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How much Bt toxin do genetically engineered MON810 maize plants actually produce?

Bt concentration in field plants from Germany and Spain

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Table of Contents

Executive Summary

In the growing season 2006, Greenpeace took leaf samples of commercially cultivated MON810 maize plants in Germany and Spain to determine the Bt toxin (Cry1Ab) concentration. A total of 619 samples from 12 fields were analysed using ELISA tests.

MON810 maize is genetically engineered to produce a modified insecticide (Cry1Ab) that naturally occurs in the soil bacterium *Bacillus thuringiensis* (Bt). The production of this toxin is supposed to protect the maize plants from European corn borer larvae (ECB, *Ostrinia nubilalis*).

This Greenpeace study shows a surprising pattern of plants that contained only very low Bt toxin levels. However, high levels could be observed in some plants. The variation found on the same field on the same day was considerable, and could differ by a factor of as much as 100. This is in agreement with the results of a new study published in April 2007[1] that concludes that "the monitoring of Cry1Ab expression [of MON810 plants] showed that the Cry1Ab concentrations varied strongly between different plant individuals."

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

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

1. The variation of Bt concentrations

Since the Bt concentration on the field can vary greatly even between neighbouring plants, the MON810 plants do not appear to be sufficiently stable in their biological traits. The reasons for the high variation in Bt contents could be related to genetic or environmental factors (e.g. weather or soil conditions), or both. Nguyen & Jehle (2007) not only found high variation between plants on a field, but also statistically significant differences between different locations in Germany. Since the reasons for such differences and the range of variation cannot be identified, the commercial cultivation of the crops should be stopped to avoid interactions with the environment that could lead to adverse and unpredictable effects.

To investigate these questions further, studies should be conducted under contained conditions (such as glasshouse experiments) to study the environmental effects (e.g. drought, moisture, temperature, soil, nutrients) on the plants. Next to no studies of this type have yet been published.

2. The risk assessment of the plants

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

3. The actual Bt toxin concentrations

If the Bt toxin in GE Bt plants were more effective in considerably lower concentrations than previously described, this would not be identical with the naturally occurring Bt toxin. This would annul a central aspect of the EU cultivation approval, which is based on the assumption that the Bt toxin in plants could in general be equated with the natural Bt protein from soil bacteria.

[1] Nguyen & Jehle 2007.

However, if the toxin is not effective in such low concentrations as we have recorded, then serious concerns about the effectiveness of the plants in controlling ECB larvae need to be raised. Additional problems would then also concern insect resistance management, as resistance development could be accelerated by sub-lethal toxin doses.

4. The methods for determining Bt concentrations

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

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

Background

An overview of the publicly available scientific literature on Bt maize MON810 shows that the actual Bt concentration that is produced by MON810 plants is largely unknown today – even after more than 12 years of commercial cultivation in the US, and 10 years after MON810 was granted cultivation approval in the EU. Detailed data on the Bt production of MON810 plants were first published in April 2007, shortly before this report was finished.[2]

It is now known that different parts of the plant produce different levels of Bt toxin, and that there are differences in Bt production throughout the season. However, it is not yet known how high these different levels actually are or what influences them. A comparison of studies[3] even shows contradictory findings on the trends of decrease or increase of Bt concentration during the vegetation period in different plant tissues. A study by scientists at the US Environmental Protection Agency (EPA) even comes to the conclusion that different parts of a single leaf produce different Bt levels.[4] Most studies on Bt concentrations have however been performed with Bt maize Bt176 which has by now been taken off the market, among other reasons reportedly due to the high variability of its Bt toxin production.

Nguyen & Jehle (2007) conducted their study on Bt production as part of a three-year project financed by the German Federal Ministry of Education and Research (BMBF) on "Post-market safety research on transgenic maize with new Bt genes". They came to the conclusion that „the monitoring of Cry1Ab expression showed that the Cry1Ab concentrations varied strongly between different plant individuals. According to the end report of the project[5], there were significant differences in Bt concentration between plants grown in two different locations in Germany (Halle and Bonn). They came to the conclusion that the toxin concentration varied seasonally as well as between different parts of the plant, and that the toxin concentrations differ in part considerably from those known from publications from the US, even though Nguyen & Jehle (2007) conclude that their results confirm the trend of these publications. According to the authors, all in all the average toxin concentration of leaves in the three years of the study was about 32-40% lower than those they used for comparison from the literature[6] (see also Table 2).

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[2] Nguyen, H.T. & Jehle, J.A. 2007.

[3] see for example Dutton et al. 2004, Abel & Adamczysk (2004), Mendelsohn 2003, as well as a number of studies on MON810 expression in other tissues or on Bt176 Bt expression.

