Are Bt toxins killing aquatic insects ?

Klaus Ammann, open link version 20090806

THE INCIDENT:

In an article of PNAS (Proceedings of the National Academy of Sciences, USA) it is reported that aquatic organisms are potentially harmed by residues and toxins of Bt maize

Rosi-Marshall, E.J., Tank, J.L., Royer, T.V., Whiles, M.R., Evans-White, M., Chambers, C., Griffiths, N.A., Pokelsek, J., & Stephen, M.L. (2007)
Toxins in transgenic crop byproducts may affect headwater stream ecosystems. Proceedings of the National Academy of Sciences of the United States of America, 104, pp 16204-16208
open link: 10.1073/pnas.0707177104 AND http://www.pnas.org/cgi/content/abstract/0707177104v2 AND
http://www.botanischergarten.ch/Bt/Rosi-Marschall-Bt-Aquatic-2007.pdf
The abstract:
"Corn (Zea mays L.) that has been genetically engineered to produce the Cry1Ab protein (Bt corn) is resistant to lepidopteran pests. Bt corn is widely planted in the mid-western United States, often adjacent to headwater streams. We show that corn byproducts, such as pollen and detritus, enter headwater streams and are subject to storage, consumption, and transport to downstream water bodies. Laboratory feeding trials showed that consumption of Bt corn byproducts reduced growth and increased mortality of non-target stream insects. Stream insects are important prey for aquatic and riparian predators, and widespread planting of Bt crops has unexpected ecosystem-scale consequences."

Comment

The news of potential harm of Bt crops to aquatic organisms has spread rapidly on many websites, Greenpeace also supports the arguments of the authors, and the EU commissioner for the environment Stavros Dimas is opposing the new maize traits from Pioneer and Syngenta on grounds of the precautionary principle and referring to a comment by Greenpeace on the above study: .
http://uk.reuters.com/articlePrint?articleId=UKL2524238420071025

Comments and letter to the editors of PNAS by panel of scientists

A consortium of scientists signing this comment in a letter to the editors below has analyzed the paper and came to critical conclusions, which seriously question the conclusions of the paper.

*"We are deeply concerned by the appearance in PNAS of a recent article, "Toxins in transgenic crop byproducts may affect headwater stream ecosystems," (**10**,1073 (2007)), by (Rosi-Marshall et al., 2007) apparently funded by NSF. We recognize that it is not unusual for papers to be published with minor flaws or infelicities, even after peer review and revision, but the article by (Rosi-Marshall et al., 2007) contains egregious methodological flaws and omissions, and presents conclusions not supported by the data.*
We call your attention, in particular, to the following:

1) There is extensive evidence in the literature that corn pollen produced by currently available Bt corn varieties contain extremely low amounts of Bt toxin. This was shown in a series of six papers by top scientists published in PNAS after the Losey Bt corn pollen-Monarch debacle, an intensive and time-consuming effort to try to set the science straight (Hellmich et al., 2001; Oberhauser et al., 2001; Pleasants et al., 2001; Scriber, 2001; Sears et al., 2001; Stanley-Horn et al., 2001; Zangerl et al., 2001). How many busy scientists and how much scarce money will we need to divert to calm this new scare?

2) The authors extrapolated from a laboratory test to a field system based on a single study. Such extrapolation is problematic to begin with; not only did the authors lack the statistical confidence necessary for a valid extrapolation, in another venue (Pokelsek et al., 2007) they reported they did not find these effects in the field [including also Hydropsyche borealis], a salient fact not mentioned in the PNAS paper. This discrepancy should have been disclosed and discussed. In addition, earlier relevant studies concluded that Bacillus thuringiensis (Bt) endotoxin concentrations in aquatic systems are extremely low and are metabolized rapidly in water (Douville et al., 2007; Douville et al., 2005).

3) The title implies transgenic crops are the only source of Bt toxins, but endotoxins in commercial Bt insecticides such as Dipel, Xentari, Foray, and Thuricide are also used by farmers, including organic farmers, to control insects, and in some areas intensively. If the authors are measuring the effect of Bt toxin at all, how do they know the toxin comes from the transgenic Bt crops rather than from these organic Bt insecticides? If they lack data to distinguish the sources, isn't the term `transgenic' in the title simply gratuitous and sensationalistic?

4) The authors seem unaware that there are several variant forms of Bt endotoxin, as they failed to disclose which one(s) they were seeking and measuring. Toxicological studies use known quantities of known toxins, and look for a dose response. If their study included specific assays, they were not reported. If they were not conducted, the report was, at best, premature.

5) The authors do not disclose which Bt-corn isolines were tested. Different hybrids can differ significantly in both secondary metabolites and in antinutrient quantity (as well as in kind and amount of Bt toxin expressed). By not using isolines, they could have been seeing the effect of different concentrations in different hybrids of anti-nutrients or of other factors unrelated to Bt toxin. Similarly, the authors do not disclose quantitative measurements of tissue sampled, e.g., "Leaves were added… as needed." This lack of detail precludes others from replicating their study.

