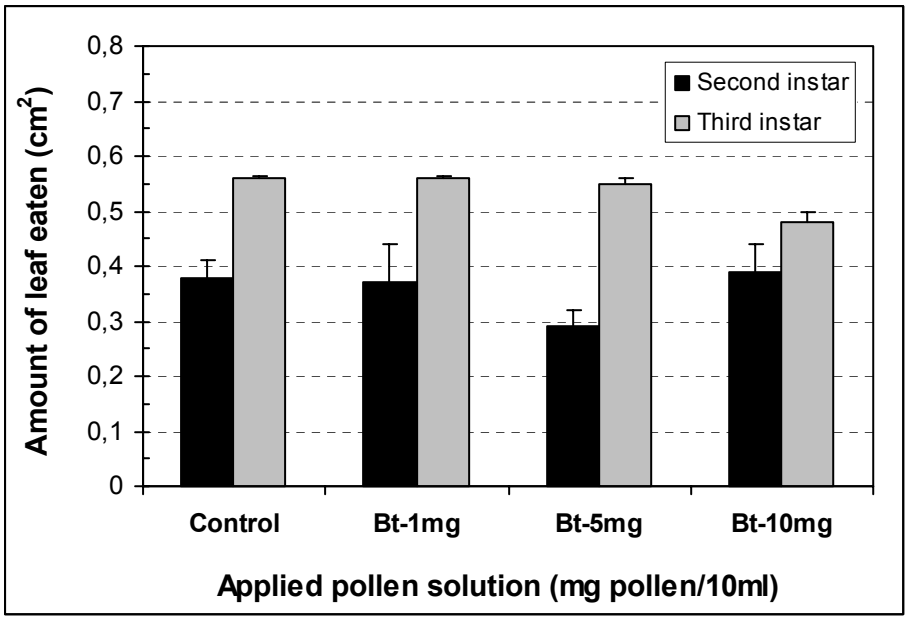

Fig. 14: Laboratory experiment on leaves eaten (in 48h) von L2 and L3 caterpillars of the woolly bear (*A. caja*). The diagram shows the mean + SD for different treatments (mg Bt corn pollen per 10ml aqueous solution applied to a dandelion leaf) separately, for the second and third larval stage. For the number of corn pollen grains eaten see Fig. 13. Sample size is 10 – 20 caterpillars per treatment and larval stage.

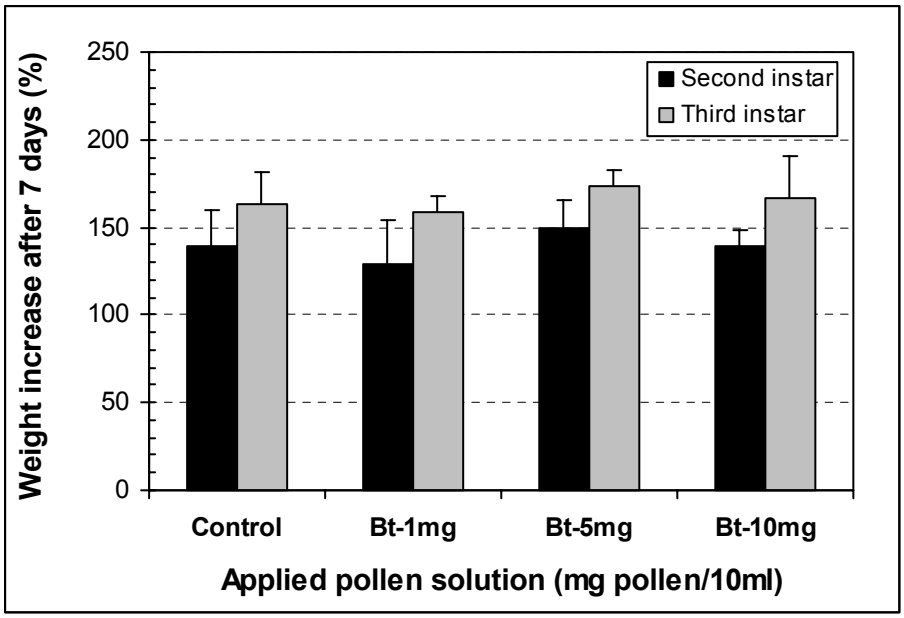

Fig. 15: Weight increase (%) of L2 and L3 caterpillars of the woolly bear (*A. caja*) within 7 days after eating Bt176 corn pollen. The diagram shows the mean + SD for different treatments (mg Bt corn pollen per 10ml aqueous solution applied to a dandelion leaf) separately, for the second and third larval stage. For the number of corn pollen eaten see Fig. 13. Sample size is 10 – 20 caterpillars per treatment and larval stage.

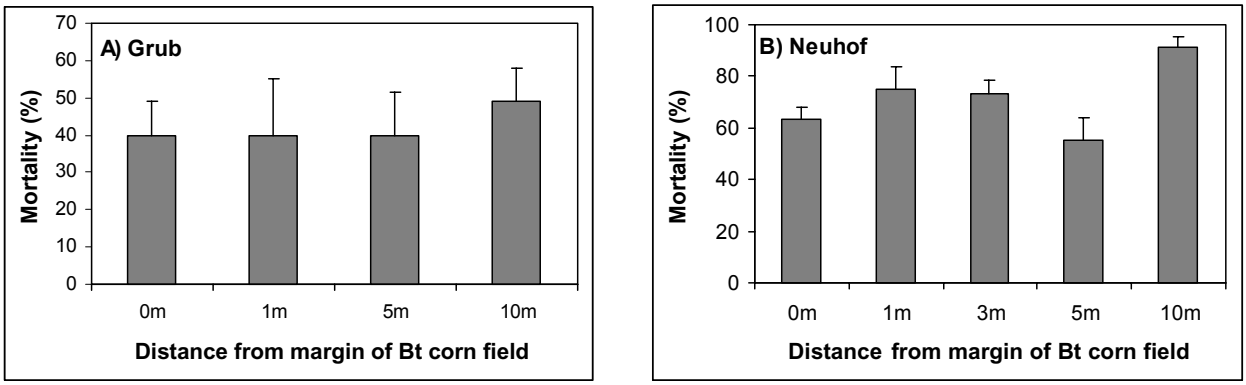

Fig. 16: Field experiments with L1 caterpillars of the swallowtail (*P. machaon*) at Grub (A) and Neuhof (B).- Caterpillars were placed on wild carrots at different distances from a Bt176 field during corn flowering and thereby exposed to a pollen density gradient (see also Fig. 2). The graph shows the mortality (%) of the caterpillars after 7 days (mean + SD).

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3.2 Nematodes

3.2.1 The Task

Nematodes are present in almost all soils with high population density and high numbers of species. Besides the plant-parasitic threadworms that cause considerable economic damage worldwide to many types of crops, other trophic groups (in particular bacteriophagous and mycophagous nematodes) play an important role in the metabolic activity of the soil and thus in soil fertility. So on the one hand we needed to study the possible effects of Bt corn [4] on endoparasitic root nematodes of the genus *Pratylenchus*, and on the other hand we needed to determine if cultivation of Bt corn causes quantitative and/or qualitative changes in "saprophagous nematodes." While a negative impact on destructive nematodes is actually desirable or tolerable from the standpoint of plant protection as well as environmental protection, any impact on non-plant parasitic nematodes and their interactions with the soil microorganism community should be avoided if possible.

3.2.2 Extensive Documentation and Evaluation of the Literature Used

There is a relatively short list of available literature on the specific task because nematodes, as non-target organisms of Bt corn, up to now have attracted little interest from researchers. Only two recent papers (Saxena & Stotzky 2001, Manachini et al. 2002) deal directly with possible effects of Bt corn on nematode fauna. We will consider these two papers in more detail in Sections 3.2.5 and 3.3.7. The relatively little interest in interactions between plants with Bt genes and nematodes is rather difficult to understand, especially since several earlier papers reported on the effects of *Bacillus thuringiensis* on nematodes (Osman et al. 1988, Meadows et al. 1990, Zuckerman et al. 1993, Borgonie et al. 1996) and a specific strain with nematicidal properties was patented 10 years ago. Genetically engineered plants with nematode resistance based on Bt genes are therefore conceivable and could contribute to solving the worldwide control problem (Urwin et al. 1998, Jung 1998, Vrain 1999, Atkinson et al. 2003).

In this project, we used Bt corn varieties that exclusively express the endotoxin Cry1Ab. This is not identical to the protoxins of *B. thuringiensis* preparations that have been used for decades, and hence we cannot categorically assume it has comparable specificity or toxicity. In this respect, regarding the efficacy of Bt crystal proteins against nematodes, we should note recent studies in which six phylogenetically diverse nematodes were shown to be susceptible to four out of seven tested toxins (Wei et al. 2003). Since only five bacteria-eating nematode species and one animal-parasitic nematode species were used and Cry1Ab itself was not tested, these studies do not let us say anything about the nematicidal effect of the tested Bt corn varieties on plant-parasitic species. It is interesting that in the studied nematodes, morphological effects of the crystal protein were found to be comparable to effects known in insects (Marroquin et al. 2000, Huffman et al. 2004). This presumes uptake of *B. thuringiensis* or crystal proteins through food. In phytonematodes, however, this is unlikely because of the very small diameter (only 0.2 µm) of their stylet (feeding spear). So it is unclear how efficacies were achieved that were comparable with a nematicide in a trial using commercial Bt preparations for nematode control in tomatoes

[4] Translator's Note: The German "Mais" will be translated according to US practice as "corn" throughout these sections. "Corn" and "maize" are used interchangeably in the references and original English figures to mean *Zea mays*.

(Osman et al. 1988). Probably during sporulation of *B. thuringiensis*, in addition to several endotoxins, other metabolites are formed for which not much is known about their mechanism of action on nematodes. For example, there have been reports of reduced hatching (Devidas & Rehberger 1992) or ovicidal properties (Bottjer & Bone 1987). The longer persistence or possible accumulation of Bt toxins in the soil (Tapp & Stotzky 1998), which reach the soil through crop residues and root exudates (Saxena et al. 1999), means we cannot absolutely rule out potential risk for nematode fauna.

Various studies have shown that anthropogenic influences can lead to shifts in the relative numbers of nematode trophic groups (Freckman 1993, Ruess 1993, Leliveldt & Sturhan 1994) and conclusions about the decomposition pathway in the soil can be drawn from the ratio of fungal and bacterial feeders (Freckmann 1988, Ingham 1985). In plant-parasitic nematodes, the dependence on farming practices has been known for a long time, and because of special host plant requirements some species are regarded as typical pests that can be controlled by crop rotation. Interest in non-plant parasitic nematodes has increased considerably in recent years for ecotoxicological studies, especially as bioindicators in the soil (Bongers et al. 1990, Freckmann & Ettema 1993, Sturhan 1996, Eckschmitt & Bongers 2000). For this purpose, special bioassay methods using nematodes have been developed to detect the effects of soil loading with toxic substances such as heavy metals or plant protection agents (Donking 1994, Höss 2001, Wilms 1992). The suitability of a life cycle test with *Caenorhabditis elegans* for monitoring bioavailability of Bt toxins in soil will be discussed in more detail in Sections 3.2.6 and 3.2.8.

We drew on literature sources that are generally well known in nematology for the test procedures and evaluation methods (Goodey 1963, Seinhorst 1988, Maaßen 1977, Yeates [5] 1993). The *C. elegans* test was performed according to an Ecossa [6] laboratory protocol. Quantitative and qualitative analyses for Cry1Ab in Bt corn samples were carried out by ELISA (Fearing et al. 1997) according to the manufacturer's instructions for the diagnostic kit.

Since there are diverse reports in the literature on the lignin content of transgenic corn (Bt+) compared with the isogenic line (Bt-) (Saxena & Stotzky 2002), which could be a reason for an altered host-parasite relationship for herbivorous nematodes and altered degradation behavior for the corn residues in the soil (Flores et al. 2000, Escher et al. 2000), we carried out some of our own laboratory studies on this matter. Even substantial equivalence, which is assumed in principle to be valid for isogenic and transgenic plants, must be reconsidered in various respects (Scheukelaars 2002). Literature references to the greater susceptibility of Bt cotton to root gall nematodes (Colyer et al. 2000) or the complete loss of nematode resistance in Bt potatoes (Brodie 2003) indicate that unexpected events can occur in gene transfer. So in addition to monitoring ecologically relevant nontarget organisms, direct pests should not be ignored.

3.2.3 Conditions Under Which the Work Was Conducted

The nematological studies of soil and root samples were carried out on fields under continuous cultivation with corn. For this purpose, two mixed soil samples were taken every year before planting the seed and after harvesting from each of 8 plots. Because of the known nonuniform nematode distribution within a field, soil sampling was restricted to small sampling sites marked off by magnets (4x5 = 20 m^2). About four liters of soil

[5] Translator's Note: Corrected misprint: Yeats should be Yeates.
[6] Translator's Note: Ecossa or ECOSSA is a company providing Ecological Sediment and Soil Assessment.

were removed with a sampling corer, with 20 insertions into the soil down to a depth of 20 cm in each case. The samples were stored at 5°C in plastic bags until the laboratory tests. In addition, roots of 20 plants were sampled from two sites on different dates for each corn variety, in order to determine the *Pratylenchus* spp. root population (Table 1). The nematodes were isolated from the soil samples using the Baermann funnel procedure from 2 x 100 mL soil per mixed sample. For extraction from the corn roots, a misting unit was available in which up to 20 grams max fresh root weight per plant was treated. Simultaneous study of all the samples was not possible because of the large sample size and the required extraction time of 4 or 12 days respectively for the soil or plant samples. We also were unable to sample all five trial sites on all scheduled dates. In particular, these operations could not be properly carried out in the very wet Fall of 2002.

Because of the sometimes very different soil textures at the sites (for example, Baumannshof 6% clay, 7% silt, and 87% sand compared with Schwarzenau 26% clay, 57% silt, and 17% sand), based just on experience we would have to expect large differences in the diversity and abundance of the nematode fauna. Since soil parameters such as clay and humus content also affect the persistence or bioavailability of Bt toxins (Koskella & Stotzky 1997, Crechio & Stotzky 1998), the permanent continuous corn trials best met the prerequisites for detecting possible effects without interference from the effects of rotating crops, as on the long-term soil observation (LSO) fields.

Since the very costly [7] studies on diversity and dominance structure of nematode fauna could not be done at the Bavarian State Research Center for Agriculture (LfL) itself, this work was subcontracted to Dr. Ruess, who has proven herself to be an expert through her several publications in this field (Ruess 1993). Fixed nematode suspensions from soil samples taken from the Baumannshof and Schwarzenau sites in Spring 2001 and from Baumannshof and Neuhof in Spring 2002 were used to determine the generic composition of the nematodes.

Dr. Höss (Ecossa) was subcontracted to carry out studies on the bioavailability of Bt toxins in selected soil samples; these studies were conducted with the bacteria-eating nematodes *Caenorhabditis elegans*. This bioassay, which was developed especially for detection of heavy metals in sediment and soil samples (Donking 1994, Traunspurger et al. 1997), is now undergoing a DIN/ISO certification process. The test has also been used at LfL. In order to assess the dose-response relations, the toxin content in some soil samples was determined by ELISA in parallel with the bioassay. These studies were kindly undertaken by a working group of the Braunschweig Federal Agricultural Research Center (FAL Braunschweig), which is involved in a Federal Ministry of Education and Research (BMBF) research project on degradation of Bt corn in soils and effects on microorganisms (Baumgarte & Tebbe 2003).

According to Hatfield et al. (1999), no chemical analysis methods were available for our own laboratory studies of corn lignin content. So instead we carried out histological studies of stalk and root cross sections with various staining techniques. In addition, the working group on Raw Materials Quality of Plant Products at LfL has experimented with

[7] Translator's Note: The German "aufwändigen" here usually means costly and the later use of Untersuchungsaufwand (study cost) and also references to Aufwand (cost) increasing as the numbers of samples increases (for better statistics) suggests this interpretation here. But aufwändig might also possibly refer to the complexity/extensiveness of the studies. In earlier text, they also used aufwändig and the related aufwendig together with zeits-(time) and arbeits-(labor) to indicate time-consuming and labor-intensive operations. Time is money, so it might not matter, but there may be some ambiguous distinction here.

near infrared (NIR) spectroscopy to examine differences in lignin content between Bt+ and Bt-.

3.2.4 Planning and Progression of the Work

The scheduled annual sampling before planting the seed and after incorporating the corn straw, as already mentioned, could not be done for all five sites. In Spring, there was not always enough laboratory capacity for processing all 80 samples, and the Fall sampling sometimes was not possible due to very unfavorable soil or weather conditions and lack of timely tillage. Also in studying corn root samples for their current *Pratylenchus* spp. population, for methodological reasons it was necessary to restrict ourselves to selected sites, especially because of the high number of replicates required (80 individual samples per site). Table 1 in the Appendix shows the sample size [8] for each of the studies performed.

3.2.5 Overview of the Overall Issues, and Currently Known Findings in the Area of the Task at Hand

Phytonematodes

For some crop species, such as potatoes or sugar beets, conventional breeding has developed varieties resistant to certain nematode species. A variety is then considered as nematode resistant if reproduction of the respective nematode species cannot reproduce or its reproduction is suppressed to a large extent. In corn or wheat also, differences have been found between varieties with regard to susceptibility to (for example) stem nematodes *Ditylenchus dipsaci* (Knuth 2001) or root lesion nematodes *Pratylenchus thornei*. No reports have yet been published on differences in host plant quality between Bt+ and Bt- corn varieties with regard to phytonematodes, although such observations are available for other transgenic Bt plant species (Colyer et al. 2000, Brodie [9] 2003). In order to resolve this issue within the monitoring project, we used a conventional procedure for testing nematode resistance where the nematode population densities are compared before and after cultivation of the respective variety. We are limited in our evaluation of the results by the fact that varieties can very specifically respond to a certain species of nematode, but at some trial sites there were mixed populations of *Pratylenchus penetrans, P. neglectus*, and *P. crenatus* which were not separately counted.

Saprophagous nematodes

Detection of the effects of Bt+ and Bt- on the total nematode fauna or certain trophic groups was the subject of pot culture tests by Saxena & Stotzky (2001) and field tests by Manachini & Lozzia (2002). The results of both papers are based on a short trial duration and thus would not detect any possible long-term effects. The pot culture tests were only 40 days long. In that case, field soil from one site was used (58% sand, 41% silt, 1% clay), and Bt corn (NK4640Bt) and the isogenic line were cultivated or else chopped material from the respective corn plants was incorporated as biomass in the pots (five replicates). The nematodes were extracted from the soil samples by a method comparable with the one we used here. There were no significant differences from the control for either nematodes or the other investigated organisms. Possible changes in the composition and diversity of

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[8] Translator's Note: The German "Probenumfang" generally means "sample size", but the indicated Table 1 seems to show only the sampling schedule and not the actual number of samples taken. They may be referring just to the number of times the required samples were taken at each site.

[9] Translator's Note: Corrected misprint: Brody should be Brodie.

these groups, however, were not recorded. A tendency toward a lower number of bacteria (-20%) for Bt+ could, for example, have resulted in a decrease in bacteriophagous nematodes, with a simultaneous increase in other trophic groups. The field studies from Italy (Manachini 2002), differentiating according to nematode genera or trophic groups, are therefore considerably more meaningful. It is interesting that for these comparative tests, averaged over eight sites, after Bt+ the bacteriophagous nematodes are less abundant and the mycophagous nematodes are more abundant, but this was not statistically validated. This might be explained by, in addition to a reduced food supply (see above), a direct effect from Cry1Ab. Dominance of bacterial feeders with a tendency toward lower numbers for Bt+ was also found in our monitoring study (see Section 3.2.8) as well as an increase in fungal feeders. Since this applied only to Bt176 and not to MON810, the overall problem in monitoring is clearly whether or not the corn varieties differ with respect to quality and quantity of Bt toxins. The studies from Italy relate exclusively to Bt176. In contrast to MON810, these varieties do not express toxin in the roots but rather in the pollen (internal studies, Jehle & Nguyen 2003). Studies by Manachini (2002) showed no effects with regard to the range of nematodes, with 37 genera for each of the corn variants.