[4] Abel,C. A. & Adamczyk, J.J. 2004.

[5] Jehle (no date given): End report.

[6] Nguyen, H.T. 2004.

The risk assessment of MON810 plants is usually based on the implicit assumption that MON810 on average produces specific (high) Bt concentrations, and that small amounts of Bt toxin are less dangerous than large amounts. Furthermore, the risk assessment is based on the assumption that Bt production is so uniform that small numbers of samples and short or medium-term studies are sufficient. These assumptions need to be questioned. This also leads to more far-reaching questions concerning risk assessment, monitoring and variety approval procedures.

To clarify these questions, Greenpeace took MON810 plant samples from fields in Germany and Spain in 2006 and had their Bt concentrations measured.

Published values of Bt concentrations

Even today, more than a decade after the first commercial growing of MON810 maize, there are next to no scientific publications about the Cry1Ab expression in MON810.[7] The few available data come from Monsanto, describing the deregulation procedure in the US and the cultivation approval in the EU. There are a few studies in which specific parts of the plant such as stems, anthers or roots were studied.[8] It is worth noting that the Bt concentration of roots, pollen and anthers were first studied only after MON810 was already approved for commercial cultivation in the US and the EU. What is usually not studied is *why* the different plant parts produce different Bt levels, and why different Bt concentrations in different parts of an individual plant are not correlated.[9] Risk assessment studies investigate some effects of the Bt plant on the environment, but next to no studies have been conducted on the effect of the environment on Bt plants.

In general there is a lack of data providing information on the average Bt production, the way in which Bt production develops during the growing season, or why different parts of the plant contain different levels of Bt. To some degree, these data are even contradictory. In general, Bt concentration is not measured continuously, but only on specific dates, for example to compare it to degradation levels after harvest.

In most studies the mean Bt concentration is given, in part with information about the standard deviation.[10] More detailed data about variations in Bt content owing to development stages, genetic conditions or environmental impacts are mostly lacking. This gives the impression that Bt plants usually produce Bt levels that are steady and consistent and more or less independent from environmental impact or specific genetic conditions.

Table 1 shows the data published by Monsanto, while Table 2 (p. 7) provides data from the scientific literature.

MON810 Bt concentrations according to Monsanto

As far as we know, the summarized data from a few locations in the US and Europe are the only Monsanto material publicly available. Monsanto's *Product Safety Description*[11] from March 2002 only lists a summary of the results of a small number of field trials over one or two years. These Bt concentrations were determined using ELISA but the actual protocol for the method is not published.

Most studies that refer to information from Monsanto, as well as the database of agbios.com[12], give an average Bt concentration in MON810 leaves of 9.35 µg Bt/g fw (standard deviation 1.03 µg,

[7] Nguyen & Jehle 2007

[8] Nguyen & Jehle 2007

[9] Nguyen 2004.

[10] The standard deviation is a statistic that describes how tightly the samples are clustered around the arithmetic mean in a set of data. One standard deviation away from the mean in either direction accounts for about 68% of the samples. Two standard deviations away from the mean account for roughly 95%.

[11] Monsanto 2002.

[12] agbios.com.

range: 7.93-10.34 µg Bt/g fw). Table 1 shows the original table as published by Monsanto in 2002. The Bt concentrations in four sets of trails range from 5.21-10.34 µg Bt/g fw in the US, and 7.59-15.06 µg Bt/g fw in the EU. The trials took place on between three to six sites, and it is not possible to deduce from the data whether some of the field trials were conducted over two years, or whether they took place over one year only on each site.

The trials show that on a changing number of fields Bt concentration was measured different plant tissues over one or two years. Surprisingly, Monsanto comes to the following conclusion:

> Cry1Ab protein levels were measured on samples from four different field trials: 1994 and 1995 trials in the U.S. and 1995 and 1996 trials in Europe. [...] The Cry1Ab protein levels in tissues collected from plants of YieldGard corn event MON810 have been consistent across several years of evaluation in the U.S. and Europe (Table 1). The consistency of Cry1Ab protein levels through years of breeding supports the stability of the insert, an important component of product performance.[13]

Plant tissue	Parameter	1994 US[1] (6 sites)	1995 US (5 sites)	1995 EU[1] (4 sites)	1996 EU (3 sites)
Leaf[2]	Mean	9.35	8.95	8.60	12.15
	Std. Dev.	1.03	2.17	0.74	3.86
	Range	7.93-10.34	5.21-10.61	7.59-9.39	7.77-15.06
Forage/ whole plant[3]	Mean	4.15	3.34	4.80	4.88
	Std. Dev.	0.71	1.09	0.75	0.52
	Range	3.65-4.65	2.31-4.48	4.11-5.56	4.32-5.34
Grain[2]	Mean	0.31	0.57	0.53	0.41
	Std. Dev.	0.09	0.21	0.12	0.06
	Range	0.19-0.39	0.39-0.91	0.42-0.69	0.35-0.46
Overseason leaf[4]	(1st) Mean 9.78	(2nd) Mean 8.43	(3rd) Mean 4.91		

