# Toxin in GM maize

## *New research reveals risks of Bt maize grown in Europe*

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## Executive Summary

The overview presented here shows the many ways Bt maize impacts the environment. Even after more then a decade of commercial growing of Bt maize crops, the risk assessment studies are still few and most of them tend to raise more open questions than solving concerns.

### The cycle of the toxin in the environment

Contrary to earlier assumptions Bt maize releases its toxin into the environment in many more ways than just by being taken up by the pest organisms or by animals using the Bt maize as feed after harvest. Bt toxin gets into the plant through living and degrading plant material but is also exudated from the plant roots. Evidence exists that the Bt toxin persists in the soil for as long as into the next growing season, but not sufficient methods exist to determine the actual amount, neither has it been determined until when the degrading Bt toxin has toxic effects on soil organisms.

Once taken up by any organism, the Bt toxin can be passed on to other organisms: either to predators or parasites or it can be released as faeces. In a number of cases the Bt have been found to be passed on in the food network without an apparent effect on the organism of the first level.

### The mode of action of Bt toxins

A new study (Broderick et al. 2006) even shows that the assumed mode of action of the Bt toxin so far has not been understood, and that in fact gut bacteria might be required for the toxic effect. This however means that there is no simple dose-response relationship between toxin and effect.

It is also not clear whether the different versions of the Bt protein in the different Bt maize events have the same effects or whether the differences in size of the protein have different effects.

In general the concentration of the Bt toxin in specific parts of the plant is not determined or monitored.

### Effects on soil organism

Effects on the soil have only been studied since the mid/late 1990s, after several Bt maize varieties were already approved for cultivation. Since then adverse effects could be observed on mycorrhiza, the symbiotic partner of a number of plants; fungus gnats, an important organism in soil ecology; nematodes, earthworms and predatory beetle larvae.

### Effects on bees, butterflies and other organisms

Non-target studies are sketchy: some species get more attention while for other organisms whole groups are left unattended. The reasons why some species are chosen and others not remain often unclear. Nevertheless it becomes more and more evident that adverse effects can be both direct and indirect, and that they can on higher trophic levels in the food network, even if the first organism to take them up might not be affected.

Research on non-target butterflies only started after an accidental discovery that butterfly larvae could take up the anti-Lepidopteran Bt toxin from Bt maize pollen that was blown of the fields. Most studies since then have focused on the Monarch butterfly in the US, even though the butterfly fauna in European agricultural landscapes is very different. The few studies that were conducted about European butterflies revealed a number of potentially affected butterflies, a number of which are already threatened, and they also revealed that some of these are just as susceptible to the Bt toxin as the target pest *Ostrinia nubilalis* (ECB). Among them are for example  Common Swallow tail, Small Tortoiseshelll or Peacock.

Studies with bees are often conducted in unrealistic conditions. A field study in Germany however came to the accidental findings that bee colonizes infected with a parasite were significantly more affected when they were feeding on Bt maize pollen. Bees treated with antibiotics in the next season however showed no effect. Besides the potential effects of Bt toxin on bees, this study also showed the differences between controlled lab studies, where common parasites can be excluded, even if they cannot in commercial growing. With new evidence however, that gut bacteria might be needed in order for the Bt toxin to unfold its toxicity, the lack of adverse effects on bees treated with antibiotics cannot be considered as  a proof that the Bt toxin will not adversely affect bees.

Other affected non-target organisms also include the Green Lacewing or the beneficial insect *Trichogramma* that is used as biological pest control

## Basic technical information is missing

It is astonishing how much basic technical information about Bt GE maize is still missing:

- It is not understood why different plant tissues produce different Bt concentrations and how environmental factors might influence the Bt plants.
- There is not even a standard method available, evaluated by authorities and independent laboratories to determine Bt concentrations in plant material or in the soil.
- Bt toxins differ from the original toxin produced by *Bacillus thuringiensis*, but they also differe from each other in size. It is therefore possible that these different proteins also have different effects.

Other open questions include that neither the number of gene sequences nor the location where the new genes are inserted can be controlled, that the interactions with other genes and the metabolism of the plants cannot be predicted, and that eco-systemic effects are complex and can only be partially assessed scientifically. Also health impacts cannot be ruled out, and pollen migration and contaminants in the harvest contaminate food products and seed.

All in all, the available studies on different Bt maize events (such as MON810, Bt176 or Bt11) show more questions then answers, and they show that the risks of Bt maize for the environment are real.

In this context, the European framework legislation (Directive 2001/18 and Regulation 1829/2003) gives high priority to the precautionary principle. Directive 2001/18, Article 4(1) states:

> "Member States shall, in accordance with the precautionary principle, ensure that all appropriate measures are taken to avoid adverse effects on human health and the environment which might arise from the deliberate release or the placing on the market of GMOs."

In the light of the many uncertainties, the requirements for EU marketing approval are not in met. While the cultivation of these plants and their use for animal feed serves the financial interests of a few companies, the potential long-term effects make such cultivation unacceptable.

# Introduction

The GM maize MON810 that is grown in Europe is a problematic plant.

- Genetic modification causes unplanned and unwanted changes to the genotype and to plant metabolism.
- Pollination and admixture contaminates harvests and food products.
- The maize produces a toxin against insects that normally only exists in bacteria but now is becoming a permanent part of food and feed and can persist in fields.

Bt maize was developed in the United States primarily to control the European corn borer (ECB, *Ostrinia nubilalis*). This moth lays its eggs on maize leaves, damaging the plant. The larvae then tunnel into the leaves and the stalk. Into the autumn they migrate down the stalk and then spend the winter in the lowest part of the stalk or the top part of the roots. The stalks of the infected plants often break off.

The European corn borer was introduced to North America between 1910 and 1920 and then spread rapidly as a pest. In Europe it is found naturally on a number of different plants. Only one of the two corn borer strains in Europe actually attacks maize. This strain is native only to some parts of Europe; for example it is not found in northern Germany or Great Britain. However, the ECB strain that attacks maize is slowly spreading northward and in Germany has been found as far as Brandenburg. In conventional agriculture ECB is usually controlled simply by ploughing the fields.

MON810 was developed using particle bombardment and the actual transgenic DNA sequences that were inserted still remain unclear (Mertens 2006). Two plasmids were used for the particle bombardement in order to insert transgenes for Bt production, herbicide tolerance and antibiotic resistance. It was however stated only parts of one plasmid were inserted so that MON810 apparently only produces a Bt toxin. Tests were used to show indirectly that no other DNA was inserted or expressed, and in 1998 the Scientific Committee on Plants (SCP), who was then responsible for the approval of GM crops in the EU, approved of MON810 even after admitting that data were missing by estimating that the integration of additional fragments was extremely remote (SCP 1998, quoted in Merthens 2006). Since then, independent studies (de Schrijver & Moens 2003, Hernandez et al. 2003) found discrepancies between the submitted data and their analysis of the MON810 DNA. (For further details see Merthens 2006).

In April 2007, the approval to grow the GM maize MON810 in the EU will expire, which means the authorities will need to re-appraise this maize. Therefore Greenpeace has compiled the latest research from Germany and other countries, drawn up a list of open questions and clarified possible risks.

For Germany, the authors mostly used an analysis of research findings from a project of the German Ministry of Education and Research (BMBF 2006), "Safety research and monitoring for Bt maize cultivation 2001-2004", the results of which have only been published in part. The studies investigated MON810 and another type of GM maize (Bt 176) that is no longer being cultivated.

A report published in April 2006 by the European Commission shows that safety problems with GM crops have become more and more obvious over the last years (European Communities 2005). The new data confirm this alarming finding. The wide range of indications that are available now show that the problems with GM maize are even more complex than originally assumed. The risks apply to the smallest soil organisms, to protected species such as butterflies and to beneficial insects such as bees and even extend to health risks for humans and animals.

The latest findings and the list of open questions clearly show that approval for commercial cultivation of the GM maize was granted prematurely and contradicts the precautionary principle that is part of EU legislation. The EU’s approval of the GM maize therefore must be withdrawn.

# 1. The cycle of the toxin in the environment

Normally the *Bacillus thuringiensis* toxin only exists in soil bacteria. This toxin has been used for many years to control agricultural pests. It is considered so harmless that it is even allowed in organic agriculture. But by genetically engineering the toxin into maize plants its characteristics have been changed fundamentally.

1. In nature, the toxin only exists in very low concentrations. If it is sprayed for pest infestations, then it is used selectively and for a very short period of time.
2. The toxin in its natural form only kills certain insects. It comes in a non-active form (crystalline protoxin) and it is not turned into the active form until it is in the insect's gut.
3. The Bt toxin in sprays is degraded within a few days by UV light.

Genetic engineering however changes the characteristics of the toxin:

1. It is produced during the whole vegetation period of the plant and it is released through the roots, parts of the plant and pollen into the environment.
2. The toxin binds to soil particles and can survive in the soil for months. It can be passed on in the food chain and can even be passed through the gut of farm animals.
3. In Bt plants, the toxin is not present in the inactive form, but in a more active version (smaller and not crystalline). This changes the range of possibly sensitive organisms.
4. Although the different toxic proteins are all called Cry1Ab, they are fundamentally different from the natural protein, and they are different from each other (Andow & Hilbeck 2004).

Cultivating Bt maize creates a completely new cycle of distribution and concentration of Bt toxin in the environment and in the food chain. This has been confirmed by the latest research (Zwahlen & andow 2005). Newest research also shows that so far the mode of action of the Bt toxin has not been understood at all (Broderick et al. 2006).

Effects of Bt plants on the soil have only been investigated since the mid/late 1990s, i.e. only after Bt maize had already been cultivated in the USA, and only after Bt176 and MON810 had been approved for cultivation in the EU.

Many of the studies that have been published since the end of the 1990s on the topic of 'Bt crops and soil' reveal unexpected effects, particularly negative environmental effects. These results also show that most areas have not been studied at all – and that nearly everywhere where research is done, indications of negative effects can be found.

## *How does the toxin get into the environment?*

Bt toxins can get into the soil via different routes: as a living plant material (roots, biosicherheit.de 2005b), as dead fine roots and root exudates during the growth period (Saxena et al. 1999, Saxena & Stotzky 2000), pollen (Losey 1999) that is washed into the soil, harvest residues (roots, stalks, leaves) after harvest (Tapp & Stotzky 1998, Stotzky 2000, Zwahlen et al. 2003b, Baumgarte & Tebbe 2005), and in animal excrement (Einspanier et al. 2004).