Determinations of nematode genera are especially costly [10]. Experience shows that determinations of infestation densities also show high variances, which is why research costs for statistical validation are unusually high. A standardizable bioassay with appropriately susceptible nematodes would therefore be advantageous in order to determine the bioavailability of Bt toxins in field samples. The tests frequently used to determine soil loading with toxic substances, for example using earthworms (*Eisenia fetida)* or collembolans, are obviously not suitable or not sensitive enough for Cry1Ab (Zwahlen et al. 2003), which is why insect bioassays have been used so far (for example, using larvae of the tobacco hornworm *Manduca sexta*). Mortality was determined three or seven days after providing feed contaminated with soil suspensions (Sims & Holden 1996). The test duration could be considerably shortened with *C. elegans*, whose life cycle is only about 72 hours. This nematode is also easy to grow, and soil is its natural habitat. So it meets important preconditions for a test system to be used to detect ecological risks from Bt toxins in the soil (Jepson et al. 1994). In Section 3.2.8, we will discuss the ECOSSA results and our own experiences with the *C. elegans* test in more detail.

3.2.6 Scientific and technical methods used

We will discuss the study methods used in more detail in Section 3.2.3. For microscopic examination of the nematodes extracted from the corn roots, in addition to visual counting we also used an automatic image analysis system (Leica Qwin). Because the nematode suspensions of some samples were contaminated by residual soil and fine roots, the labor-saving image analysis approach unfortunately was only suitable to a limited extent. However, the system could be easily used for this purpose by optimizing sample preparation. We have also experimented with image analysis for the *C. elegans* test, where the increase in length of the nematodes can be measured as a sublethal parameter.

For statistical data analysis, the pi and pf values (pi = initial population density, pf = final population density) were separately calculated site by site for *Pratylenchus* spp. and the saprophagous nematodes. Each corn variant provided eight individual values (four mixed samples with two extractions each) for this purpose. The means were compared by

[10] Translator's Note: See previous footnote concerning "aufwändig" = costly or possibly difficult/labor-intensive.

ANOVA using the *t* test and the Newman-Keuls test. It seemed feasible to average over the plots with and without chemical control of corn borers, since as expected no effect on the nematodes was detected from the insecticide (Baythroid). We proceeded similarly with the root populations. To evaluate the nematode dominance structure, we used the Wilcoxon-Mann-Whitney U-Test, which is used to compare two independent random samples with values that are not normally distributed.

3.2.7 Progress Made in Other Places That Became Known While This Study Was Conducted

For the particular problem of how much Bt corn affects the nematode fauna, no new results became known that have not already been mentioned in previous sections. However, transgenic rape expressing Cry1Ac was studied for the first time with regard to effects on the trophic structure of the nematode fauna (Manachini et al. 2003). Those studies found a significantly higher proportion of mycophagous nematodes and a lower proportion of phytophagous nematodes compared with the conventional variety. There are also some indications of antibacterial effects from Bt toxins (Escher et al. 2000, Zalunin et al. 2003), where an indirect effect on nematodes is possible. Multiple-year toxin analyses for Bt corn fields also confirm the suspicion of possible accumulation of Cry1Ab (Hopkins & Gregorich 2003), which is why suitable bioassays for evaluation of bioavailability have become more important.

3.2.8 Presentation and Evaluation of the Results

Phytonematodes

As expected, at all sites the population density of *Pratylenchus* spp. more or less clearly increases on the average for all varieties during four corn cultivation cycles (Fig. 1), since corn is a good host plant (Knuth 2000). The different initial levels and growth of the nematodes at the five sites are attributed to the different previous crops grown before the trials began and differences in the soils, since *Pratylenchus* spp. prefers lighter soils. With regard to host plant quality for *Pratylenchus* spp., only the Baumannshof site showed lower nematode reproduction with Nobilis/Novelis-Bt compared with Antares/Navares-Bt during two years (2002 and 2003) (Fig. 2), which also could be seen in the correspondingly lower root populations (Fig. 5). Such different responses of corn varieties are known, for example, for infestation with stem and bulb nematodes (*Ditylenchus dipsaci*) (Knuth 2001), and can possibly be connected with the different ripening times of these varieties and also with the nonuniformity of the trial fields. As measured by *Pratylenchus* spp. abundances for all sampling times, there were no other significant differences between varieties at any site (Fig. 3). So no effects from Bt toxins could be detected for either MON-810 or Bt-176. However, Novelis-Bt repeatedly had markedly lower infestations in Fall soil samples (Fig. 4), presumably because of a higher lignin content in this variety (Saxena & Stotzky 2002). Due to slower degradation of plant or root residues, we should expect delayed nematode emigration. Also for a lignin content of 6.2% compared with 3.7%, as was found for MON-810 hybrids compared with isogenic lines, altered host-parasite behavior cannot be ruled out in herbivores. In our histological studies on lignin content in stalk and root cross sections, we could identify structural differences but they could not be quantified. Using near infrared spectroscopy, which among other things is suitable for studies of plant components or the composition of organic matter in soil samples (Schulz 2002, Couteaux et al. 2003), we found differences in lignin content between stalk and root but not between varieties. In contrast to the study by Saxena & Stotzky (2002), there are reports in the literature about the lower lignin

content of Bt corn and its faster degradation (Escher et al. 2000). Studies of the substantial equivalence of transgenic and isogenic varieties are therefore of particular importance.

The first indications of different responses of Bt corn to *Pratylenchus* spp. appeared back in 1998. In LBP [Bavarian State Institute of Soil and Plant Cultivation] variety trials (without appreciable European corn borer infestation), the relative silage corn yields based on 80% dry matter for Navares-Bt and Pactol-Bt, another Bt corn variety, were 109 and 107 respectively compared with conventional comparison varieties. The greater growth of these Bt varieties was reproducible in pot culture tests in a greenhouse with *Pratylenchus* spp. contaminated soil. The obvious question was whether Bt corn has a lower susceptibility to nematodes or better nematode tolerance, and our studies addressed this question even before the monitoring project (Arndt 2000); the harmful effect of *Pratylenchus* spp. on corn was supported in particular by older nematicide trials (Küthe & Rössner 1978). Unfortunately, within the monitoring project it was not possible to determine yields, and so we could not examine tolerance differences (as also exist in the conventional range of varieties).

Saprophagous nematodes

The population density of saprophagous nematodes mainly depends on the soil conditions at the time the samples are taken. In addition, supplying organic matter, such as by incorporating harvest residues or applying liquid manure, causes a short-term population increase. Partly because of the large amounts of corn straw accumulating yearly in the continuous corn trials, except for the Baumannshof site there was a more or less considerable increase in the population density for all corn variants (Fig. 6). The opposite development at Baumannshof, the site with the lowest microbial biomass content, was possibly due to premature sampling in Spring 2003. Breaking down the data according to individual varieties, there was no effect from Bt corn at any site, as shown by the Neuhof example (Fig. 7). This also applied to studies on nematode diversity, since there were only slight differences between the number of detected genera in the different trophic groups for Bt+ and Bt- (Table 2). Regarding the dominance structure, in the Spring 2002 samples for Bt176 we did indeed observe an increase in fungal feeders and a drop in bacterial feeders (Table 3) that were comparable with the previous year's data and results from Italy (Manachini 2002) but could not be statistically validated. For future monitoring surveys accompanying cultivation, these studies are too costly [[11]] if significant effects are to be detected.

***Caenorhabditis elegans* bioassay**

The bioassay, which tested the effect of Bt toxins on sublethal parameters (growth, number of eggs, reproduction efficiency) for *C. elegans*, was carried out with soil clinging to the roots from Baumannshof and Neuhof corn plots in 2002. Additional tests with soil samples and rhizosphere soil from 2003 were carried out in some cases in parallel with the LfL working group and Ecossa. The 2002 results in some cases showed significant differences between Bt+ and Bt- and also a clear influence of the site on the parameters of body length, number of eggs, and reproduction rate (Fig. 8). Since two subsamples from a non-Bt corn plot also showed significant differences, an edge effect due to improper incorporation of corn straw could possibly be responsible for this. Also in Bt toxin analysis of soil samples, isolated positive values were found in adjacent control plots which presumably were due to entry of Bt corn pollen (Baumgarte 2003).

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[11] Translator's Note: See previous footnote concerning "aufwändig" = costly or possibly difficult/labor-intensive.

In samples from 2003, which came exclusively from Baumannshof, the nematode assay with both unoccupied soil before corn cultivation (Fig. 9) and with soil clinging to the roots showed effects from Bt corn, especially for Novelis-Bt. Some determinations of toxin content in the soil (kindly done by FAL Braunschweig using ELISA) support the indications of possible susceptibility of *C. elegans* to Cry1Ab. In comparison samples of soil clinging to the roots with clear effects on growth and reproduction rate, over 0.5 ng Cry1Ab per gram soil was detected (Höss et al. 2004). This method will be included in a new BMBF joint project starting in 2005 for further testing and validation of the nematode assay to determine the bioavailability of Bt toxins.

3.2.9 Summary

Insecticides based on *B. thuringiensis* have been approved for agricultural use for decades, and may be also used in organic farming. Commercial preparations, depending on the Bt strain used, are distinguished by high specificity (for example, against lepidopterans or beetles) and are regarded as especially environmentally friendly (Krieg 1983). Bt corn (to which a *B. thuringiensis* gene was transferred) expresses a toxin which the plant uses to protect itself against European corn borer infestation, but this is not comparable qualitatively or quantitatively to a conventional Bt spray. The range of nontarget organisms that can be affected by Bt corn cultivation is estimated as considerably greater, partly because more toxins reach the soil through the harvest residues and the efficacy does not depend on special receptors in the target organisms.

Among the Metazoa, soil nematodes are the group with the highest number of species and individuals. With their different feeding types (fungal feeders, bacterial feeders, plant parasites), they are involved in metabolic processes in the soil ecosystem and have attracted increased interest for biological soil analysis. There are several references in the literature to the effects of *B. thuringiensis* on nematodes, which however do not permit any conclusions concerning any nematicidal effect from transgenic Bt plants. The soil samples studied within the monitoring project did not show any significant differences between Bt+ and Bt- plots for plant-parasitic nematodes of the genus *Pratylenchus*, other than a somewhat higher reproduction rate with Antares/Navares-Bt compared with Nobilis/Novelis-Bt at only one out of the five sites. Likewise no effects from Bt corn could be detected for mycophagous and bacteriophagous nematodes. A slight increase in fungal feeders at the expense of bacterial feeders for Bt176 is consistent with the results of one-year field trials from Italy. Since lower reproduction efficiencies could be detected during a bioassay with the bacteriophagous nematode *Caenorhabditis elegans* for Bt corn, this could be a sign of a direct toxic effect from Cry1Ab. Because we have only a few comparison values for toxin contents in the soil samples, which were measured in parallel using ELISA, we cannot make any definitive statement concerning the sensitivity of this nematode assay. In a subsequent project, this test (which already has been standardized for ecotoxicological studies) should be validated for Bt corn monitoring with other Cry toxins.

3.2.10 Appendix with Tables, Figures, References

Tables and Figures

Table 1: Overview of sampling and tests performed

Site **Soil samples**[2)]	Sp[1)] 2000	F 2000	Sp 2001	F 2001	Sp 2002	F 2002	Sp 2003	F 2003
Baumannshof	x	x	**x**	**x**	**x**	sampling not possible	**x**	x
Neuhof	x	x	**x**	x	**x**		x	x
Schwarzenau	x		**x**	x	x		x	x
Grub	x				x		x	x
Puch	x				x		x	x
Root samples [3)]	2000	2000	2001	2001	2002	2002	2003	2003
Baumannshof	Aug.	Sept.	July	Aug.	**July**	Sept.	June	**Sept.**
Neuhof		Oct.			**June**			

[1)] Sp = Spring, F = Fall before corn cultivation and after harvesting, respectively; [2)] testing for *Pratylenchus* spp. and saprophagous nematodes; **x** = additional determination of genera and/or *C .elegans* bioassay*;* [3)] boldface = additional soil clinging to the roots for *C. elegans* test

Table 2: Number of nematode genera by trophic group in samples from 2002

	NEUHOF				BAUMANNSHOF			
	Antares	Navares-Bt	Nobilis	Novelis-Bt	Antares	Navares-Bt	Nobilis	Novelis-Bt
Bacterial feeders	9	9	12	10	15	14	15	14
Fungal feeders	2	2	3	3	3	3	3	3
Plant feeders	3	3	4	3	2	3	2	2
Obligate plant parasites	2	2	4	5	1	1	4	2
Predators	-	-	-	1	1	-	-	-
Omnivores	2	3	4	2	2	1	1	1
Total genera	18	19	27	24	24	22	25	22

Table 3: Proportions (%) of trophic groups in samples of Antares and Navares-Bt from the Baumannshof site in 2002

Trophic groups	Antares	Navares-Bt
Bacterial feeders	76.9 (61.8)	73.5 (57.5)
Fungal feeders	16.8 (4.7)	19.1 (9.4)
Plant parasites	5.0 (30.6)	5.9 (28.7)
Other	1.2 (2.9)	1.6 (4.4)

Values in parentheses come from field trials in Italy (Manachini et al. 2002)

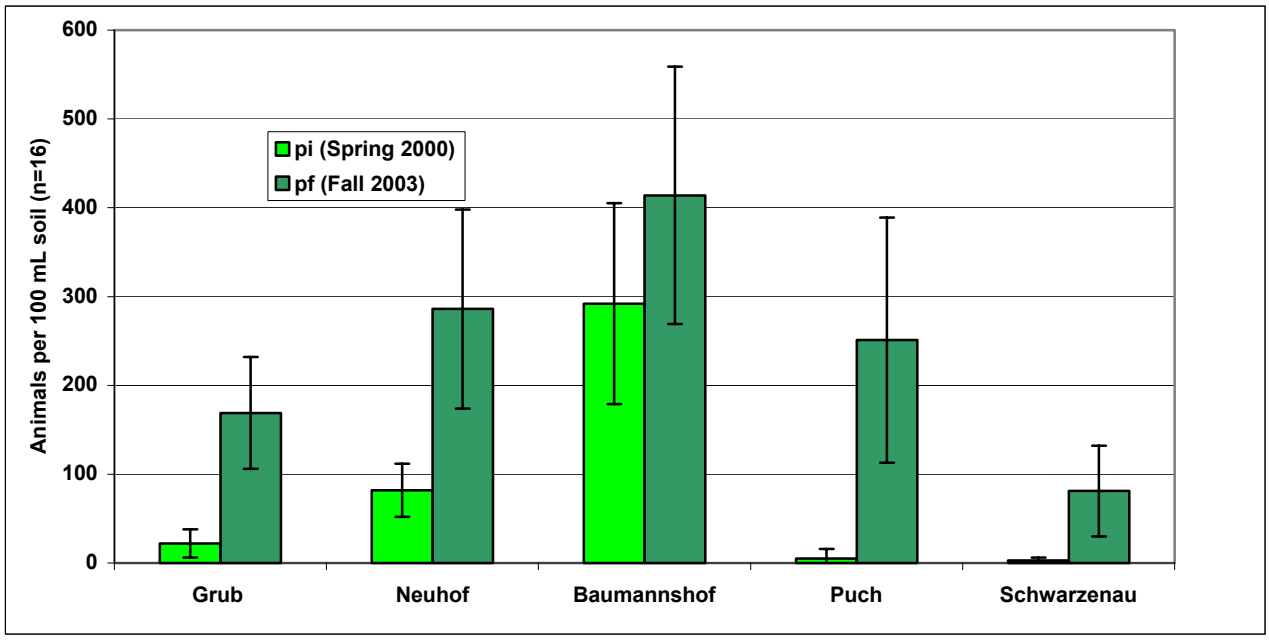


Fig. 1:Initial infestation (pi) and final infestation (pf) with *Pratylenchus* spp. after four corn cultivation cycles (means with standard deviation)

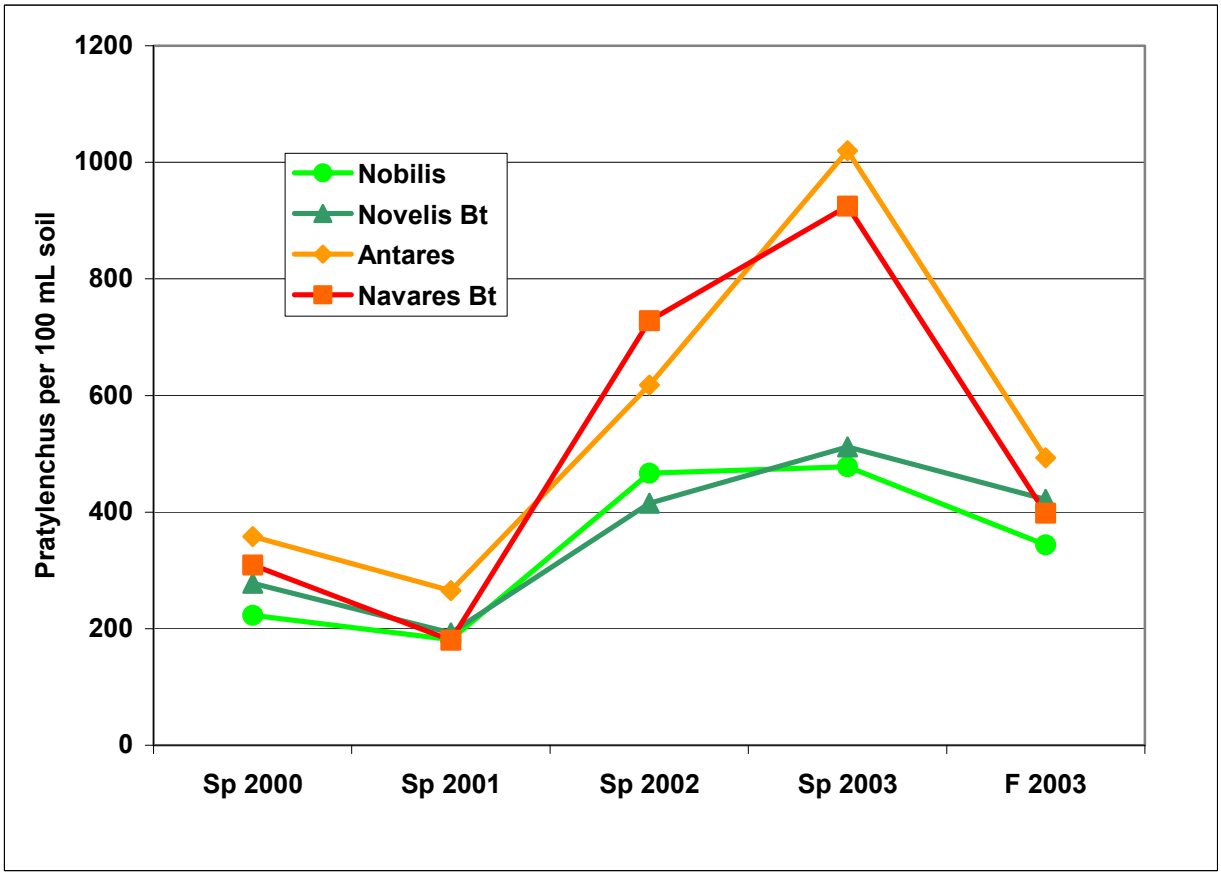


Fig. 2: Population dynamics for *Pratylenchus* spp. at the Baumannshof site

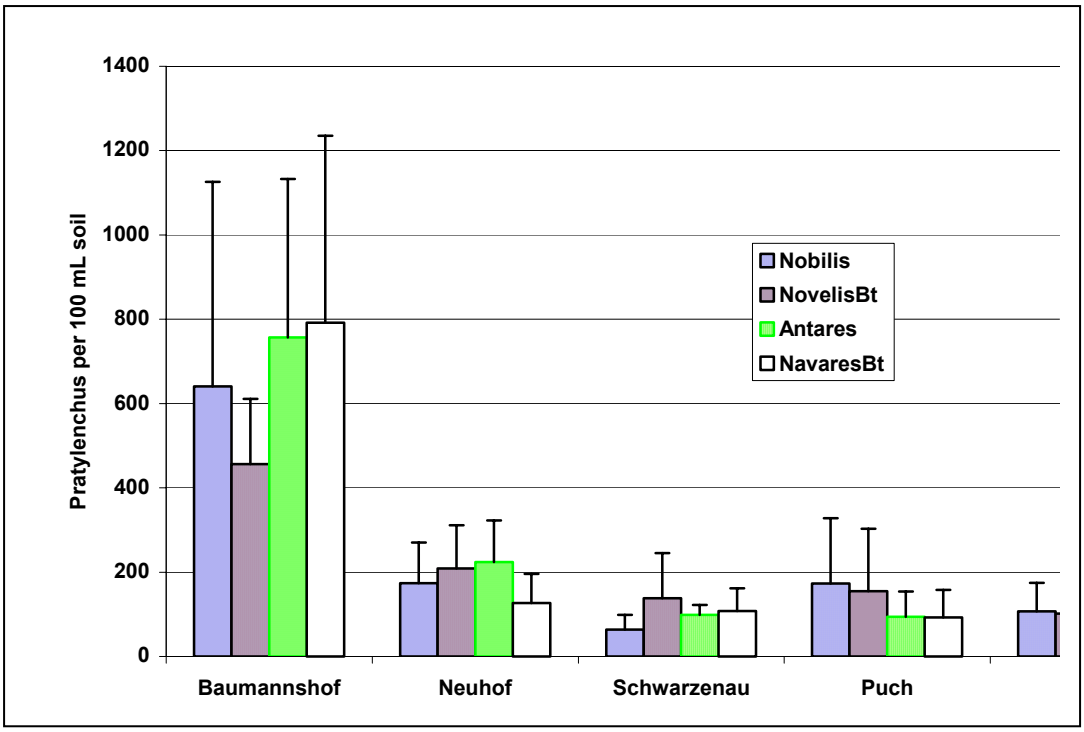


Fig. 3: Abundances of *Pratylenchus* spp. (mean + standard deviation for all sampling dates; Baumannshof and Neuhof n = 6, Schwarzenau n = 5, Puch and Grub n = 3). Varieties at the same site marked by different letters are significantly different (Newman-Keuls test, $p < 0.05$).