Table 1: Original table from Monsanto on the Bt concentration of MON810 plants:

Cry1Ab Protein Levels in YieldGard Corn Plants (µg/g fwt issue). [1] US is United States; EU is European Union. [2] The mean was calculated from the analyses of plant samples from each field site. [3] For the 1994 US trials, values represent the analysis of whole plants; for the remaining trials, values represent the analysis of forage tissue. Whole plants were collected two weeks after pollination; forage samples were collected at the soft dough or early dent stage. Means were determined from the analysis of plant samples from one site in the US and all sites in the EU. A plant sample was a pool of two individual plants. [4] Mean of a pooled leaf sample collected at two week intervals, from V4 stage until pollination, at one site.

Note: The table contains no data on Bt concentrations in either roots or pollen. Source: Monsanto 2002. Safety assessment of YieldGard insect-protected event MON810. Published on agbios.com[12]

[13] Monsanto 2002.

Bt concentrations in Germany (BMBF project)

Nguyen & Jehle (2007) studied the Bt concentration of Bt maize plants as part of the German project on Bt maize financed by the BMBF (2001-2004). Their results are currently published in different formats and with different focuses.[14]

They determined the Bt concentrations of MON810 and Bt176 plants over three years on locations in Bonn and Halle, but unfortunately not at the other locations of the project as well, where a number of risk assessment studies have taken place. The MON810 variety used was Novelis. Nguyen & Jehle (2007) found the highest Bt concentrations in the leaves of the plants. For MON810 the average Bt concentration ranged across the season between 2.4-6.4 µg Bt/g fw in the top leaf, and 3.8-5.7 µg Bt/g fw in the bottom leaf during the four development stages tested. The authors came to the conclusion that the Bt concentration in the leaves significantly increases during the growing season, and is highest in the ripening stage, with Bt contents approximately twofold[15] or 1.5 to 4 times[16] higher than at the beginning of the growing season.

Comparing the three years, the trend in Bt production was lower in 2002 than in 2001 and 2003. The plants in both locations showed a similar pattern in the production of different Bt levels in different parts of the plant.

Nguyen (2004) also found that the Bt concentrations in different plant tissues are not correlated.

The authors' main conclusion is that there is an increase of Bt contents in the leaves towards ripening as well as considerable variation in the expression levels of Bt toxin. However, they also found significant differences between the two fields. The Bt levels in plants in one field (Halle) were predominantly higher than those from the other field (Bonn). Even though both locations are in Germany, they differ considerably in their weather and other abiotic conditions.[17] In 15 out of 17 tissue samples, the mean Bt contents of plants growing in Halle were higher then those detected in Bonn,[18] with differences of 6-49%.[19] The increase in Bt levels during the vegetation period was most distinct in the top leaf. The greatest difference between the two locations (49%) was in the top leaves.[20]

The summary of the results[21] therefore states:

> The toxin concentration fluctuates both seasonally and between different plant parts. In some cases, the measured toxin concentration values differ considerably from those familiar from similar experiments in the United States, but the general trends were confirmed. This finding highlights the importance of carrying out such experiments under local climatic conditions and with local varieties.

[14] The results of these studies are published in four different publications – however, the German and English summaries published on biosicherheit.de were not written by Ngyun and Jehle.

[15] Nguyen & Jehle 2007.

[16] Nguyen, H.T. 2004

[17] Jehle, May 2007. pers. communication.

[18] Nguyen & Jehle 2007

[19] Jehle (no date given)

[20] Nguyen & Jehle 2007.

[21] http://www.gmo-safety.eu/en/safety_science/31.docu.html

variety	leaf	year	coun-try	loca-tion	Bt concentration [µg Bt/g fw]	standard deviation	range [µg Bt/g fw]	source
		1994	USA	6	9.35	1.03	7.93-10.34	Monsanto: Product Safety Description
		1995	USA	5	8.95	2.17	5.21-10.61	
		1995	EU	4	8.60	0.74	7.59-9.39	
		1996	EU	3	12.15	3.86	7.77-15.06	
		1995	USA				5.2-10.6	US EPA
		1995	France				7.6-9.4	US EPA
		2003	review		10.34			Mendelsohn 2003
Novelis	top leaf	2001-03	Ger-many	2 (at 4 dates)	2.4-6.4	0.15		end report, Jehle
Novelis	top leaf	2001-03	Bonn	BBCH19	2.45	0.15	0.32-4.62	Nguyen & Jehle 2007
				BBCH30	3.24	0.24	0.70-6.59	
				BBCH61	2.72	0.21	1.24-4.52	
				BBCH83	5.52	0.24	3.59-8.60	
			Halle	BBCH19	3.33	0.19	1.96-4.71	
				BBCH30	4.62	0.29		
				BBCH61	5.06	0.36	1.96-8.58	
				BBCH83	6.37	0.44	1.88-11.07	