6) The authors conclude that growing Bt-corn may cause downstream adverse effects in waterways, but they fail to consider alternative explanations. Moreover, they analyze their results in a vacuum. In the real world, the choices are not `Bt-corn' versus `no intervention', and to imply that that is the case displays a remarkable ignorance of agriculture. Farmers grow more than one species and cultivar, and often use more than one pesticide strategy. For example, if a farmer were to control insects using conventional pesticides (that is, absent Bt corn plants), how would those pesticidal treatments affect caddisflies? For all we know, Bt corn may be environmentally preferable to traditional pesticides or other strategies to control insects. The authors imply otherwise without providing the comparative evidence.

The points above illustrate sloppy experimental design and interpretation that should have been detected by even a cursory peer review. Where were the crucial qualitative and quantitative data on source tissue, distinction of diverse types of Bt toxins, and discussion of alternate explanations for their results? We are at a loss to explain how qualified reviewers and editors could be unaware of flaws of this magnitude. Publication of this flawed paper has seriously jeopardized the credibility of PNAS as a high quality, scientific forum.

Sincerely,
Alan McHughen, Professor, University of California, Riverside.
Brian Federici, Professor, University of California, Riverside.
Henry Miller, M.D., The Hoover Institution, Stanford University.
Klaus Ammann, Prof. emerit. Delft University of Technology, the Netherlands
C. Kameswara Rao, Professor. Foundation for Biotechnology Awareness and Education,

Bangalore, India.
Prof. Dr. Ingo Potrykus, Chairman, Humanitarian Golden Rice Board & Network
Dr. Piero Morandini, Dept. of Biology, University of Milan, Italy
C. J. Leaver, CBE, FRS, FRSE, Sibthorpian Professor of Plant Science,
University of Oxford, UK
S. Shantharam, Director, Biotechnology Education Programs, Asian Institute of Technology, Bangkok, Thailand
Mark Sears, University of Guelph, Ontario, Canada.
C. S. Prakash, Professor, Plant Molecular Genetics, Tuskegee University, USA"

Citations within the letter are moved to the end of the full text of the ASK-FORCE contribution.

Two of the undersigned have also written to the journal with similar contents, (Beachy et al., 2008; Parrott, 2008) and an answer has been published by (Rosi-Marshall et al., 2008), where the authors admitted that due to methodological flaws the results cannot be addressed properly to the environmental impact of Bt maize.

Beachy, R.N., Fedoroff, N.V., Goldberg, R.B., & McHughen, A. (2008)
The burden of proof: A response to Rosi-Marshall et al. Proceedings of the National Academy of Sciences, pp 0711431105
openlink: http://www.pnas.org AND http://www.botanischergarten.ch/Bt/Beachy-Rosi-Marshall-Burden-2008.pdf

"To the Editor: A recent paper in PNAS (1) purports to show that insect-resistant crops have unexpected effects on nontarget insects in streams. A sentence in the Abstract reads ''Stream insects are important prey for aquatic and riparian predators, and widespread planting of Bt crops has unexpected ecosystems-scale consequences.'' The data presented in the paper do not support this statement. Because previous studies reported no significant effects on caddisflies (Glare & O'Callaghan, 2000), 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: (i) 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. (ii) 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. Publications that report studies lacking appropriate controls and include unfounded summary statements on a topic such as this can cause significant damage. It is unfortunate that this paper, like the previous claim of effects on Monarch butterflies (Hellmich et al., 2001; Losey, 1999) is being used to fuel the contentious debate over the safety of genetically modified crops." (Beachy et al., 2008)

Parrott, W. (2008)
Study of Bt impact on caddisflies overstates its conclusions: Response to Rosi-Marshall et al. Proceedings of the National Academy of Sciences, pp --
http://www.pnas.org AND http://www.botanischergarten.ch/Bt/Parrott-Rosi-Marshall-2008.pdf

"To the Editor: Ecological studies can help ensure that new biotechnologies provide maximum benefit while minimizing detrimental effects. Accordingly, a recent study (1) published in PNAS is appropriate but lacks the genetic and toxicological components necessary for proper execution and interpretation. The study used different maize hybrids. Because all maize hybrids differ in many traits, any trait that differs between the hybrids, e.g., the level of trypsin inhibitors present, could easily explain the results. Because isogenic lines were not used, it is impossible to attribute the observed effect to Bt as opposed to any other factor that differed.
The study assumed that pollen from currently grown Bt maize contains toxic levels of Bt when the levels in pollen are negligible (Mendelsohn et al., 2003) and innocuous (Hellmich et al., 2001). The presence and type of Bt toxin was never verified or quantified. If any Bt was present, the level administered to the larvae is unknown. Yet, dose–response measurements are key to

establishing toxicity. Even if their results were really due to Bt, it is impossible to extrapolate with any confidence from an aquarium to a whole ecosystem where many more variables come into play. Given these limitations, the conclusion that ''widespread planting of Bt crops has unexpected ecosystem-scale consequences'' is untenable. The data cannot even support the more tentative conclusion that ''Bt corn byproducts may have negative effects,'' because no cause and effect was shown specific to Bt." (Parrott, 2008).

The answer of E. Rosi-Marshall: (Rosi-Marshall et al., 2008):

"Beachy et al. (Beachy et al., 2008) and Parrott (Parrott, 2008) have questioned some findings reported in our recent paper (Rosi-Marshall et al., 2007); here, we respond to issues raised by these authors. All tissues identified as ''Bt'' in our paper (Rosi-Marshall et al., 2007) were verified to contain Cry1Ab protein by using Bt Cry1Ab protein Immuno-Strips (Agdia materials identified as ''non-Bt'' were similarly confirmed to lack Cry1Ab protein.