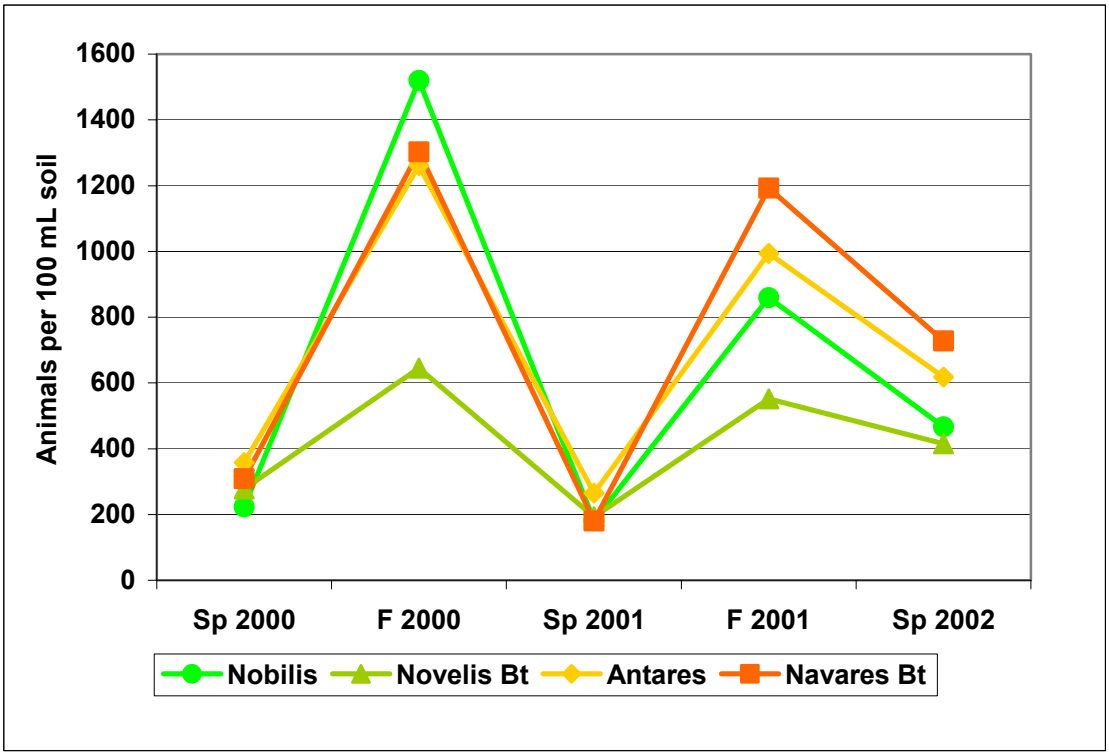


Fig. 4: Population dynamics of *Pratylenchus* spp. between Spring and Fall samples at Baumannshof site

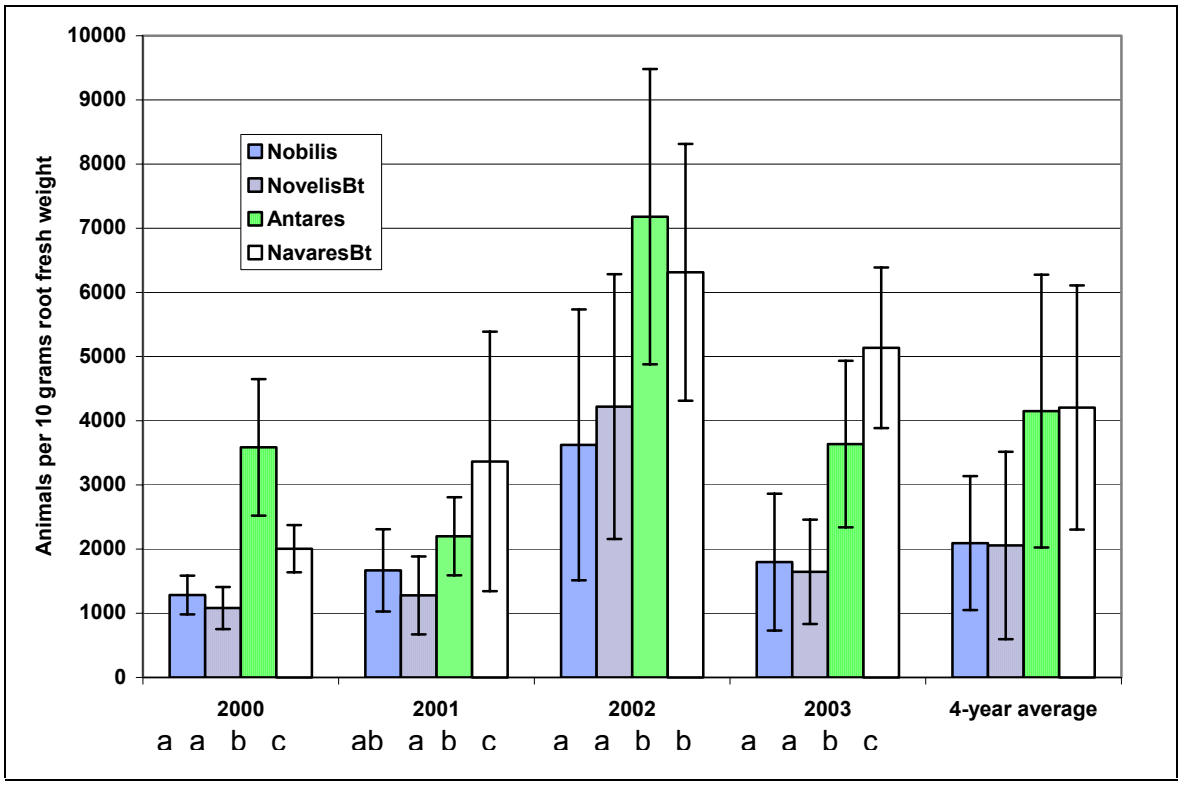


Fig. 5: *Pratylenchus* spp. corn root population at Baumannshof site (mean and standard deviation of 20 plants each); varieties marked by different letters in the same year are significantly different (Newman-Keuls test, $p < 0.05$).

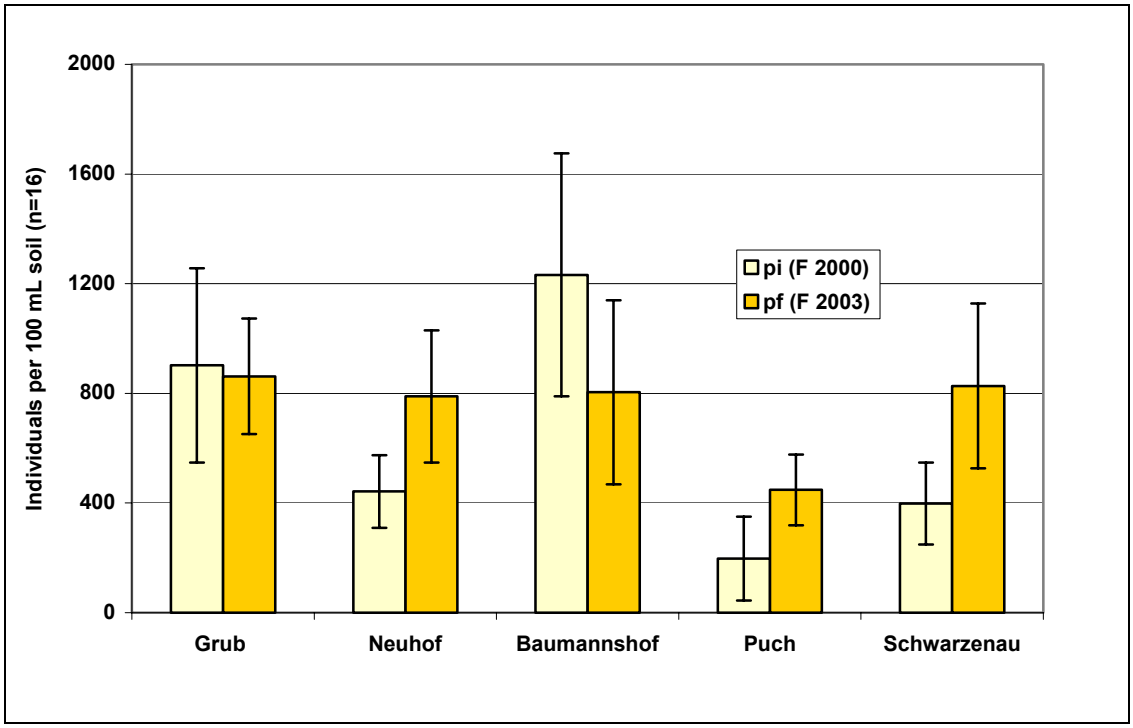


Fig. 6: Initial infestation (pi) and final infestation (pf) with saprophagous nematodes after three corn cultivation cycles (means with standard deviation)

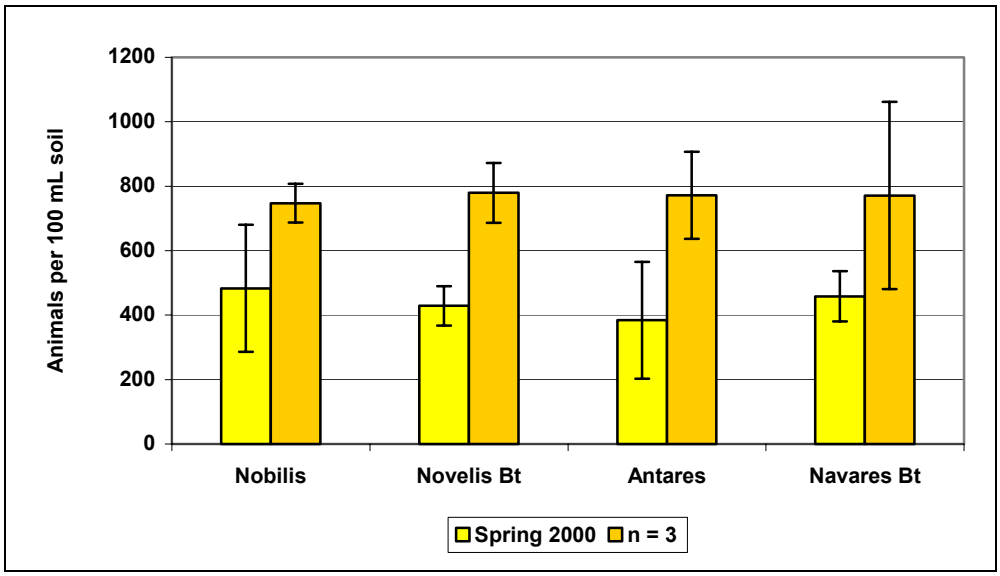


Fig. 7: Change in population of saprophagous nematodes by variety at the Neuhof site (mean and standard deviation at start of trial and for three Spring samplings)

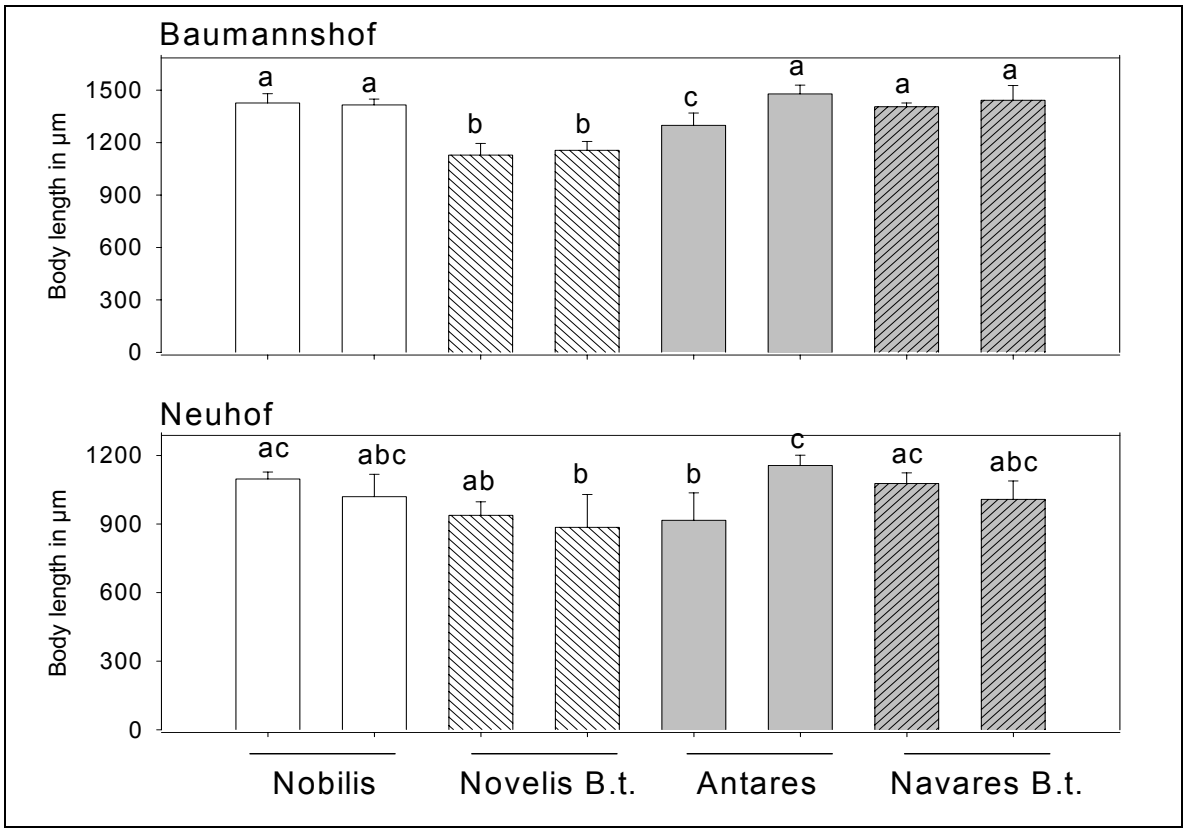


Fig. 8: Body length of *C. elegans* after 96-hour exposure in soil clinging to the roots from the Baumannshof and Neuhof sites (2002) and eggs in the body; different letters mean significantly different values (one-way ANOVA, posthoc: Tukey, $p < 0.05$)

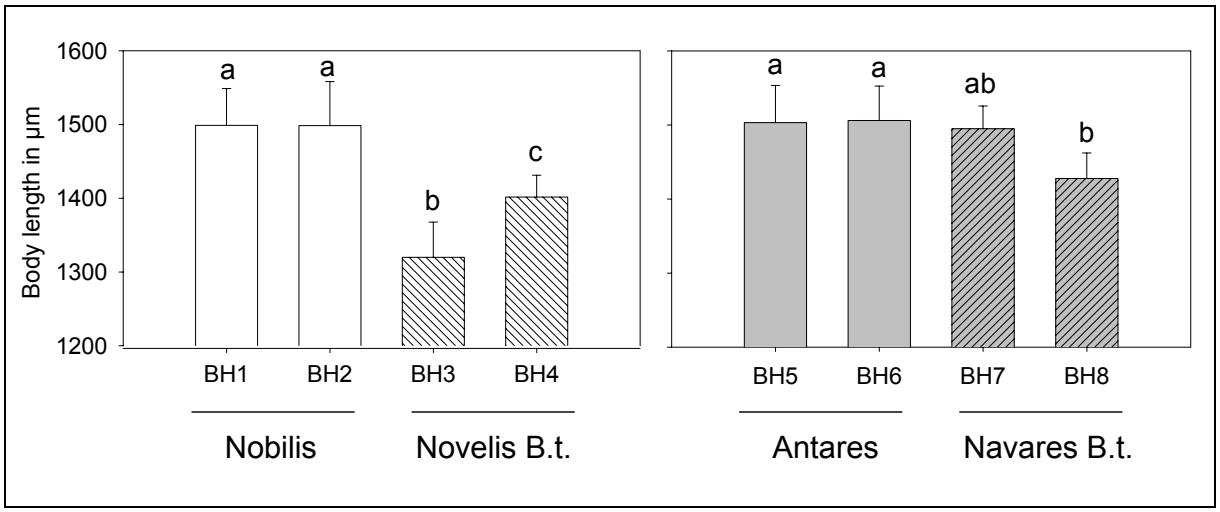
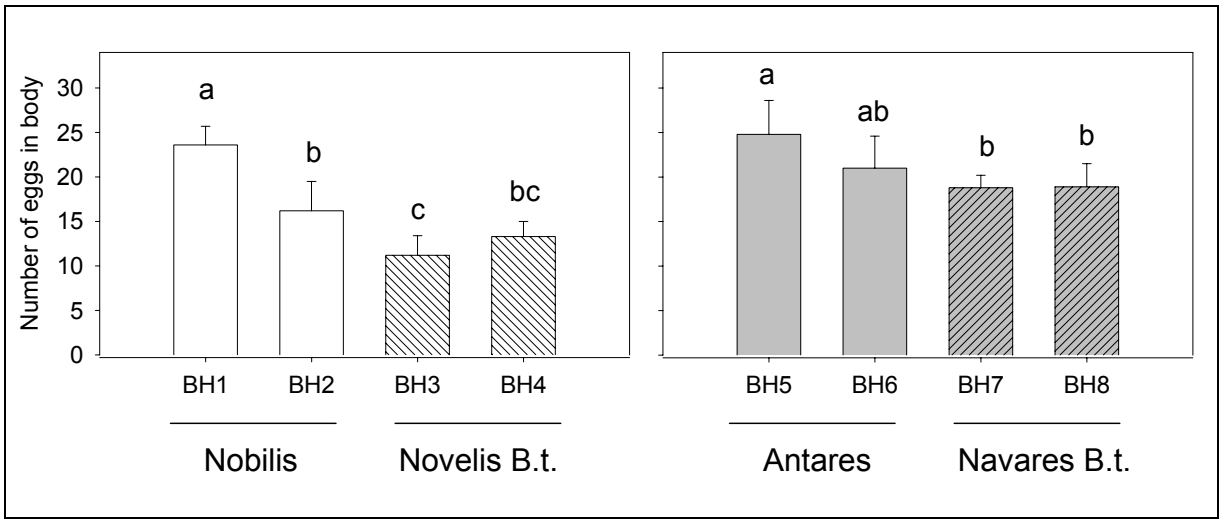
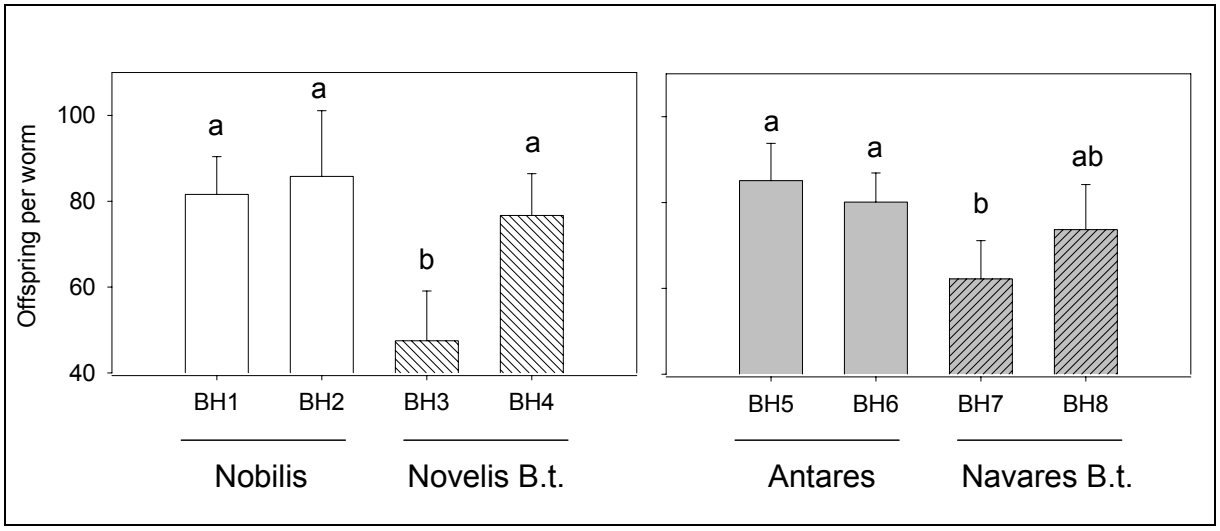


Fig. 9 : Body length of *C. elegans* after 96-hour exposure in samples (Spring 2003) from different plots of the Baumannshof site; different letters mean significantly different values (one-way ANOVA, posthoc: Bonferroni, $p < 0.05$)





Figs. 10, 11: Number of eggs and reproduction rate for C. elegans

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3.3 Soil microbiology

3.3.1 The Task

Soil microorganisms are essential for cycling nutrients and nearly every metabolic process in soil begins with them. The special importance of soil microorganisms for plant nutrition lies in their mineralization of harvest residues, making a supply of nutrients available to the plants. In addition, soil microorganisms regulate the humus content and create the chemical and physical properties of the soil which promote plant growth. Therefore microbial activity is of fundamental importance for soil fertility. The aim of the studies was to identify all the soil microbial metabolic activities that are most important for long-term soil fertility at various agricultural sites.