Table 2: Bt concentration of MON810 leaves in publicly available literature

Bt concentrations of different varieties

There are indications that different varieties of the same GE event produce different Bt levels due to the crossbreeding of the GE event into different backgrounds. An attempt to determine whether a new GE variety produces enough Bt toxin to control the target organism reliably therefore appears to be an essential part of the variety approval procedure. In fact, the Bt concentration not only has to be high enough to kill the target organism, but for the Insect Resistance Management (IRM) requirements, the Bt concentration needs to be 25 times higher than the amount needed to kill 99% of the target organisms.[22] (There is however no information on how much the absolute toxin concentration in MON810 would need to be in order to fulfil this criterion.)

On would therefore expect that the Bt concentration of MON810 and other Bt maize varieties would be measured as part of the variety approval procedure. In fact, Bt production is the key characteristic for which these new maize varieties are approved. Just as any other new variety approved under the UPOV criteria, a GE variety has to display homogeneity, be distinct from other varieties, and display its trait in a stable way. But (at least) in the German variety approval procedure, Bt concentration is not measured. Instead, it it is only determined indirectly in the laboratory where its efficiency against the target organism is tested. This means that no data for Bt concentrations are available for the variety trials.

Method

Sampling

Samples were taken between May and September/October 2006 on a weekly basis on two fields in Bavaria and on four fields in Brandenburg, as well as biweekly at five locations in Spain. As far as

[22] agbios.com. MON810 - Insect Resistance Management. http://agbios.com/cstudies.php?book=ESA&ev=MON810&chapter=IRM&lang=#. EPA 1999. EPA and USDA Position Paper on Insect Resistance Management in Bt Crops. http://www.agbios.com/docroot/articles/epa_irm.pdf

we know, MON810 was grown on the two fields in Bavaria for a feeding study. The locations in Brandenburg and Spain were used for commercial maize cultivation. In addition, samples were taken from a field trial in Borken (North Rhine-Westphalia) three times during a three-week period in July/August 2006.

The locations of the fields were known from information on official sites and from farmers, and confirmed by Cry1Ab stick qualitative ELISA TraitChek test strips from Strategic Diagnostics.[23] No plant material was sampled from the edge of the fields in case non-Bt maize had been planted as a border. The top (or second top-most) leaf was taken from five individual plants, resulting in more than 800 leaves. The leaves from each field were sent directly by courier to the lab for analysis. More than 600 samples were analysed.

The samples we took are not samples from plants that would have been grown for analysis under known and controlled conditions, but rather samples from plants as they were growing under actual conditions in summer 2006 from commercially available seed (with the exception of the field trial in Borken). They therefore represent the actual post-market plants and cultivation of GE event MON810.

Analysis of the Bt concentration

The Bt toxin in the maize leaf samples was quantified by the Ecostrat laboratory (in Switzerland) by Double Antibody Sandwich ELISA (DAS-ELISA), as described by Zwahlen et al. (2003).[24] (For more details, see Annex 1: ELISA)

Statistical analysis

A statistical analysis of the data was commissioned to clarify whether there are trends and/or statistical differences.[25]

Results

ELISA Protocol

During analysis, it became obvious that there is no standardised method for measuring the Bt (Cry1Ab) concentration of Bt plants, and that those ELISA protocols published differ.

Even though ELISA as such is a standard method, there is no standardised protocol for the determination of Bt toxin levels that would enable an inter-laboratory comparison of accuracy. It is therefore also not possible to know whether for example different sampling methods or slight differences in extraction methods[26] would lead to relevant differences in the result. Talking to scientists working in this field leads us to the conclusion that this problem is known but is rarely, if ever, voiced in the scientific literature.

Fresh weight or dry weight?

Another problem of the lack of standardisation is that there is no agreement on whether Bt concentrations should be determined in relation to the fresh or the dry weight of the plants. In most studies on this issue the Bt concentration is measured per g fresh weight, but sometimes even the information on whether it has been measured in fresh or dry weight is missing.

Fresh weight depends on the water concentration of the plants and thus also on local and seasonal conditions that can vary every year. Water concentration also depends on irrigation on the field, so

[23] www.sdix.com

[24] Zwahen, C., Hilbeck, A., Gugerli, P. & Nentwig, W. 2003.