The quantity of Cry1Ab protein actually consumed (in pollen or leaf tissue) by an individual insect could not be determined because of variation in feeding rates among individuals in any particular experiment. Our goals for the research did not include developing a traditional dose–response relationship *because (i) the dose depended on individual feeding rates, and (ii) a dose–response relationship would have little relevance in assessing the effect of Cry1Ab containing materials on actual stream ecosystems in which organisms select among multiple food resources, not all of which would contain Cry1Ab protein. The goal of our feeding experiments was to determine whether trichopterans were at all susceptible to the effects of Cry1Ab protein, not to determine a safe level of exposure in a toxicological context. Growth of trichopterans can be affected by many factors, including nutritional quality of food resources. As we stated (Rosi-Marshall et al., 2007) we paired ''Bt'' and ''non-Bt'' materials on the basis of nutritional quality (carbon: nitrogen ratios and lignin content). The use of isogenic hybrids would have resulted in food resources of different nutritional quality (Saxena & Stotzky, 2001b) and Cry1Ab content, and this would have confounded the experiments.* ***We cannot fully disregard the unlikely possibility that some other leaf constituent was responsible for observed differences between the ''Bt'' and ''non-Bt'' treatments****. However, we argue that the presence or absence of Cry1Ab protein is the most likely explanation for observed differences in trichopteran growth and mortality. We encourage others to pursue further research to develop a broader body of knowledge on the effects of Cry1Ab protein on aquatic insects.*

We agree that extrapolation from laboratory experiments to ecosystems is unjustified without supporting evidence from field measurements*. We (Rosi-Marshall et al., 2007) presented several lines of evidence suggesting that Cry1Ab-containing materials could potentially affect headwater stream ecosystems: (i) inputs of corn pollen and detritus to streams were documented and quantified, (ii) trichopterans collected from streams contained pollen in their guts or often were found associated with decaying corn detritus, and (iii) laboratory feeding trials indicated trichopterans are susceptible to the effects of Cry1Ab.* ***Further study may reveal that the potential for detrimental effects is not realized in situ in streams or that effects are limited spatially or temporally and thus may not outweigh the benefits associated with the planting of Bt corn****—only further study will reveal whether this is the case. Regarding the concern of Beachy et al. (1) and Parrott (2) that the final sentence of our abstract overstated the conclusions of the paper, we agree that the sentence should have articulated the potential for ecosystem-scale consequences within streams, rather than suggesting that such consequences were observed in situ.*

Lastly, Beachy et al. imply that our publication (3) and statements therein could ''cause significant damage.'' We are unsure what Beachy et al. believe to have been significantly damaged. We argue that the wise use of any new technology requires a full understanding of both the benefits and the potential costs. In the case of corn genetically modified to express the Bt _-endotoxin, the environmental costs appeared not to have been fully assessed, and we believe the studies we reported (Rosi-Marshall et al., 2007) contribute to a better understanding of potential effects on aquatic ecosystems. (Rosi-Marshall et al., 2008).

In their reply (Rosi-Marshall et al., 2008) admitted a few critical points and call for more research. However, their generalizations at the end are not justified, since they do not take into account recent literatue about Bt toxin impact on aquatic systems, and the *potential* effects described in this article merit further research from the point of view of basic research, but they clearly belong into the category of the "nice-to-knows", not really relevant to modern agriculture, since one really important problem of the original paper is its lack of a true baseline comparison, namely the situation *in reality* that one has to compare Bt maize fields and non-Bt maize fields with all its implications, and this means that a comparative toxicological research should include many other factors, such as fertilizer, pesticides and other cultivation factors, which would then lead to a more holistic view of the whole issue. This is certainly not the case in the paper of (Rosi-Marshall et al., 2007), which is also acknowledged by the authors themselves.

There is no space here for an extensive literature review on comparative experiments related to the ecological impact of Bt toxins, here just one typical example with abstract, it relates to soil biota:

Griffiths, B.S., Caul, S., Thompson, J., Birch, A.N.E., Scrimgeour, C., Andersen, M.N., Cortet, J., Messean, A., Sausse, C., Lacroix, B., & Krogh, P.H. (2005)
A comparison of soil microbial community structure, protozoa and nematodes in field plots of conventional and genetically modified maize expressing the Bacillus thuringiens is CryIAb toxin. Plant and Soil, 275, 1-2, pp 135-146
<Go to ISI>://000233381600013 AND http://www.botanischergarten.ch/Bt/Griffiths-Comparison-protozoa-2005.pdf