By comparative studies of various important soil microbial properties on fields cultivated with Bt corn or with comparable conventional corn varieties, we should be able to establish any effects on soil microorganisms from Bt toxin in root and plant residues and in the soil.

3.3.2 Extensive Documentation and Evaluation of the Literature Used

For this project, we exclusively used the literature for the methodological portion of the work. This literature is found in the Materials and Methods section (3.3.6.).

3.3.3 Conditions Under Which the Work Was Conducted

At the time of the application, we allowed for the equivalent of three full-time personnel to do the work. Because of unforeseeable circumstances, during the research project our work area lost two and a half positions, which meant we had to drop some of the planned studies. However, the essential aspects of the project were completed as planned.

3.3.4 Planning and Progression of the Work

The studies were carried out in continuous trials with grain corn on the state experimental farms at Baumannshof, Grub, Neuhof, Puch, and Schwarzenau as well as on available long-term soil observation (LSO) fields with corn.

In order to establish possible changes during the growing season, in addition to the usual sampling dates in Spring, we also collected samples in Summer during corn flowering and in Fall after the corn was harvested. Sampling began in Summer 2000 and ended with the sample collection in Fall 2003. So there were eleven test dates for each of the continuous cultivation trials. For logistical reasons, we could not do the sample collection ourselves at the Schwarzenau site; it was carried out by a third party.

Because of the nonuniformity of the individual samples and their inconsistent analytical data, the Schwarzenau site was no longer sampled starting in the year 2003. In addition to the microbial biomass as a measure of the microbial soil vitality we determined four enzyme activities which characterize essential metabolic activities in soil.

For the long-term soil observation fields, sampling was skipped in the summer and, due to lack of personnel, had to be entirely abandoned in Fall 2002. A total of 8 LSO fields were sampled but only 6 were analyzed, since only one test date was possible at two sites. Likewise, the studies of potential and current nitrification were abandoned in 2002 due to lack of personnel.

3.3.5 Overview of the Overall Issues, and Currently Known Findings in the Area of the Task at Hand

At the moment there have only been a few studies of the effect of Bt toxin on the activity of soil microorganisms (FIFRA SAP Report 2001). But these dealt with either *in vitro* laboratory tests (Saxena and Stotzky 2001) or field trials at one site with only one study parameter: the dimethyl sulfoxide reductase that we also studied (Mücher 2004). In both cases, no effect on soil microorganisms from Bt toxin or Bt corn could be established.

All other soil microbial research in connection with cultivation of Bt corn has concerned possible Bt corn → soil microorganisms gene transfer and possible shifts in the population dynamics of the soil microorganisms.

In both areas, no definitive results have been obtained. With regard to gene transfer, we should bear in mind that more than 10^9 soil microorganisms live in one gram of agricultural soil, corresponding to 10^{17} soil microorganisms per hectare for a soil depth of 10 cm. In view of the astronomically high number of soil microorganisms, searching for a rare event is therefore futile. In addition, *Bacillus thuringiensis* is a typical soil bacteria and "natural" gene transfer from *Bacillus thuringiensis* to other soil microorganisms is much more likely.

Also studies of population dynamics for soil microorganisms are not very promising since every soil shows daily changes in the composition of soil microorganisms due to altered environmental influences (temperature, water, incoming supply of materials) (verbal communication, Dr. Schloter, National Research Center for Environment and Health (GSF)).

3.3.6 Scientific and Technical Methods Used

Sample collection, processing, and storage

From each area of the field, a mixed sample was made up from 50 individual samples of the topsoil (0-10 cm). The homogenized samples were air-dried and passed through a 2 mm sieve. Some of the samples were stored deep frozen at –18°C until analysis. Reserve samples for retesting and more extensive analysis were also stored at –18 °C. For analysis, the samples were thawed in the refrigerator and adjusted to 50% maximum water holding capacity.

Analyses

All samples were analyzed for soil microbial characteristics of biomass, catalase activity, DMSO reductase, β-glucosidase, and arginine ammonification. In addition, the activity of nitrification microorganisms could be checked if their presence was suspected. All tests were carried out according to international standardized procedures (Methods in Soil Biology, F. Schinner et al. 1993).

Microbial biomass

- Substrate-induced respiration (SIR), according to the method of Anderson and Domsch 1978. Glucose was added to soil samples and the subsequent respiration reaction (CO_2 production) was determined using an infrared gas analyzer. By calibration, we can convert to microbially bound carbon (= Cmic) (µg C/g dry wt).

Enzyme activities

- Catalase turnover number: After hydrogen peroxide was added to the soil sample, the amount of O_2 enzymatically cleaved within three minutes was measured volumetrically and reported in percent (Beck 1971).
- β-Glucosidase, carbon cycle: Soil samples were incubated at 37°C for 3 hours with the substrate saligenin β-glucoside (salicin). The saligenin [12] liberated by β-glucosidase activity was determined (after converting to a colored form) colorimetrically at 578 nm (modified method of Hoffmann and Dedeken 1966).
- Dimethyl sulfoxide reduction, sulfur cycle: After addition of dimethyl sulfoxide, soil samples were incubated for 3 hours at 30°C in a gas-tight vessel. The dimethyl sulfide formed was detected by gas chromatography (Alef and Kleiner 1989).
- Arginine ammonification, nitrogen cycle: After three hour incubation of the soil samples with an aqueous arginine solution, the ammonium formed was extracted with a 2 M potassium chloride solution and determined colorimetrically (modified method of Alef and Kleiner 1986).

Analysis

The statistical analysis was carried out using the SAS software package. Then after analysis of variance (taking into consideration the influence factors of sampling date and varieties as well as, when the entire series was included, also the site), the conventional and transgenic varieties were compared pairwise with the *t* test. This comparison was carried out site by site and over the sites for the pairs Novelis-Bt/Nobilis-conventional or Navares-Bt/Antares-conventional. In addition, a multivariate analysis of variance was also carried out, where all the study parameters together were drawn on to determine differences between the comparison pairs. With this method, when the effects of the parameters are in the same direction we expect that even smaller differences can be statistically validated than with the univariate approach.

For all the comparisons, the insecticide-treated plots were used as replicates, since we found that the factor “without insecticide” and “with insecticide” had no effect on the microbial soil parameters.

3.3.7 Progress Made in Other Places That Became Known While This Study Was Conducted

In the year 2004, results from Bt corn field trials on soil microbiology were published for the first time in a dissertation from the Rhein-Westphalian Institute of Technology at Aachen (RWTH-Aachen) (Mücher 2004). That dissertation compared the dimethyl sulfoxide reductase activity on one trial site with Bt corn and an isogenic comparison corn during two test years. In each case, the samples were collected in Fall (September or October) and the analyses were carried out by the method of Alef and Kleiner 1986, which corresponds to the method we used. In both test years, no significant difference in DMSO

[12] Translator's Note: "Saliginin" in German text is apparently a misprint for "Saligenin".

reductase activity could be established between the Bt corn and the isogenic comparison variety. In his work, Mücher pointed out that other soil microbial cumulative parameters such as soil respiration, ammonification, and dehydrogenase activity could also be used for monitoring.

3.3.8 Presentation and Evaluation of the Results

Continuous Bt corn cultivation trials

Fig. 1 shows the initial biomass content for all five trial sites. The clear differences in microbial soil vitality (µg microbial carbon/g dry wt.) on the individual trial sites reflect quite well the range in soil vitality for Bavarian agricultural soil. Thus the biomass content at the acidic Baumannshof site at 156 µg Cmic, is very low; while the biomass content at Grub at 465 µg Cmic is clearly above average. The other three sites correspond to about the average biomass content for Bavarian agricultural fields, 278 µg Cmic, as reported from the Bavarian LSO project.

Due to inconsistent analytical data, the Schwarzenau site was left out; so there were eleven sampling dates (Summer 2000 to Fall 2003) at four sites for analysis.

If we consider the microbial characteristics for all eleven test dates, then we see a significant increase in microbial activity during the growing season. At all the sites and on all the trial plots, microbial activity increased from Spring through Summer to Fall. This "rhizosphere effect," due to increased root formation during the growing season, has been known in the literature for a long time. Therefore for analysis, the results for individual seasons (3 Springs, 4 Summers, and 4 Falls) were pooled into groups. Statistical analysis did not show any significant differences within the groups sampled at the same time.

Figs. 2 to Fig. 6 show all the study parameters at the individual trial sites as Bt corn/conventional variety pairwise comparisons, giving the statistical results (*t* test).

The microbial biomass and the catalase turnover number are regarded as important parameters for determining the general soil vitality, and are less affected by environmental factors than other soil enzyme parameters. For both study parameters, no significant differences were found at the four trial sites for the Bt corn/conventional corn pairwise comparisons (Fig. 2 and Fig. 3). The catalase turnover number and the microbial biomass were not significantly different in all sampling date groups without exception, and likewise for both pairwise comparisons (Navares-Bt/Antares; Novelis-Bt/Nobilis). Also no dependence on the site or the variety of transgenic corn could been identified in the measured non-significant differences.

We also saw a similar result in the other three soil enzyme studies (Fig. 4 to Fig. 6).

For the most part, the specific soil enzyme pairwise comparisons did not give any significant differences. Only four pairwise comparisons, one at the Neuhof site and three at Puch, showed significant differences. For the glucosidase measurement, at Neuhof a significantly smaller value could be established for the Bt corn variety Navares than for the comparison variety Antares. At the Puch site, the DMSO reductase activity for the Bt variety Navares was significantly higher than for the comparison variety, and for the Bt variety Novelis it was significantly lower than for the comparison variety. At that site, significantly higher arginine deaminase could also be measured for the Bt variety Navares. There was a total of four significant differences, where twice the values for the Bt varieties were higher and twice they were lower than for the comparison varieties. Looking at all the sites, no significant differences were seen in the five individual study parameters (see Table 1). This result is also supported by the multivariate analysis of variance, where all 5

study parameters were used together so statistical differences could be identified (Table 2). To claim a difference between the Bt corn variety and the isogenic comparison variety, according to all four calculated statistics we would have to accept a probability of error of just under 85% for Novelis/Nobilis and 99 % for Navares/Antares.

All in all, in the trial we saw very good agreement between the soil enzyme parameters and the biomass results.

LSO comparison fields

Because of the different trial preconditions at each LSO site (experimental facility and maintenance by the farmer, differences in nonuniformity of the trial fields), the results were presented as a before/after comparison. For this the Spring sampling (before cultivation of the corn) was compared with the Fall sampling (after cultivation of the corn) and the isogenic comparison variety was set as equal to 100%. For example, if the Bt field had 110% of the activity of the comparison field in Spring and 112% in Fall, then there was a change in activity of +2% because of Bt corn cultivation. This procedure was necessary because the comparison plots sometimes differed by up to 30% in activity numbers in Spring before the trial started.

In Table 3, the changes in microbial study parameters due to cultivation of Bt corn are summarized for the six studied LSO sites. Positive numbers indicate an increase in the activities for Bt corn and negative numbers accordingly indicate a decline compared with the isogenic comparison variety. From the Bavarian LSO program, we know that individual samples have an average scatter of 6% in microbial activity numbers. So activity changes less than 12% (twice the scatter) were not considered as distinguishable. In the summary, we see only two large changes in the study parameters, and both have positive values. Once at LSO site B, the catalase turnover number in the Bt variant was 12% higher than for the comparison variant; and another time, at the C2001 site, the DMSO reductase was 15% higher. The next year, this difference could no longer be observed at site C2002. For the most part, the established differences were clearly below the 12% level for all other study parameters and sites. The results from the LSO sites thus support the conclusion from the continuous cultivation trials, according to which no differences can be established in soil microbial activity due to Bt corn cultivation.

3.3.9 Summary

In this monitoring report, for the first time we have conducted a comparative study at several sites on all the essential soil microbial cumulative parameters, comparing Bt corn and isogenic comparison varieties within a long study timeframe and for the entire growing season.

After eleven sampling dates over a four year period, we cannot establish any change in the studied soil microbial characteristics at the four evaluated sites under continuous Bt corn cultivation. The occasional significant differences appearing in the pairwise comparisons were both positive and negative; and according to our numerical data, no dependence on transgenic corn plants can be identified.

Additional support for this result also comes from the analytical values for the selected LSO fields. According to our numerical data, we can rule out any adverse effect on soil fertility and microbial soil activity from cultivation of the Bt corn varieties Navares and

Novelis. From a soil microbial standpoint, we have no reservations concerning cultivation of the two studied transgenic corn varieties.

3.3.10 Tables, Figures, and References

Table 1: Statistical pairwise comparison of Bt corn and conventional varieties (Navares/Antares and Novelis/Nobilis) for all dates and insecticide levels

Site	Comparison[1)]	Biomass	Catalase	DMSO	Glucosidase	Arginine
Baumannshof	Nov./Nob.	no[2)]	no	no	no	no
	Nav./Ant.	no	no	no	no	no
Grub	Nov./Nob.	no	no	no	no	no
	Nav./Ant.	no	no	no	no	no
Neuhof	Nov./Nob.	no	no	no	<[3)]	no
	Nav./Ant.	no	no	no	no	no
Puch	Nov./Nob.	no	no	>[4)]	no	>[4)]
	Nav./Ant.	no	no	<[3)]	no	no
All sites	Nov./Nob.	no	no	no	no	no
	Nav./Ant.	no	no	no	no	no

[1)] Nov./Nob. = Novelis-Bt/Nobilis-conventional; Nav./Ant. = Navares-Bt/Antares-conventional.

no[2)] = no significant difference

<[3)] = significantly smaller value for Bt corn

>[4)] = significantly higher value for Bt corn

Table 2 Multivariate analysis of variance with biomass, catalase, DMSO, glucosidase, arginine

- **Novares/Antares and Novelis/Nobilis Comparison**		
Statistic (from SAS)	Exceedance probability *)	
	Navares/Antares	Novelis/Nobilis
Wilks' Lambda	0.9916	0.8486
Pillai's Trace [13]	0.9916	0.8486
Hotelling-Lawley Trace	0.9916	0.8486
Roy's Greatest Root	0.9916	0.8486

*) All four statistics support the null hypothesis, i.e., the Bt variety does not differ from the conventional variety ($P > 0.05$).

Example, 0.9916: To claim a difference between the two varieties, we would have to accept a 99.16% probability of error.

Table 3: Change (%) in soil microbial parameters for the Bt corn/conventional corn comparison on the LSO (long-term soil observation) fields (for explanations, see text)

LSO fields/Year	Microbial biomass µg C/g dry wt	Catalase turnover number	DMSO reductase DMS/g dry wt/h	β-Glucosidase saligenin/g dry wt/3 h [14]	Arginine deaminase µg N/g dry wt/h
LSO A / 2002	6	-4	-1	-3	3
LSO B / 2002	3	12	2	0	5
LSO C / 2001	0	-2	15	-5	0
LSO C / 2002	-4	1	-2	-7	2
LSO D / 2001	-6	-6	-5	-3	-3
LSO E / 2001	1	-2	5	1	-3
LSO F / 2001	4	6	7	2	-2

[13] Translator's Note: Corrected misprint: "Pillais Trace" should be "Pillai's Trace".
[14] Translator's Note: Corrected misprint: "saliginin" should be saligenin.