[25] Statcon 2007. Statistical evaluation of Bt maize data

[26] Nguyen & Jehle (2007) for example describe a slight modification of the extraction protocols from the manufacturer's instruction of the Cry1Ab/Cry1Ac Quantiplate kit that they used.

that there could for example be differences between (small) well-managed field trials and large-scale commercial cultivation.

If no information is given on these abiotic factors, it then becomes difficult to compare the fresh weight data from different studies, even though the Bt concentration per fresh weight is likely to be an important factor for the non-target organisms on the field.

This problem was also mentioned in the BMBF project.[27] To compare the results from different summers, the authors related the Bt concentration of fresh weight to the dry weight of the samples. Unfortunately the Bt concentration for dry weight was not given, so that there are no available data that we could compare with our results.

In this study, Bt concentration was determined in both fresh and dried weight plant material. The data per fresh weight therefore allow some comparability with the data published in the literature, while the Bt concentrations per dry weight can be more reliably compared with the samples of this study. The differences between Bt concentrations in fresh and dry weight are shown in Table 4 (p. 12).

Variability of Bt concentrations in the field

The plants sampled showed in general very low Bt concentrations in the leaves, but a high degree of variation. The analyses of the leaf samples show very strong variability in Bt production on all fields. Bt concentrations were measured in a range from the detection limit (0.1 µg Bt/g fw) to high levels of more than 10 µg Bt/g fw. The highest Bt concentration of 14.8 µg Bt/ g fw was measured in Spain. On the test field in Borken the highest Bt concentration measured was as low as 3.4 µg Bt/g fw. Some plants were detected that did not produce any Bt in their leaves on all fields with the exception of Bavaria. On the fields in Brandenburg, 8% of the plants did not produce any Bt toxin. (For more details, see Table 3.)

High levels of above 10 µg Bt/g fw could be measured in leaves from the same day and field as low levels of 0.1 µg Bt/g fw. This means that two individual plants could show a hundred-fold difference in Bt concentrations.

However, although some individual plants were observed with high Bt content, in general the Bt concentration was low, with means generally below 2 µg Bt/g fw, with means reaching 2.08 to 4.50 µg Bt/g fw on only a few fields. Table 5 (p. 22) gives a detailed overview of the mean Bt concentrations and standard deviations of all fields per month.

The Bt concentrations can still be characterised as following a normal distribution, but right-skewed with the median below the arithmetic mean.[28] This means that more than half of the leaves sampled had Bt concentrations lower than the arithmetic mean. The higher mean is thereby caused by individual samples with high Bt concentrations that have a greater influence on the mean.

Pooled over the whole season, the means in all regions were lower then those of Nguyen & Jehle (2007) or Monsanto (2002). With the exception of the results from Borken, the median is lower, indicating that the majority of samples had Bt concentrations that were lower than the mean. More than half of all the plant samples from Bavaria analysed had a Bt content that was lower than 1.3 µg Bt/g fw. In Brandenburg this median was 0.7 µg Bt/g fw, and in Spain, 0.6 µg Bt/g fw.

Region	Number of samples	Mean	Median	Minimum	Maximum	Number of samples with no detectable Bt
		[µg Bt/g fw]				
Bavaria	116	2.2	1.3	0.1	10.9	0

[27] Nguyen 2004.

[28] The median describes that value below and above which 50% of all samples can be found.

Brandenburg	207	1.3	0.7	0	13.0	28
Spain	160	1.6	0.6	0	14.8	7
Borken	136	0.5	0.4	0	3.4	1
Monsanto		9.4 (8.6-12.2)		7.9 (5.21-7.6)	10.3 (9.4-15.1)	
Jehle & Nguyen (top leaf)		2.4-6.4		0.32	11.07	1

Table 3: Minimum and maximum Bt concentrations, by region.

Figure 1 shows the distribution of the Bt concentrations in the top leaf of MON810 plants in Bavaria. The data from two fields are pooled in this graphic for simplification because the fields were close together, and to our knowledge planted with the same MON810 variety and managed in the same way. (See Figure 3, p. 25 for details of each individual field.)

Figures 3 to 5 (Annex 2, p. 25) show similar impressions for individual fields in Bavaria, Brandenburg and Spain for each week. Similar results were also obtained when Bt concentration was measured in dry weight of the plant material.

Figure 2 shows the results of 136 leaf samples taken on three dates within three weeks from 12 plots at a test field in Borken. Here the Bt concentrations are much more uniform, albeit very low, with a mean of only 0.5 µg Bt/g fw (maximum Bt concentration: 3.4 µg Bt/g fw).

