

2.3. Controversial Cases

Special note on scientific papers on an experimental basis which seemingly demonstrate negative effects of GM crops on NTOs, *but which can be criticised on methodological grounds*:

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We concentrate on well known controversial cases here:

2.3.1. Bt toxicity on lacewings

Several papers of the research group of Hilbeck set a clear negative signal and it was accepted by part of the scientific community, that there are clearcut detrimental effects of Bt toxins on lacewings. (Hilbeck et al., 1998a; Hilbeck et al., 1998b, 1999; Hilbeck & Schmidt, 2006; Meier & Hilbeck, 2001). In those papers, the authors have shown that larvae of the green lacewing predator *Chrysoperla carnea* are negatively affected when preying on lepidopteran larvae that had been fed with transgenic maize expressing the cry1Ab gene from *Bacillus thuringiensis*.

here just one example with abstract:

Hilbeck, A., W. J. Moar, M. Pusztai-Carey, A. Filippini and F. Bigler (1998). "Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera : Chrysopidae)." *Environmental Entomology* 27(5): 1255-1263. <Go to ISI>://000076639500024 AND <http://www.botanischergarten.ch/Bt/Hilbeck-Toxicity-Bt-Chrysoperla-1998.pdf>

Laboratory feeding studies were carried out to determine the effects of the *Bacillus thuringiensis* (Berliner) Cry1Ab toxin on developmental time and mortality of *Chrysoperla carnea* (Stephens) larvae. A bioassay technique was developed that allowed for incorporation of the Cry1Ab toxin into a liquid diet that was then encapsulated within small paraffin spheres. Because only 2nd and 3rd instars can penetrate the

surface of the paraffin spheres, 2 different methods were used to rear chrysopid larvae through the 1st instar. The 1st method used small foam cubes soaked in non-encapsulated, liquid diet (with or without Cry1Ab). The 2nd method used *Ephestia kuehniella* (Hubner) eggs as prey during the first instar (no Cry1Ab exposure). After reaching the 2nd instar, all larvae received encapsulated, artificial diet with or without Cry1Ab, respectively. When reared only on artificial diet containing Cry1Ab toxin, total immature mortality was significantly higher (57%) than in the respective untreated control (30%). Also, significantly more chrysopid larvae died (29%) that received Cry1Ab later during their larval development compared with the respective control (17%). Although mortality was consistently higher, no or only small differences in developmental times were observed between Cry1Ab-treated and untreated *C. carnea* larvae. *C. carnea* larvae required significantly more time to complete larval development when reared on artificial diet only than when reared first on *E. kuehniella* eggs followed by encapsulated artificial diet or on only *E. kuehniella* eggs, regardless of exposure to Cry1Ab. These results demonstrate that Cry1Ab is toxic to *C. carnea* at 100 $\mu\text{g/ml}$ of diet by using encapsulated artificial diet.

However, (Romeis et al., 2004), in order to test whether those effects were really caused by the Bt toxin, they developed a bioassay, which allows them to feed high concentrations of the toxin directly to the predator. By doing so, they discovered another explanation for the detrimental effects on *Chrysoperla*:

Romeis, J., A. Dutton and F. Bigler (2004). "Bacillus thuringiensis toxin (Cry1Ab) has no direct effect on larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae)." *Journal of Insect Physiology* **50**(2-3): 175-183. <http://www.sciencedirect.com/science/article/B6T3F-4B84T3T-1/2/041f2a2f6f5410dd5ec2748a70707ec6> or <http://www.botanischergarten.ch/Bt/Romeis-et-al-04-Chrysoperla.pdf> and evaluation F1000: <http://www.facultyof1000.com/article/15019519/evaluation>

Earlier studies have shown that larvae of the green lacewing predator *Chrysoperla carnea* are negatively affected when preying on lepidopteran larvae that had been fed with transgenic maize expressing the cry1Ab gene from *Bacillus thuringiensis*. To test whether the observed effects were directly caused by the Cry1Ab toxin, we have developed a bioassay which allows us to feed high concentrations of the toxin directly to the predator. The results of these feeding studies show no direct toxic effect of Cry1Ab on *C. carnea* larvae. The amount of toxin ingested by first instar *C. carnea* in the present study was found to be a factor 10,000 higher than the concentration ingested when feeding on Bt-reared lepidopteran larvae, a treatment that was previously shown to have a negative impact on the predator. In addition, feeding first instar *C. carnea* with the Cry1Ab toxin did not affect the utilisation of subsequently provided prey. Furthermore, the quality of the prey provided to first instars did not affect the sensitivity of second and third instar *C. carnea* to the Bt-toxin. The presented results strongly suggest that *C. carnea* larvae are not sensitive to Cry1Ab and that earlier reported negative effects of Bt-maize were prey-quality mediated rather than direct toxic effects. These results, together with the fact that lepidopteran larvae are not regarded as an important prey for *C. carnea* in the field, led us to conclude that transgenic maize expressing Cry1Ab poses a negligible risk for this predator. (Romeis et al., 2004)

In an array of scientific journal publications, the case was extensively discussed, here just one example which also came to the conclusion that lacewings are not harmed through Bt toxins:

Rodrigo-Simon, A., R. A. de Maagd, C. Avilla, P. L. Bakker, J. Molthoff, J. E. Gonzalez-Zamora and J. Ferre (2006). "Lack of detrimental effects of *Bacillus thuringiensis* cry toxins on the insect predator *Chrysoperla carnea*: a toxicological, histopathological, and biochemical analysis." *Applied and Environmental Microbiology* **72**(2): 1595-1603. <Go to ISI>://000235353100078 AND <http://www.botanischergarten.ch/Bt/Rodrigo-Simon-Lack-Effect-Chrysoperla-2006.pdf>

The effect of Cry proteins of *Bacillus thuringiensis* on the green lacewing (*Chrysoperla carnea*) was studied by using a holistic approach which consisted of independent, complementary experimental strategies. Tritrophic experiments were performed, in which lacewing larvae were fed *Helicoverpa armigera* larvae reared on Cry1Ac, Cry1Ab, or Cry2Ab toxins. In complementary experiments, a predetermined amount of purified Cry1Ac was directly fed to lacewing larvae. In both experiments no effects on prey utilization or fitness parameters were found. Since binding to the midgut is an indispensable step for toxicity of Cry proteins to known target insects, we hypothesized that specific binding of the Cry1A proteins should be found if the proteins were toxic to the green lacewing. In control experiments, Cry1Ac was detected bound to the midgut epithelium of intoxicated *H. armigera* larvae, and cell damage was observed. However, no binding or histopathological effects of the toxin were found in tissue sections of lacewing larvae. Similarly, Cry1Ab or Cry1Ac bound in a specific manner to brush border membrane vesicles from *Spodoptera exigua* but not to similar fractions from green lacewing larvae. The in vivo and in vitro binding results strongly suggest that the lacewing larval midgut lacks specific receptors for Cry1Ab or Cry1Ac. These results agree with those obtained in bioassays, and we concluded that the Cry toxins tested, even at concentrations higher than those expected in real-life situations, do not have a detrimental effect on the green lacewing when they are ingested either directly or through the prey. (Rodrigo-Simon et al., 2006)

Other examples:

(Dutton et al., 2002, 2003; Lozzia et al., 1999; Mochizuki & Mitsunaga, 2004; Obrist et al., 2006; Villenave et al., 2006)

2.3.2. Field experiments with monarch larvae of Hansen-Obrycki

(Hansen-Jesse & Obrycki, 2000) (– sometimes cited as Jesse-Hansen & Obrycki)

„present the first evidence that transgenic *Bacillus thuringiensis* (Bt) corn pollen naturally deposited on *Asclepias syriaca*; common milkweed, in a corn field causes significant mortality of *Danaus plexippus* L. (Lepidoptera: Danaidae) larvae.”