"Field trials were established at three European sites (Denmark, Eastern France, South-West France) of genetically modified maize (Zea mays L.) expressing the CryIAb Bacillus thuringiensis toxin (Bt), the near isogenic non-Bt cultivar, another conventional maize cultivar and grass. Soil from Denmark was sampled at sowing (May) and harvest (October) over two years (2002, 2003); from E France at harvest 2002, sowing and harvest 2003; and from SW France at sowing and harvest 2003. Samples were analysed for microbial community structure (2003 samples only) by community-level physiological-profiling (CLPP) and phospholipid fatty acid analysis (PLFA), and protozoa and nematodes in all samples. Individual differences within a site resulted from: greater nematode numbers under grass than maize on three occasions; different nematode populations under the conventional maize cultivars once; and two occasions when there was a reduced protozoan population under Bt maize compared to non-Bt maize. Microbial community structure within the sites only varied with grass compared to maize, with one occurrence of CLPP varying between maize cultivars (Bt versus a conventional cultivar). ***An overall comparison of Bt versus non-Bt maize across all three sites only revealed differences for nematodes, with a smaller population under the Bt maize. Nematode community structure was different at each site and the Bt effect was not confined to specific nematode taxa. The effect of the Bt maize was small and within the normal variation expected in these agricultural systems****." (Griffiths et al., 2005).*

In essence this paper demonstrates the considerable effort which is necessary to come to true comparison within agricultural reality, the paper (Griffiths et al., 2005) works with randomized field plots, is extended over two seasons with observations already starting the year before, and makes sure that every possible effort is made to take into account as many relevant agricultural and environmental factors as possible, thus assuring that the results have maximum agricultural and ecological relevance.

The German decision about the Bt maize MON810 influenced by the paper

Also Minister of Agriculture of Germany, Ilse Aigner, did not hesitate to use this paper in her scientific justification of the rejection of the MON810 Bt Maize in Germany.

This premature decision is full of errors in its argumentation and does not really reflect the status of scientific research on the biosafety of Bt Maize,
http://www.botanischergarten.ch/Bt/BLV-BUND-mon_810_bescheid-20090417.pdf

Therein the comments on the impact of Bt toxins on aquatic ecosystems are likewise premature and they are based also on a superficial analysis of the original paper of Rosi-Marshall. German:

„Rosi-Marshall et al. (2007) wiesen nach [sic], dass beim Anbau von transgenem Mais Köcherfliegenlarven Bt ausgesetzt sein können. Es wurde auch gezeigt, dass bei Bt-Pollen-Dichten, die in der gleichen Größenordnung lagen wie sie im Feld vorliegen [sic], Köcherfliegenlarven auf Cry1Ab empfindlich reagierten (höhere Sterblichkeit und bis zu 50% längere Entwicklungszeiten). Trichoptera (Köcherfliegen), die Teil der meisten aquatischen Ökosysteme sind, spielen eine Hauptrolle in aquatischen Nahrungsnetzen und können in den meisten Binnengewässern gefunden werden."

English:

“Rosi-Marshall et al. (2007) demonstrated [sic] that due to the growing of transgenic maize larvae of caddisflies can be exposed to Bt. It was also demonstrated, that the caddisfly larvae reacted sensitively to the Cry1Ab Bt toxin concentrations similar to those in the field [sic] (higher mortality and prolonged developing times up to 50%. Trichoptera (caddisflies) are part of most aquatic ecosystems and can be found in most headwater streams.”

Contradiction by the experts on biotechnology of the German government

The argument about aquatic organisms allegedly harmed by Bt toxins is clearly contradicted (among many other false, pseudoscientific arguments in this decision) by a report published beginning of July: http://www.botanischergarten.ch/Bt/BLV-ZKBS-MON810-20090707.pdf

The paragraph referring to the Rosi-Marshall paper is fully cited in German and English:

„(Rosi-Marshall et al., 2007):

Die Autoren haben Eintrag und Transport von Pollen und Ernterückständen aus Bt- und Nicht-Bt-Maisbeständen in feldnahe Gewässer gemessen. Zusätzlich wurden Pollen oder Rückstände von Mais, der das Bt-Protein exprimiert, in Laborversuchen an die sich detritivor ernährenden Larven der Köcherfliegenart Lepidostoma liba sowie an die sich filtrierend ernährenden Larven der Köcherfliegenart Hydropsyche borealis verfüttert. Im Ergebnis führte die Aufnahme von Bt-Mais-Pflanzenmaterial zu einer geringeren Wachstumsrate bei L. liba und die Aufnahme von Bt-Mais-Pollen zu einer erhöhten Mortalität bei H. borealis.

Die Verwendung von Köcherfliegenlarven als Testorganismen für das lepidopteren-spezifische Cry1Ab-Protein ist nachvollziehbar, da Köcherfliegen in relativ enger Verwandtschaft zu Schmetterlingen stehen. Effekte des Cry1Ab-Proteins auf Köcherfliegen wären somit denkbar.