In recent years a series of studies with varying approaches was conducted to study the persistence of Bt toxins in the soil, but there are only very few studies that investigate the amount and form of Bt toxin during and after the growth period. In 2005 it was still unknown how much toxin is actually exudated by the roots.

> "To our knowledge, it is not known how much Cry1Ab protein is produced in the rhizosphere of Bt maize under agricultural practice and how much of that protein remains in the soil after harvesting" (Baumgarte & Tebbe 2005).

Apparently rather high toxin levels can be found in the soil close to the roots. Some of the toxin is found in the soil even months after the harvest, even though higher levels are found in the remaining plant residues:

> "The amount of Cry1Ab protein in bulk soil of MON810 field plots was always lower than in the rhizosphere, the latter ranging from 0.1 to 10 ng/g soil. Immunoreactive Cry1Ab protein was also detected at 0.21 ng/g bulk soil 7 months after harvesting, i.e. in April of the following year. At this time, however, higher values were found in residues of leaves (21 ng/g) and of roots (183 ng/g), the latter corresponding to 12% of the Cry1Ab protein present in intact roots" (Baumgarte & Tebbe 2005).

Even though it is known that roots contain Bt toxin and can exudate it into the soil, this issue is not considered a factor at all in some risk assessments of Bt maize. For example, in the approval application for Bt maize 1507 that is currently pending at the EU, the Bt concentrations for different parts of the plant are given – but not that of the roots. Nevertheless, the EU authority EFSA gave a positive opinion for the commercial cultivation of this Bt maize.

The path of the toxin through roots, pollen and plant material is not the only path through which Bt toxin is released into the environment. Initial research into the degradation of Bt protein in the gut of cows shows that "remarkable" amounts of Bt toxin are found in the gastrointestinal tract, and that the animals' faeces contains the toxin (Einspanier et al. 2004. Bt toxins as well as fragments of the *cry1Ab* transgene could also be found in Bt maize silage. However, the biological activity of these degraded biochemical forms was not tested, so that they cannot be excluded from further risk assessments (Lutz et al. 2004).

## *How long does the toxin stay in the soil?*

A number of studies are investigating the persistence, activity and degradation of Bt toxin in the soil, but because different issues are being studied (persistence of complete or partly degraded toxins, activity of the target organism etc.) and because of different methods used (lab studies, use of isolated bacterial Cry1Ab, dried and pulverized leaves, plant residues from the field) the results cannot be compared readily.

Field studies and monitoring show in any case that the toxin can still be detected several months after the harvest in plant residues and in soil, and it is active. Earlier studies, which extrapolated the results of unrealistic laboratory studies, are therefore refuted.

Different soil types influence the persistence of Bt toxins in the soil. The toxin can persist in clay soils for a particularly long time.

According to Dolezel et al. (2005), "persistence of Bt toxins released into the soil is a function of

- the amount of the toxin present,
- the rate of consumption and inactivation by insect larvae,
- the rate of degradation by micro-organisms, and
- the rate of abiotic inactivation (Stotzky 2004)."

Saxena et al. (2002a) were able to detect Bt toxin in Bt maize exudates and in degrading Bt maize plant material in the soil after 350 days (the longest period studied). In other studies, isolated Bt toxin could still be found after 234 days (when the study was stopped; Tapp & Stotzky 1995, Palm et al. 1996, Koskella & Stotzky 1997, Tapp & Stotzky 1998). This also refutes earlier studies.

Sims & Holden (1996) had calculated from lab studies that 90% of the toxin would be degraded after 41 days and concluded that the Cry1Ab toxin in Bt maize would be unstable under field conditions and would be degraded fast under growing conditions. Applicants and authorities still frequently refer to this study (Sims & Holden 1996), even though it clearly does not reflect reality. As explained below, this is an example of how unrealistic studies are often used for risk assessments of Bt maize then - but still today.

Sims & Holden assumed for their lab study a constant soil temperature of 24-27ºC. This is completely unrealistic for soil temperatures in European maize cultivation areas. Zwahlen et al. (2003a) recorded in field trials soil temperatures of 8.5ºC. As Zwahlen et al. (2003b) explain, the degradation of Bt toxins depends largely on microbial activities (Palm et al. 1996, Koskella & Stotzky 1997) which are reduced at lower temperatures.

Therefore, it must be expected that the degradation in temperate regions differs substantially from that observed in laboratory trials with high constant temperatures (Zwahlen et al. 2003b). The authors (Zwahlen et al. 2003a) also showed in a comparative study that Bt plant material under (comparable) field and lab conditions is degraded slower in the fields and stays toxic longer.

This trend is also confirmed by the most recent studies on this topic in a project of the German Ministry of Education and Research (BMBF). Baumgarte & Tebbe (2005) observed that surface roots of MON810 maize still contained 12% of the toxin levels of intact roots seven months after the harvest, that is, shortly before the next sowing. This level dropped then in the following two months.

## *Does the toxin accumulate in the soil?*

Bt toxins bind to surface-active soil particles and are therefore protected from biological degradation (Saxena & Stotzky 2001a, Saxena & Stotzky 2000). The complete binding is completed within 30 minutes (Schröder undated)  Once bound, the Bt toxins do not detach easily (Lee et al. 2003).

Saxena & Stotzky (2002) observed that soil with Bt plant material showed different toxicity after 120 to 180 days depending on the composition of soil minerals. Bt toxins bind better to soil particles with higher cation exchange capacity and with a more distinct surface structure.

Lee et al. (2003) confirm this results. They showed that the majority of the Bt toxins (88-98%) bind firmly to clay particles, and that even with great amounts of Cry1Ab protein no saturation effect occurred. They did not observe any structural changes for the bound Bt toxins, but there was persistent toxic activity. After 45 days the toxicity of bound Bt proteins was even higher than of free toxins.

It is therefore clear that Bt toxin accumulates to different degrees in different soil types, depending on climatic conditions, and that it can then can show different levels of activity. However, these effects have t been sufficiently investigated, something that is confirmed by the latest studies  from Germany.

In a three-year study at three sites (Halle and in the Rhineland) all soils had similar mineralogical composition with high clay content. But the bedrock and the climate were different so that according to the scientists "important soil characteristics differed" (biosicherheit.de 2005m) They observed that the mobility of the Bt toxin Cry1Ab at the different locations varied greatly (Schröder undated).

> “The following general statements can be made regarding the chemical parameters of the soil: - The higher the level of organic substances, particularly in the topsoil, the lower the binding of Bt toxin. [..] The greater the surface area of the soil particles, the more Bt toxin is bound to the soil particles.” (biosicherheit.de 2005m). The authors (Schröder undated) conclude  that "this information has to be taken into account when evaluating our results in terms of a monitoring method for the release of genetically modified plants."

In general one has to assume that the toxin concentrates in the soil, and can accumulate for years (Hopkins & Gregorich 2003, Lang & Arnd 2005). There is a great need to conduct research about toxin accumulation in soil: "In the second year of Bt maize cultivation at both locations, the observed Bt levels [in the soil] were clearly higher than those from 2002. The increase in toxin levels on the different locations was five to seven times higher than in the previous year, depending on the location. The toxin could even be detected in soil samples that were taken in April 2003, i.e. before the next sowing" (biosicherheit.de 2005l).

## *Bt toxin in the food chain*

When the Bt toxin is ingested by insects and other animals it has not disappeared from the food chain. In some cases the Bt toxin was found in animals that had fed on the Bt maize plants without being acutely harmed by it.

When these animals were eaten by others then their predators also took up the toxin. In spider mites the Bt toxin became so concentrated that they contained more Bt toxin then the Bt maize plants themselves. Studies of the spider mite *Tetranychus urticae* found the Bt concentrations of the animals ranged from Bt levels similar to those of Bt maize leaves (Dutton et al. 2002) to levels that were three times higher than those of the leaves (Obrist et al. 2005).

Compared with other herbivorous insects (thrips, aphids and grasshoppers) the Bt levels in spider mites were the highest (Dutton et al. 2004b). These levels were up to 33 times higher then those of cicadas that feed on the same parts of the plant (Dolezel et al. 2005).

Therefore, we should not only assume that the toxin is passed on by the (unaffected) spider mites, but also that the Bt toxin concentrates in spider mites, whereby predators are subjected to higher Bt concentrations then those found in the Bt plants themselves.

In other animals, the Bt toxin was found both in the gut and in the faeces (e.g. in earthworms, caterpillars, spiders and woodlice; Saxena & Stotzky 2001a, Harwood et al. 2005, Wandeler et al. 2002). These faeces are part of the soil so that the Bt toxin can be ingested by further organisms.

Bt toxin from maize plants even survives the stomachs of ruminants and is excreted with the faeces (Einspanier et al. 2004, Lutz et al 2005). In this way the Bt toxin can be returned to the fields through animal dung. The Bt toxin found so far could be detected by laboratory methods, and because the protein found is not the partly broken down, it is assumed that there is no (relevant) biological activity. However, this has not been confirmed by bioactivity studies. In fact, there is no reason to believe that a smaller protein should have no toxic effects on non-target organisms, just because it is smaller.

## 2. How does the Bt toxin work?

Broderick et al. (2006) describe the current knowledge about how Bt toxins work. What had been studied so far was how specific enzymes in the insect gut metabolized the protoxin, turning it into a toxin. Then, by a process that remains unclear, the toxin appear to be inserted in the membrane of the gut cells, where they form pores that lead to the cell being disslolved. Two different mechanisms have been discussed about whether the insects then die of starvation or by septicemia when the bacteria enter the blood stream. Although these two models have been cited many times in the literature, according to Broderick et al. (2006), neither is entirely consistent with experimental observations.[1]

Bt crops were then developed without understanding of how the Bt toxin actually works, and in most Bt plants the toxins are shorted so that they do not need to be activated by enzymes in the insect gut anymore, even though this potentially increase the number of susceptible (non-target) organisms. For the risk assessment of Bt crops, a dose-response relationship is generally assumed, meaning that less toxin will lead to lesser effects - even independent of the different Cry1Ab variations used in different Bt crops such as MON810, Bt176 and others. Some of the non-target studies however found greater effects even in Bt events and/or plant tissues with lower Bt contents (see chapter 3 and 4). So far this often called 'inconsistent' findings could not be appropriately assessed.