The results show clear detrimental effects of the Bt 176 event of Novartis (which is ruled out today for its higher toxicity values anyway (Sears et al., 2001b)) on the Monarch larvae. Graphs and statistics are certainly correct and leave seemingly no doubt, until one detects through careful reading, that the experiment was not truly following the rules of a real field experiment, insofar as the pollen collection was done on leaves from plants which have been risen as potplants and deposited for the time of the pollen shed of the maize in the field. The field-deposited pollen sampling was done in the following way:

“To assess mortality of *D. plexippus* larvae exposed to field-deposited transgenic and non-transformed pollen, 143 leaf disks (0.79 cm²) were removed on 1 and 4 August 1998 from *A. syriaca* plants located within and at the edge of non-Bt (hybrid 4494) and event 176 (MAX 454) corn plots. Pollen was washed off 72 leaf disks. These leaf disks were examined under a dissecting microscope to determine that all pollen grains had been removed. The number of pollen grains on the unwashed leaf disks was counted and each disk was placed in a 5.2-cm-diameter petri dish on moistened filter paper.

One first-instar *D. plexippus* was placed on each leaf disk (transgenic $n=35$, non-transgenic $n=36$, or washed $n=72$) for 48 h. Although larvae were placed on top of the leaf disk, their movement was not restricted: they could feed from either leaf surface. The *D. plexippus* larvae were from a 2-month-old laboratory colony started from field-collected individuals.”

It is obvious that with the meticulously described procedure you get a high standard of comparability which is desirable and interesting for scientific questions, but at the same time you sacrifice a lot of elements of real field conditions, where, according to wind and

weather, and through various other circumstances the contact with toxic pollen deposited on the *Asclepias* leaves in agricultural reality will definitely be reduced. This has been shown by the field assessments of (Sears et al., 2001b), who criticize the studies of (Losey, 1999) and (Hansen-Jesse & Obrycki, 2000) in the following way:

“Previous reports indicating the hazard of *Bt* corn pollen to monarch butterfly are inadequate to assess risk, because assigning risk can be accomplished only when the likelihood of toxic response can be properly expressed and the likelihood of exposure is estimated through appropriate observations. We have used a comprehensive set of new data and a formalized approach to risk assessment that integrates aspects of exposure to characterize the risk posed to monarchs from *Bt* corn pollen. Characterization of acute toxic effects alone indicates that the potential for hazard to monarchs is currently restricted to event 176 hybrids, which express Cry1Ab protein in pollen at a level sufficient to show measurable effects. Event 176 hybrids have always had a minor presence in the corn market and current plantings, which comprise ,2% of corn acres, are rapidly declining.

Other events either express negligible Cry1Ab protein in corn pollen (Mon810 and Bt11) or express Cry protein of significantly less toxicity to monarch (Dbt418, Cbh351, and Tc1507 expressing Cry1Ac, Cry9c, and Cry1F proteins, respectively). These corn hybrids have little or no effect on monarch populations, although sublethal effects due to chronic exposure to *Bt* pollen over the entire larval growth of monarchs has not been accounted for in these studies. Should chronic effects be documented, the impact on monarch populations will remain low or negligible, because overall exposure of monarch larvae to *Bt* pollen is low.”

More details and criticism of this paper are given in (Shelton & Sears, 2001), details here cited:

“While the authors indicate their study was a field study, others thought differently, as only the collection of the leaf samples with the pollen itself was carried out in the field while exposure was measured under laboratory conditions (Ammann, 2000). This study also made the assumptions that the placement of the potted plants simulates the natural distribution of milkweed plants in and near corn fields; and that adult monarchs would and lay their eggs on these plants. These assumptions, as well as the assumption that monarch populations occur at the same time as pollen shed, go to the heart of the matter of whether monarch larvae will be exposed to lethal concentrations of natural deposits of *Bt* pollen.

Criticism of the study also focused on the bioassays. As the authors noted in their paper, there were large discrepancies between the toxin levels in pollen that they measured and those from replicated measurements accepted by the EPA. For example, for Bt 11 corn their level was over four times higher (0.39 versus 0.09 mg g \pm 1). and for event 176 corn their toxin levels were less than four times lower than the EPA-accepted figures. Furthermore, they detected *Bt*-toxin concentrations of 0.052 mg g \pm 1 pollen, half the accepted figure for Bt 11, in pollen collected from the ‘non-*Bt*’ variety 4494. reported that, due to the methods they employed in extracting pollen from corn tassels, the resulting pollen samples contained 43% of plant debris. The impact of this debris has been shown by Hellmich, Siegfried, Sears, Stanley-Horn, Mattila, Spences, Bidne and Lewis (unpublished results) to cause significant mortality, and reduce weight gain by >80%, of larvae exposed to contaminated versus uncontaminated pollen samples. Such debris is an artifact of the collection method. Thus it was not surprising that high doses of this non-*Bt* variety caused the same mortality to larvae as high doses of event Bt 11 transgenic corn. Furthermore, it was odd that there was 40% survival of larvae exposed to event Bt 11 pollen at doses of both 135 and 1300 grains cm 2 . These questions about the study, along with the fact that event 176, which has a higher concentration of *Bt* in the pollen, constitutes <2% of the corn acreage planted in 2000, did not seem to enter the public discussion of this report. However, on the day the report was published Obrycki received 44 calls from the press.”