Allerdings weist die Arbeit von (Rosi-Marshall et al., 2007) erhebliche methodische Schwächen auf. So wird die Quelle des Bt-Mais-Pollens und des Nicht-Bt-Mais-Pollens nicht angegeben. Unklar bleiben auch Sorte, Linie bzw. Isogenität der Bt-Mais-Einträge. Dosis-Wirkungsbeziehungen, wie für toxikologische Untersuchungen üblich, werden für das Bt-Protein nicht erhoben. Außerdem wurde bei den Untersuchungen zum Eintrag von Pollen und Maisrückständen in die feldnahen Gewässer versäumt, deren Bt-Protein-Gehalte zu messen. Es wurden keine eindeutigen Unterschiede zwischen den Zersetzungsraten von Bt- und Nicht-Bt-Maisabfällen festgestellt. Es fehlen weiterhin Informationen zur potenziellen Exposition der Köcherfliegenlarven gegenüber dem Bt-Protein im Gewässer. Lediglich aus den in einer Abbildung gemachten Angaben zum Eintrag von Bt-Pollen in die Gewässer und den bekannten Gehalten von Bt-Protein in Maispollen lässt sich überschlägig kalkulieren, dass ein sehr geringer jährlicher Eintrag (9 – 90 ng/m² Wasseroberfläche) besteht. Auch die jährliche Menge an eingetragenem Pflanzenmaterial ist mit max. 8 g/m2 als gering einzuschätzen. In beiden Fällen sind die entsprechenden Mengen an Bt-Protein, auch bei kurzfristigem zeitlichen Auftreten der Expositionsquellen (Blühphase), angesichts des sogleich einsetzenden Proteinabbaues als vernachlässigbar für Köcherfliegenlarven BVL Stellungnahme der ZKBS zur Risikobewertung von MON810 einzustufen (Douville et al., 2007; Douville et al., 2005).

Fragen werfen auch die Fütterungsversuche mit der Köcherfliegenart L. liba auf. Die Herkunft der Bt-Maisblätter und der Nicht-Bt-Maisblätter wurde ebenfalls nicht angegeben. Es ist jedoch sicher, dass als Nicht-Bt-Variante nicht die Blätter einer isogenen Maislinie verwendet wurden. Begründet wird dies mit der Studie von (Saxena & Stotzky, 2001b), die bei ihren Versuchen in den Blättern der isogenen Bt-Linie einen zwischen 33 bis 97% höheren Ligningehalt fanden. Nach Auffassung der Autoren verschlechtert der höhere Ligningehalt die nutritive Qualität der Blätter. (Rosi-Marshall et al., 2007) wählten zum Vergleich daher Blätter einer anderen Maislinie mit einem im Vergleich zum Bt-Mais vermeintlich ähnlichem Ligningehalt und C/N-Verhältnis aus. Allerdings werden keine quantitativen Angaben über die Inhaltsstoffe (Lignin, C/N oder weitere) der in den Labortest verwendeten Maispflanzen gemacht. Aufgrund der nicht angegebenen Herkunft und der fehlenden Charakterisierung des verwendeten Pflanzenmaterials ist nicht auszuschließen, dass sich die in den Fütterungsversuchen verwendeten Bt- und Nicht-Bt-Pollen bzw. Bt- und Nicht-Bt-Blätter nicht nur im Hinblick auf die Anwesenheit von Bt- Protein unterschieden. Zudem wurde die Konzentration von Bt-Protein im Blattmaterial nicht bestimmt. Auch die in den Fütterungsversuchen verabreichten Blattmengen wurden nicht angegeben (Zitat: „Leaves were added to aquaria as needed“).

Die fehlende Standardisierung ist ein kardinaler Mangel, der vor allem für die von den Autoren durchgeführten Laboruntersuchungen gilt. Unabhängig davon misst die ZKBS den beobachteten in vitro Effekten auf Köcherfliegenlarven trotz u.U. großer Mengen in Gewässer eingetragenen Bt-Maismaterials geringe Bedeutung zu. Gründe für die Einschätzung sind folgende: Die natürliche Exposition von Köcherfliegenlarven gegenüber dem Bt-Protein in Gewässern, die an Bt-Maisfelder *grenzen, ist sowohl räumlich (Abstand zu Gewässern und Verteilung von Maisfeldern in der Landschaft) als auch zeitlich (kurze Blütezeit) begrenzt. Darüber hinaus wird die potenzielle Exposition der Wasserorganismen erheblich eingeschränkt durch die geringen Mengen und Konzentrationen des Bt-Proteins im Pflanzenmaterial sowie dessen vergleichsweise raschem Abbau in Gewässern. Die im Labor beobachteten Mortalitätseffekte wurden nur bei unnatürlich hoher Exposition und nur einer Spezies gefunden. Zwar wiesen die Autoren im Falle von der Köcherfliegenart H. borealis eine signifikant erhöhte Mortalität bei Verfütterung von Bt-Maispollen im Vergleich zu Nicht-Bt-Maispollen nach, doch lag die Pollenmenge zwei- bis dreimal höher als der maximal gemessene jährliche Polleneintrag in ein Gewässer. In den Versuchen mit der Köcherfliegenart L. liba wurde keine erhöhte Mortalität bei Fütterung mit Pflanzenmaterial von Bt-Mais festgestellt, jedoch eine verminderte Wachstumsrate. Angesichts der in der Natur zeitlich beschränkten Exposition (Blühzeit des Mais) bei gleichzeitig meist niedrigerer Bt-Konzentration am Wirkort ist auch die im Laborexperiment gezeigte verminderte Wachstumsrate als nicht relevante Umweltwirkung einzustufen.*

Fazit:

Die ZKBS stellt fest, dass in den Untersuchungen an Köcherfliegenlarven von Rosi-Marshall et al. (2007), nicht eindeutig der kausale Zusammenhang zwischen Bt-Protein oder der gentechnischen Veränderung und negativen Wirkungen hergeleitet wurde. Die Studie wurde auch von anderen Autoren hinsichtlich ihrer Durchführung und der getroffenen Schlussfolgerungen kritisiert ((Beachy et al., 2008); (Parrott, 2008). Die Autoren räumen selbst ein, dass sie nicht ausschließen können, dass Unterschiede zwischen den verwendeten Maissorten und nicht das Bt-Protein Ursache der beobachteten Wirkungen sind (Rosi-Marshall et al., 2008). Darüber hinaus stellt die ZKBS fest, dass die von (Rosi-Marshall et al., 2007) in Laborexperimenten erhaltenen Ergebnisse unter Berücksichtigung der anzunehmenden Exposition unter Freilandbedingungen nicht relevant sein dürften. Diese Schlussfolgerung wurde von den Autoren in ihrer Replik ebenfalls erwogen (Rosi-Marshall et al., 2008).