1 "*Bacillus thuringiensis* is an opportunistic insect pathogen that was discovered almost a century ago. The salient feature of this species is accumulation of crystalline parasporal inclusions during sporulation. These inclusions are composed of one or more protoxins, known as delta-endotoxins, each of which is specific primarily at the level of insect orders, particularly Lepidoptera, Diptera, and Coleoptera. In Lepidoptera, specificity is due in part to the extremely alkaline midgut environment that is required to solubilize the protoxin into the active form. Solubilized protoxins are activated by midgut proteases and bind to receptors on the epithelial surface. Then, by a process that remains unclear, the toxins appear to insert into the membranes of gut cells, where they form pores that lead to cell lysis. It has been proposed that disruption of the midgut epithelium results in a prolonged cessation of feeding and eventual death by starvation. An alternative proposed mechanism of killing is that extensive cell lysis provides spores access to the more favorable environment of the hemocoel, where they germinate and reproduce, leading to septicemia and death. Although these two models have been cited many times in the literature, neither is entirely consistent with experimental observations. " (Broderick et al. 2006)

Broderick et al. (2006) studied the effect of the presence or absences of gut bacteria of gypsy moth larvae by raising larvae either on sterile artificial diets or treating them with antibiotics before feeding them with *Bacillus thuringiensis* bacteria. Without gut bacteria, the Bt protein was not toxic to the lavae, but when the bacterial gut flora was restored by feeding them a *Enterobacter* sp. strain Bt became toxic again. Other gut bacteria did not have this effect. In addition to natural *B. thuringiensis* bacteria. Broderick et al. (2006) also tested GM *E. coli* bacteria, modified to produce Bt toxin. Such bacteria are routinely used in labs to produce Bt toxin for risk assessment instead of using Bt plant material. Broderick et al. (2006) found that these living GM *E. coli* were toxic to gypsy moths without a normal bacterial gut flora.

Broderick et al. (2006) come to the conclusion "that the Bt toxin alone is not sufficient to cause larval mortality, because an enteric bacterium (a member of the Enterobacteriaceae family) such as *E. coli* or *Enterobacter* must also be present to induce septicemic death." Even if this study gives no final explanation of how Bt toxin from bacteria and GM plants deploys its toxic activity in different organisms, it shows the lack of basic knowledge around Bt plants, and the wrong assumptions to which this has lead so far.

Generally a dose-response relationship is assumed for Bt toxin activity, while in fact not ony the presence of specific enzymes but also of specific bacteria groups play an key role. However, the bacterial gut flora of insects and other organisms varies greatly. In addition, non-target studies in which isolated test organisms are raised on artificial diets or studies in which non-target organisms are treated with antibiotics thereby interact and disturb with the way how the Bt toxins functions normally and cannot give appropriate answers in a risk assessment.

Hilbeck & Schmidt (2006) raised concerns that complex interactions are involved in the mode of action of the Bt toxin, and it would be impossible to keep such processes apart experimentally. A study of Bt effects on honey bees (biosicherheit.de 2005i, see below) give a good example how inadvertedly and unknowingly a key factor in the Bt toxin activity might have been removed in the effort to have a standardized study design.

# 3. Effects on soil organisms

In principle, Bt toxins can affect all parts of the soil ecosystem. One has to take into account that Bt toxins get into the soil in different ways (living and dead roots, root exudate, dead stalks and leaves, pollen, faeces and liquid manure) and that it is present at different times in different forms and different concentrations, but so far little data exists.

Effects of Bt plants on non-target organisms in the soil were not studied at all before the end of the 1990s. Apparently there was no clear idea that Bt toxin would be produced in the roots, that there are non-target organisms in the soil of agricultural fields, and that in agricultural practice (in contrast to scientific studies) Bt plant material resides in the soil. The lack of such studies is glaring.

## *Effects on micro-organisms*

The importance of micro-organisms is beyond doubt as they are responsible for 90% of the carbon turnover in the soil. More than 109 micro-organisms live in 1 gram of field soil. For a depth of 10 cm, this amounts to 1017 micro-organisms per hectare. Micro-organisms are also directly associated with specific insect groups. Insects such as the larvae of fungus gnats decompose decaying plant material that has already been partly degraded by micro-organisms.

Several studies describe the effects of Bt maize on soil micro-organisms, which vary depending on soil type. For several years there has been "some indication of anti-bacterial effects of Bt toxins" (Escher et al. 2000, Zalunin et al. 2003; cited in Lang 2005).

In greenhouse experiments, Castaldini et al. (2005) found differences in the bacteria communities of the rhizosphere (root area) of three maize varieties (Bt176, Bt11 and a non-Bt maize). For up to four months, plant residues affected bacterial communities, soil respiration, and mycorrhizal symbiosis (Castaldini et al. 2005).

Baumgarte & Tebbe (2005) also observed that plant age and field heterogeneity had a strong influence on the bacterial communities of MON810 cultivation. In two of the three years they found structural changes in the bacteria communities of the rhizosphere of Bt maize (Dolezel et al. 2005). Baumgarte & Tebbe (2005) concluded that "there is presumably an effect of the presence of the Cry1Ab protein on the structure of the bacterial community but this effect was masked by more selective factors", and they "emphasize the importance of considering post-harvest effects on non target organisms."

## *Effects on mycorrhiza*

Mycorrhiza is a symbiosis between fungi and plants in which the fungus is in contact with the system of fine roots of a plant. The mycorrhiza fungi help the plant to take up nutrients and water more easily from the soil. In addition the symbiosis gives the plant some protection against diseases and enables the plant to grow better during drought. Mycorrhiza of crop plants is an important ecological parameter and should be part of any risk assessment. However, the current approval applications for Bt maize do not take mycorrhiza colonization into account.

Two studies show that the roots of Bt maize plants are less colonized with mycorrhiza. If this happens, the Bt maize not only loses a symbiotic partner and its contribution to plant nutrition, but the plants might even be more susceptible to pest insects because without mycorrhiza colonization maize attracts fewer natural enemies of the pests.

Turrini et al. (2004) were the first to study mycorrhiza colonization of Bt plants. They found that the fungi were not able to develop viable structures on the roots of Bt176.

Castaldini et al. (2005) conducted a second study on this issue and also found a significantly lower mycorrhiza colonization of Bt maize roots. In laboratory experiments a significant decrease of mycorrhiza colonization was found at Bt176 roots, but not for Bt11 maize. A healthy mycorrhiza makes crop plants more attractive to natural enemies of aphids which are a maize pest (Guerrieri et al. 2004 cited in Dolezel et al. 2005). Lower mycorrhiza colonization as described by Turrini et al. (2004) and Castaldini et al. (2005) would make Bt maize more susceptible to pest insects because fewer natural enemies of the pests come to the maize plant (Dolezel et al. 2005 p.37). However, so far no studies have investigated the issue of a higher susceptibility to pest insects.

## *Bt maize harms fungus gnats*

Fungus gnats (Sciaridae) are 2 to 3 mm long gnats that, just like their 1mm long larvae, feed on dead plant material. They live in the upper layers of soils where they hatch in rates up to 6,000 individuals per $m^2$. They play an important role in soil ecology and for soil fertility as decomposers of plant material in the soil.

According to a new study in Germany (biosicherheit.de 2005f)[2], Bt maize MON810 harms fungus gnats. Their mortality rate is higher and their pupation rate is lower. In addition, the Bt toxin can harm the beetles that feed on the larvae (Langenbruch et al. 2006).

Larvae of the fungus gnat *Lycoriella castanescens* feeding on MON810 maize needed significantly longer to pupate than larvae feeding on non-Bt maize (Büchs et al. 2004, biosicherheit.de 2005f). The period until pupation is a very important factor for the larvae because they do not have a hard-shelled chitin cover and have a limited ability to move. The longer it takes till pupation, the higher the chance that the larvae can be attacked by parasites or diseases. Larvae that only fed on MON810 material were eaten

2 The results of the whole project "Sicherheitsforschung und Monitoring zum Anbau von Bt-Mais 2001-2004" (Post-market safety research on transgenic maize with new Bt genes (2001-2004)) are partly available as final reports of the individual project, and partly only as publication on the website www.biosicherheit.de (www.gmo-safety.eu). However, to our knowledge, the texts on the websites have not necessarily been written by the scientists who conducted the studies.

more often by other insects because the larval period lasted longer (Langebruch et al. 2006). Therefore the time until pupation is a key parameter for assessing negative effects for these insects that are particularly important for degradation and soil fertility. Particularly if Bt maize is grown over years on the same field, the decomposers' community can be altered, affecting the formation of compost and soil development (Langebruch et al. 2006).

The effects on the population of fungus gnat larvae only become visible after some time. Fungus gnat larvae feed by degrading plant residues in the soil. Therefore negative effects in the field are not necessarily obvious in the first year of Bt maize cultivation, and may only appear when the Bt plant material has actually been incorporated into the soil. This was observed in a three-year study. During the first year on MON810 fields, there were even increased number of species, hatching and decomposition activity, but this changed in the second year. In the third year the degradation rate on MON810 fields was significantly lower than on the control field. This decrease coincided with an increase in the Bt toxin levels in the plant material by a factor of more than 2.5 (Langenbruch et al. 2006).

How difficult it is to study effects of the Bt maize on organisms such as fungus gnat larvae is shown by the finding that the amount of Bt toxin alone apparently is not the only contributing factor. The Bt toxin Cry1Ab in the Bt maize variants Bt176 and MON810 is claimed to be the same, but the toxins might have different biological effects: When fungus gnat larvae *Lycoriella castanescens* were fed maize pollen, a slower development was observed with MON810 but not with Bt176 even though the Bt176 variety Valmont used in this study contains 2962 ng/g Pollen – 30 times more then the Bt toxin content of the MON810 variety Novelis (97 ng/g; biosicherheit.de 2005f). On the one hand the different Bt toxin used in different Bt crops might be all be called 'Cry1Ab' but the proteins have different sizes and therefore also might differ in their effect. In addition, newer research (Broderick et al. 2006) shows that the mode of action of Bt toxin is not simply a dose-response-relationship, but mediated by gut bacteria as well.

The negative effects of Bt maize on fungus gnat larvae can influence the next levels of the food web in two ways. Firstly, the changes in the life span of the larvae also has an effect on the predators that feed on these larvae. Initially the predators can find more prey when the larvae need longer till pupation. Over the long term however, this effect could reverse, so that soil fertility could be fundamentally negatively affected (Langenbruch et al. 2006). However, there are no studies on such long-term effects on the food web in the soil of repeated Bt maize cultivation.

Secondly, the negative effect is reproduced directly in the food chain. When the fungus gnat larvae raised on MON810 plant material were given as prey to the larvae of two beetle varieties (Atheta coriaria and Poecilius cupreus), which naturally feed on these larvae, the beetle larvae also experienced developmental delays (biosicherheit.de 2005f).