It is also important to read all the details in the paper of Shelton and Shears about the whole controversy, notably the bias demonstrated by newspapers and more seriously the questionable publication policy of reputed scientific journals like *Nature*, insofar as

they published the first Monarch study of Losey, although there was clear evidence that the paper did not have a lot of scientific value. This criticism is detailed extensively in (Shelton & Sears, 2001), see also (Shelton & Roush, 1999; Shelton et al., 2000)

2.3.3. Butterfly feeding experiments of Felke and Langenbruch, with some remarks about the monarch butterfly case.

Felke, M. and G. A. Langenbruch (2005). Auswirkungen des Pollens von transgenem Bt-Mais auf ausgewählte Schmetterlingslarven. BfN-Skripten. V. G. Dr. Mathias Otto; Fachgebiet II 2.3 „Bewertung gentechnisch veränderter Organismen. Bonn - Bad Godesberg 2005, Bundesamt für Naturschutz, Konstantinstr. 110, 53179 Bonn. **157:** pp 143. www.bfn.de AND <http://www.botanischergarten.ch/Bt/Felke-Langenbruch-Bt-Mais-BfN-2005.pdf>

Aim of the study presented here was to assess potential side effects on non-target-butterflies due to cultivation of transgenic BT-maize in a number of laboratory and field experiments. Experiments were conducted with the two transgenic events Bt-176 (variety: PACTOL CB) of SYNGENTA-SEEDS and MON810 (variety NOVELIS) of MONSANTO. BT-maize expresses the lepidopteran-specific Cry1Ab toxin in various amounts also in pollen, so cultivation of these genetically modified plants must be considered as a potential threat for non-target-butterflies. After a suitable bioassay-method was established the impact of pollen could be determined. LD50-values for larvae of 7 indigenous butterfly-species, regarding to the amount of applied pollen grains (Bt-176-maize), could be calculated. LD50-values regarding to pollen of Bt-176-maize ranged between 8 pollen grains for the diamond back moth (4th instar) and 61 pollen grains for the peacock butterfly (2nd instar). Larval susceptibility to Bt-pollen varies considerably between different species. Within the same species neonate larvae are much more susceptible than older caterpillars. Toxicity of Bt-176-maize pollen does not decrease within the first three weeks after pollen shed. Bioassays for assessing sublethal effects were conducted with larvae of the diamond back moth and the peacock butterfly. Ingestion of low Bt-176-pollen numbers can lead to a delay in larval development. Only if individuals are harmed in an early larval stage caterpillars are able to compensate an initial decrease in weight gain before they pupate. Impacts of sublethal effects on population dynamics are discussed. MON810 maize contains comparable low amount of Bt-toxin in pollen. Larvae of the diamond back moth, which had been proved to be highly Bt-susceptible were not harmed after consumption of 80 MON810 pollen grains. On the other hand ingestion of MON810 anther fragments caused a significant increase in mortality. From the aspect of butterfly conservation cultivation of the event MON810 seems to be less problematic than cultivation of the event Bt-176, because of lower Cry1Ab-toxin expression in pollen. Nevertheless it has to be proved if there is variation in pollen toxin expression between different transgenic cultivars or due to different abiotic factors. Also it has to be cleared to which extent high toxin concentration in anthers can pose a risk for non-target-butterflies. During 2 years of field collections in a part of Bavaria (Germany) butterfly-species inhabiting agricultural areas were recorded. Because of their life cycle and preferences of habitat, larvae of 26 diurnal and 53 nocturnal species will be exposed to maize pollen with high probability. Summarizing the results of the presented study we recommend only to permit cultivation of Bt-maize with negligible toxin expression in pollen to minimize potential risks for nontarget butterflies. Despite this, transgenic maize fields should be surrounded by at least 10 rows of a non-transgenic maize hybrid to prevent dispersion of Bt-toxin containing pollen. Also cultivation of genetically modified maize should be prohibited near nature reserves. (Felke & Langenbruch, 2005)

Felke, M., N. Lorenz and G. A. Langenbruch (2002). "Laboratory studies on the effects of pollen from Bt-maize on larvae of some butterfly species." Journal of Applied Entomology-Zeitschrift Fur Angewandte

Entomologie **126**(6): 320-325.<Go to ISI>://000177281800010 AND
<http://www.botanischergarten.ch/Bt/Felke-Langenbruch-Bt-Mais-Journal-2002.pdf>

Three lepidopteran species were tested to determine their susceptibility against the ingestion of pollen from genetically modified maize plants. To prove the existence of dose-response relations between the applied amount of pollen (Bt-maize) and the damages on the tested larvae, a method was developed which makes it possible to feed caterpillars with defined amounts of pollen. If their food plants were contaminated with pollen of a cultivar of the Bt-176 maize-line *Pieris brassicae*, *Pieris rapae* and *Plutella xylostella* -larvae fed less, grew more slowly and showed a higher mortality than caterpillars of an untreated control group. The 50% lethality (LD50)-values were calculated for *P.xylostella* (L-4) with 19.2, for *P. rapae* (L-2) with 39.0 and also for *P. brassicae* (L-2) with 139.2 pollen of the transgenic maize Pactol CB. Studies with *P. brassicae* -caterpillars of different larval stages indicated, that older individuals showed a higher tolerance against pollen from Bt-maize than younger ones. It must be stated on the basis of the present studies, that ingestion of non-transgenic maize pollen has neither a positive nor a negative effect on caterpillars. It becomes clear from the information presented here that it is still difficult to make general statements about the endangering of butterflies, arising from cultivation of genetically modified maize lines. Further investigations on this issue are needed. Initially, the LD50-values concerning the larvae of certain butterfly species have to be determined to anticipate the risks, and in addition the distances between habitats with caterpillar host plants and maize fields, and the abundance of these 2.

Comment on the studies: There is no doubt that Bt maize events with high Bt toxicity in the pollen grains like the almost completely outruled Bt 176 can cause some damage on a range of butterfly species, which come into contact with Bt pollen. But the studies of the research group of Langenbruch fail to meet any baseline comparison with realistic field conditions, and in addition to this also fail to compare with pesticide spraying. These omissions devaluate the studies of the Langenbruch study group unfortunately to the point where a fair judgement of the situation is impossible.

It is also necessary to say that already at the time of the bio-assays done by Felke et al. extensive field studies in a similar case related to the larvae of the monarch butterfly in the United States have revealed that it is scientifically unfeasible to conclude directly from laboratory studies to field conditions. Already in the year 2000 there was a widely publized report made available from the University of Guelph in Canada uncovering the fact that in the field the monarch larvae were not harmed (Sears, 2000), contrary of what was stated in a short letter to Nature by (Losey, 1999). In addition, also in the same year a study was published in the the scientific literature confirming the absence of toxicity of *Bacillus thuringiensis* pollen to black swallowtails under field conditions (Wraight et al., 2000).

2.3.4. The case of the monarch butterfly

The monarch case, prematurely made public by Nature in an alarmist letter (Losey, 1999) caused a considerable setback and largely unnecessary concerns related to the development and spread of Bt crops, which is summarized in a populist article in the *Scientific American* (Stix, 1999). NGOs like Greenpeace and Friends of the Earth protested sharply against Novartis, at that time responsible for the development of an early Bt maize variety (event 176), protests which were e.g. rejected by EPA: (Dove, 2000).

But the short letter of Losey also triggered major field research, precipitating in classic field investigation papers, dismissing the monarch case as false alarm, interestingly enough also with the co-authorship of Losey. (Anderson et al., 2005; Anderson et al.,

2004; Dively et al., 2004; Gatehouse et al., 2002; Hartzler & Buhler, 2000; Hellmich et al., 2001; Oberhauser et al., 2001; Pimentel & Raven, 2000; Prasifka et al., 2007; Scriber, 2001; Sears et al., 2001a; Sears et al., 2001b; Stanley-Horn et al., 2001; Zangerl et al., 2001). See also the comments in Nature Biotechnology of Hodgson (Hodgson, 1999, 2000)

It is interesting to note that the company Novartis wrote May 3, 1999 a letter to John Losey (Stein, 1999) with arguments available in 1999 and earlier against the conclusions including critical notes on the methodology of the Losey manuscript which was nevertheless published on May 20 – arguments which were fully confirmed later by the subsequent field studies. This is another example of peer review having failed, which happens increasingly often in well-known journals as Lancet, Nature, Science and PNAS (Miller et al., 2008), for more comments see chapter on aquatic non-target organisms below.