English:

The authors have analyzed input and transport of pollen and detritus from Bt and non- Bt maize fields into nearby headwater rivers. In addition pollen and detritus of maize expressing Bt toxin was fed to detritivorous larvae of the caddisfly Lepidostoma liba as well as the larvae of Hydropsyche borealis with its filtering feeding habits. The result was a lower growing rate with L. liba by taking up Bt maize detritus and a higher mortality with H. borealis by the uptake of Bt pollen.

The experimental use of the larvae of caddisflies bares some logic, since they are relatively close relatives of Lepidoptera, and effects on the larvae are therefore imaginable.

However, the work of (Rosi-Marshall et al., 2007) shows considerable methodological weakness: the pollen source of the Bt- and non-Bt maize remains unknown – and further on the traits, lines, respectively it is also unclear whether the experimental input is isogenic or not. And there is no dose-effect relationship established as it is usually done with all toxicological research.

Furthermore, quantitative data on the pollen and maize detritus input of Bt proteins. There were no clear cut differences in the decay of Bt- and non-Bt maize materials. In addition information is missing on the potential Bt protein exposure of caddisfly larvae in the water. Only from the indications contained in one figure it is possible to calculate approximately a very low yearly Bt-protein input (9-90ng /m^2 of water surface. Also the yearly input of detritus is with max. 8g/m^2 to be estimated as very low. In both cases the respective amount of Bt protein, with regard to the short flowering phase and with regard to the immediately starting decomposition can be estimated as negligible for caddisfly larvae (Douville et al., 2007; Douville et al., 2005). Also the feeding experiments with the larvae of the caddisfly L. liba should be questioned: the origin of the leafs of Bt- and non-Bt maize again has not been given. On the other hand it is certain, that the material of non-Bt maize is not isogenic. This is justified with a paper of (Saxena & Stotzky, 2001b) who found a 33% to 90% higher lignin content, which means a lower nutritive value of the leafs due to lower digestibility. This is why (Rosi-Marshall et al., 2007) chose leafs of another line of maize with a seemingly similar lignin content and C/N ratio. However, quantitative data on ingredients (lignin, C/N ratio or others) of the maize used in the experiments. Due to missing data on origin and characterization it cannot be excluded that the Bt- and non-Bt Pollen, respectively Bt- and non-Bt maize leafs contain also differences other than those regarding Bt proteins. In addition, the Bt protein content of the maize leaves has not been determined. Further on the amount of leafs added to the aquariums has not been indictated: (citations: "leaves were added to the aquaria as needed").

The missing standardization is a cardinal mistake mainly referring to the laboratory experiments of the authors. Apart from those facts the ZKBS does not regard the observed in vitro effects to be important, even though the input of maize Bt material could be rather high. This for the following reasons: The exposition of caddisfly larvae in nature with Bt protein in the waters adjacient to maize fields is spacially limited (distribution of maize fields in the landscape) and temporally limited (short flowering time).

Further on the potential exposition of aquatic organisms is considerably limited due to the low amounts and concentration of Bt protein and its comparably rapid decomposition in the water.
The mortality values found in the lab were only possible due to unnaturally high concentrations and only found with one species. It is true that the authors demonstrated with the caddisfly larvae of H. borealis a significanly higher mortality in the feeding experiments with Bt maize pollen compared to non-Bt pollen, but the amount of pollen was 2-3 times higher than the maximum pollen input in the water. The experiments with the caddisfly larvae of L. liba did not result in higher mortality, but in a lower growth rate. In the face of the short exposition time and at the same time lower concentration in nature the lower growth rate in the laboratory experiment can be neglected as an environmental impact.
Conclusion:
The ZKBS emphasizes that in the experiments with larvae of caddisflies of (Rosi-Marshall et al., 2007) the causal connection between Bt proteins or the genetical transformation of maize and its negative impact could not be demonstrated. The study has also been criticized by other authors regarding methodology and conclusions: (Beachy et al., 2008; Parrott, 2008). It has also been admitted by (Rosi-Marshall et al., 2008) in their reply that they cannot exclude the differences of the used maize lines to be responsible for the effects described in the original paper. Again, the ZKBS emphasizes that the laboratory results obtained by (Rosi-Marshall et al., 2007) are not relevant, confronted with the reasonably accepted environmental exposition in nature – a conclusion which has also been drawn by (Rosi-Marshall et al., 2008).

EFSA rebuttal of Rosi-Marshall paper on Bt-impact on Aquatic Organism

Also the EFSA (EFSA, 2007) commented and refuted the paper of (Rosi-Marshall et al., 2007) with clear words:

"In summary, the conclusions of the paper Rosi-Marshall et al. (2007) 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."