## *Nematodes neglected*

Nematodes (roundworms) are the most numerous organism group in the soil besides bacteria and fungi. They only have limited mobility, are relatively susceptible to stress and include groups with very diverse feeding types. They can be affected directly and indirectly by Bt toxins, because there are herbivorous and decomposing nematodes, and also parasites and nematodes that feed on insects (Manachini et al. 2004).

Several studies have described the adverse effects of the Bt toxin on nematodes (references in Land & Arndt 2005 p. 62). Nevertheless there is little interest in the interaction of Bt plants and nematodes.

In the early 1990s, different studies showed that the toxins of several *Bacillus thuringiensis* strains negatively affect the eggs and larvae of nematodes (Meadows et al. 1990, Bottjer et al. 1985; cited in Manachini et al. 2004). When Bt toxins persists in the soil for longer or when Bt toxins from harvest residues or root exudates accumulate (Tapp & Stotzky 1998, Saxena et al. 1999), a risk for the nematode fauna cannot be excluded (Lang & Arndt 2005).

Negative effects on nematode under field conditions are difficult to study because, due to the high diversity of feeding forms, little can be concluded from the total number of nematodes. The numbers of nematodes of the different feeding types need to be measured.

Only a few studies investigated individual nematode species, but these lab studies showed negative effects. Soil from the rhizosphere of MON810 and Bt176 negatively affected the growth and reproduction rate of the nematode *Caenorhabditis elegans* (Lang & Arndt 2005). *C. elegans* also showed a possible sensitivity to the Bt toxin in the field, especially in the soil from the rhizosphere of the MON810 variety Novelis (Mananchini & Lozzia 2003).

In the field, differences in the composition of nematode populations were observable. In a field with Bt176 maize plants - while there was no difference in the abundance of the major nematode genera - some nematode species that feed on bacteria were not found, but some nematode species that feed on fungi were found here and not in the control field (Manachini & Lozzia 2002). The scientists concluded that “the decreased number of bacteria-eating nematodes in the Bt maize field could have been caused by a direct effect of the Bt toxin on the nematodes or by an indirect effect on another level of the food web (bacteria, fungi, predator).”

## *Earthworms not factored in*

Earthworms are important and beneficial organisms in agricultural fields, which makes it all the more astonishing that they get almost no attention in the risk assessment of Bt maize. Earthworms degrade plant material, their tunnels contribute greatly to soil movement, the walls of their tunnel form niches that are rich in oxygen in otherwise oxygen-poor soils, and their faeces add to soil fertility.

Nevertheless there are only a few studies (Ahl Goy et al. 1995, Saxena & Stotzky 2001a, Zwahlen et al. 2003a, Lang & Arndt 2005, Vercesi et al. 2006) that also only focus on three different species of earthworms (*Eisenia fetida*, *Lumbricus terrestris*, *Aporrectodea caliginosa*). Only one of these species – the one studied in the most recent study from June 2006 (Vercesi et al. 2006) – is of relevance for agricultural land. In three of the studies (Ahl Goy et al. 1995, Saxena & Stotzky 2001a, Lang & Arndt 2005) the main focus was on the absolute mortality rate or the abundance of the animals.

Even in studies (Ahl Goy et al. 1995) that could not detect an acute effect of Bt maize, the Cry1Ab toxin could be detected in the gut and faeces of the earthworms. Nevertheless, there are no further studies about the question of how the Bt toxin is spread via the faeces of earthworms and through the soil movement that they cause.

Zwahlen et al. (2003a) report that the mortality rate and the growth of juvenile and adult *L. terrestris* was largely unaffected over 160 days of feeding on Bt maize. However, for the final measurement after 200 days, the adult earthworms that were fed Bt maize weighed significantly less. This study gives an important indication of what the long-term or chronic effects of Bt maize on earthworms might be, even though *L. terrestris* is an earthworm species that is not very common in agricultural soils (Vercesi et al. 2006). It shows that long-term studies are absolutely necessary.

Vercesi et al. (2006) studied for the first time different life history parameters (such as survival rate, hatching rate, reproduction, growth). They studied a species (*A. caliginosa*) that is probably the most abundant species in agricultural soil in the temperate climate zone, and the MON810 variety Monumental (Vercesi et al. 2006).

The majority of the parameters studied were not negatively affected by the Bt maize, but there was a significant decrease in the number of earthworms hatching from their cocoons. This is a negative effect that could greatly reduce the population numbers of this earthworms in a Bt maize field and this could also influence other soil organisms that depend on the manifold interactions of earthworms.

### *Predators in the soil: Ground beetles*

At least three newer studies also show that ground beetle take up Bt toxin and that it has adverse effects on them. In laboratory studies the predatory larvae of the beelte *Poecilus cupreus* had a significantly higher mortality when they fed on *Spodoptera littoralis* from Bt maize plants (Meissle et al. 2005). In Germany, Büchs et al. (biosicherheit.de 2005f) found that predatory beetle larvae *Poecilus cupreus* and *Atheta coriaria* showed development delays when feeding on fungus gnat larvae raised on MON810 litter. Zwahlen & Andow (2005) found studied seven species of ground beetles in fields with Bt maize residues. They found Bt toxin in all of them. This shows that not only soil organisms feeding on plant residues are permantely subjected to Bt toxin but also predatory soil organisms such as beetle and their larvae. (Mertens 2006)

## 4. Effects on bees, butterflies and other organisms

Not enough lab, field and monitoring studies have been done to rule out effects on non-target organisms. Especially indirect and long-term effects have only been studied rarely. Nevertheless a majority of these studies show individual negative effects, that in general have not been investigated in follow-up studies, let alone refuted. This points to a "remarkable number of cases" (Lövei & Arpaia 2005) with negative effects.

Hilbeck & Schmidt (2006) come to similar conclusions. In their detailed review they compared the reports that document adverse effects in non-target organisms. They conclude that the evidence for adverse effects in non-target organisms is compelling enough to merit more research. They also found that the key experiments to explain the mode of action of the Bt toxin in most affected non-target organisms are still missing. Since then Broderick et al. (2006) showed that not even for the target organisms the toxic effect of the Bt toxin has been properly understood, and that in fact gut bacteria seem to play an important role. For this background the concerns raised by Lövei & Arpaia (2005) and Hilbeck & Schmidt (2006) in their reviews are even more important.

Only in the rarest cases did studies bother to first assess which species are present in the (European) agricultural landscape and then chose their test organisms from them. The few studies that did such a survey show how necessary this is: A survey of the butterfly fauna around maize fields, for example, produced a list of 79 species with diverse abundance and different potential for endangerment (Lang 2005). A survey of bees and wasps on a MON810 field listed 200 species of which 39 species are on the German Red List of endangered species (Gathmann 2005).

Of the numerous groups of non-target organisms none or only few species have been studied, and most of these are species from North America. Only in the last years – years after MON810 and Bt176 where approved for cultivation in the EU – were studies conducted for the first time on European species; a lot of these in Germany.

The US agriculture and agricultural landscape are fundamentally different from those in Europe – but nevertheless the ecological conditions in the US are used as basis for the risk assessment of GM crops in Europe.

Lang (2005) gives a good overview of the published peer-reviewed scientific field studies. In the course of 2005, this situation basically did not change even though a few more studies have been published, most of which show negative effects.

> "These studies are rather diverse with regard to the animal groups studied, the research period, the maize varieties, the field sizes, the sample size, the detection method used, the geographic location, and more, which makes a direct comparison of these studies difficult (Orr & Landis 1997, Pilcher et al. 1997a, Lozzia 1999, Manachini 2000, Wold et al. 2001, Bourguet et al. 2002, Hassel & Shepard 2002, Jansinski & Eisley 2003, Kiss et al. 2003, Musser & Shelton 2003, Dively & Rose 2003, Mayne et al. 1997, Rathinasabapathi 2000, Volkmar & Freier 2003). The majority of the studies are from the USA (47%), 13% (i.e. 2 publications each) from France, Italy and Spain, and one each from Hungary and Germany (Volkmar & Freier 2003). Six studies

> (40%) were done over a period of only one year, eight studies over two years (53%) and only one study from Spain was done over a period of three years. Mainly predator arthropods that feed on aphids, such as lady bugs, lace wings, parasitic wasps and predatory bugs were studied." (Lang 2005)

However, it is now known that the phloem that aphids eat does not contain Bt toxin so no direct effect on aphids and those animals that feed on aphids can be expected. The whole design of these studies appears questionable, in addition to the fact that so few studies are undertaken in Europe or with European species, and that no real long-term studies were undertaken.

A newest study by Marvier et al. (2007) undertook a meta-analysis of three different types of Bt-crops and comes to similar conclusions about the quality of the available studies: "in the case of GM crops, scientific analyses have been [...] deficient. In particular, many experiments used to test the environmental safety of GM crops were poorly replicated, were of short duration , and/or assessed only a few of the possible response variables." While assembling a database of non-target field studies they "found that numerous studies that die not report measures of variance to accompany treatment means [...], did not clearly present the sample size [...], or improperly uses subsamples to calculate measures of variance [...]. By corresponding with the authors, [Marvier et al.] were often able to resolve these issues." They further conclude that "if regulatory agencies were to require researchers to enter details regarding their study methods and results into a similar database, it would be easy to spot omitted information and postpone approval of pesticidal crops until complete records were submitted." (Marvier et al. 2007).

To be able to compare the available peer-reviewed studies, Marvier et al. (2007) focussed only on the abundance of non-target organisms. All other factors that for example could information on sub-lethal or chronic adverse effects had to be left out. (What is also left out are of course all the groups of non-target organisms that are not studied in the first place or where no peer-reviewed articles are published.)

Nevertheless, they come to the conclusion that non-target organisms in Bt fields, including Bt-maize against ECB, are less abundant then in fields without pesticide use. Pesticide use however had more adverse effects on non-target organism then Bt-crops. They relativate this further by pointing out that in most maize production pesticide usage is already restricted (Marvier et al. 2007). On the other hand, the cultivation of a Bt-crop does not automatically exclude the application of pesticides against other pest.

## *Butterflies*

Butterflies (Lepidoptera) are an important non-target group to study because the pests in questions (such as ECB) are Lepidoptera themselves, and the Bt toxin Cry1Ab works against Lepidoptera in general. Nevertheless, Marvier et al. (2007) come to the conclusion that there is "insufficient data" to test whether the abundance of non-target butterflies is reduced in Bt maize as it is in other Bt crops.

The majority of studies on the effects of the Bt toxin on butterflies and butterfly larvae are studies with the Monarch in the United States. First studies in Europe of European butterflies have identified butterflies that are present in or around maize fields, among them a number of endangered species. These studies show that species such as Peacock and Swallowtail can be damaged lethally or sublethally by even small amounts of Bt toxin (Felke & Langenbruch 2003, 2004, 2005).