2.3.5. Endangered Karner blue butterfly larvae and Bt maize pollen

Here again we have a study which is methodologically per se ok, although it works with modeling and consequently should be judged with caution. It would be imperative to know what impact the normal pesticide management has on this rare butterfly.

Peterson, R. K. D., S. J. Meyer, A. T. Wolf, J. D. Wolt and P. M. Davis (2006). "Genetically engineered plants, endangered species, and risk: A temporal and spatial exposure assessment for Karner blue butterfly larvae and Bt maize pollen." *Risk Analysis* **26**(3): 845-858. <Go to ISI>://WOS:000238442900025 AND <http://www.botanischergarten.ch/Bt/Peterson-Karner-Blue-Butterfly-2006.pdf>

Genetically engineered maize (*Zea mays*) containing insecticidal endotoxin proteins from *Bacillus thuringiensis* (Bt) delta-endotoxin proteins has been adopted widely in the Midwestern United States. The proteins are toxic to several lepidopteran species and because a variety of maize tissues, including pollen, may express the endotoxins, the probability of exposure to nontarget species, including endangered species, needs to be understood. The objective of this study was to assess the potential temporal and spatial exposure of endangered Karner blue butterfly larvae (*Lycaeides melissa samuelis*) to Bt maize pollen in Wisconsin using probabilistic exposure techniques and geographic information systems analysis. Based on degree-day modeling of butterfly phenology and maize pollen shed, there is some potential for temporal exposure of larvae to maize pollen. However, in the majority of years and locations, maize pollen shed most likely will occur after the majority of larval feeding on wild lupine (*Lupinus perennis*). The spatial analysis indicates that some Karner blue butterfly populations occur in close proximity to maize fields, but in the vast majority of cases the butterfly's host plant and maize fields are separated by more than 500 m. A small number of potential or existing Karner blue butterfly sites are located near maize fields, including sites in two of the four counties where temporal overlap is most likely. The exposure assessment indicates that these two counties should receive the highest priority to determine if Karner blue butterfly larvae are actually at risk and then, if needed, to reduce or prevent exposure. (Peterson et al., 2006)

2.3.6. Laboratory assessment of the effects of *Bacillus thuringiensis* on native Lepidoptera

This assessment lacks completely an agronomic baseline comparison with the conventional pesticide involved management systems, which certainly has more impact

on indigenous lepidoptera. The conclusions are therefore devaluated because of a not very realistic scenario.

Peacock, J. W., D. F. Schweitzer, J. L. Carter and N. R. Dubois (1998). "Laboratory assessment of the effects of *Bacillus thuringiensis* on native Lepidoptera." *Environmental Entomology* **27**(2): 450-457. <Go to ISI>://000073520800039 AND <http://www.botanischergarten.ch/Bt/Peacock-Effects-Lepidoptera-1998.pdf>

The effect of 2 formulations of *Bacillus thuringiensis* subsp. *kurstaki* (Foray 48B and Dipel 8AF) was evaluated on 42, species of native Lepidoptera in laboratory bioassays using instars that are present in the field at the time of gypsy moth suppression applications. Mortality was significant for 27 of the 42 species evaluated against Foray 48B, and 8 of 14 species evaluated against Dipel 8AF. Susceptible species were noted in 5 of 6 families assayed-Papilionidae, Nymphalidae, Geometridae, Lasiocampidae, Saturniidae, and Noctuidae. The 1 species treated in the Lymantriidae family was not susceptible to *B. thuringiensis*. Treated individuals that survived for a week were likely to reach adulthood. Intrageneric differences in susceptibility to *B. thuringiensis* were recorded among 8 species of *Catocala* and 3 species of *Lithophane* assayed. Of the 18 species assayed as 1st or 2nd instars, mortality was significant, usually exceeding 95%. By contrast, 9 of 11 species not susceptible to *B. thuringiensis* were assayed as penultimate or ultimate instars. However, species susceptible to *B. thuringiensis* were found in both early and late instars.

2.3.7. Bt toxicity on a moth parasitoid *Cotesia marginiventris*

Under clearly artificial laboratory conditions feeding experiments have been carried through and commented with care. Emphasis has been put by the authors on effects which are dose related and can only be demonstrated at high doses, which is described in detail in the paper (Ramirez-Romero et al., 2007)

Ramirez-Romero, R., J. S. Bernal, J. Chaufaux and L. Kaiser (2007). "Impact assessment of Bt-maize on a moth parasitoid, *Cotesia marginiventris* (Hymenoptera : Braconidae), via host exposure to purified Cry1Ab protein or Bt-plants." *Crop Protection* **26**(7): 953-962. <Go to ISI>://000247186300012 AND <http://www.botanischergarten.ch/Bt/Ramirez-Romero-Impact-Assessment-Cotesia-2007.pdf>

Addressing whether Cry1Ab protein produced by Bt-maize affects non-target insects, including parasitoids, is a necessary component in the risk assessment of this crop protection alternative. This study assessed host-mediated effects of Cry1Ab protein on the parasitoid *Cotesia marginiventris* Cresson (Hymenoptera) via two delivery methods: delivery of purified Cry1Ab protein via artificial diet, and delivery of Cry1Ab protein via Bt-maize plant tissue. In the first case, lethal and sublethal effects of purified Cry1Ab protein on the host, *Spodoptera frugiperda* (Lepidoptera), were evaluated prior to evaluating effects on the parasitoid. Unparasitized host larvae were exposed to one of three Cry1Ab concentrations, 0.46 (C1), 9.13 (C2), and 182.6 (C3) μg Cry1Ab/ml diet. The C3 concentration proved highly toxic to host larvae, so only host-mediated effects of C1 and C2 concentrations on the parasitoid *C. marginiventris* were studied. As expected, purified Cry1Ab affected survival, developmental times, and growth rates of *S. frugiperda* larvae at all three Cry1Ab concentrations. In contrast, host-mediated effects of purified Cry1Ab protein on *C. marginiventris* were not evident at the two concentrations that were evaluated, C1 and C2. However, several host-mediated effects on *C. marginiventris* were detected when Cry1Ab protein was delivered via Bt-maize tissue. Exposure to Cry1Ab protein via Bt-maize tissue affected parasitoid developmental times, adult size, and fecundity. Though effects on parasitoids of direct exposure (i.e. not mediated by the host) to Cry1Ab protein were not evaluated, the results of the present study suggested a direct effect of the protein, delivered via host feeding on Bt-maize, on *C. marginiventris*. (c) 2006 Elsevier Ltd. All rights reserved. (Ramirez-Romero et al., 2007)