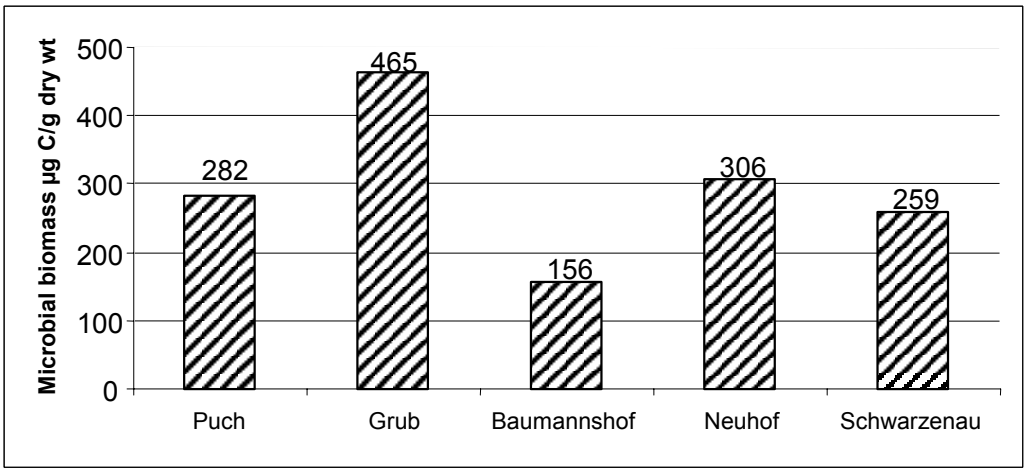

Fig. 1: Average microbial biomass content at the trial sites

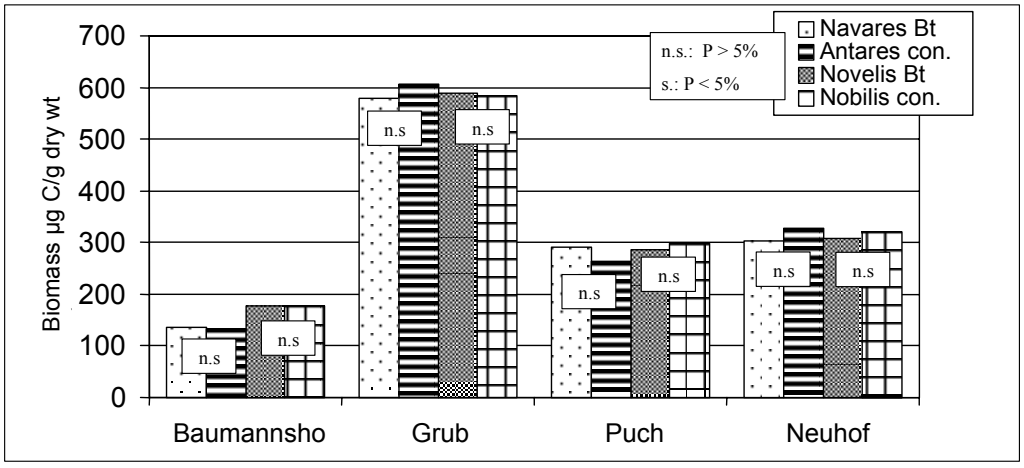

Fig. 2: Average values for the biomass and the *t* test for Bt corn/conventional pairwise comparison, for all sampling date groups and both insecticide levels

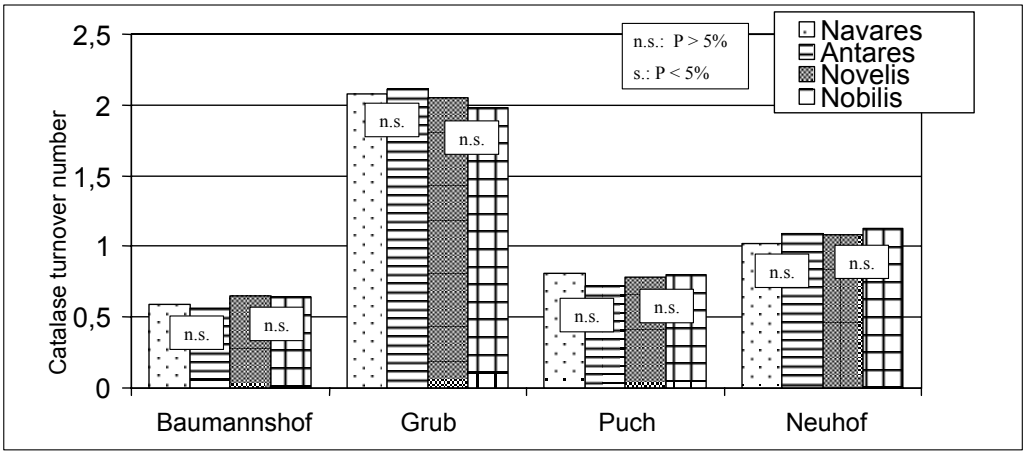

Fig. 3: Average values for catalase turnover number and the *t* test for Bt corn/conventional pairwise comparison, for all sampling date groups and both insecticide levels

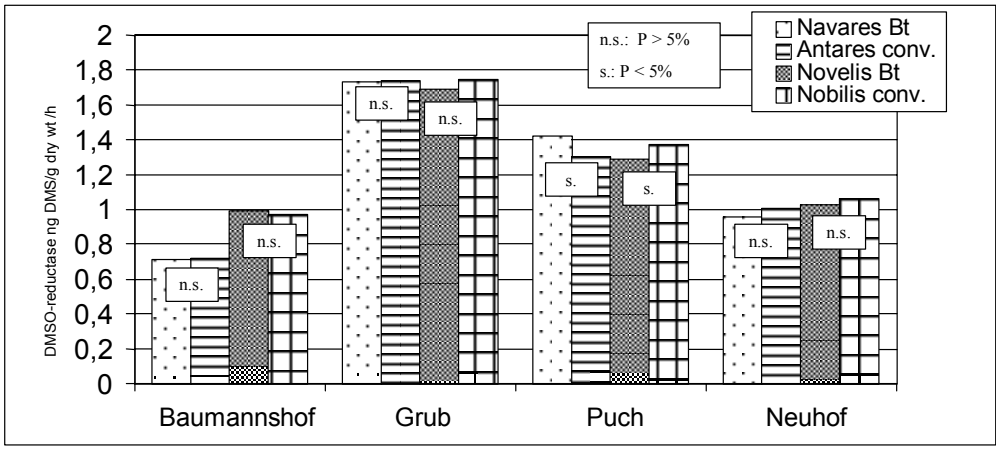

Fig. 4: Average values for DMSO and the *t* test for Bt corn/conventional pairwise comparison for all sampling date groups and both insecticide levels

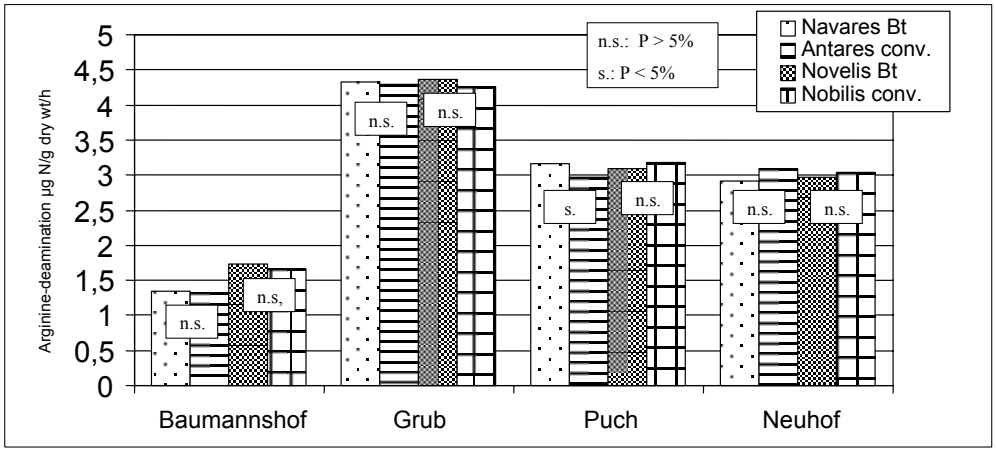

Fig. 5: Average values for arginine and the *t* test for Bt corn/conventional pairwise comparison for all sampling date groups and both insecticide levels

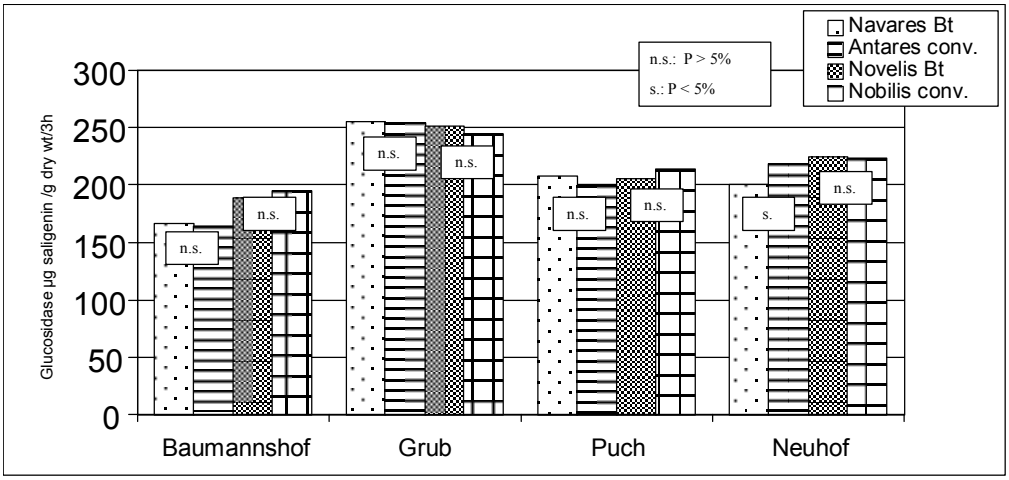

Fig. 6: Average values for β-glucosidase and the *t* test for Bt corn/conventional pairwise comparison for all sampling date groups and both insecticide levels

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Schinner,F., Öhlinger, R., Kandeler, E. und Margesin,R. (1993): Bodenbiologische Arbeitsmethoden. 2. Aufl., Springer Verlag Berlin, Heidelberg.

3.4 *Collembola* and *Lumbricidae*

3.4.1 The Task

The aim of the studies was to test whether cultivation of Bt corn compared with non-genetically engineered varieties as well as insecticide treatment for the European corn borer compared with insecticide-free cultivation have any negative effects on soil fauna. As the indicator organisms, we used *Collembola* (springtails) and *Lumbricidae* (earthworms): two groups of animals whose high abundance of individuals and high species diversity make them good indicators of any quantitative or qualitative change in the soil and nutrient supply, which result in changes in their taxonomic community structure. The studies were carried out in five large field trials at different sites under continuous cultivation with corn.

3.4.2 Extensive Documentation and Evaluation of the Literature Used

Up to now, no results on the effects of Bt corn on soil fauna have been available from true field trials with natural populations under real farming conditions. The few investigational approaches described in the literature come from laboratory or "semifield" studies on only one species in each case, and thus are only partially transferable to agricultural fields. In laboratory feeding tests, Goy et al. (1995) as well as Saxena and Stotzky (2001) studied the effect of Bt corn on the compost worm *Eisenia foetida* or on the field variety *Lumbricus terrestris*. Goy et al. kept *E. foetida* in artificial soil to which they added leaf extracts from Bt and non-Bt corn. After 14 days, all the animals were still alive and had gained the same amount of weight. Saxena and Stotzky used the mineral soil dwelling species *L. terrestris* for their studies. They kept 20 sexually mature animals in each plastic pot filled with mineral soil and planted with Bt corn or non-Bt corn. After a test period of 40 days at 24°C and day/night illumination, no difference was found in mortality and biomass. Pot tests with mineral soil where Bt corn straw or non-Bt corn straw were mixed into the soil gave the same results. The Bt toxin was detected immediately after the end of the test in the intestines and castings of the earthworms, but within 2-3 days after transfer of the worms to fresh soil the toxin could no longer be detected.

Zwahlen et al. (2003) tested the effect of Bt toxin on *L. terrestris* in laboratory and "semifield" tests. Feeding tests were conducted in the laboratory for this purpose on adult animals in small glass tubes. In the field, subadult animals were buried 60 cm deep in the field inside mesh bags filled with corn straw and soil. The earthworm feeding tests in the laboratory and in the field with non-Bt or Bt corn straw lasted 200 days. The mortality and weight development of the animals were examined. Bt corn did not lead to higher mortality in either the laboratory or in the field test. In the laboratory test, after 160 days of the test (for adult animals), a significant weight loss was seen in the group fed with Bt corn. This could not be confirmed in the field test (with subadult animals). Differences between the laboratory and field tests were also seen in the Bt toxin degradation rate. In the laboratory, the toxin content of the corn straw first decreased considerably and then stayed at a constant low level until the end of the test. In the field, the toxin content of the corn straw at least until the 80th day of the test was still high enough to kill corn borer larvae. At the end of the test, after 240 days, the toxin was detectable in only low amounts. So the test conducted by Zwahlen et al. (2003) is quite important because in that work, the

response of the earthworm field species *L. terrestris* was tested not only in a laboratory experiment, but also in a "semifield trial" for the first time.

There are only a few papers on the response of *Collembola* to Bt corn and non-Bt corn. In field trials, Deeb et al. (2003) found no significant differences between plots with Bt corn and non-Bt corn. However, that work involved a variety with Cry3b toxin that had been genetically engineered to combat corn rootworm.

3.4.3 Conditions Under Which the Work Was Conducted

In contrast to the work presented in Section 3.4.2, these studies were carried out in the field under real farming conditions. Bt corn and non-Bt corn and also insecticide-treated and untreated (no insecticide) plots were compared on fields under continuous corn cultivation. Continuous cultivation with corn is regarded as the "worst case" scenario for Bt or insecticide loading in agricultural practice. These studies were carried out in the second and third year of cultivation. So this ensured that toxin-containing root exudates, pollen, and degradation products of plant residues had already reached the natural food chain. Another advantage of field trials is that the naturally present taxonomic community structures, with an abundance of species, can be tested. Disadvantages of field studies are the sometimes very heterogeneous distribution of animals, a sometimes low population density, and the effects of site-specific and weather factors.

The trials were carried out on large plots (30 x 50 m) at five sites on the Baumannshof, Grub, Neuhof, Puch, and Schwarzenau state farms. Because the trial sites were scattered widely over Bavaria, it was possible to test any effects of Bt corn and insecticide treatments under various soil and weather conditions. The trial layouts were the same at all the sites (Fig. 1). In the first block, there were four plots side by side with corn varieties Bt 176 Navares, the isogenic variety Antares, as well as Bt MON 810 Novelis and the isogenic variety Nobilis. In the second block, there were the same varieties with application of insecticide. The trial fields were surrounded by a larger corn crop, so we did not have to be concerned about any edge effects due to other cultivated plants.

For evaluation of the studies, it would have been better to have had replicate trials at individual trial sites. Since this was not possible for technical reasons, the trials at different sites were regarded as replicates.

Since the taxonomic community structures of *Collembola* and *Lumbricidae* had already been studied at LfL in various projects (Bauchhenss, 1977, 1989, 1997, 1998), the technical prerequisites and know-how were available for this work.

3.4.4 Planning and Progression of the Work

For technical reasons, the studies were limited to five large plots under continuous cultivation with corn, since this provides the best comparison between the effects of the Bt toxin and the conventional crop rotation used in farming practice.

To test the *Collembola*, the samples in 2001 and 2002 in each case were taken in Summer or in Fall (Table 1). In the summer, the sampling was done on plots without insecticide, and in Fall sampling was done on plots with insecticide (Novelis+I; Nobilis+I). The

Summer samples thus were used to compare Bt corn and non-Bt corn, while the Fall samples were primarily used to compare insecticide vs. no insecticide.

For the *Lumbricidae* studies, samples were taken only in Fall 2001 and 2002 on selected trial sections, specifically on the following plots: Navares, Antares, Novelis, and Nobilis as well as Nobilis+I. Since it was not expected that *Lumbricidae* would be damaged by the insecticide, studying only one insecticide-treated plot seemed sufficient.

3.4.5 Overview of the Overall Issues, and Currently Known Findings in the Area of the Task at Hand

For cultivation of economically useful transgenic plants, it is essential to know their effect on the environment. This is the only way to avoid damage to the ecosystem in large-scale and long-term cultivation. So multiple-year studies of the total ecosystem under real farming conditions are necessary. The research cited in Section 3.4.2 covers only some aspects by means of laboratory or semifield tests, which is why additional studies under real farming conditions are needed. These field trials with continuous corn cultivation can achieve this goal. The *Collembola* and *Lumbricidae* studies were carried out in the 2nd and 3rd year of continuous corn cultivation. By continuous cultivation with corn, we ensured that its degradation products were already in the soil at the beginning of the studies, and so they would be available to the *Collembola* and *Lumbricidae* as food sources in various stages of decay, together with their accompanying fungal and bacterial growths. Inevitably, therefore, the Bt construct or Cry1ab toxin was incorporated via food.

Collembola and *Lumbricidae* are suitable test objects because they play an important role in the nutrient cycle, and improve soil structure; and as detritus feeders, they feed exclusively on decaying plant matter in various stages of decomposition.

3.4.6 Scientific and Technical Methods Used

In order to rule out subjective effects in the study from laboratory personnel, all the counts and determination of species for the samples were done with blind coding.

***Collembola* samples**

For the *Collembola* studies, 20 samples were taken per plot in two 7 meter wide strips, 5 meters to the right and to the left of the center line and 15 m from the lower and upper edge of the plots. The samples were each taken with a 100 mL corer tube customarily used in soil physics, in undisturbed spots between the seed rows. The 4 cm tall corer tube was hammered in deep enough so that the upper edge was flush with the soil surface. The corer tubes, sealed with two lids, were brought to the laboratory in insulated bags. On the same day, we began to force the animals out by warming the samples for two days at 25°C, two more days at 30°C, and then the last day at 35°C. The temperature inside the funnel was always about 10°C lower. The expelled animals were killed and preserved with 96% alcohol in collection vessels and sorted under a binocular microscope and classified by family. We used the method of Gisin (1960) and Palissa (1964) to make the determination under the microscope with illuminated specimens. Juveniles were generally not assigned to any species. They are indicated in the species lists as one group.

***Lumbricidae* samples**

We took 10 samples per plot for the *Lumbricidae* studies. The samples were taken from the central area of the plots, up to 5 m to the right and to the left of the central line and 15 m from the lower or upper edge. We took 10 samples of one square meter each from this interior portion per plot. The samples were collected by the formalin method (2 x 10 L, 0.2% solution). The animals were killed and preserved in 96% alcohol. The species were determined under a binocular microscope by the method of Wilcke (1967), Bouche (1972), and Herr (1987). The biomass was calculated by the method of Bauchhenss (1981) from the volume. Juveniles were generally not classified. They are indicated in the species lists as one group.

Statistics

Soil animals are generally not normally distributed. So we selected nonparametric statistical methods to validate any possible differences. For further analysis, we calculated the median from 20 measurement replicates for *Collembola* and 10 measurement replicates for *Lumbricidae* for each date and site as well as for each large plot.

The hypothesis (H_0) that there is no difference between Bt corn and non-Bt corn with respect to the number of *Collembola* and the number as well as the weight of *Lumbricidae* was tested with the Wilcoxon test for pairwise differences (Sachs 1992). The difference of the medians for Bt corn and the corresponding non-Bt corn variant was tested against $\mu = 0$. We used this test to additionally test whether there is any significant difference between the insecticide-treated trial sections and the corresponding variants without insecticide.

We similarly tested the secondary hypothesis of a difference between Bt corn and non-Bt corn as well as between insecticide vs. no insecticide for the overall *Collembola* population density as well as within the *Collembola* families and *Collembola* species, and the overall population density of *Lumbricidae* as well as for individual *Lumbricidae* species and their biomasses. We also tested for differences between the variants using the Kruskal-Wallis test (Sachs 1992) for each test date for *Collembola*, the *Collembola* families and species, as well as for *Lumbricidae* and the *Lumbricidae* biomasses and species. The results for the secondary hypotheses are presented graphically and should not be considered as definitive statistics. We used subsequent analysis of variance to assess the weight of the factors year, site, Bt corn, and insecticide.

I would like to thank Peter Eiblmeier for his advice concerning statistical analysis of the trial results and review of the manuscript.

3.4.7 Progress Made in Other Places That Became Known While This Study Was Conducted

During the term of this research project, the papers cited in Section 3.4.2 by Saxena and Stotzky (2001) and by Zwahlen et al. (2003) became known. Since those were laboratory and "semifield" studies, in each case on only one species, these results do not represent any important gain in knowledge relative to our project. Our results were supported by the paper by Deeb et al., which appeared in 2003 and presented field studies on *Collembola*. However, that paper dealt with a genetically engineered corn variety with another Bt toxin conferring corn rootworm resistance.

3.4.8 Presentation and Evaluation of the Results

Range of *Collembola* families and species

A total of 21,614 *Collembola* were sorted and classified by family. The *Isotomidae* were most abundantly represented with 9548 individuals, followed by *Onychiuridae* [15] with 7319 individuals, *Entomobryidae* with 3289 individuals, *Poduridae* with 869 individuals, and *Sminthuridae* with 589 individuals. See Table 3 and Fig. 3. The highest population density was found at Baumannshof, with 7466 animals in 320 100-mL samples, followed by Grub with 4950 animals, Puch with 3717 animals, Schwarzenau with 2941 animals, and Neuhof with 2549 animals. The species were determined for the *Isotomidae* in the Summer and Fall samples from the year 2001 (6152 individuals). A total of 14 species were represented, although with quite dissimilar distributions (see Table 4). The species *I. notabilis*, *F. fimetaria*, and *F. listeri* occurred at all the trial sites: *I. notabilis* with a total of 2204 individuals, *F. fimetaria* with 116 individuals, and *F. listeri* with 190 individuals. The following species occurred at four sites: *F. candida*, *F. quadrioculata*, *I. viridis*, *I. minor*, and *I. palustris*.

Range of earthworm species

A total of 18,655 earthworms were counted and classified. 10 species were represented (see Table 4). The most abundant species was *Allolobophora caliginosa*, with 2478 animals. It is represented at all the study sites. This species occurs most abundantly at Puch, Grub, and Neuhof. *Lumbricus terrestris* was the second most abundant species with a total of 690 animals, and likewise was represented at all the study sites. *L. terrestris* was most abundant at Grub, Neuhof, and Puch. It was followed by *A. chlorotica*, *A. rosea*, and *O. lacteum* with a total of 578, 365, and 39 animals respectively, which in each case were found at 3 sites. The population density (total from 50 1-m^2 samples) was highest at Grub with 7592 animals, followed by Puch with 5713 animals, Baumannshof with 2641 animals, Neuhof with 2236 animals, and Schwarzenau with 473 animals.