Important longitudinal studies such as the monitoring of butterfly larvae in and around Bt maize fields have so far not (or not sufficiently) been conducted – due to a lack of money for such studies.

> "Based on what we know now, it is impossible to predict whether specific butterfly species could be endangered on the species level by the cultivation of transgenic Bt maize. At least on the population level negative effects cannot be ruled out. Indigenous butterflies are endangered by a number of anthropogenic influences. The biggest danger stems from loss of habitat. The cultivation of Bt maize is an additional threat whose impacts on numerous species has not been determined. [...] A negative effect of pollen of Bt maize on butterfly larvae should [...] be expected especially where a bigger maize field borders on a much smaller butterfly habitat such

> as a hedge or the edge of a field. [...] Particularly populations of those species whose larval habitats are mainly grassland or other areas in the agricultural landscape and that are regarded as regionally endangered have to be considered as potentially endangered. Especially for species that are distributed in patches, damaging a single population can impact the total population of a specific region." (Felke & Langenbruch 2005)

Studies in the United States have primarily been of the Monarch butterfly (*Danaus plexippus*). At the end of the 1990s it was accidentally observed that the Monarch could be affected by Bt maize (Losey 1999). Since then, it has been shown repeatedly that Monarch caterpillars can be affected by cultivation of Bt maize and its pollen. "Interestingly the effect was found using the Bt11 maize variety N4640. Bt11 is known to have less Bt toxin then the pollen of Bt176." (Felke & Langebruch 2005).

In the meantime, studies have shown that Bt pollen is not necessarily always acutely toxic for monarch butterflies. However, longitudinal studies have shown clear negative effects on monarch caterpillars (Dively et al. 2004). In this study, too, MON810 and Bt11, whose pollen contains much less Bt toxin than Bt176, were used. Before Dively et al. published their study it was assumed that MON810 would have next to no effects on butterfly larvae.

## *Studies with European butterflies*

When German scientists identified butterflies in the immediate surroundings of a Bt maize field, they found 26 day- and 53 night-active butterfly species (Felke & Langenbruch 2005). According to their data, the risk to 33 of the listed species cannot currently be assessed because it is unknown how sensitive they are to the Cry1Ab toxin. All of these species are owlets, belonging to the moth family.

For 16 species a minimal risk is assumed because they are common, widespread species. Twenty-three species are classified as slightly threatened, because they are not present all over the country; their population densities are lower then those of the 16 species mentioned earlier. Five butterfly species are only present sporadically in a lot of areas and are therefore considered to be highly threatened. These species already have a decline in population or are already considered endangered species – at least in some parts of Germany (Felke & Langenbruch 2005).

Hilbeck & Meier (2006) showed how it is possible to identify Lepidoptera that could be affected by Bt crop cultivation and used as indicator. One of species identified was the 'Queen of Spain' (*Issoria lathonia*) that was also identified by Lang (2005).

A lab study with seven butterfly species native to Germany showed that the caterpillars of six species were sensitive to the Bt toxin in Bt176 maize. When this pollen was on their feeding plants, the caterpillars fed less and their weight increased more slowly; there also was a higher mortality rate (Felke & Langenbruch 2005).

| **Species** | | **$LD_{50}$ [number of pollen grains]** |
|---|---|---|
| Diamondback moth | *Plutella xylostella* | 8 |
| Common swallowtail | *Papilio machaon* | 14 |
| European corn borer | *Ostrinia nubilalis* | 32 |
| Small tortoiseshell | *Aglais urticae* | 32 |
| Peacock | *Inachis io* | 37 |
| Small white | *Pieris rapae* | 39 |

**Table 1: $LD_{50}$ value of Bt176 pollen for butterflies native to Germany.** The $LD_{50}$ value describes the amount that if consumed once causes the death of half of the test animals (source: Felke & Langenbruch 2005; Lang & Vojtech 2006)

In a second part of the study the scientists determined more exactly how sensitively the different butterfly species reacted and they found there were major differences. They determined the so-called LD50 values, i.e. the amount of Bt toxin that will kill half of the caterpillars when they consume it once. Three of the species (Peacock, Small tortoiseshell and Small white) were as sensitive as the European corn borer that is supposed to be killed by the Bt maize. The Diamondback moths reacted even more sensitively (Felke & Langenbruch 2005). Another study showed a similarly high sensitivity for the Common swallowtail (Lang & Voijtech 2006)

Even below the $LD_{50}$ threshold, delays in development have been measured for the Peacock and the Diamondback moth among others problems (Felke et al. 2002). Feeding on Bt pollen made caterpillars lethargic so they stayed on the top of the leaf instead of feeding on it from below, which made it harder to hide from predators (Felke et al. 2002). Even small, non-lethal effects of the Bt pollen can cause the butterfly pupae or hatched butterflies to weigh less, so they lay fewer eggs and die earlier (Dolezel et al. 2005 p.16).

One has to take into account that butterfly larvae in the lab are usually kept under optimal conditions. Here, the caterpillars are not subjected to any other stress factor likely to be encountered in the wild (agrochemicals, parasites, weather conditions, suboptimal feeding due to a lack of specific feeding plants, etc). In the field, Bt maize is an added stress factor for butterfly species that are already endangered.

Apparently some of the findings in the lab are valid in the field as well: "Besides their lab experiments, M. Felke and G. A. Langenbruch also conducted field experiments with Peacock larvae and Bt176 maize. This unpublished results show that under field conditions the flight of Bt176 pollen also has negative effects on Peacock larvae." (Lang & Arndt 2005).

Based on their $LD_{50}$ values, Felke & Langenbruch (2005) estimated the distance from the edge of the field at which the butterfly larvae could be damaged: "If one counts in a safety margin of the factor 100, then the Diamondback moth (*Plutella xylostella*) should only be exposed to 0.08 pollen grains. At a distance of 32 metres from the edge of a flowering maize field an average of 3 to 5 pollen were counted per square centimetre. The maximum number at that distance was 34. This means that negative effects on species that react as sensitively as the Diamondback can not be ruled out at a distance of 32 metres from a Bt176 maize field. This is also true for the neonate larvae of Peacock and Small tortoiseshell butterflies." (Felke & Langenbruch 2005).

There is no EU regulation that requires a safety buffer between Bt maize and the habitat of butterflies or other protected animal species.

## *Aphids*

The effect of Bt maize on aphids has been studied several times. No special effects could be observed (Manachini et al. 1999, Vidal undated, biosicherheit.de 2005d). The lack of such effects is often used as a proof that in general there are no negative effects on non-target organisms. A subsequent study (Raps et al. 2001), however, showed that the phloem, on which aphids feed, does not contain any Bt toxin.

Aphids were also used to study effects on predators of maize pests such as *Chrysoperla carnae* (lacewing; Manachini et al. 1999). Neither negative effects on the development, nor a higher mortality of *C. carnae* could be observed (Vidal undated, biosicherheit.de 2005d). This is not surprising since there is no Bt toxin in the phloem of maize plants, the aphids’ food source. (However in other Bt plants with different promotoer, for example in Bt cotton, Bt toxin can also be found in the phloem; Bernal et al. 2001).

## *Bees illustrate research problems*

In bee larvae feeding studies for approval applications, unrealistic conditions are often used that fail to meet scientific criteria. For the approval application for 1507 maize, for example, bee larvae were fed with Bt maize pollen only once before their acute mortality was measured. Most of the few scientific studies that were conducted with bees and Bt maize did not show negative effects. This is explained in large part by the way that bees or bee larvae were feed.

In a field study Kaatz et al. (biosicherheit.de 2005i) show a more differentiated result. He and his colleagues could not show a chronic toxic effect for Bt maize (Bt176 and MON810) on honey bee colonies treated with antibiotics, but they found a significant negative effect on bees that were weakened by other factors.

> "The first year the bee colonies happened to be infested with parasites (microsporida). This infection led to a decrease in the number of bees and to reduced breeding activity in the hives that were fed with Bt pollen, as well as in those fed with non-Bt pollen. As a result, the experiments were stopped prematurely. The effect was much stronger on the bee colonies fed with Bt maize pollen. (The significant differences indicate an interaction of toxin and pathogen on the epithelial cells in the gut of the honey bee. The underlying mechanism of action is unknown.)" (biosicherheit.de 2005i)

In the second year of the study, the bee hives were preventively treated with antibiotics to avoid parasite infections. In this year no effect could be observed: "Overall it was not possible to prove the existence of any chronic toxic effects of [...] Mon810 Bt maize varieties on healthy honeybee colonies." (biosicherheit.de 2005i). Unfortunately there is still no scientific article published on this study – only the summary by biosicherheit.de. On that website, the overall project leader further simplifies the outcome of the study by stating that "no effect could be found for bees." - such a simplified statement however cannot be drawn out of the results of this study (Lorch 2007).

Newer scientific research (Broderick et al. 2006) now shows that the activity of gut bacteria are required for the toxic effect of Bt toxin. The attempt to eradicate parasites by using antibiotics therefore is likely to also have eradicated the normally occurring bacteria in the gut. In this case the bees weren't "healthy bees" with a preventive medication, but bees deprived of their gut flora and deprived of a normally occurring required factor for the mode of action of the Bt toxin.

This observation reveals three important problems for research: On the one hand, studies, especially lab studies, are conducted with healthy test organisms isolated from further external influences. However, if the mode of action of a toxin is unknown, the artificial conditions used to set up standardised studies might inadvertently change other important factor – such as in this case, possibly eradicating a then unknown factor in the mode of action of the toxin. Furthermore, while standardized conditions may be a valid scientific approach to gain comparable data, but it disregards the fact the once a GM crop is cultivated the non-target organisms are subject to these, possibly cumulative factors.

But even if additional factors were to be studied, this could not happen unless the organisms and their pathogens could be bred and kept in the lab. For example, in the bee study described (biosicherheit.de 2005i), it was not possible to repeat and study the parasite infection under controlled conditions because the parasites could not be bred in the lab.

The results of Kaatz et al. (biosicherheit.de 2005i) that untreated bees under stress conditions are adversely affected by Bt toxins, show the need for further risk assessment studies with bees, especially in conditions where they might not only be subject to one Bt maize field with a short pollination period, but also to scenarios, where bees are located in agricultural landscapes with more and different Bt crops.