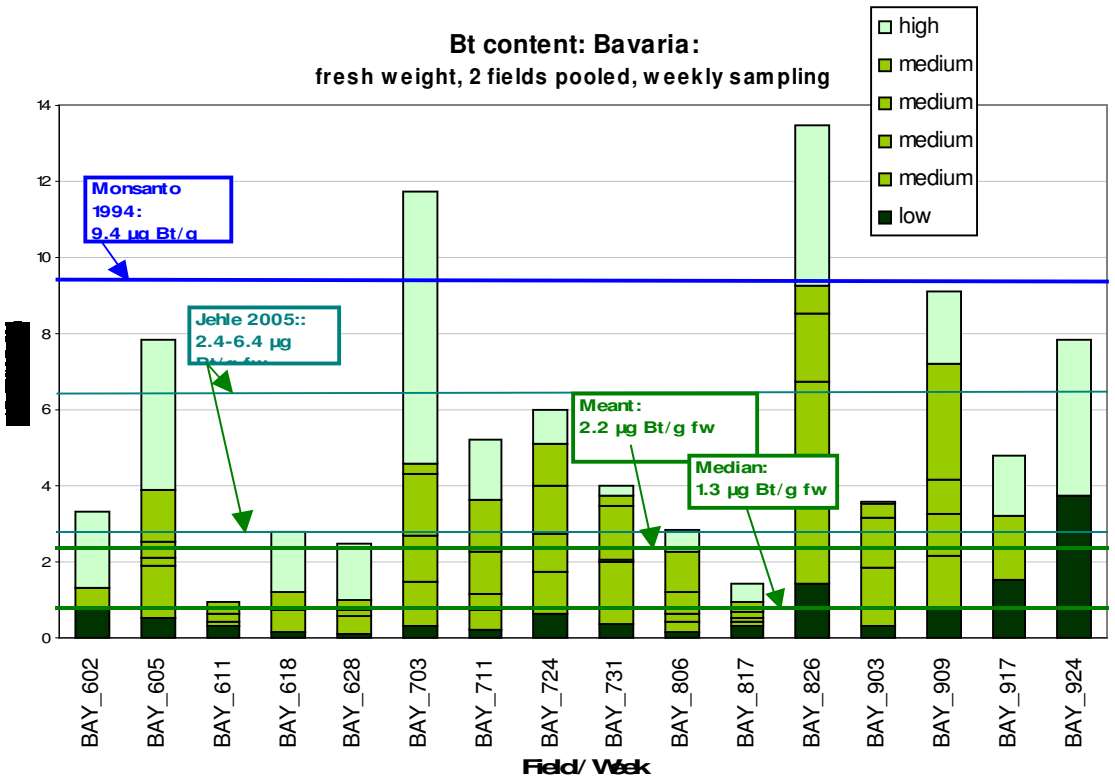

Fig. 1: Bt contents of MON810 leaves in Bavaria. Every bar shows the levels of six (or three) plants pooled from two fields and sampled onthe same day. Dates are indicated as month and day, e.g. BAY_602 was sampled on 2 June. The levels are stacked so that the lowest line (above the dark part of the bar) indicates the lowest Bt content of that day. The top end of the bar indicates the maximum value, while additional lines indicate the different levels measured in the other samples. See also Figure 3, p. 25.