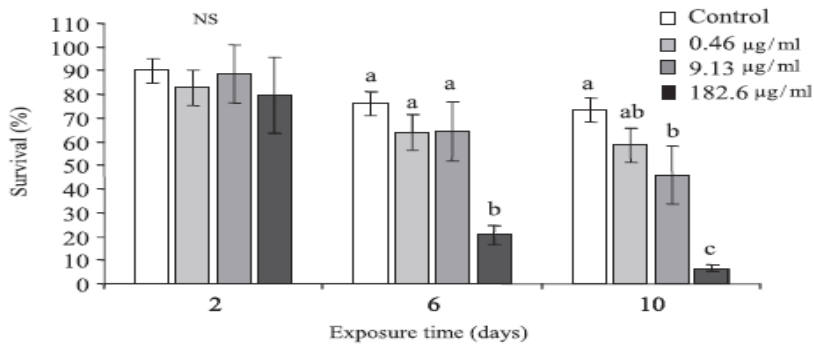


Fig. 1. Survival (%) of *S. frugiperda* larvae on artificial diet containing 0.46, 9.13, or 182.6 µg Cry1Ab protein/ml diet, or control diet (without Cry1Ab protein), after 2, 6, and 10 d of exposure. Within exposure times, columns sharing lower-case letters are not significantly different ($P < 0.05$) (2 d exposure, $\chi^2 = 6.13$, 3 df, $P = 0.106$; 6 d, $\chi^2 = 78.90$, 3 df, $P < 0.001$; 10 d, $\chi^2 = 107.75$, 3 df, $P < 0.001$).

Fig. 1 Caption see above, from (Ramirez-Romero et al., 2007)

comments of (Ramirez-Romero et al., 2007) to the figure above:

"Survivorship of *S. frugiperda* larvae after 2 d of exposure (i.e. 5 d-old larvae) was not significantly affected by the different Cry1Ab protein treatments relative to the control ($P \geq 0.106$) (Fig. 1). However, significant reductions of survivorship ($P < 0.001$) were evident on C3 diet after 6 d of exposure, and on C2 and C3 diets after 10 d of exposure (Fig. 1). Weights of *S. frugiperda* larvae were significantly lower relative to the control on C1, C2, and C3 diets after 2, 6, and 10 d of exposure ($P < 0.001$) (Fig. 2). Larvae exposed to C2 and C3 diets gained little weight during the observation period (15- and 4-fold, respectively), relative to the control (139-fold) or C1 diet (68-fold). Head capsule widths were significantly smaller ($P < 0.001$) on C2"

True field experiments will most probably reveal only small impact of Bt toxin on *S. frugiperda* in view of agro-ecological comparisons with conventional crops and their pesticide management.

2.3.8. Quantification of Bt-protein digestion and excretion by the primary decomposer *Porcellio scaber*, fed with two Bt-corn varieties

The project is done with artificial feeding experiments, and no attempt is made to check the data in agricultural field reality.

Pont, B. and W. Nentwig (2005). "Quantification of Bt-protein digestion and excretion by the primary decomposer *Porcellio scaber*, fed with two Bt-corn varieties." *Biocontrol Science and Technology* **15**(4): 341-352. <Go to ISI>://BIOSIS:PREV200510174135 AND <http://www.botanischergarten.ch/Bt/Pont-Quantification-Bt-2005.pdf>

Despite increasing use and fabrication of new transgenic plants, there are still concerns about a potential accumulation of Bt-proteins in the soils. Even previous studies have revealed that some phytophagous animals ingest and excrete Bt-protein. Neither the digested proportion of Bt protein nor the potential amount of an insecticidal activity of the feces excreted by soil arthropods is known. For a period of 15 days, we fed the primary decomposer woodlouse *Porcellio scaber* (Latreille) with leaves of two transgenic Bt-corn varieties (N4640Bt and Max88Bt) and one non-transgenic control variety (N4640). The Bt-protein in the leaves and in feces was quantified using ELISA. The Bt-protein digestion rate was obtained as a

ratio of the Bt-protein ingested to the Bt-protein excreted. Additionally we quantified the naturally occurring Bt-protein dissipation in the leaves of the two transgenic corn varieties over a period of 15 days. Finally bioassays using the susceptible species *Ostrinia nubilalis* (Hübner) (Lepidoptera, Pyralidae) were carried out to determine if the Bt-protein in the decomposer feces is still active. We calculated that *P. scaber* feeding on N4640Bt corn leaves digests a mean of $61.1 \pm 16.8\%$ of the Bt-protein they ingest, while *P. scaber* feeding on Max88Bt corn leaves digests $80.5 \pm 14.4\%$, which is significantly more ($P < 0.05$). At an average temperature of 18.3°C , the Bt-protein concentration in the leaves shows a rapid and constant Bt-protein decrease of approximately $40 \pm 15\%$ per 3 days in both transgenic corn plants. This accumulates to a Bt-protein loss of more than 90% after 15 days. The bioassays indicate that the Bt-protein excreted with the feces is still insecticidally active. Our study suggests that a part of the Bt-protein taken up by primary decomposers is not digested and is released in its active form into the soils. Under the much cooler field conditions in autumn and winter, it stays active and remains available to soil organisms until the next field season. (Pont & Nentwig, 2005)

2.3.9. Impact of Bt crops and its residues on aquatic non-target organisms

A recent publication in PNAS has stirred up environmentalists and opponents of GM crops likewise: (Rosi-Marshall et al., 2007a) It provoked reaction from the science community and from politics. The scientific and political situation is best described by an editorial of John Hodgson in Nature Biotechnology: (Hodgson, 2008)

The original publication was subsequently heavily criticized by scientists in a letter to the editor of PNAS (McHughen et al., 2007) with many details. The massive criticism was then re-emphasized by two shorter letters to PNAS which were published (Beachy et al., 2008; Parrott, 2008). The main points of the criticism (from Beachy et al. 2008)

“Because previous studies reported no significant effects on caddisflies (Douville & Gagne, 2003; Douville et al., 2007), the topic of the present study leads the reader to reconsider the issue. However, the authors of the recent paper made fundamental errors in experimental design that make it impossible to draw the conclusion that Bt crops have impacts on aquatic insects:

They failed to use proper control materials, which would have to have been isogenic, nontransgenic tissues. It is well known that the chemical composition of leaves varies widely between different maize genotypes. It is possible that the claimed negative impacts on larval growth were attributable to chemical components in the tissue and not to the Bt protein.

They failed to identify and to quantify the Bt protein, other leaf chemicals, and agricultural chemicals in stream waters, making it impossible to repeat the study or to draw conclusions from the data.”

And (Parrott, 2008) agrees with this criticism, since maize hybrids differ in many traits, e.g. the variation in trypsin inhibitor levels could have been responsible just as well for observed effects, attributed uncritically to Bt alone.