We could not establish any significant difference between the Bt variants and the non-Bt variants for the overall *Collembola* population density, the overall *Lambricidae* population density, or the *Lumbricidae* biomass. Comparison of the insecticide and no-insecticide variants also did not yield any significant difference for the overall *Collembola* population density, the overall *Lumbricidae* population density, and the *Lumbricidae* biomass (Fig. 2). Differential analysis of the *Collembola* families, *Collembola* species, and *Lumbricidae* species only indicated a significant difference for the comparison of the insecticide variant and the no-insecticide variant for *Isotomidae* and *Entomobryidae*. For both *Isotomidae* and *Entomobryidae*, higher abundances were found in the insecticide variant (Figs. 3-7). In an analysis of individual Bt variants and insecticide variants at different measurement times, we saw no indication of significant differences (Figs. 8-21).

Additional analyses showed that both the site and the sampling year had a significant influence on the overall *Collembola* population density. For the overall *Lumbricidae* population density and the *Lumbricidae* biomass, we could see a significant influence from the sampling site in the year of the survey. Table 6 summarizes the analysis of variance results.

Determination of the taxonomic community structures of *Lumbricidae* and *Collembola* is very labor-intensive due to procedural difficulties (Bauchhenss, 1977). So with the work we performed (species determination for 18,600 earthworms and determination of the families for 21,000 *Collembola*), we could properly test only a narrow range of possibly relevant cultivation scenarios for Bt corn and possible accumulation of Bt toxin with

[15] Translator's Note: Corrected misprint: German *Onychuriden* should be *Onychiuriden*.

respect to *Lumbricidae* and *Collembola*. Under these conditions, both the site and the year had an influence on the taxonomic community structure for *Collembola*. For the taxonomic community structure of *Lumbricidae*, we could establish a significant influence of the site in the sampling year. There were also indications that application of insecticide had a positive influence on the taxonomic community structure of *Collembola*. With regard to Bt corn, at present we have not been able to establish any influence on the taxonomic community structures of *Lumbricidae* and *Collembola* from the two varieties Navares and Nobilis.

We would like to continue the studies, especially on *Collembola* and *Lumbricidae*. In this case, it would be important to be able to work on plots with true replicates.

3.4.7 Summary

The aim of this project was to establish whether and to what extent large-scale cultivation of Bt corn (Navares, Novelis) compared with non-Bt corn (Antares and Nobilis) and application of insecticide (Baythroid, to control the European corn borer) compared with no insecticide can have quantitative and qualitative effects on the taxonomic community structures of *Collembola* and *Lumbricidae*.

Collembola and *Lumbricidae* are regarded as indicator organisms for soil fauna. Changes in the soil and especially the range of nutrients are very easily identified from the change in the taxonomic community structures of these animals (Bauchhenss 1977, 1989, 1997, 1998). They feed on plant decomposition products and associated microbial populations. Their diet ensures that the animals have to feed on decomposition products of the respective corn variety in the second and third year of continuous corn cultivation.

The studies were carried out with large plot trials which were repeated at five different sites on state farms in Bavaria. In each trial, the varieties Bt-Navares, Antares, Bt-Novelis and Nobilis were cultivated continuously on 30x50 meter plots with and without application of insecticide.

Collembola samples were collected with 100 mL coring tubes that were brought to the laboratory in insulated bags and sorted the same day. The *Lumbricidae* were forced out using a 0.2% formalin solution at the time the animals were fully active.

The *Collembola* samples were collected in the years 2001 and 2002 in Summer and Fall; the *Lumbricidae* samples were collected either in 2001 (Schwarzenau) or 2002 (all other sites) in Fall.

For *Collembola*, we collected twenty 100-mL samples per sampling date and per plot; for *Lumbricidae*, we collected ten 1-m^2 samples. The *Collembola* (21,600 animals) were sorted from Berlese collection vessels and classified by family. The *Isotomidae* for study year 2001 (6150 animals) were classified by species. For *Lumbricidae* (18,600 animals), all the animals were classified by species.

For the analysis, we calculated the median using 20 parallel samples from one plot for *Collembola* and using 10 parallel samples for *Lumbricidae*. Based on this median, we carried out the statistical treatment of the results according to the Wilcoxon test for pairwise differences and the Kruskal-Wallis H-test (Sachs 1992).

The plots of Bt corn and non-Bt corn showed no significant differences with respect to abundances for *Collembola* or *Lumbricidae*. For the *Collembola* families *Isotomidae* and *Entomobryidae*, the population density on the insecticide plots was significantly higher than on the no-insecticide plots. But this could not be verified on a species basis.

According to the H-test, in no case did we establish any significant negative effects from Bt corn cultivation and insecticide application on the taxonomic community structures of *Collembola* and *Lumbricidae* on the test fields. The study shows the development after three years under continuous corn cultivation. It should be repeated after a few years and further continuous cultivation with corn, and completed with simultaneous measurements of soil toxin content.

3.4.10 Appendix with Tables, Figures, References

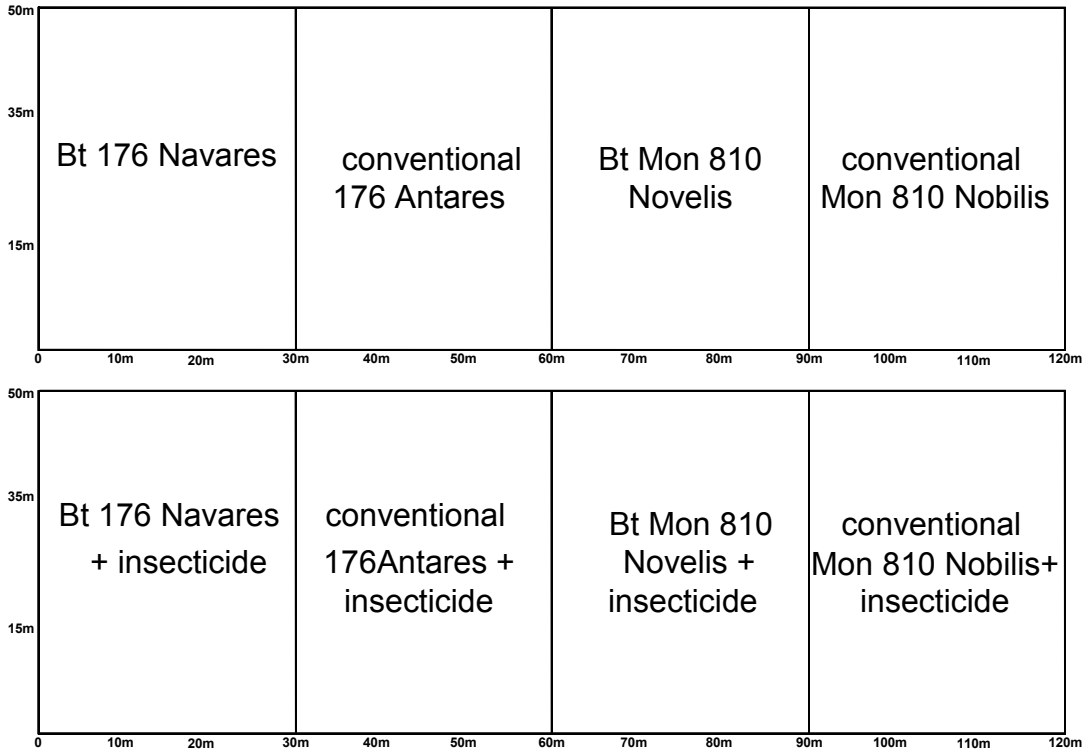

Figure 1: Experimental design

Table 1: *Collembola* sampling dates

	Baumannshof	Grub	Neuhof	Puch	Schwarzenau
Summer 2001	15 Jul 01	11 Jul 01	01 Aug 01	25 Jul 01	07 Aug 01
Fall 2001	18 Sep 01	25 Sep 01	02 Oct 01	10 Oct 01	19 Oct 01
Summer 2002	09 Jul 02	22 Jun 02	23 Jul 02	16 Jul 02	29 Jul 02
Fall 2002	11 Sep 02	04 Sep 02	18 Sep 02	01 Oct 02	----------

Table 2: *Lumbricidae* sampling dates

	Baumannshof	Grub	Neuhof	Puch	Schwarzenau
Earthworm samples	18 Oct 02	16 Oct 02	23 Oct 02	11 Oct 02	15 Nov 01

Table 3: Abundances of *Collembola* families 2001/2002 (totals from 320 100-mL coring tube samples)

Collembola families	Baumannshof	Grub	Neuhof	Puch	Schwarzenau	Total
Isotomidae	2306	3052	1212	2139	839	9548
Onychuridae	4475	790	705	1170	179	7319
Entomobryidae	399	656	147	219	1868	3289
Poduridae	147	381	336	4	1	869
Sminthuridae	139	71	140	185	54	589
Total	7466	4950	2540	3717	2941	21614

Table 4: Abundances of *Collembola* species 2001 (totals from 160 100-mL coring tube samples)

Collembola species	Baumannshof	Grub	Neuhof	Puch	Schwarzenau	Total
Isotoma notabilis	733	467	424	49	531	2204
Isotomurus palustris	0	278	142	1275	1	1696
Isotomina	473	7	3	0	0	483
Folsomides parvulus	0	318	0	7	0	325
Folsomia listeri	152	3	3	16	16	190
Isotoma viridis	22	16	129	0	4	171
Folsomia fimetaria	69	3	8	23	13	116
Folsomia quadrioculata	2	1	32	63	0	98
Folsomia candida	13	76	2	4	0	95
Isotoma olivacea	0	0	0	1	91	92
Isotomiella	44	0	9	1	1	55
Folsomia multiseta	0	0	0	34	0	34
Folsomia spinosa	16	0	0	1	0	17
Isotoma violacea	0	9	7	0	0	16
juvenil	251	180	33	74	22	560
Total	1775	1358	792	1548	679	6152

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Table 5: Abundances of earthworm species 2001 or 2002 (totals from 50 1-m^2 samples)

Earthwormspecies	Baumannshof	Grub	Neuhof	Puch	Schwarzenau	Total
A. caliginosa	8	959	522	987	2	2478
L. terrestris	2	296	232	119	41	690
A. chlorotica	386	0	68	0	124	578
A. rosea	0	164	190	11	0	365
L. rubellus	54	0	0	119	0	173
D. rubida	71	4	0	0	0	75
O. lacteum	0	13	0	9	17	39
A. longa	0	2	0	0	0	2
O. cyaneum	0	0	0	0	1	1
L. castaneus	0	0	0	1	0	1
juvenile	2120	6154	1224	4467	288	14253
Total	2641	7592	2236	5713	473	18655

Table 6: *A posteriori* analysis of variance

Parameter	Year	Site	Bt	Insecticide
Collembola, total individuals	*	*	-	-
Lumbricidae, total individuals	*	0	-	-
Lumbricidae, biomass	*	0	-	-

*) $p < 0.05$

-) $p \geq 0.05$

0) no result

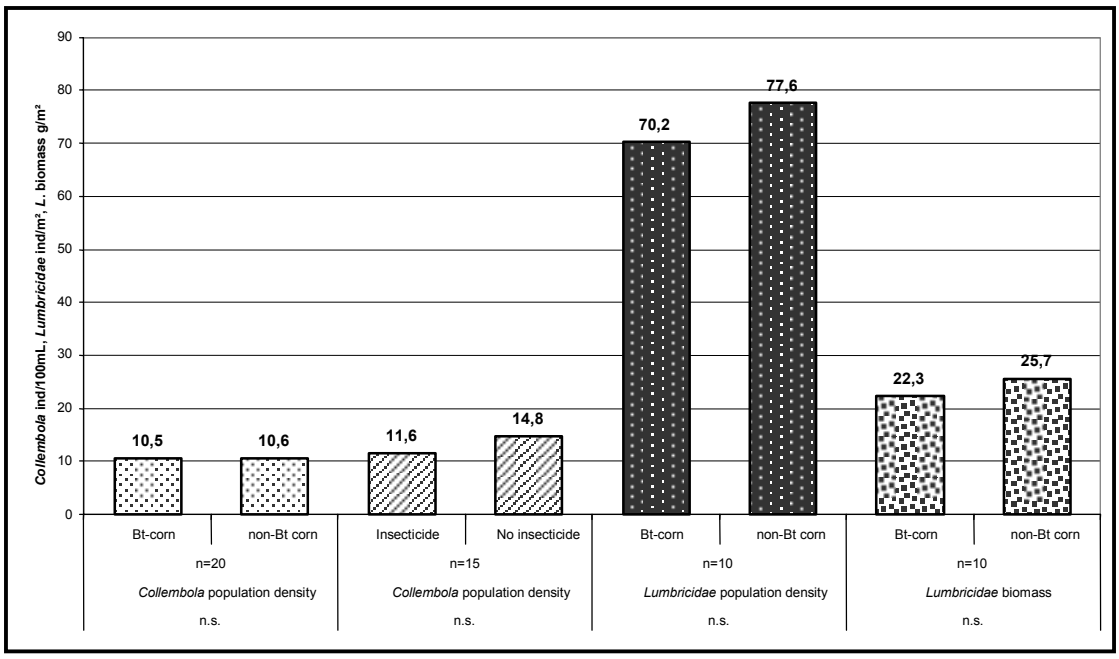

Fig. 2: Average population density and biomass for *Collembola* and *Lumbricidae* on Bt and non-Bt plots as well as on insecticide and no-insecticide plots. No significant differences (Wilcoxon test, p = 0.05)

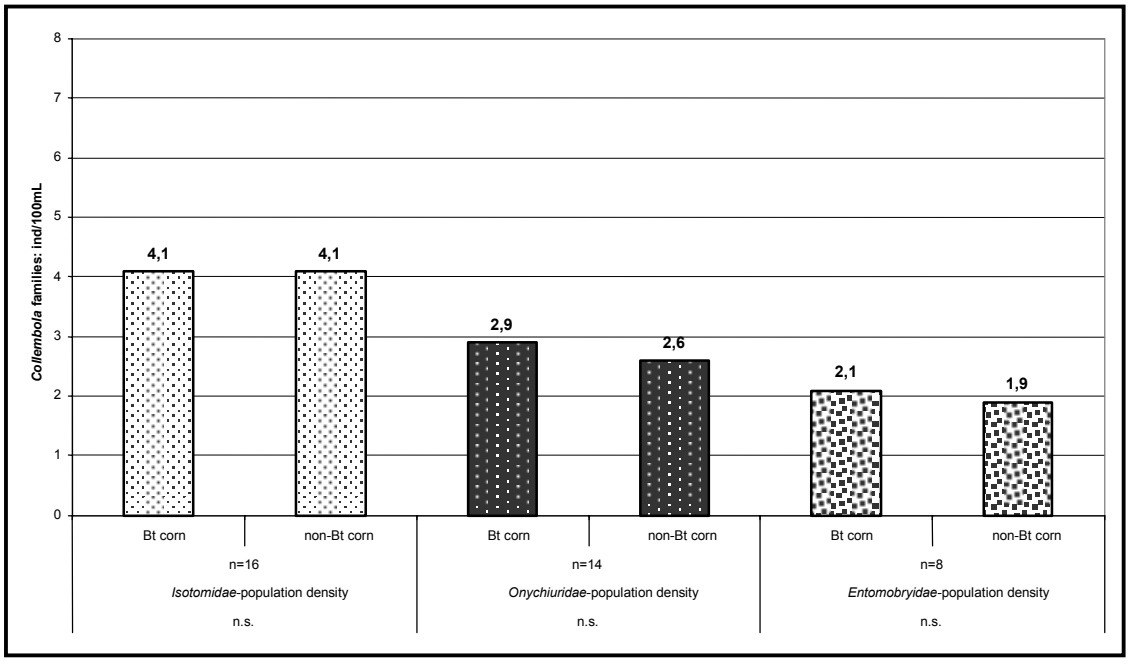

Fig. 3: Average population density and biomass for *Collembola* families Isotomidae, Onychiuridae and Entomobryidae (families with the highest population density) on Bt and non-Bt plots. No significant differences (Wilcoxon test, p = 0.05).

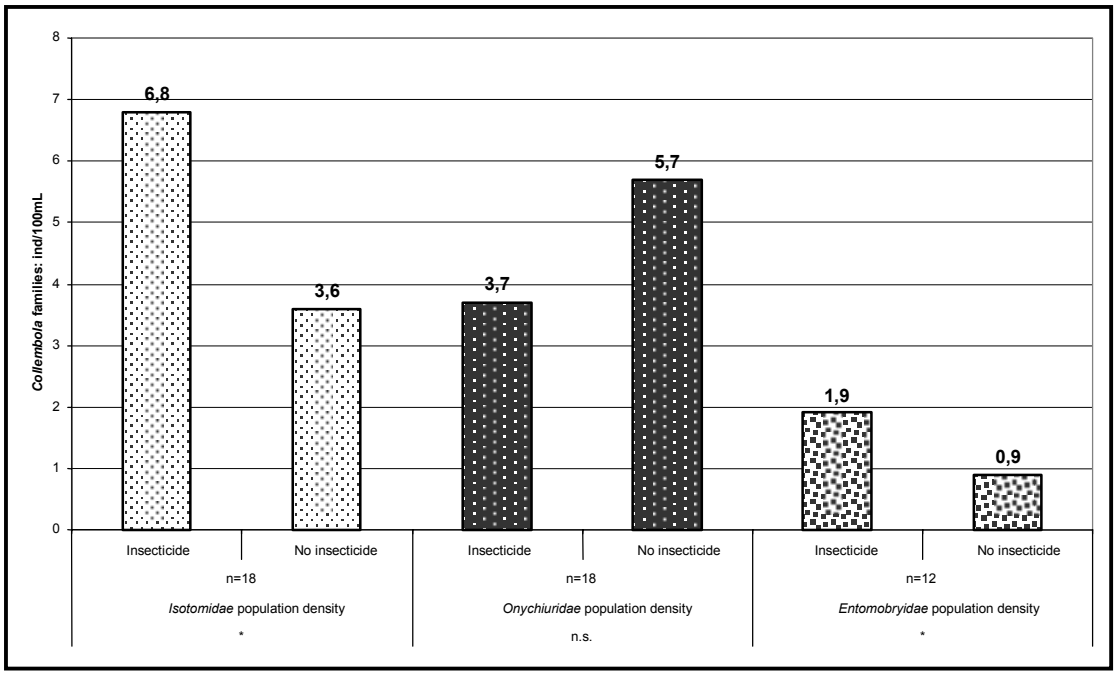

Fig. 4: Average population density for *Collembola* families *Isotomidae*, *Onychiuridae*, and *Entomobryidae* on insecticide and no-insecticide plots. The values for *Isotomidae* and *Entomobryidae* show significant differences; no significant differences for *Onychiuridae* (Wilcoxon test; p = 0.05).