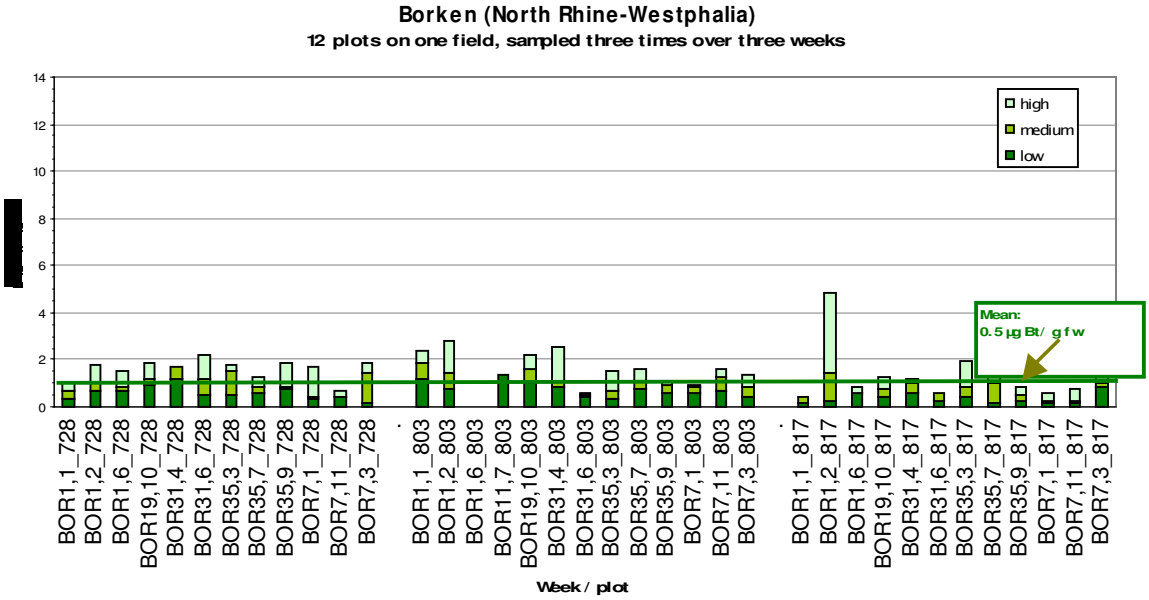

Fig. 2: Bt contents in MON810 leaves in Borken (North-Rhine Westphalia). Each bar shows the levels of three plants taken from one of twelve plots in the same field. Samples were taken on three dates within three weeks. The scale of the graphics is the same as Fig. 1. Compared to the results from the other fields, these plans produced a much more uniform Bt content with a mean average of only 0.5 µg Bt/g fw in these three weeks.

Differences between fields and during the cultivation period

The large number of samples taken during the cultivation period 2006 consist of small numbers of samples (three leaves) taken at short intervals (weekly or biweekly, on 17 dates). Other studies such as Nguyen & Jehle (2007), however, took a larger number of samples (16 samples) on fewer dates (four growth stages). Looking at these four growth stages, Nguyen & Jehle (2007) observe greater Bt production towards the ripening of the MON810 plants. In our investigation we see an increase in Bt content in July and August compared to May and June. For statistical purposes, May and June were classified as one season (S1, spring/summer), July and August as summer (S2) and September and October as fall (S3). With data pooled from all fields over the regions, then we can an increase from spring to summer and again a decrease towards autumn. This result should however be seen as preliminary since the weather conditions – with a hot dry July and a wet and fairly cold August - are not particularly representative of typical climate conditions in Germany during these months.

Plants without Bt toxin

Besides a number of plants with very low Bt content, we also found that in nearly 8% of all plant samples, mainly from Brandenburg, no Bt toxin could be detected. These samples were mainly taken in June and July 2007 (see Figure 4, p. 26). On the two fields in Bavaria, however, all samples contained some Bt toxin, even though sometimes only very small amounts (0.1 µg Bt/g fw).

According to Monsanto, about 2% of all plants do not produce Bt toxin.[29] This could be explained by the fact that commercially available seed is allowed to contain low levels of contamination with seed of other varieties.

Nguyen & Jehle (2007) found only one plant in which no Bt toxin could be detected. A number of other samples that were found to contain no Bt toxin had to be excluded as a sampling error.[30]

Our results however show that a large number of plants only produce very low Bt levels. These plants cannot be considered as exceptions from a field of maize plants that otherwise produce Bt toxin, but they are part of a continuum of plants: from plants that produce no Bt toxin (or only amounts under the detection level), to a high number of plants that produce little Bt, to a handful of plants that produce very high levels.

Bt content in relation to fresh and dry weight

In this study, the Bt content was measured in relation to both the fresh as well as the dry weight of the leaf material. As expected, the dry weight Bt concentration is higher. Table 4 shows a comparison of the medians of the concentrations. The Bt content in dry weight in our samples is on average about 2.5 to 3.5 times higher then the fresh weight Bt content.

Region	Median	
	[µg Bt/g fw]	[µg Bt/g dw]
Bavaria	1.3	4.5
Brandenburg	0.7	2.0
Spain	0.6	1.5
Borken	0.5	3.1

Table 4: Bt content of MON810 leaves from four different regions in Germany and Spain, by fresh and dry weight (fw, dw) of the plant material.

Discussion

Our data clearly reveal some unresolved issues and a lack of appropriate studies, and many of the questions raised so far are far from resolved. Nevertheless, the currently available data make it possible for a number of conclusions to be drawn and some additional questions to be asked.

High variability in the field

Unfortunately no original data are available on the approval procedure for MON810, only summarised data. The first, more detailed description of Bt concentrations in different plant tissues from different growing stages over three years was only published in April 2007.[31] The authors come to the conclusion that "the monitoring of Cry1Ab expression showed that the Cry1Ab concentrations varied strongly between different plant individuals". They go on to say that:

> Although our studies corroborate the tendencies of reported Cry1Ab contents of MON810,[32] a considerable variation in the expression levels of Cry1Ab was observed. The observed variation exceeds variation levels reported previously and may be due to the large number of analysed samples and different growing years. They suggest a certain plant to plant variation in Cry1Ab expression.