In a recent publication (Miller et al., 2008), the most extensive rebuttal of the paper has been published, co-authored by the writer of this report, here the full citation:

“The most recent example of egregious failures of editorial and peer review occurred in a ‘research’ article published in September 2007 in the PNAS. Rosi-Marshall et al claimed to show that pollen from Bt-maize was injurious to caddisflies in a laboratory aquatic ecosystem (Rosi-Marshall et al., 2007b). However, their conclusions are dubious for numerous reasons.

- First, pollen produced by currently available varieties of Bt-maize contains very low concentrations of Bt toxin.

- Second, the authors extrapolated from a laboratory experiment to a field system based on a single study, an extrapolation that is problematic, especially given that they used pollen in doses higher than the maximum encountered under field conditions.
- Third, and most damning of all, they reported elsewhere that they had failed to find these effects in the field (<http://www.benthos.org/database/allabstracts.cfm/db/Columbia2007abstracts/id/370>), an important fact that should have been disclosed in the PNAS paper. The omission of those contrary findings arguably amounts to investigator misconduct.
- Fourth, earlier studies reported in the literature concluded that Bt-endotoxin concentrations in aquatic systems are extremely low and are metabolized rapidly in water [18].
- Fifth, intact Bt organisms (which contain pesticidal toxins) are used to control insects by farmers and home gardeners and for mosquito abatement in ditches, ponds and lakes. Even if the authors were actually measuring the effects of Bt toxin (which is uncertain, inasmuch as they did not use isogenic lines), they appear to have had no way to know whether the toxin came from the transgenic Bt crops or whether it came from the Bt organisms applied exogenously.
- Sixth, the authors seem unaware that there are several variant forms of Bt endotoxin and that different Bt-maize transgenes carry different isoforms, inasmuch as there is no indication in the paper as to which they were using
- Seventh, because they failed to measure the levels of Bt protein, there is no direct evidence for a dose-dependent effect of the Bt toxin.
- Eighth, the authors conclude that growing Bt-maize could cause downstream adverse effects in waterways, but they fail to consider alternative explanations. Under actual field conditions, any deleterious environmental effects from Bt toxin(s) (which, it should be emphasized, have not been demonstrated) could be derived from Bt-maize or from the exogenous application of Bt spores (vide supra).
- Finally, they analyze their results in a vacuum: in the real world, the choices are not 'Bt-maize' versus 'no intervention against pests'. The cultivation of Bt-maize could well be environmentally preferable to traditional pesticides or other strategies for controlling insects, but the authors fail to consider that possibility.

Once again, we are at a loss to explain how qualified reviewers and editors could be unaware of flaws of this magnitude. Editorial negligence or a failure of the peer review process?

Equally as shocking as the publication of such a shoddy paper in a major journal was the lack of an appropriate response from Professor Randy Schekman, the editor of PNAS, to numerous complaints about it (including from members of the Academy), some of them quite detailed. After promising to discuss the paper during the regular conference of the journal's associate editors, he appears to have decided to ignore the problem in the hope that it would go away. The only concession from PNAS was to agree to publish online a 250-word letter to the editor –along with a rebuttal of the same length from the original authors! We would remind Professor Schekman that in science, as in politics, the cover-up is often as bad as – or worse than – the original transgression”.

All scientists opposing the PNAS paper got full support by EFSA:

Rejection of the conclusions in the Rosi-Marshall paper by the EFSA GMO panel:

The GMO panel of the EFSA (European Food Safety Agency) analysed the publication as well and came to the following conclusion:

“In summary, the conclusions of the paper (Rosi-Marshall et al., 2007b) are not supported by the data presented in this paper. The GMO Panel is of the opinion that based on the available information such a low level of exposure to Trichoptera in aquatic ecosystems is unlikely to cause a toxic effect. The arguments are roughly the same as in the above cited rebuttals. The panel's argumentation is based on two publications: (Icoz & Stotzky, 2007) conclude: The protein was detected for only 21 days in the 3M soil and for 14 days in the 6M soil, which were not adjusted in pH. These results indicate that the Cry3Bb1 protein does not persist or accumulate in soil and is degraded rapidly. And (Nguyen & Jehle, 2007) give in their summary again the same picture of rapid degradation:”

"The tissue-specific expression and seasonal abundance of Cry1Ab protein were determined in transgenic maize plants (Mon810, variety 'Novelis') from two field trials located near Bonn and Halle, Germany. A total of 1085 samples were analysed by using Double Antiserum-Enzyme Linked Immunosorbent Assay (DAS-ELISA). The Cry1Ab contents of various plant tissues (root, stem, upper leaf, lower leaf, anther, pollen and kernel) were determined at four different growth stages (BBCH19, BBCH30, BBCH61 and BBCH83) collected in 2001, 2002 and 2003. Mon810 showed the highest Cry1Ab contents in the leaves (5.5 - 6.4 $\mu\text{g g}^{-1}$) fresh weight [fw] at BBCH83, whereas the lowest Cry1Ab contents were detected in the pollen (1 - 97 ng g^{-1} fw). Cry1Ab content of residual root stocks collected in the field nine months after harvest was 15 - 17 ng g^{-1} fw. This demonstrated that the Cry1Ab concentration in residual root stocks was reduced to about one-hundredth of the fresh roots. The monitoring of Cry1Ab expression showed that the Cry1Ab contents varied strongly between different plant individuals."

Additional comments by the author of this report

The GMO panel could have also cited the following papers confirming rapid degradation of Bt toxin levels in water:

Although not directly comparable, the study of (Kreutzweiser et al., 1996) confirmed, that residues from sprays based on *Bacillus thuringiensis kurstaki* are rapidly degraded and do not pose a problem to aquatic organisms. Contamination of watercourses with Btk is unlikely to result in significant adverse effects on microbial community function in terms of detrimental composition.

If one compares other insecticides in their impact, it is necessary to take more restrictive management measures in order to protect aquifers from detrimental effects (Maltby et al., 2005; van Wijngaarden et al., 2004).

A closer comparability is offered with the study of (Prihoda & Coats, 2007), again the Bt based coleopteran insecticidal Cry3Bb1 Protein has no impact on aquatic organisms:

Prihoda, K. R. and J. R. Coats (2007). "Aquatic fate and effects of *Bacillus thuringiensis* Cry3Bb1 protein: Toward risk assessment." *Environ Toxicol Chem electronic prepublication*(--): --
<http://www.botanischergarten.ch/Bt/Prihoda-Fate-Effects-2007.pdf>

Genetically engineered crops expressing *Bacillus thuringiensis* (Bt) insecticidal crystalline (Cry) proteins became commercially available in the US in 1996. In 2006, 19 million hectare (ha) of Bt corn were planted worldwide, which represents a 10 million ha increase in 10 years. The sustainability of Bt crops is important because their use has significantly reduced the amount of chemical insecticides necessary to control agricultural pests. Despite the high adoption rates of this novel insecticide, little is known about the aquatic fate of transgenic Bt proteins and their non-target effects on aquatic invertebrates although several potential routes exist for their transport to aquatic systems. Methods were developed to investigate the aquatic fate of transgenic Bt proteins and to determine their potential effects on non-target aquatic invertebrates. Laboratory microcosms containing pond water only or pond water and sediment were used to examine the fate of the coleopteran-active Bt Cry3Bb1 protein in decomposing transgenic corn event MON863 (hereafter referred to as MON863 corn) leaf, stalk, and root. A half-life of less than three days was found for Bt Cry3Bb1 from decomposing MON863 corn residue. No Bt Cry3Bb1 was measured in the pond water or sediment extracts of microcosms containing MON863 corn. In an acute, static, partial-renewal toxicity test, Bt Cry3Bb1 protein from MON863 root extracts was fed to *Chironomus dilutus* larvae for ten days. A significant decrease in *C. dilutus* survival at nominal concentrations of 30 ng/ml was found, however, no effect on growth among the surviving larvae was observed.