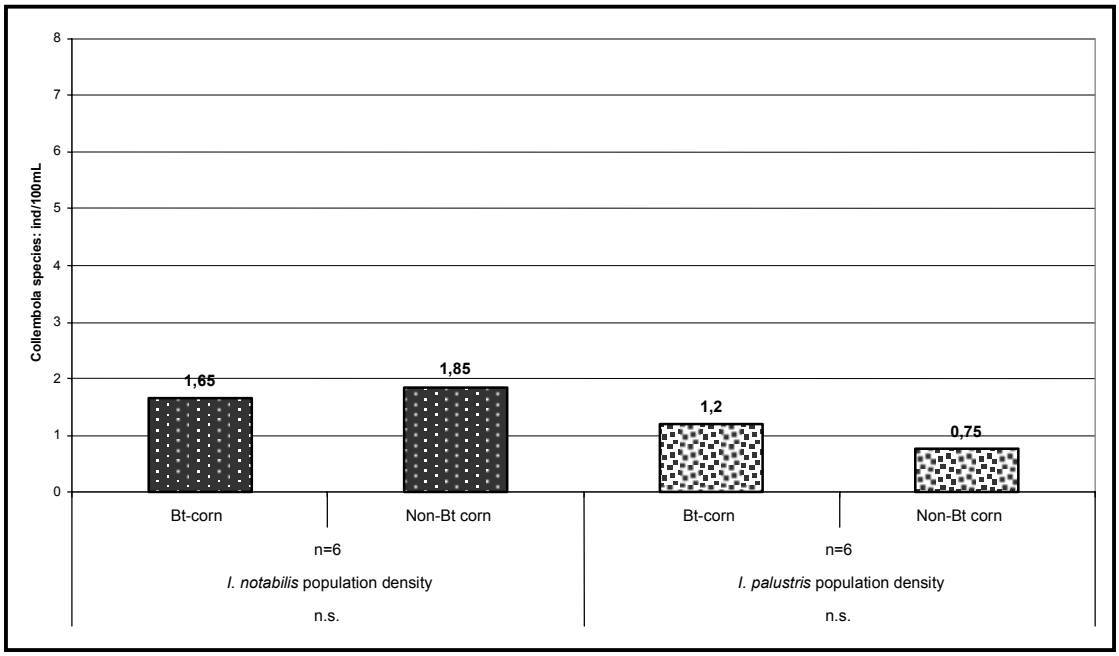

Fig. 5: Average population density for *Collembola* species *I. notabilis* and *I. palustris* (the species with the highest abundances) in Bt corn and non-Bt corn fields. No significant differences (Wilcoxon test, p = 0.05).

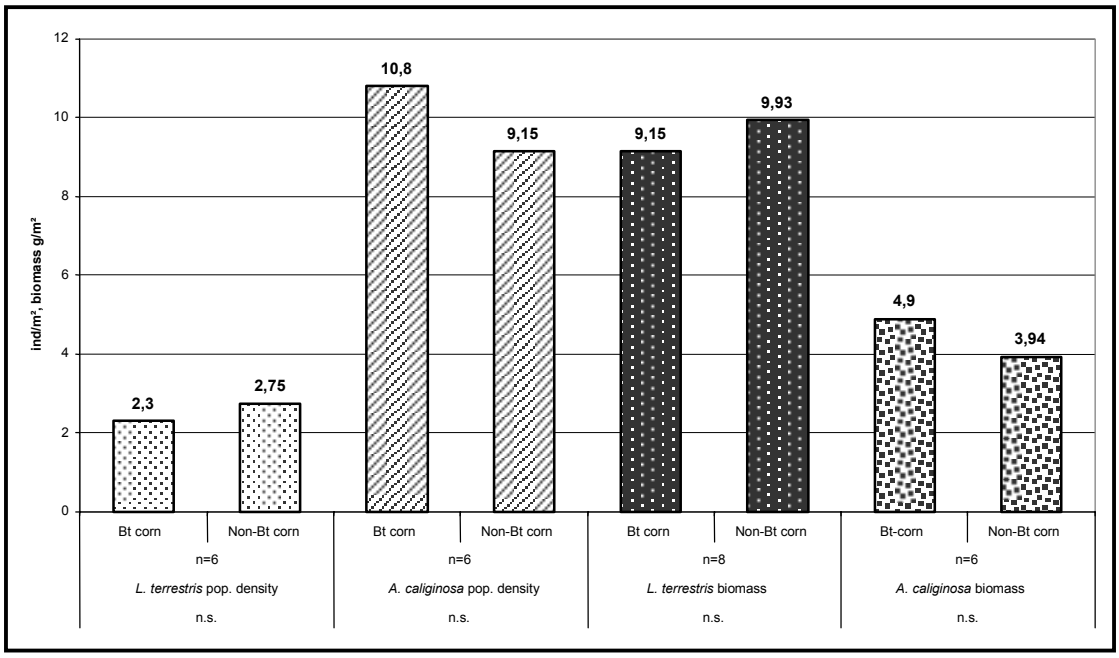

Fig. 6: Average population density and biomass for *Lumbricidae* species *A. caliginosa* and *L. terrestris* in Bt corn and non-Bt corn fields. The abundances and biomasses show no significant differences. (Wilcoxon test; p = 0.05).

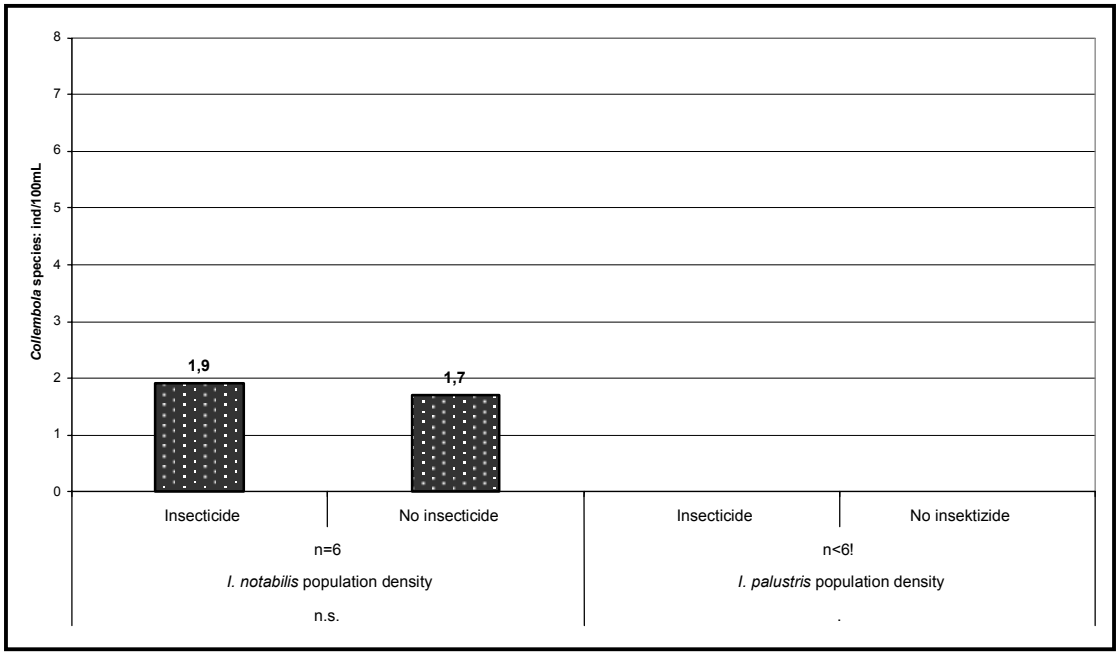

Fig. 7: Average population density for *Collembola* species *I. notabilis* and *I. palustris* on insecticide and no-insecticide plots (for *I. palustris*, there were not enough replicates for an assessment). No significant differences (Wilcoxon test, p = 0.05).

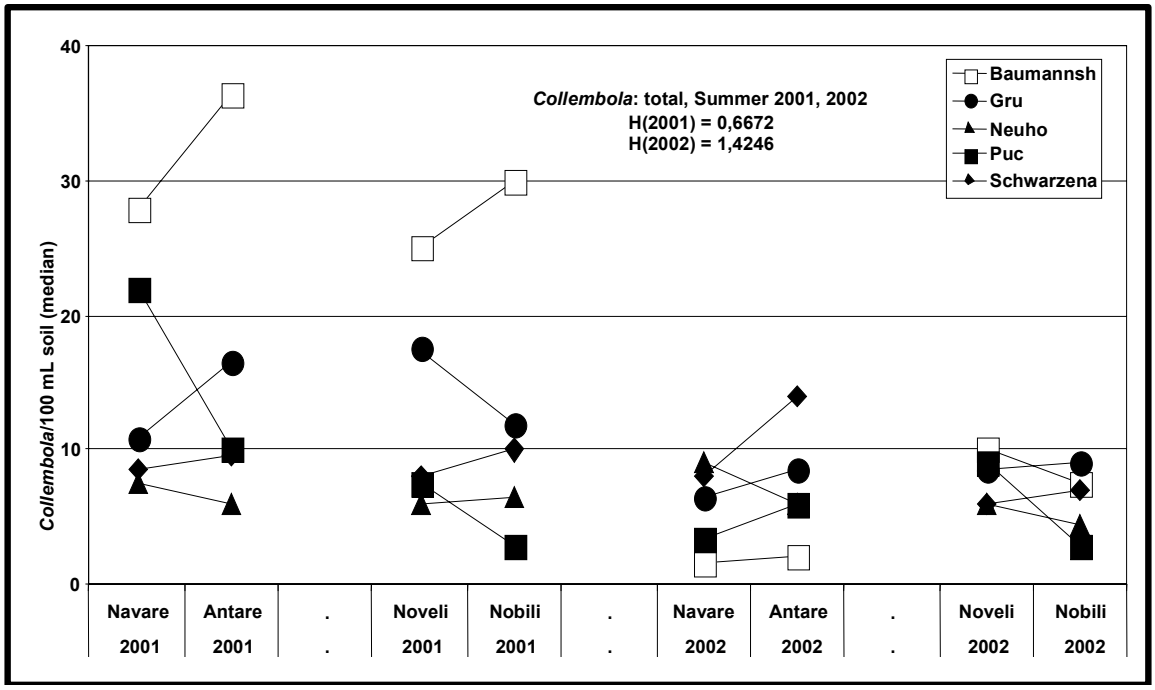

Fig. 8: *Collembola*: overall population density on Bt-Navares, Antares, Bt-Novelis, and Nobilis plots, medians from 20 individual samples, Summer 2001 (left), Summer 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

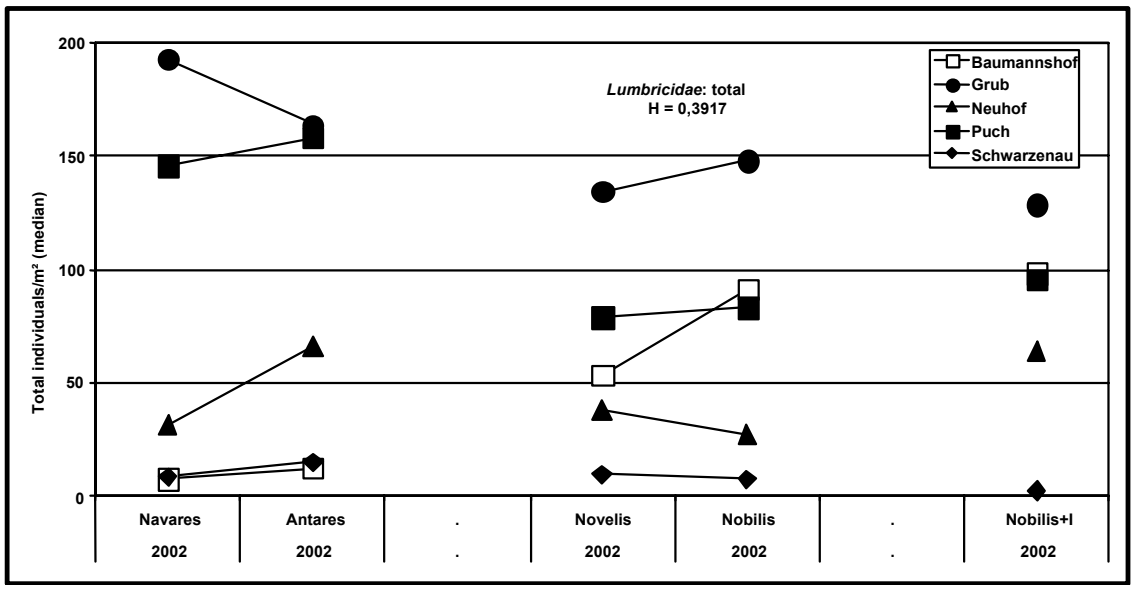

Fig. 9: *Lumbricidae*: overall population density on Bt-Navares, Antares, Bt-Novelis, Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 10 individual samples, Fall 2002.

No significant differences (Kruskal-Wallis test, p = 0.05).

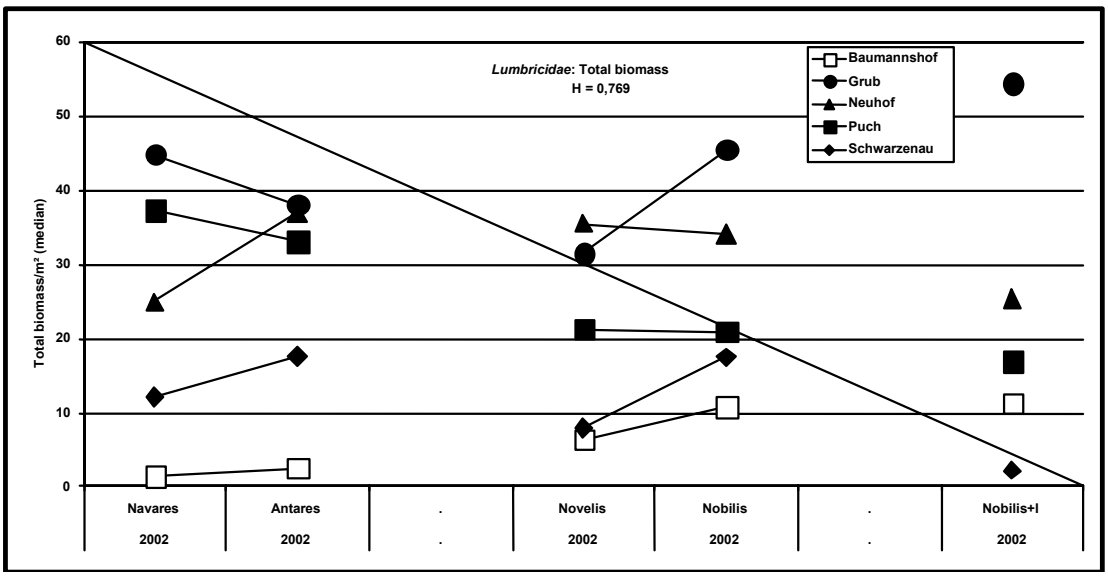

Fig. 10: *Lumbricidae*: Total biomass, on Bt-Navares, Antares, Bt-Novelis, Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 10 individual samples, Fall 2002.

No significant differences (Kruskal-Wallis test, p = 0.05).

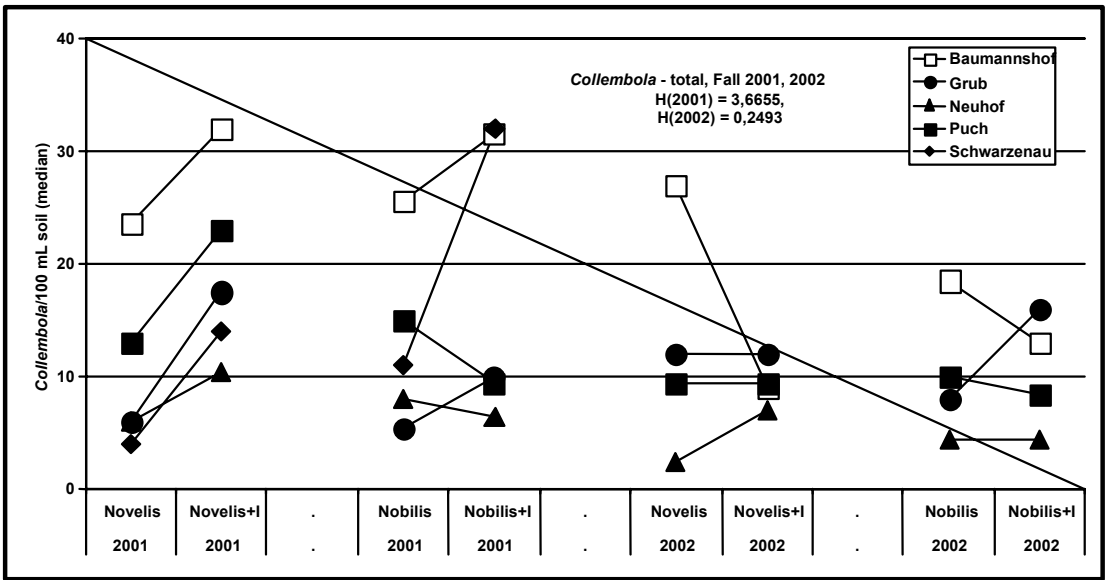

Fig. 11: *Collembola*: total population density on Bt-Novelis, Bt-Novelis with insecticide (Novelis+I), Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 20 individual samples, Fall 2001 (left), Fall 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

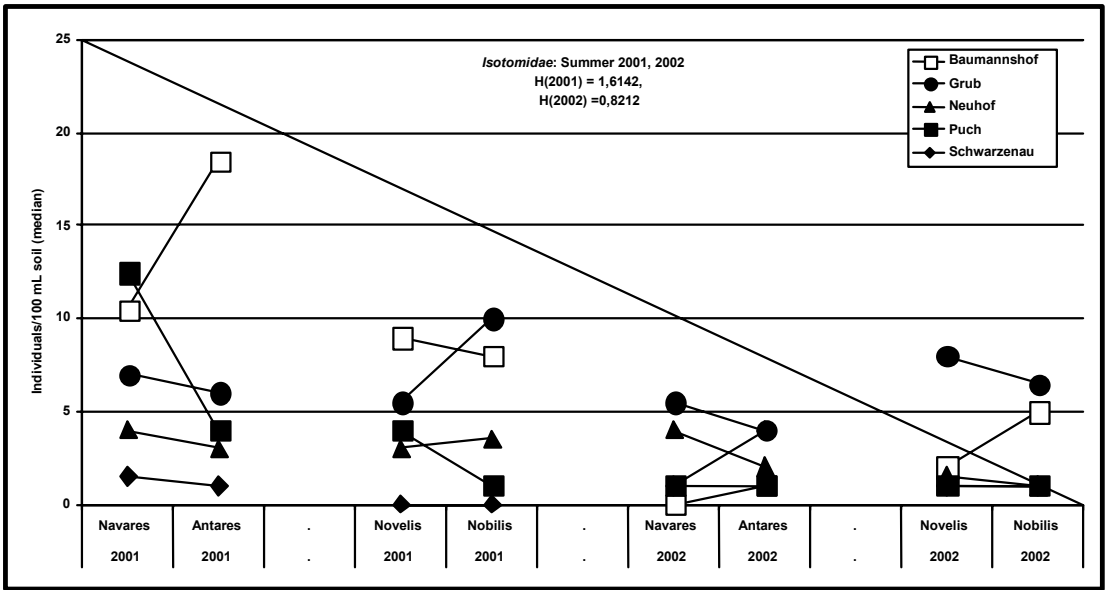

Fig. 12: *Isotomidae*: overall population density on Bt-Navares, Antares, Bt-Novelis, and Nobilis plots, medians from 20 individual samples Summer 2001 (left), Summer 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

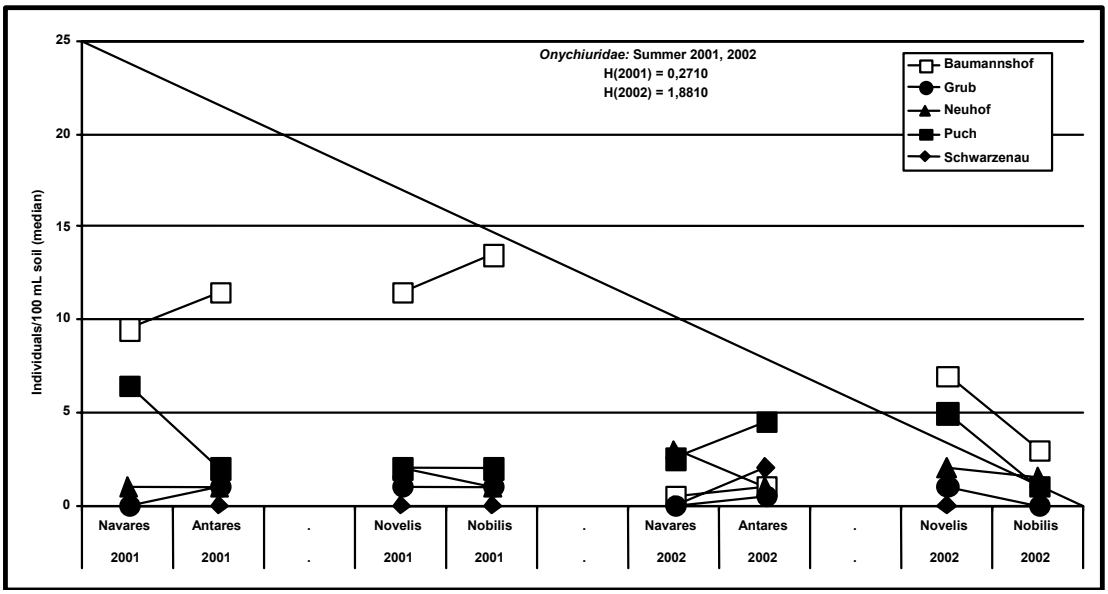

Fig. 13: *Onychiuridae*: population density on Bt-Navares, Antares, Bt-Novelis, and Nobilis plots, medians from 20 individual samples, Summer 2001 (left), Summer 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