More critical remarks about the paper Rosi-Marshall 2007

In vitro mortality of L. liba fed with Bt toxins at realistic concentrations found in the environment is lower than when fed with non-Bt pollen

An additional comment to figure 3 in the paper about the potential ecological effects of *L. liba*. The mortality of *L. liba* when fed with mean ambient concentrations *are lower for Bt maize* compared to non-Bt maize – a fact which is not mentioned in the paper: B: left black column non-Bt.

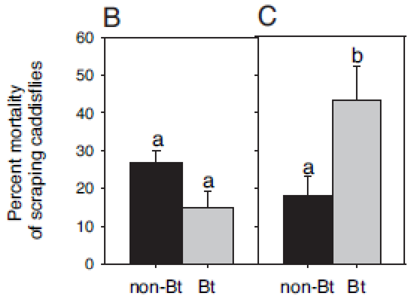

Fig. 3. Potential ecological effects of Bt corn. (*A*) Growth rates of the shredding caddisfly *L. liba* fed non-Bt and Bt corn leaves ($P = 0.008$, Student's *t* test). (*B* and *C*) Mortality rates of the scraping caddisfly *H. borealis* when fed non-Bt and Bt corn pollen at mean ambient concentrations (0.055 g m^{-2}) ($P = 0.42$, Tukey's post hoc test) (*B*) and high concentrations (2.75 g m^{-2}) ($P = 0.059$; Tukey's post hoc test) (*C*). Error bars represent standard errors, and significant differences are indicated by different letters.

Fig. 3 from (Rosi-Marshall et al., 2007)

Field research of the same research group reveals other results than in vitro experiments:

In a short conference abstract there are some interesting details communicated:

Chambers, C.P., Whiles, M.R., Griffiths, N.A., Evans-White, M., Rosi-Marshall, E.J., Tank, J.L., & Todd, V.R. (2007)

Assessing the impacts of transgenic Bt corn detritus on macroinvertebrate communities in agricultural streams. In North American Bentological Society, 55th Annual Meeting NABS 2007. NABS http://nabs.confex.com/nabs/2007/techprogram/P1698.HTM AND http://www.botanischergarten.ch/Bt/Chambers-NABS-Assessing-Impacts-2007.pdf

Full abstract:

"Streams draining agricultural landscapes may receive significant inputs of crop detritus, including transgenic materials containing Bacillus thuringiensis (Bt) δ-endotoxin proteins. However, potential influences of these materials on stream invertebrate communities have received little attention. We quantified crop detritus inputs to streams draining both Bt and traditional corn fields in Indiana and assessed potential impacts on macroinvertebrates using quantitative field sampling, litterbag studies, and laboratory feeding trials. Crop detritus comprised up to 40% of allochthonous litter entering these streams during fall harvest. Laboratory experiments showed that Lepidostoma, trichopterans that are related to the lepidopteran targets of Bt toxins, grew slower when fed Bt corn (instantaneous growth = 0.022 d-1) compared to traditional corn (0.049 d-1) (p=0.049). ***However, trichopteran biomass and total shredder biomass was similar in Bt and traditional corn litterbags, and there was no difference in decay rates of Bt and traditional corn litter. Macroinvertebrate communities were similar between the two stream types, and trichopterans were poorly represented in all streams (1% of total biomass). Results demonstrate that Bt corn detritus can slow growth of shredding caddisflies, but in situ it did not have significant adverse effects on invertebrates in these highly degraded streams."*** *(Chambers et al., 2007).*

The litter bag experiments which already come close to true field experiments, demonstrate on the caddisfly larvae of *Lepistoma* (the same organism as used in the PNAS publication) no significant adverse effects of Bt toxins in these highly degraded streams **IN SITU**. This conference paper (Chambers et al., 2007) is not mentioned in the PNAS paper (Rosi-Marshall et al., 2007), nor are there results mentioned obtained with the litter bag (not litter trap) method.

Bacillus thuringiensis occurs in nature in many different substrates and environments.

(Damgaard et al., 1997) isolated *Bacillus thuringiensis* from the phylloplane of cabbage foliage. The same authors have previously shown (Pedersen et al., 1995) that spores of *B. thuringiensis* serovar *kurstaki* can readily be dispersed from soil to the lower leaves of cabbage plants. Therefore they could expect that the population studied now would not differ from that normally found in soil.

The relatively high proportion of isolates from the phylloplane with lepidopteran activity both in the studies above and in that of (Smith & Couche, 1991) is in contrast to the findings of most surveys on the natural occurrence of *B. thuringiensis* in soil, which have shown 'non-toxic' strains to be the most common types (Hastowo et al., 1992; Ohba & Aizawa, 1986a, b; Ohba & Aratake, 1994). The serotyping of the isolates in (Damgaard et al., 1997) showed that the majority of the isolates belonged to serovar kurstaki. Isolation of *B. thuringiensis* from soil has shown to contain a very diverse population of serovars, but never with a frequency of the insecticidal serovar kurstaki above 50% (Delucca et al., 1981; Ohba & Aratake, 1994; Rongsen et al., 1990).