Best comparability with the paper of Rosi-Marshall is given by (Douville et al., 2007), therefore the full abstract is cited:

Douville, M., F. Gagne, C. Blaise and C. Andre (2007). "Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn cry1Ab gene from an aquatic environment." *Ecotoxicology and*

Environmental Safety **66**(2): 195-203.<Go to ISI>://000243187000009 AND <http://www.botanischergarten.ch/Bt/Douville-Occurrence-Persistence-2007.pdf>

Genetically modified corn crops and suspensions of *Bacillus thuringiensis* (Bt) are currently used to control pest infestations of insects of the Lepidoptera family. For this purpose, the cry1Ab gene coding for protein delta-endotoxin derived from *B. thuringiensis* *kierstaki* (Btk), which is highly toxic to these insects, was inserted and expressed in corn. The aims of this study were to examine the occurrence and persistence of the cry1Ab gene from Btk and Bt corn in aquatic environments near fields where Bt corn was cultivated. First, an optimal DNA preparation and extraction methodology was developed to allow for quantitative gene analysis by real-time polymerase chain reaction (qPCR) in various environmental matrices. Second, surface water and sediment were spiked in vitro with genomic DNA from Bt or Bt corn to evaluate the persistence of cry1Ab genes. Third, soil, sediment, and water samples were collected before seeding, 2 weeks after pollen release, and after corn harvesting and mechanical root remixing in soils to assess cry1Ab gene content. DNA was extracted with sufficient purity (i.e., low absorbance at 230 nm and absence of PCR-inhibiting substances) from soil, sediment, and surface water. The cry1Ab gene persisted for more than 21 and 40 days in surface water and sediment, respectively. The removal of bacteria by filtration of surface water samples did not significantly increase the half-life of the transgene, but the levels were fivefold more abundant than those in unfiltered water at the end of the exposure period. In sediments, the cry1Ab gene from Bt corn was still detected after 40 days in clay- and sand-rich sediments. Field surveys revealed that the cry1Ab gene from transgenic corn and from naturally occurring Bt was more abundant in the sediment than in the surface water. The cry1Ab transgene was detected as far away as the Richelieu and St. Lawrence rivers (82 km downstream from the corn cultivation plot), suggesting that there were multiple sources of this gene and/or that it undergoes transport by the water column. Sediment-associated cry1Ab gene from Bt corn tended to decrease with distance from the Bt cornfield. Sediment concentrations of the cry1Ab gene were significantly correlated with those of the cry1Ab gene in surface water ($R = 0.83$; $P = 0.04$). The data indicate that DNA from Bt corn and Bt were persistent in aquatic environments and were detected in rivers draining farming areas.

The authors conclude explicitly:

“However, the levels of Cry1Ab protein in our samples *were below the detection limit most of the time*, although it was detected at concentrations ranging from 0.1 to 1 ng/g or ng/ mL in sediment and surface water, respectively (Douville et al., 2005). And:

This finding suggests that environmental concentrations were low but the null hypothesis (i.e., that the cry1Ab transgene was not expressed in aquatic environments) cannot be accepted at the present time and further research and monitoring efforts are required.”

In their earlier paper (Douville et al., 2005) they made it quite clear, that it is necessary to distinguish between crystal Bt (source: Bt sprays like Dipel) and non-crystal Bt endotoxin coming from the transgenic Bt crops, a distinction which was done through isotopic pattern analysis. The results showed that Bt-corn endotoxin is degraded more rapidly in water than in soils ($t(1/2)$: 4 and 9 days, respectively), while crystals appeared to be more resilient, as expected.

The group of Rosi-Marshall would have had at hand a method to distinguish between Bt originating from external use and Bt endotoxine, but they failed to use the methods published earlier by Douville.

Douville, M., F. Gagne, L. Masson, J. McKay and C. Blaise (2005). "Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment." Biochemical Systematics and Ecology **33**(3): 219-232.<Go to ISI>://000227414300001 AND <http://www.botanischergarten.ch/Bt/Douville-Tracking-Source-2005.pdf>

The application of *Bacillus thuringiensis* (Bt) and the growing of genetically-modified crops are currently practised to control infestations of crop-eating insects. The increasing use of these biopesticides could

lead to an increase in Cry1Ab endotoxin in both terrestrial and aquatic environments. The aim of this study was to quantify levels of Cry1Ab endotoxin and locate its source in the environment. Agricultural soils and surface waters were spiked with crystals (biopesticide-Dipel(R)) or with pure Bt-corn endotoxin. Cry1Ab concentrations were then determined with immunoassays. Additionally, surface water, soils and sediments were sampled in an area sprayed with Bt kurstaki and at a site where genetically-modified corn expressing Cry1Ab is grown. Isotopic analysis was performed on the endotoxin from Bt and Bt corn to characterize the proportions of C-13/C-12 and N-15/N-14. The results showed that Bt-corn endotoxin is degraded more rapidly in water than in soils ($t(1/2)$: 4 and 9 days, respectively), while crystals appeared to be more resilient, as expected. The isotopic patterns of C-13 and N-15 in Bt-corn endotoxin differed markedly from Bt, making it possible to track the source of Cry1Ab in the environment. Preliminary field surveys indicate that Cry1Ab is fairly uncommon in aquatic environments, being found only at trace concentrations when it is detected. (Douville et al., 2005)

In a later paper (Douville et al., 2007) went again into the study of the fate of Bt proteins from crops finding their way into the adjacent waters:

Douville, M., F. Gagne, C. Blaise and C. Andre (2007). "Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn cry1Ab gene from an aquatic environment." *Ecotoxicology and Environmental Safety* **66**(2): 195-203. <Go to ISI>://000243187000009 AND <http://www.botanischergarten.ch/Bt/Douville-Occurrence-Persistence-2007.pdf>