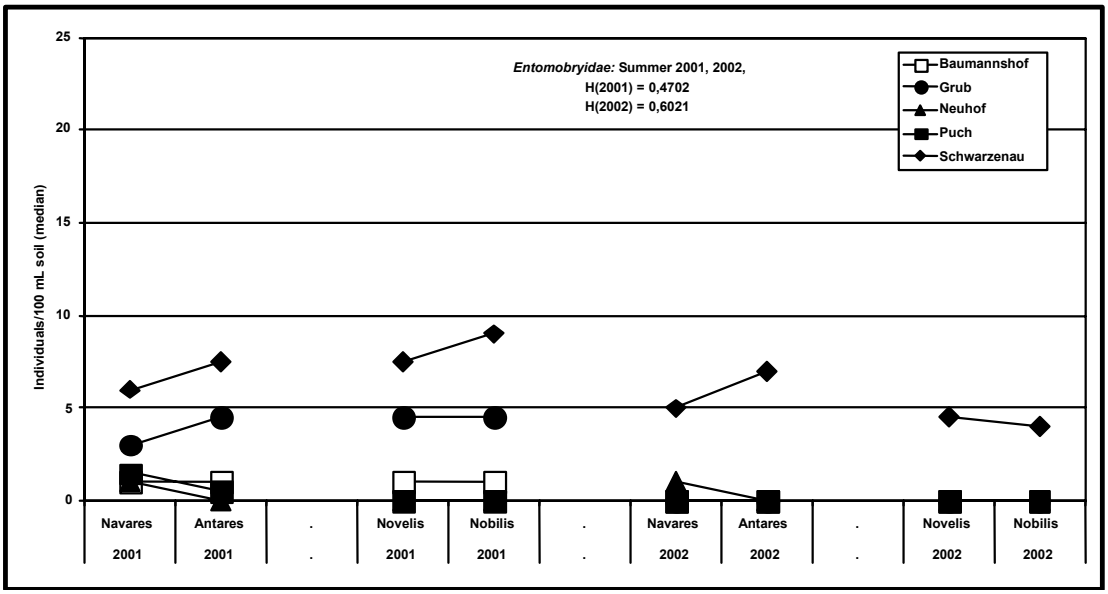

Fig. 14: *Entomobryidae*: population density on Bt-Navares, Antares, Bt-Novelis, and Nobilis plots, medians from 20 individual samples, Summer 2001 (left), Summer 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

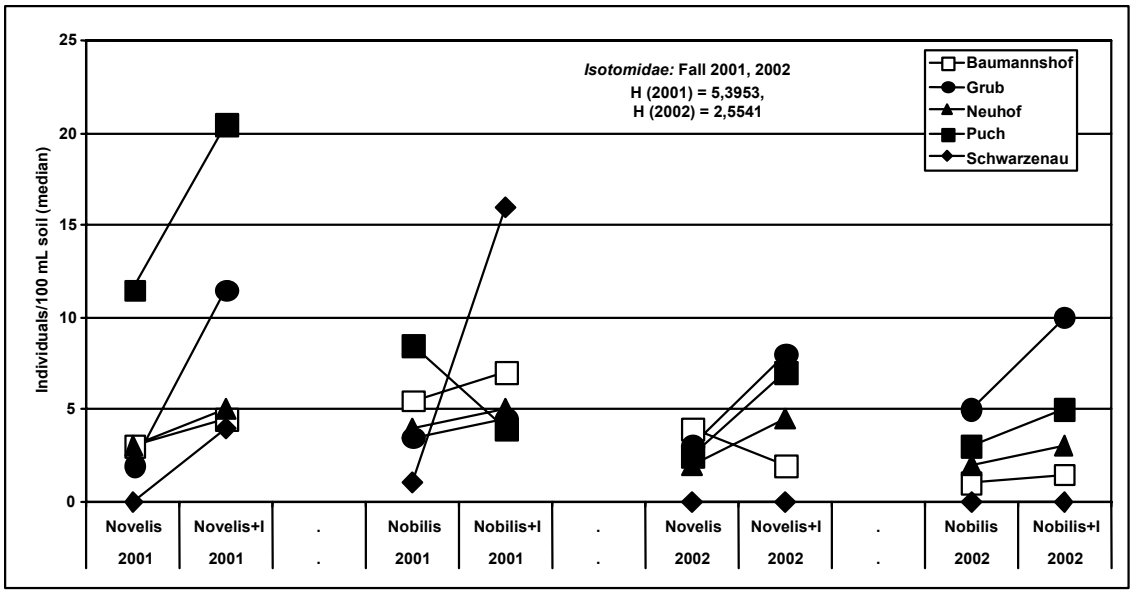

Fig. 15: *Isotomidae*: population density on Bt-Novelis, Bt-Novelis with insecticide (Novelis+I), Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 20 individual samples, Fall 2001 (left), Fall 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

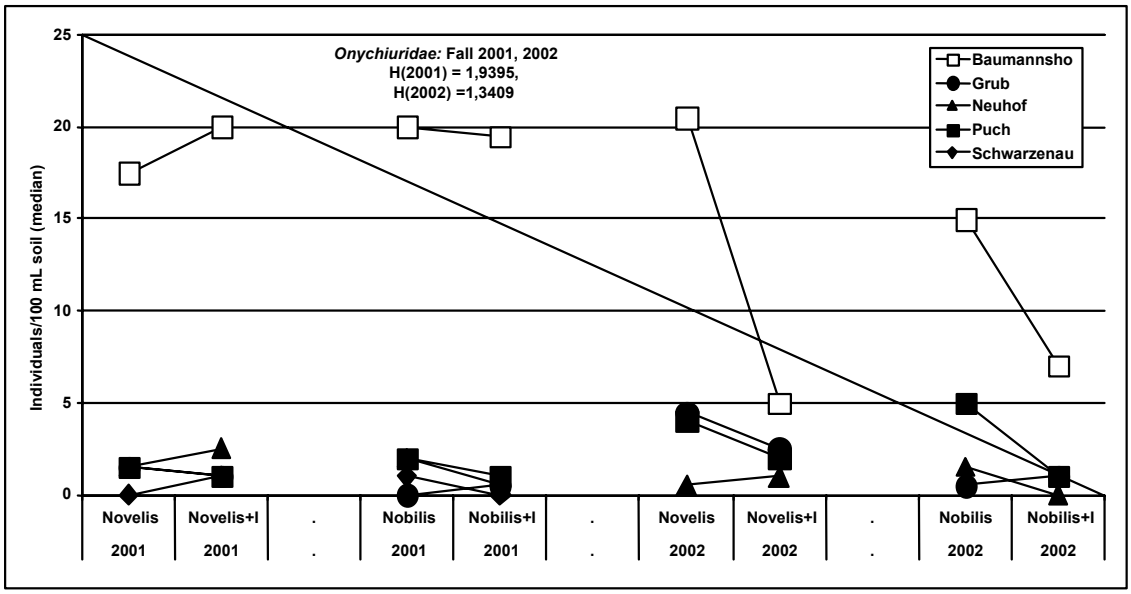

Fig. 16: *Onychiuridae*: population density on Bt-Novelis, Bt-Novelis with insecticide (Novelis+I), Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 20 individual samples, Fall 2001 (left), Fall 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

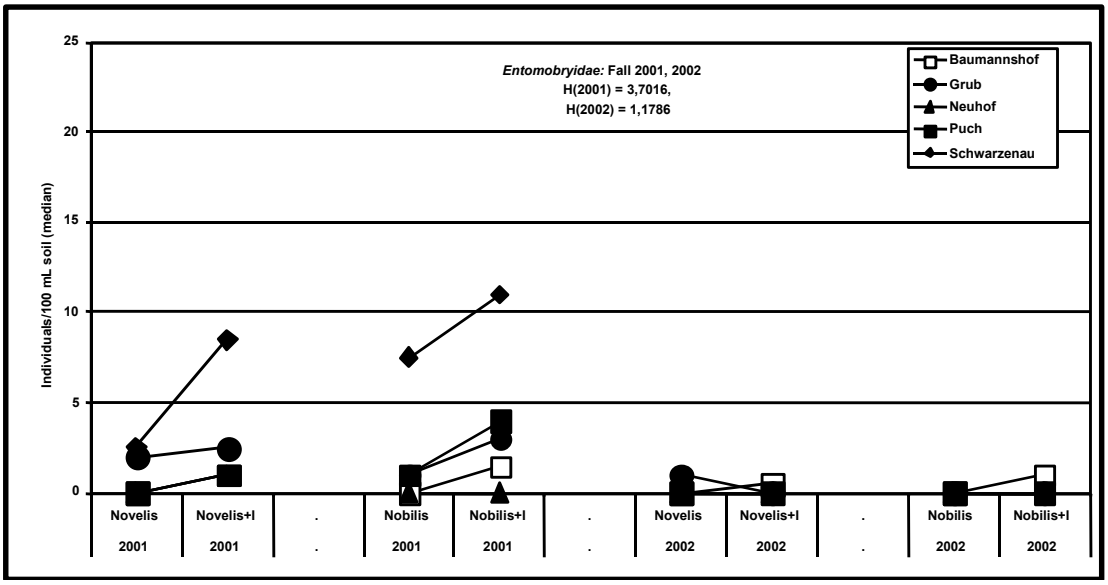

Fig. 17: *Entomobryidae*: population density on Bt-Novelis, Bt-Novelis with insecticide (Novelis+I), Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 20 individual samples, Fall 2001 (left), Fall 2002 (right).

No significant differences (Kruskal-Wallis test, p = 0.05)

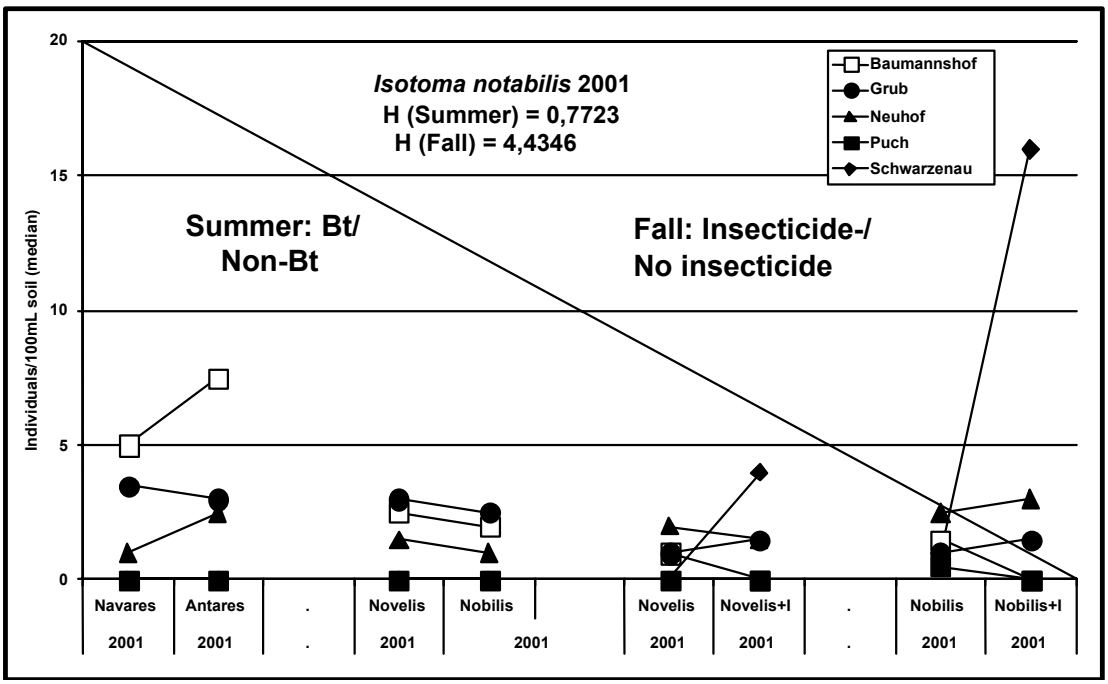

Fig. 18: *Isotoma notabilis*: population density on Bt-Navares, Antares, Bt-Novelis, and Nobilis plots, Summer 2001 (left) and on Bt-Novelis, Bt-Novelis with insecticide (Novelis+I), Nobilis, and Nobilis with insecticide (Nobilis+I) plots, Fall 2001 (right), medians from 20 individual samples.

No significant differences (Kruskal-Wallis test, p = 0.05)

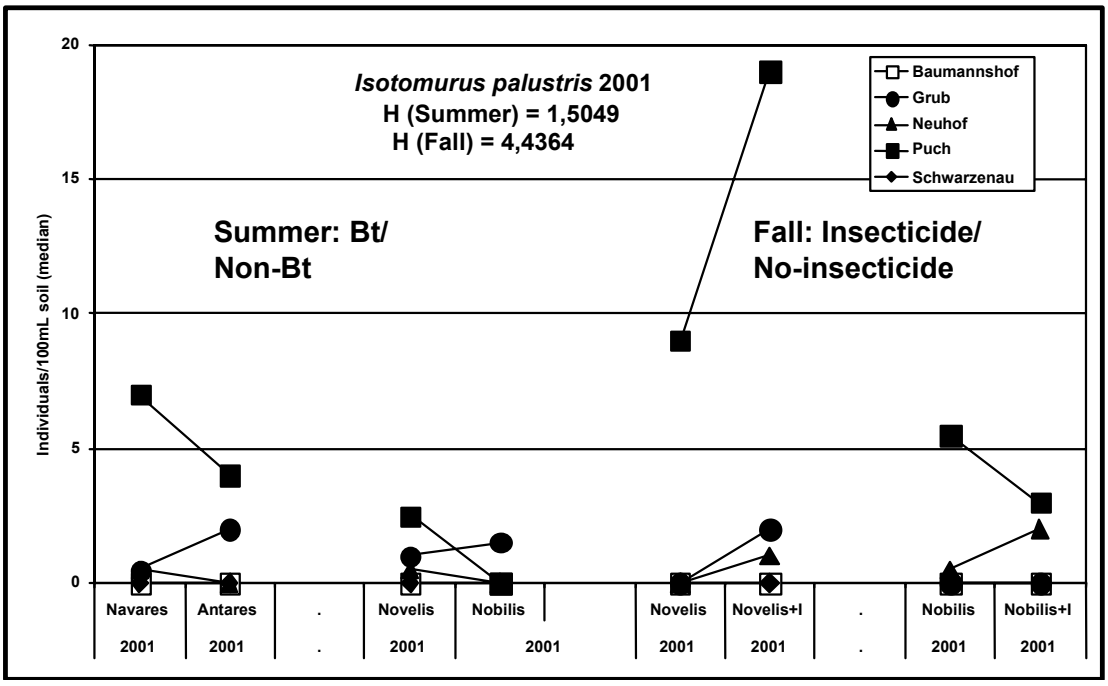

Fig. 19: *Isotomurus palustris*: population density on Bt-Navares, Antares, Bt-Novelis, and Nobilis plots, Summer 2001 (left) and on Bt-Novelis, Bt-Novelis with insecticide (Novelis+I), Nobilis, and Nobilis with insecticide (Nobilis+I) plots, Fall 2001 (right), medians from 20 individual samples.

No significant differences (Kruskal-Wallis test, p = 0.05)

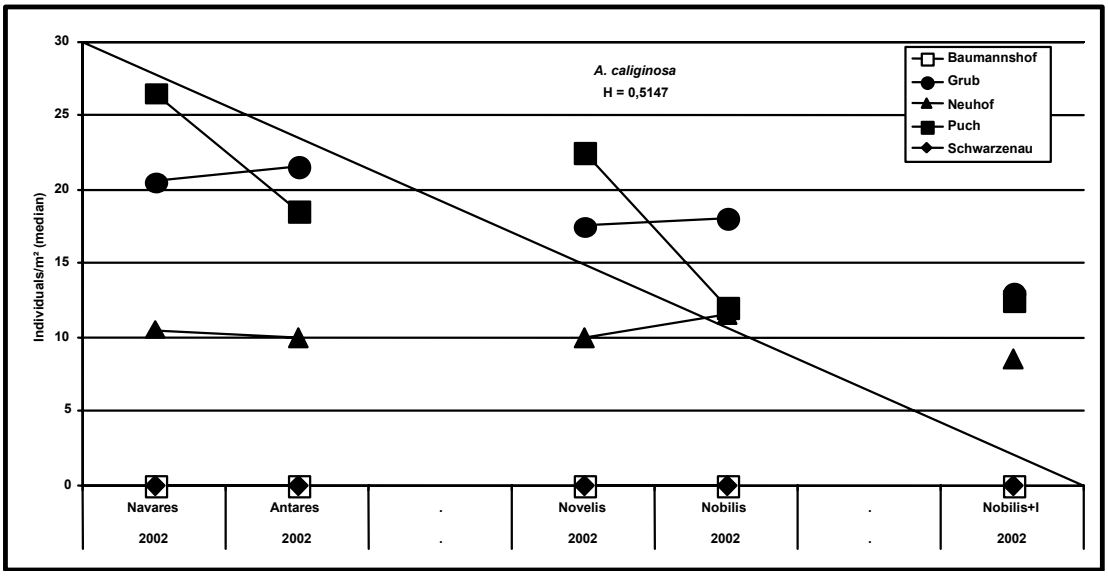

Fig. 20: *Aporrectodea caliginosa*: population density on Bt-Navares, Antares, Bt-Novelis, Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 10 individual samples, Fall 2002.

No significant differences (Kruskal-Wallis test, p = 0.05)

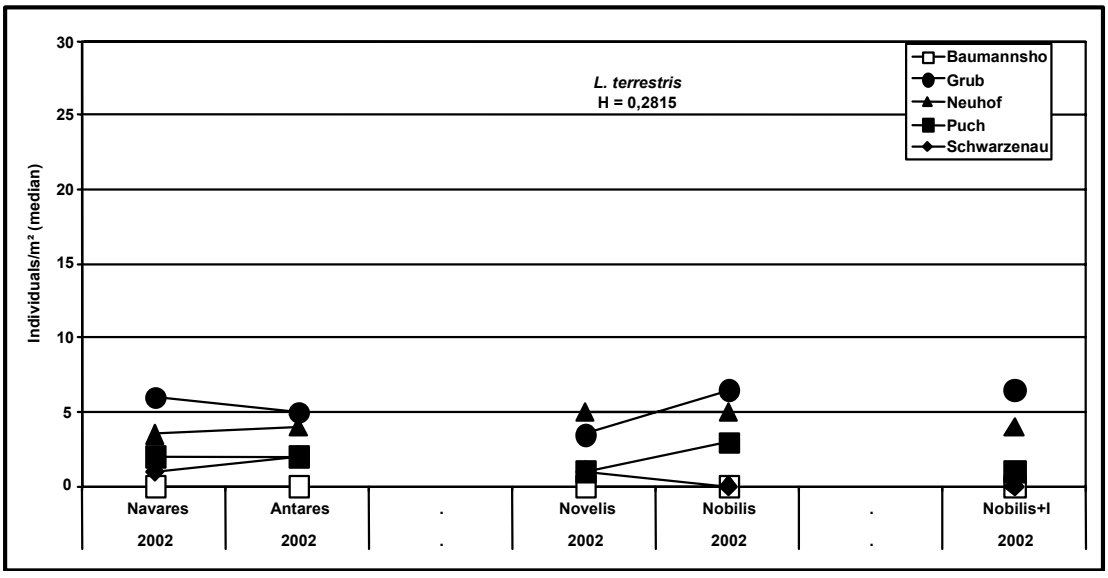

Fig. 21: *Lumbricus terrestris*: population density on Bt-Navares, Antares, Bt-Novelis, Nobilis, and Nobilis with insecticide (Nobilis+I) plots, medians from 10 individual samples, Fall 2002.

No significant differences (Kruskal-Wallis test, p = 0.05)

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