With the results of Broederick et al. (2006) the reassuring statement, that bees without parasites would not be affected by Bt toxin (biosicherheit.de 2005i), does not hold anymore, because it appears to be based on a wrong assumption about the mode of action of the toxin which no appears to be mediated by naturally occurring gut bacteria.

## *Spiders: Neglected persistently*

Only a few studies have investigated the possible effects of Bt maize on spiders. (Details for these studies can be found in Lang 2005). Bt maize appears to threaten orb-weaving spiders as a result of several different factors. They ingest the Bt toxin either directly as pollen (e.g. through the recycling of their web) or indirectly through prey (Lang 2005). A longitudinal study shows negative effects of Bt pollen on orb-weaving spiders (Lang 2005, Ludy & Lang 2006). According to the scientists these could be indirect effects caused by a reduced number of prey or by a lower feed quality of the prey. Similar indirect effects have already been claimed for lacewings (Hilbeck et al. 1998, Dutton et al. 2003a).

Spiders are more abundant in maize fields then expected. At the outset of a field study, Lang (2005) counted 50 species in the field and on the field margins, two of which were endangered species.

According to Ludy & Lang (2006) the exposure of orb-weaving spiders to maize pollen can be very high, but also very variable. In the maize field the exposure, with up to 6900 pollen in a web, is much higher then on the field margins.

In a lab study, 65% of the orb-weaving spiders ingested pollen from the web (Volkmar & Freier 2003), even if only in low amounts. A monitoring study (Lang 2005) showed that more than 7% of all spiders collected in the Bt maize field had Cry1Ab in their systems, which indicates long-term exposure. Ludy & Lang (2006) concluded that in their experiments the Bt toxin did not seem to have any relevant negative effects, but they point out "that the sample size sometimes was rather low and therefore possible effects of the Bt toxin could not be statistically validated" (Ludy & Lang 2006).

Under certain conditions the Bt maize even had a positive effect on the spiders. In 2003, there was a greater abundance of spiders in the Bt176 maize field (Lang 2005). The reason for this seems to lie in unplanned change to metabolism of the GM maize (Saxena & Stotzky 2001b, Magg et al. 2001, Hassel & Shepard 2002). For unknown reasons the Bt maize stays green longer during drought. This effect raises questions about whether the metabolism of GM plants is altered to a much greater extent than intended. Other indicators of this phenomenon are higher concentrations of lignin (see below).

## *Green Lacewings: Research results depend on the questions*

A first study in 1998 (Hilbeck et al. 1998a,b) showed that a predatory larvae (Green Lacewing, *Chrysoperla carnea*) was adversely affected when they they were fed *Spodoptera littoralis* larvae, one of the target pests of Bt maize. Since then Green Lacewing has become the most intensively investigated non-Lepidopteran, non-target organism to date with six studies published. These studies are often described as contradictory, but a detailed review by Hilbeck & Schmidt (2006) showed that these differences can be explained through differences in the methodologies used and the underlying research questions. Nevertheless a review of the data of these six studies confirmed complex interactions are involved in the adverse effects:

> "These could involve other modes of action of the Bt toxins and its metabolites, and altered chemistry of Bt toxins when, firstly, expressed in a plant and, secondly, passing through the gut of a herbivore prey organism, including possibly one or all of the following: a) altered nutritional prey quality, b) toxicity of the Bt toxin or its metabolites, c) toxicity of natural plant secondary metabolites interacting with the Bt toxin/metabolites." (Hilbeck & Schmidt 2006).

## *Beneficial insects: Victim of Bt maize*

The Ichneumon wasp *Trichogramma brassicae* is a natural enemy of the European corn borer (ECB). It lays its eggs in the ECB's larvae and is therefore used in organic farming for targeted pest control.

Naturally occurring Ichneumon wasps are endangered by Bt maize cultivation. If they lay their eggs in ECB larvae, substantially fewer wasps hatch than in non-Bt fields (Manachini & Lozzia 2004b). In addition, *Trichogramma* finds less prey in the Bt fields.

In regions with intensive Bt maize cultivation, the ECB’s natural enemies are at risk of becoming (locally) extinct. Specialized natural enemies can even be more threatened than the pest itself because they can only reproduce in those fields where the pest is present. The regional loss of specialized natural enemies could lead to increased pest infestation in other maize fields (Sisterson & Tabashnik 2005).

*Trichogramma* is doubly threatened because they also feed on maize pollen. The Bt toxin could be especially dangerous for them because of their small body size. This has not been proven, though. In lab studies no negative effects on the life expectancy of female wasps or on the total number of eggs laid could be determined (Langenbruch et al. 2006).

Another natural enemy of the ECB is the lacewing (*Chrysoperla carnae*). Their development is substantially delayed if the larvae feed on other larvae raised on Bt maize (Hilbeck et al. 1998a,b). This is another example of how the Bt maize toxin can affect several levels of the food web.

# 5. Basic technical information missing

The Bt concentration can be different in different parts of the plant, in one part at different moments during the growing season, and in different varieties with the same genetic modification. Even different parts of one leaf can contain different amounts of Bt toxin. There are no comprehensive studies on this issue.

Quite often Bt plant material is used in experiments without a determination of how much Bt it contains, which makes it impossible to compare these studies and it remains to be seen whether or not they describe real conditions.

The Bt concentrations of different plant parts and at different times were (and are) often not listed in sufficient detail in EU approval applications, even though it is known that the Bt content in Bt maize varies a lot (Felke & Langenbruch 2005).

Data on Bt exudates from roots or the Bt content in the soil in the rhizosphere are not required for approval applications, even though these data are important for estimating the risk to soil organisms.

Possible differences between different varieties into which the Bt gene is being incorporated are not being studied either, even though concentrations of the toxin can fluctuate depending on the variety.

## *Toxin levels depend on the variety, growth, environment and climate*

The production of Bt varies by season and by plant parts and can be influenced by environmental factors. These variations in different parts of the plant occur to different degrees. Data from other climate zones or from other varieties can therefore not be used for an environmental risk assessment.

The variations in Bt production have been known for more than 10 years, but so far there has been no serious research in the reasons. We have initial indications that higher temperatures reduce or silence Bt production and/or that Bt production is correlated with photosynthesis activity of the plant parts.

Several studies show that young and old Bt maize plants produce different amounts of Bt toxin (Fearing et al. 1997, Dutton et al. 2004b). A new study from Germany confirms this variation. According to Nguyen & Jehle biosicherheit.de 2005b), "the toxin content varies seasonally and among plant parts" and “the monitoring of Cry1Ab expression showed that the Cry1Ab contents varied strongly between different plant individuals” (Nguyen & Jehle 2007).The toxin concentrations measured "differed to some extent considerably from those known from corresponding studies in the U.S., but tendencies could be confirmed. This result underlines the importance of studies under local climatic conditions with local varieties." ( biosicherheit.de 2005b).

Within one leaf there can be different Bt concentrations (Abel & Adamczyk 2004). Nguyen & Jehle (2007) found and increase of Bt concentration in leaves during the growing seasons. When Dutton et al. (2005) studied the Bt content of different leaves of Bt11 plants, they found that the youngest leaves showed the highest variation – with the highest concentration at the tip of the leaf and lower values in the growth region of the leaf, close to the stalk. In contrast, the Bt levels in older leaves were much more consistent.

Dutton et al. (2004a) also found similar results in MON810 plants grown under different conditions in the greenhouse and in the field. Young plants had nearly twice the Bt content of the older plants.

The Bt content within one plant differs depending on the plant part. In MON810 the Bt content is highest in the leaves and lowest in the kernels (Nguyen & Jehle 2007). In MON810 leaves the Bt content is four to seven times higher than in the roots (Mendelsohn 2003, Nguyen 2004, Baumgarte & Tebbe 2005). In Bt176 plants, however, the highest concentrations can be found in the pollen and leaves (Fearing et al. 1997).

In both Bt176 and MON810 the cobs have very low Bt content (Nguyen & Jehle 2005). Maize kernels or cobs are usually used for feeding studies on possible negative effects with mammals and birds, but since the Bt content in cobs is considerably lower than in leaves, these studies can not be used to assess the risk for animals that feed on other parts of the maize plant.

Burns & Abel (2003) discovered that lower nitrogen levels coincide with reduced Bt levels in leaf tissue. Dutton et al. (2004a) also found that higher Bt levels in young plants coincide with higher nitrogen values. They assume that differences in temperature reduce Bt production or prevent it altogether. 'Gene silencing' under extreme (weather) conditions and especially when the plants are under heat stress has been discussed for other GM plants for quite a while.

Abel & Adamczyk (2004) studied the Bt content of different parts of the maize leaf. They found significantly lower concentrations of Bt toxin in the white-yellow parts of the leaf than in the green ones. Their results show that plant parts with little chlorophyll and less photosynthesis activity produce less Bt toxin (Abel & Adamczyk 2004).

Photosynthesis activity of plants is influenced by several environmental factors such as temperature, water and light, therefore it stands to reason that these factors could also influence Bt production. This is why Bt values measured in one place cannot be generalized for cultivation under diverse environmental and climatic conditions.

Indications of the effects of environmental factors and climatic conditions on Bt production were also found when the Bt concentration were measured in different years and at different locations. Even at locations in Germany, Bt concentrations can vary by nearly 50% (Nguyen & Jehle 2007, biosicherheit.de 2005b). Samples of Bt176 and MON810 maize for several sub-projects of the 'Safety research and monitoring for Bt maize cultivation 2001-2004' project of the German Ministry of Education and Research (BMBF) were tested. At the two locations in Germany (Bonn and Halle) clear differences were found. "The toxin levels at one location were about 6-49% higher than at the other location during nearly all development stages during the three years of the trial." (Nguyen & Jehle 2007, biosicherheit.de 2005b).

In two successive years, the differences can even be greater, as two examples from this project show. Büchs (biosicherheit.de 2005f) registered Bt toxin in MON810 litter that was 2.5 times higher than in the previous year. Such differences could have a considerable impact on the studies of non-target organisms or Bt persistence in the soil.

Some Bt plants do not produce any Bt toxin at all. According to information from Monsanto, this affects 2% of the maize plants in a field (N. Mülleder; Monsanto Agrar Deutschland GmbH; pers. communication cited in Magg et al. 2001). Jehle & Ngyen (Jehle undated) did not detect Bt toxin in 9% of their samples, but this was put down to sampling errors (Jehle pers. communication). In 2006, Greenpeace took samples from commercial MON810 fields in Germany and Spain and a high variability in the Bt content of plants even on the same field and the same day. Amongst the results they found plants with no detectable Bt toxin as well as a high number of plants with extremely low Bt concentrations. (Greenpeace 2007).