Genetically modified corn crops and suspensions of *Bacillus thuringiensis* (Bt) are currently used to control pest infestations of insects of the Lepidoptera family. For this purpose, the cry1Ab gene coding for protein delta-endotoxin derived from *B. thuringiensis kurstaki* (Btk), which is highly toxic to these insects, was inserted and expressed in corn. The aims of this study were to examine the occurrence and persistence of the cry1Ab gene from Btk and Bt corn in aquatic environments near fields where Bt corn was cultivated. First, an optimal DNA preparation and extraction methodology was developed to allow for quantitative gene analysis by real-time polymerase chain reaction (qPCR) in various environmental matrices. Second, surface water and sediment were spiked in vitro with genomic DNA from Bt or Bt corn to evaluate the persistence of cry1Ab genes. Third, soil, sediment, and water samples were collected before seeding, 2 weeks after pollen release, and after corn harvesting and mechanical root remixing in soils to assess cry1Ab gene content. DNA was extracted with sufficient purity (i.e., low absorbance at 230 nm and absence of PCR-inhibiting substances) from soil, sediment, and surface water. The cry1Ab gene persisted for more than 21 and 40 days in surface water and sediment, respectively. The removal of bacteria by filtration of surface water samples did not significantly increase the half-life of the transgene, but the levels were fivefold more abundant than those in unfiltered water at the end of the exposure period. In sediments, the cry1Ab gene from Bt corn was still detected after 40 days in clay- and sand-rich sediments. Field surveys revealed that the cry1Ab gene from transgenic corn and from naturally occurring Bt was more abundant in the sediment than in the surface water. The cry1Ab transgene was detected as far away as the Richelieu and St. Lawrence rivers (82 km downstream from the corn cultivation plot), suggesting that there were multiple sources of this gene and/or that it undergoes transport by the water column. Sediment-associated cry1Ab gene from Bt corn tended to decrease with distance from the Bt cornfield. Sediment concentrations of the cry1Ab gene were significantly correlated with those of the cry1Ab gene in surface water ($R = 0.83$; $P = 0.04$). The data indicate that DNA from Bt corn and Bt were persistent in aquatic environments and were detected in rivers draining farming areas. (Douville et al., 2007)

Reading this abstract one could be convinced that growing corn along waterways will pose a long term problem. But reading a bit more in the original text reveals several serious uncertainties about the abstract statements – and – environmental concentrations were low, and it is not even sure that the Bt proteins were not produced by aquatic organisms in situ or whether they stem from Bt crops [or from Bt sprays].

The increased presence of the cry1Ab transgene but not the cry1Ab gene from Bt in May before sowing of Bt corn seed was of concern, as it suggests that the transgene survives over the winter months. Whether the transgene was associated with decaying corn roots, sediment particles, bacteria, or other microorganisms remains to be established.

DNA was produced in extracellular environments by bacteria in aquatic microcosms. Genetically altered strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, and *Bradyrhizobium japonicum* were able to produce extracellular nucleic acids where the production and release of extracellular (i.e., cell-free) DNA were more strongly influenced by physicochemical factors than by biotic factors (Paul & David, 1989). Furthermore, rates of extracellular DNA production were greater in fresh than in marine waters, and ambient microbial populations were able to utilize readily materials released by these organisms. However, the levels of Cry1Ab protein in our samples were below the detection limit most of the time, although it was detected at concentrations ranging from 0.1 to 1 ng/g or ng/ mL in sediment and surface water, respectively (Douville et al., 2005). This finding suggests that environmental concentrations were low but the null hypothesis (i.e., that the cry1Ab transgene was not expressed in aquatic environments) cannot be accepted at the present time and further research and monitoring efforts are required.

Final remarks: Instead of focussing on the possible contamination of waterways near Bt maize fields, despite the fact that rapid degradation of the Bt toxins has been proven, we recommend to set the the priority to check more precisely about pesticide residues and biocides finding their way into rivers and lakes, here just two examples of publications which point to serious problems and unsolved questions:

Nawrocki, S. T., K. D. Drake, C. F. Watson, G. D. Foster and K. J. Maier (2005). "Comparative aquatic toxicity evaluation of 2-(thiocyanomethylthio)benzothiazole and selected degradation products using *Ceriodaphnia dubia*." *Archives of Environmental Contamination and Toxicology* **48**(3): 344-350.<Go to ISI>://000228103100008 AND <http://www.botanischergarten.ch/Pesticides/Nawrocki-Comparative-Aquatic-2005.pdf>

2-(Thiocyanomethylthio)benzothiazole (TCMTB) is a biocide used in the leather, pulp and paper, and water-treatment industries. TCMTB may enter aquatic ecosystems during its manufacture and use. TCMTB is environmentally unstable; therefore, it is important to evaluate the toxicity of the more persistent degradation products. This study compared the toxicity of TCMTB with its degradation products 2-mercaptobenzothiazole (2-MBT), 2-(methylthio)benzothiazole (MTBT), benzothiazole (BT), and 2-hydroxybenzothiazole (HOBT). Toxicity was determined using *Ceriodaphnia dubia* 48-hour acute and 7-day chronic test protocols. TCMTB was the most toxic compound evaluated in both the acute and chronic tests with EC50s of 15.3 and 9.64 μ g/L, respectively. 2-MBT, the first degradation product, was the second most toxic compound with acute and chronic EC50s of 4.19 and 1.25 mg/L, respectively. The toxicity of MTBT and HOBT were similar with acute EC50s of 12.7 and 15.1 mg/L and chronic EC50s of 6.36 and 8.31 mg/L, respectively. The least toxic compound was BT with acute and chronic EC50s of 24.6 and 54.9 mg/L, respectively. TCMTB was orders of magnitude more toxic than its degradation products. Toxicity data on these benzothiazole degradation products is important because of concerns regarding their release, degradation, persistence, and non-target organism effects in aquatic ecosystems. (Nawrocki et al., 2005)

Pillard, D. A., J. S. Cornell, D. L. Dufresne and M. T. Hernandez (2001). "Toxicity of benzotriazole and benzotriazole derivatives to three aquatic species." *Water Research* **35**(2): 557-560.<Go to ISI>://000166466700027 AND <http://www.botanischergarten.ch/Bt/Pillard-Toxicity-Benzotriazone-2001.pdf>

Benzotriazole and its derivatives comprise an important class of corrosion inhibitors, typically used as trace additives in industrial chemical mixtures such as coolants, deicers, surface coatings, cutting fluids, and hydraulic fluids. Recent studies have shown that benzotriazole derivatives are a major component of

aircraft deicing fluids (ADFs) responsible for toxicity to bacteria (Microtox(R)). Our current research compared the toxicity of benzotriazole (BT), two methylbenzotriazole (MeBT) isomers, and butylbenzotriazole (BBT). Acute toxicity assays were used to model the response of three common test organisms: Microtox(R) bacteria (*Vibrio fischeri*), fathead minnow (*Pimephales promelas*) and water flea (*Ceriodaphnia dubia*). The response of all the three organisms varied over two orders of magnitude among all compounds. *Vibrio fischeri* was more sensitive than either *C. dubia* or *P. promelas* to all the test materials, while *C. dubia* was less sensitive than *P. promelas*. The response of test organisms to unmethylated benzotriazole and 4-methylbenzotriazole was similar, whereas 5-methylbenzotriazole was more toxic than either of these two compounds. BET was the most toxic benzotriazole derivative tested, inducing acute toxicity at a concentration of less than or equal to 3.3 mg/l to all organisms. (Pillard et al., 2001)

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