The high frequency of lepidopteran-active serovar *kurstaki* isolates found on foliage in this study indicates that the (natural!) population of *B. thuringiensis* on phylloplane is different from that normally found in soil. It is therefore likely that the phylloplane population is not exclusively the result of transfer of soil bacteria to the foliage. Apparently some kind of propagation and/or selection of the *B. thuringiensis* population takes place on the phylloplane. *Bacillus thuringiensis* was also discovered on the surface of clover and other phylloplanes (Bizzarri & Bishop, 2007).

In soils *Bacillus thuringiensis* is ubiquitous: In a selection approach, (Travers et al., 1987) using a high acetate medium to isolate Bt semi-selectively from soil and obtained over 8000 isolates. They claimed that these isolates represented some 73 new biochemically distinct varieties of Bt. (Martin & Travers, 1989) found the insect control agent *Bacillus thuringiensis* to be a ubiquitous soil microorganism. They isolated *B. thuringiensis* in 785 of 1,115 soil samples. These samples were obtained in the United States and 29 other countries. A total of 48% of the *B. thuringiensis* isolates (8,916 isolates) fit the biochemical description of known varieties, while 52% represented undescribed *B. thuringiensis* types. Over 60% (1,052 isolates) of the isolates tested for toxicity were toxic to insects in the orders *Lepidoptera* or *Diptera*. This kind of ubiquitous occurrence was again confirmed by (Jouzani et al., 2008) and (Haddad et al., 2005) who verified that 77%, 78% and 80.5% of the effective doses (viable spores) remained on the leaf surface after the first day of external Bt treatment, respectively.

In a recent comprehensive review Swiecicka (Swiecicka, 2008) widens the picture of natural occurrence of *Bacillus thuringiensis* and its close relatives: While much is known about the taxonomic properties and molecular basis for virulence of *Bacillus thuringiensis* and *Bacillus cereus*, comparatively less is known about their ecology in natural environments. Thus, there are limited data regarding their resilience, i.e.

recycling of vegetative and sporulated phases of growth in soil, ecolgical niches including symbiotic interactions with other organisms, and the impact on ecosystems in which they proliferate.

Nevertheless, based on recent data, apicture is beginning to emerge that *B. thuringiensis* and *B. cereus* are capable of establishing mutual and commensal relationships with both animals and plants. In this

regard, these bacilli can proliferate in the digestive tracts of animals, where upon defecation they form dormant spores in the soil, and to a lesser extent on the phylloplane and rhizospheres of plants.
Bacillus thuringiensis has been found in many more and diverse habitats (Federici, 1999), such as animal feces, sludge etc. (Hwang et al., 1998; Lee et al., 2003; Mizuki et al., 2001; Okumura et al., 2001; Yu et al., 1991).
(Federici & Siegel, 2007) summarize the enormous complexity of more than 70 varieties and subspecies of *Bacillus thuringiensis*, there are more than 100’000 isolates that occur among the plasmids and insecticide protein complements detected in the Bt isolates. 120 different types of genes are encoding Cyt proteins, and at least 12 different types of genes encode Cyt proteins having been cloned and sequenced up to now.
Usually, each type of Cry protein has an extremely limited target spectrum (*lepidopteran, dipteran, coleopteran, nematodes*), and each specific protein like Cry1Ac is always much narrower than the type as a whole, and even within a target category such as *lepidoptera* there can be marked differences from species to species: Cry1Ac is highly toxic to *Heliothis virescens*, but non-sensitive to *Spodoptera exigua.*
From this paragraph it is obvious, that the source of Bt toxins for such research on aquatic organisms has to be studied carefully, and with the used detection methods in the paper of (Rosi-Marshall et al., 2007) the source of Bt proteins remain unclear.

Conclusion and Summary

Unfounded concerns about accumulation of Bt protoxins from GM crops in Water and soil

Aquatic systems

(Douville et al., 2007) tested the short time persistence of Bt proteins in aquatic systems. 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. However, the authors also refer to their own previous study (Douville et al., 2005), where the results showed that Btcorn 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 13C and 15N 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*. This will say that Bt protoxins are highly unlikely to cause any environmental problems in aquatic systems. As a whole, the publications of Douville et al. are anyway not convincing, because they lack an important scientific quality: the baseline comparison is totally lacking. As an example: There are several publications from the same river system, such as (Tall et al., 2008) and many others which clearly point to metal and phosphorus contamination of the river sediments, causing negative effects to the fauna and flora.
Critical reference is given to the paper of (Rosi-Marshall et al., 2007) on the occurrence of Bt protein in headwater stream ecosystems, written in an unnecessary alarming style and not even confirmed with hard field data in the chapter on non-target insects of this report. There is not even a hint on the nature of the Bt toxin (it could well be at least partially of natural origin), and when you compare her own (!) figure 3 B the graph with realistic concentrations, then you see that Bt shows a clearly lower mortality of the scraping caddisflies experiment – so what?? And again it shows, like the work of Douville, the unfortunately widespread sin in agricultural science of a lacking a proper baseline comparison.

Soil systems

The whole question on persistence of Bt toxins in soil is treated in a separate ASK-FORCE contributioin. There are again, after a first wistle blower phase (Saxena et al., 1999), enough long term studies to demonstrate that accumulation does not take place to a degree that it could harm soil organisms, here just as an example two papers: (Head et al., 2002; Saxena & Stotzky, 2001a)

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