## *Pollen: More toxin than expected*

In general it is assumed that the Bt content in the pollen of Bt176 plants is considerably higher than in the pollen of Bt11 or MON810 plants (Felke & Langenbruch 2005). The different varieties can produce very different levels (Nguyen 2004).

The Bt content for MON810 pollen is usually specified as very low (e.g. 0.09 µg/g pollen; Stanley-Horn et al. 2001). Results from German fields were much higher. Nguyen et al. (2001) found Bt levels in pollen as high as 0.32-6.6 µg/g – nearly as high as the Bt content in Bt176 pollen (7.1 µg/g; Stanley-Horn et al. 2002). The high variation in toxin production in MON810 maize could be caused by abiotic factors as well as by differences among different varieties.

Felke & Langenbruch (2005) conclude: "It is therefore essential that the pollen of MON810 maize be biotested further to clarify whether the different MON810 varieties have different toxin concentrations in the pollen and whether there are individual differences in toxin expression among plants of the same variety."

## *Cry1Ab Toxins differ*

The effects caused by one Bt maize cannot directly be used to assess another Bt maize, because the Bt production is regulated differently. In addition, the Bt toxins (CryAb) are different in the different Bt plants. Lower (absolute) Bt levels can therefore not necessarily be equated with less impact.

Different Bt maize plants (MON810, Bt176, Bt11 etc.) use different promoters to produce the Bt protein in the plant cell. It is known that different promoters activate the Bt production in different plant tissues (e.g. pollen, roots or phloem) differently (Dotton et al. 2003b), but as for example the review of Andow & Hilbeck (2004) states clearly: there is no safety research on this issue.

The Bt toxins in different Bt maize plants are not identical even though they are all called Cry1Ab. First of all they are all fundamentally different from the natural Bt toxin, produced in the bacteria Bacillus thuringiensis. The natural Bt protein is available in crystalline structure, much bigger and develops its toxic properties only when it is partly degraded in the insect gut by specific enzymes. However, not all insects produce the right enzyme.

Therefore the primary characteristic of the natural protein, as it is used in organic agriculture, is that it is non-toxic. It only becomes a toxin when it is ingested by specific insects. In the gut of the insect the protein is turned into a toxin when it is degraded by enzymes from is original size of 130 kDa to smalle proteins of about 60-65 kDa. However, the Bt toxins in Bt plants are present in shortened forms and is already toxic, which is why the Bt sprays used in organic agriculture cannot be compared with genetically modified Bt plants.

In addition the Bt toxins of different Bt plants are not identical. The Bt protein in MON810 is larger than that of Bt176 (92 kDa and 65 kDa, respectively, Nguyen 2004). In general it is assumed that the Bt toxins in Bt crops are identical and that only the amount of Bt toxin is relevant to possible effects on other insects. However there are indications that these different Bt proteins can cause different effects.

Studies on Monarch butterflies revealed long-term negative effects of MON810 and Bt11 even though they have lower Bt levels in the pollen than Bt176 (Dively et al. 2004).

A newer study makes this even more clear. Compared to MON810 and Bt176, pollen from MON810 harmed the fungus gnat *Lycoriella castanescens*. This negative effect could not be observed with Bt176 pollen even though it contains 30 times as much Bt toxin in the pollen . It was concluded that "apparently there is no connection between the observed effect and the absolute Bt content for different Bt maize varieties." (biosicherheit.de 2005f)

## *Disturbed metabolism in Bt maize*

Bt maize has a substantially higher lignin (wood) content than unmodified maize. This is presumably an unintended consequence of the genetic modification that is now known and has been measured (Saxena & Stotzky 2001b), but it has not been resolved why this is the case. Without doubt higher lignin levels have effects on the environment and influence whether or not Bt maize is suitable as food or animal feed (Poerschmann et al. 2005).

For MON810 and Bt176 the lignin content in stalks is considerably higher. In contrast, the levels in the leaves are not much different from the control plants (Poerschmann et al. 2005). This unexpected and unintended effect of the genetic modification could be a reason why the stems of Bt maize are harder and why harvest residues of Bt maize decompose more slowly in the soil (Poerschmann et al. 2005).

Higher lignin content also changes the soil ecology. The Bt plant material decomposes more slowly and the Bt toxin is likewise protected from degradation because it is bound to lignin in the plant material (Poerschmann and Kopinke 2001, Stotzky 2000, Saxena er al. 200b). The higher lignin content could also be a reason why the Bt plants have a lower nutritional value for organisms such as the larvae of fungus gnats. Poerschmann et al. (2005) conclude that studies of the lignin content of genetically modified maize are indispensable.

The higher lignin content is not the only unwanted difference compared to normal maize. Other studies show that the leaves of Bt maize stayed green longer then those of control plants (Lang 2005). Other pleiotropic effects of Bt maize have also been described by other scientists (Saxena & Stotzky 2001b, Magg et al. 2001, Hassel & Shepard 2002, Lumbierres et al. 2004; cited in Lang 2005). Nitrogen levels of leaves also seem to be higher (Escher et al. 2000).

During EU approval procedures Bt maize is considered 'substantially equivalent' to normal maize. This implies that the approval authorities assume that – besides the additional Bt toxin – there is no difference between Bt and non-Bt maize. It is because Bt maize is assessed in this way that further (health) risk assessment steps are dispensed with. However the examples of changes to metabolism presented here show that Bt maize is not substantially equivalent to normal maize.

MON810 was developed using a so-called gene gun. Cells are bombarded with metal particles to get the additional gene construct (transgene) into the plant. The insertion of the transgene is completely random and cannot be controlled. Apparently not only does this method disturb the normal maize genome, it can also change the transgene itself. The transgene's DNA sequence that is actually present in MON810 plants is different from the sequence described in the EU approval application (Hernandez et al. 2003).

# 6. GM maize is ecological and economic questionably

In recent years more and more of the mechanisms that plants use to naturally protect themselves against pests have been discovered. Maize plants have an indirect mechanism to protect themselves against Lepidoptera such as the European corn borer (ECB). If the plant is damaged by caterpillars feeding on them, the plant produces a scent that attracts natural enemies of the ECB such as the ichneumon fly *Trichogramma* (Degenhardt undated, biosicherheit.de 2005g).

> "Every maize variety has a typical basic scent pattern and every plant has an individual form of this pattern that is acquired during its lifetime while undergoing constant changes. A young maize plant does not release any scent. Not until the intensive growth phase does the plant produce its scent pattern, depending on local conditions and on pest and pathogen infestations. There are also differences in the release of volatile substance throughout the day. Plants that appear practically identical to the naked eye, that have similar weights and that are infested with similar numbers of Lepidoptera larvae, can still release very different levels of scent. Maybe there is also a fungal infection, root pests or other factors that influence the scent pattern." (biosicherheit.de 2005g).

Different maize varieties therefore have differences in their natural susceptibility to pests such as the ECB. Evidently the (North American) varieties used for genetic modifications are more susceptible to pests.

MON810 has been shown to produce fewer natural substances to protect itself from pests. For MON810 and unmodified control varieties significant differences were found in the amount of protective substances produced. It was shown that the difference was not caused by the genetic modification, but was due to a varietal effect (biosicherheit.de 2005g). This means that the Bt maize MON810 starts out at a natural disadvantage compared to other maize varieties in regard to the problem that it is supposed to have solved. Genetic engineering repeats the problems of the Green Revolution when it tries to replace multiple better-adapted varieties with only a few varieties.

Lower mycorrhiza levels can make the plants more susceptible to pests. Mycorrhiza symbiosis also makes crops more attractive to the natural enemies of aphids (Guerrieri et al. 2004, cited in Dolezel et al. 2005). Less mycorrhiza colonization (Turrini et al. 2004, Castaldini et al. 2005, see above) makes Bt maize plants more susceptible to pests since they cannot attract as many natural enemies of the pests (Dolezel et al. 2005 p.37).

In EU approval procedures the question is not being raised as to whether gene manipulation has an impact on these natural defence mechanisms or whether the source plant material used for the genetic modification might be more susceptible to certain pests from the outset.

## *Who needs Bt maize?*

In an issue of the ForschungsReport (1/2006), a publication of the German Ministry for Food, Agriculture and Consumer Protection (BMEVL) on the topic of genetic engineering and safety research, ECB infestation in Germany is described as follows:

> "In Germany maize is cultivated on 1.7 million hectares, of which 350,000 hectares are in the infestation area of the ECB (*Ostrinia nubilalis*), the only maize pest that is controlled year round on larger fields in Germany. ECB is mainly controlled through preventive measures as chaffing the stubbles and clean ploughing after the maize harvest. This kills the larvae in stubbles and robs them of material for pupation on the soil surface. The application of insecticides (on about 35,000 hectares per year) is laborious, because they have to be applied with special machinery due to the height of the plants. On about 14,000 hectares per year *Trichogramma* larvae are applied as an organic method of controlling the eggs of the pests." (Langenbruch et al. 2006, translated).

This means that only a fifth (20%) of the German maize acreage is infested with the European corn borer. Insecticides are applied to only 10% of this acreage, which means in turn that only 2% of the German maize acreage is treated with insecticides to control ECB.

The main argument for Bt maize cultivation is usually that farmers would not need to spray pesticides to control ECB any more, thus reducing overall applications of pesticides. Marvier et al. (2007) also point out how important it is to define the right comparator: "The general indication of our analyses is that if agriculture with insecticide application is the standard of comparison and if application of Bt crops truly reduces insecticide applications, then Bt crops may increase the abundance of nontarget invertebrates overall. Alternatively, if the comparison is made to farming systems without insecticides, some non-target groups are significantly less abundant in Bt than in control fields."

For Germany, Bt fields clearly need to be compared with fields without pesticides because only about 2% of the fields are even treated with pesticides. On the other 98% of maize acreage in Germany, Bt maize will lead to a decline in non-target organisms. Even more so when one takes into account that Marvier et al. (2007) also point out that most of the underlying studies are of poor quality and short term. Overall, it has to be concluded that the amount of pesticide released into the environment and the soil and the environmental effects increase when previously untreated fields (and especially fields that do not even have ECB infestations) are planted with Bt maize.

In addition, preventive practices to control ECB exist and are already applied: using mechanical field work such as chaffing the maize straw and clean, deep ploughing can decimate 80-90% of the ECB populations (Hurle et al. 1996, Langenbruch 2003; cited in Schorling 2006). During acute ECB infestation there is also the possibility of applying *Trichogramma* or of using Bt sprays. "With Bt sprays only members of specific insect groups in a specific development stage over a short period of time (of about a week) are harmed after the spray has been applied." (Langenbruch et al. 2006).

The amount of Bt toxin released on the field by Bt plants is considerably higher than that from Bt sprays (Szekacs et al. 2005).

## *European corn borer infestation: Not a problem in Europe*

Bt maize was developed in the United States. The situation in the United States is completely different from the situation in Germany and Europe in several respects, especially when it comes to the issue of ECB infestation. In the assessment of whether Bt maize is necessary and/or safe to grow in Germany, these issues are not usually considered sufficiently.

*Ostrinia nubilalis* (ECB) is a member of the butterfly family (Lepidoptera). It is native to Europe. Here, it has two different strains and not only colonizes maize (Liebe 2004; cited in Schorling 2006). ECB was introduced to the United States between 1910 and 1920 and spread fast as a maize pest; hence its name in English: European corn borer. In hot regions, ECB can produce two or three generations per year. In Germany (and in most of the EU), O. nublilalis only has one generation per year. The larvae spend the winter in the stems and roots left on the field and can usually be controlled by chaffing the plant residues and ploughing them under.

Furthermore, ECB infestations are cyclical, i.e. in some years there is high infestation while in other years there will be only few or no incidents. In Germany pesticides are used very infrequently to control the European corn borer. O. nubilalis has natural enemies in Europe. For example, ichneumon flies (*Trichogramma*, see above) lay their eggs in ECB larvae and thereby reduce the ECB populations.

Maize plants actively contribute to controlling ECB when they are affected by this pest by releasing volatile substances that attract Trichogramma (Degenhardt undated, biosicherheit.de 2005g). Trichogramma is also used as an organic pest control – though on only a small part of the limited acreage.

In North American varieties the ability to produce these volatile substances was lost in the course of plant breeding, while teosinte (the predecessor of maize) and European varieties use it to attract natural enemies, e.g. when the corn is infested with the corn rootworm (Rasmann et al. 2005).

With regard to MON810 this means that probably a conventional maize variety was genetically modified which has a poor interaction with beneficial insects that occur naturally or that are used intentionally as beneficial insects. Therefore MON810 is structurally more susceptible to exactly the pest that it is intended to control.

European agriculture differs significantly from North American agriculture. German agriculture is much more small scale. Field margins and hedges are an important part of the agricultural landscape. Similar structures are often missing from large-scale North American maize cultivation.

Effects on non-target organisms have to be assessed under conditions that represent the structures (and organisms) of the respective croplands. For instance, this is key to the question of whether Bt pollen negatively affects butterflies and butterfly populations. The amount of pollen deposited at field margins varies greatly and depends on a range of environmental factors (Dolezel et al. 2005). It must accordingly be assumed that the effects on the environment will be more serious in Europe than in the United States.

# 7. Conclusion

Even after more then a decade of commercial growing of Bt maize crops, the risk assessment studies are still few and most of them tend to raise more open questions than solving concerns. Non-target studies are sketchy: some species get more attention while for other organisms whole groups are left unattended. The reasons why some species are chosen and others not remain often unclear. Nevertheless it becomes more and more evident that adverse effects can be both direct and indirect, and that they can on higher trophic levels in the food network, even if the first organism to take them up might not be affected.

A new study even shows that the assumed mode of action of the Bt toxin so far has not been understood, and that in fact gut bacteria might be required for the toxic effect. This however means that there is no simple dose-response relationship between toxin and effect.

Also unclear are the persistence and effects of Bt toxins in the soil: either from living root material on which soil organisms feed, from root exudates or from degrading plant material after the harvest.

The fact that it is not understood why different plant tissues produce different Bt concentrations and how environmental factors might influence the Bt plants, makes a sufficient risk assessment even more difficult.

Other open questions include that neither the number of gene sequences nor the location where the new genes are inserted can be controlled, that the interactions with other genes and the metabolism of the plants cannot be predicted, and that eco-systemic effects are complex and can only be partially assessed scientifically. Also health impacts cannot be ruled out, and pollen migration and contaminants in the harvest contaminate food products and seed.

All in all, the available studies on different Bt maize events (such as MON810, Bt176 or Bt11) show more questions then answers, and they show that the risks of Bt maize for the environment are real.

In this context, the European framework legislation (Directive 2001/18 and Regulation 1829/2003) gives high priority to the precautionary principle. Directive 2001/18, Article 4(1) states:

> "Member States shall, in accordance with the precautionary principle, ensure that all appropriate measures are taken to avoid adverse effects on human health and the environment which might arise from the deliberate release or the placing on the market of GMOs."

In the light of the many known repercussions and because it is factually impossible to thoroughly study and assess all relevant risks, the requirements for EU marketing approval are not in place. While the cultivation of these plants and their use for animal feed serves the financial interests of a few companies, the potential long-term effects make such cultivation untenable.

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# 9. Annex 1: How much Bt toxin do MON810 plants actually produce?

The complete report can be downloaded at:
www.greenpeace.de/fileadmin/gpd/user_upload/themen/gentechnik/greenpeace_bt_maize_engl.pdf

## Executive Summary

In the growing season 2006, Greenpeace took leaf samples of commercially cultivated MON810 maize plants in Germany and Spain to determine the Bt toxin (Cry1Ab) concentration. A total of 619 samples from 12 fields were analysed using ELISA tests. MON810 maize is genetically engineered to produce a modified insecticide (Cry1Ab) that naturally occurs in the soil bacterium Bacillus thuringiensis (Bt). The production of this toxin is supposed to protect the maize plants from European corn borer larvae (ECB, Ostrinia nubilalis).

This Greenpeace study shows a surprising pattern of plants that contained only very low Bt toxin levels. However, high levels could be observed in some plants. The variation found on the same field on the same day was considerable, and could differ by a factor of as much as 100. This is in agreement with the results of a new study published in April 2007 (Nguyen & Jehle 2007) that concludes that "the monitoring of Cry1Ab expression [of MON810 plants] showed that the Cry1Ab concentrations varied strongly between different plant individuals."

In total, the Bt concentrations were much lower than those available from Monsanto for cultivation approval in the US and the EU, with a arithmetic mean of 9.35 µg Bt/ g fresh weight (fw; standard deviation 1.03; range 7.93-10.34 µg Bt/g fw). Here, our data also corroborate the results of Nguyen & Jehle (2007), who also found lower Bt concentrations (with means between 2.4 and 6.4 µg Bt/g fw) than those known from the literature. The data recorded by Greenpeace, however, deviate even more from the data published so far. The means ranged from 0.5 to 2.2 µg Bt/g fw, while Bt concentrations ranged from a minimum of no or 0.1 µg Bt/g fw to concentrations of about 14.8 µg Bt/g fw.

The results presented here raise far-reaching questions about the safety and the technical quality of the MON810 plants as well as some fundamental methodological questions.

## 1. The variation of Bt concentrations

Since the Bt concentration on the field can vary greatly even between neighbouring plants, the MON810 plants do not appear to be sufficiently stable in their biological traits. The reasons for the high variation in Bt contents could be related to genetic or environmental factors (e.g. weather or soil conditions), or both. Nguyen & Jehle (2007) not only found high variation between plants on a field, but also statistically significant differences between different locations in Germany. Since the reasons for such differences and the range of variation cannot be identified, the commercial cultivation of the crops should be stopped to avoid interactions with the environment that could lead to adverse and unpredictable effects. To investigate these questions further, studies should be conducted under contained conditions (such as greenhouse experiments) to study the environmental effects (e.g. drought, moisture, temperature, soil, nutrients) on the plants. Next to no studies of this type have yet been published.

## 2. The risk assessment of the plants

Risk assessment studies with non-target organisms or feeding studies in which the actual Bt concentration has not been determined appear to be of little use. Studies in which the toxin concentration is unknown cannot be used to give approval for the commercial growing of these plants.

## 3. The actual Bt toxin concentrations

If the Bt toxin in GE Bt plants were more effective in considerably lower concentrations than previously described, this would not be identical with the naturally occurring Bt toxin. This would annul a central aspect of the EU cultivation approval, which is based on the assumption that the Bt toxin in plants could in general be equated with the natural Bt protein from soil bacteria. However, if the toxin is not effective in such low concentrations as we have recorded, then serious concerns about the effectiveness of the plants in controlling ECB larvae need to be raised. Additional problems would then also concern insect resistance management, as resistance development could be accelerated by sub-lethal toxin doses.

### 4. The methods for determining Bt concentrations

The methods used by Monsanto to determine the Bt concentration of their original MON810 plants are not available from the publicly available documents. In order to make a reliable comparison of new data with Monsanto's data, it is essential that the test protocols as well as the original data are published. All interested laboratories need unrestricted access to relevant sample material. The authorities need to define standardised and sufficiently reliable methods for determining Bt concentrations in plants for risk assessment studies and for post-market monitoring.

Until the open questions regarding risk assessment, monitoring and product quality have been satisfactorily answered, the commercial cultivation of MON810 needs to be stopped, because the legal basis for approving MON810 for cultivation has not been fulfilled.

The complete report can be downloaded at:
www.greenpeace.de/fileadmin/gpd/user_upload/themen/gentechnik/greenpeace_bt_maize_engl.pdf

## 10. Annex 2: BVL requirements for a MON810 monitoring plan

In May 2006, the German authority for Consumer protection and Food safety (BVL) ordered to hold the further selling of MON810 until a new monitoring plan would be developed, based on new scientific findings since the original approval.

In a letter to Monsanto, the BVL ordered:

> "The release of seed of genetically modified maize of the event MON810 to third parties for commercial cultivation may only take place, after the permission holder has submitted a monitoring plan for the environmental effects in terms of Annex VII of Directive 2001/18/EG to the Federal Office for Consumer Protection and Food Safety. The plan needs to comply with Annex VII of Directive 2001/18/EG as well as decision 2002/811/EG and needs to take especially the following checkpoints into account:
>
> a) exposition of germinable maize seeds in the environment (loss during harvest, transport and processing),
>
> b) exposition of the Bt toxin in the environment (e.g. through pollen, silage, plant residues in the soil),
>
> c) fate of the Bt toxin in the soil of the area under cultivation; effects on soil organisms and soil fucntions,
>
> d) effect on non-target organisms on the area under cultivation and in affected habitats in the surroundings of the area under cultivation,
>
> e) long-term and large-scale effect effects on the biodiversity,
>
> f) fate of transgenes (persistence and accumulation) in organisms and in environmental media,
>
> g) development of secondary pests,
>
> h) changes in pesticide applications (type of the pesticide, volume, frequency and date),
>
> i) effects on food webs." (BVL 2007, translated)