[29] Magg, T., Melchinger, A.E., Klein, D. & Bohn, M. 2001. Comparison of Bt maize hybrids with their non-transgenic counterparts and commercial varieties for resistance to European corn borer and for agronomic traits. Plant Breeding 120: 397-403.

[30] Jehle, May 2007, personal communication.

[31] Nguyen & Jehle 2007

[32] agbios 2001, Mendelsohn 2003

Nguyen[33] suspects that the high variability of the toxin expression could be caused by epigenetic effects. This effect could possibly be due to the number of copies of the inserted construct, the position of the insert, the strength of the expression of the 35S promoter, and environmental factors, mainly temperature.[34] Other studies suspect a correlation with photosynthetic activity.[35]

The considerable variability and differences of up to hundred-fold in Bt concentration (0.1 and 10 µg Bt/g fw on the same day in the same field) challenge the significance of trends and average Bt levels published in other studies.

Consider high and low Bt levels

The question whether high Bt concentrations are outliers caused by sampling or measuring errors or truly caused by biological factors is not merely a question about trends, but is of key biological relevance.

First of all, it is more likely that Bt concentrations will decrease due to degradation in the samples if there are sampling errors, so repeatedly occurring individual higher Bt contents should not easily be disregarded as outliers. But if they are real, then this has an impact on the non-target organisms in the field.

Target and non-target organisms in the field usually feed on a little amount of plant material, and some only feed on one plant. Individual plants with high Bt content could accordingly have considerable effects on individual organisms even if the average Bt content of all plants in the field is lower, and even if that average could be considered to be too low to cause adverse effects. High individual Bt levels must therefore not be disregarded as outliers. At the same time, low levels of individual plants are not outliers either, because the target organisms on them are subject to low and sub-lethal Bt concentrations.

We have to conclude that for organisms which only feed on a little amount of plant material or have a low level of mobility, average Bt content and trends (which could possibly be achieved with much higher numbers of samples) are not permissible for an environmental risk assessment. Due to the high variation of plants in the field, plants with high as well as low Bt levels need to be taken into account.

Is the gene expression unstable owing to environmental factors?

The more or less tacit assumption of the current approval and monitoring practice for Bt maize, and the expectation of the farms that grow it, is that a GE crop like MON810 should continuously produce consistent and similar concentrations of Bt toxin. However, our data and the data from Nguyen and Jahle (2007) show that this expectation does not really tally with the actual Bt concentrations in the leaves. To explain these findings, several scenarios can be offered, such as the impact of environmental factors. Yet there are next to no studies on the effects of such factors on the transgenic expression of Bt plants. For example, the transgenic expression could be down-regulated by variable degrees by environmental factors, thereby leading to high variation of Bt concentration in individual plants

The other possibility, however, is that the genetic modification itself might not be stable. MON810 is by now quite an old GE event, and it might be possible that the genetic modification has changed since it was first made and approved for cultivation. The original MON810 plants have been parent to a number of generations over more than a decade, and the event has been crossbred into different maize varieties. Possibly the transgenic construct has changed further, or maybe it is

[33] Nguyen 2004

[34] Moch 2004, cited in Nguyen 2004

[35] Abel & Adamczyk 2004

regulated differently in the background of different varieties. Different maize varieties might for example vary in the degree to which they are able to silence the transgenic construct.

Basically, our results as well as those of Nguyen & Jehle[36] contradict Monsanto's statement that "the consistency of Cry1Ab protein levels through years of breeding supports the stability of the insert"[37]. If Cry1Ab levels are not consistent, then there can be no support for the postulated stability.

Controlled test conditions vs. variable field conditions

The statistical analysis of the data shows that the variation in Bt concentration is high, and that some statistic differences can be detected. However, it was not possible to determine causes for these differences. Non-measured variables in our samples were, for example, weather and climate conditions, soil, fertilisation, water, agricultural practices and possibly variety differences. Both the "field" and "time" factors were therefore not determinant. In a laboratory study or greenhouse experiment, however, as many factors as possible are (at least attempted to be) fixed to be able to compare the effects of one or few factors. This is not possible when samples are taken from "real" fields that are representative of some of the actual cultivation conditions applied by different farmers under different conditions.

Scientifically there is clearly a lack of studies that investigate the environmental effects and possible stress effects on Bt plants under controlled conditions such as in greenhouse experiments. These controlled experimental cultivations would need inter alia to take into account different types of soil, climate conditions, certain stress conditions, different varieties and different agricultural practices, in order to assess their impact on the GE plants. So far the data for EU authorisation regarding Bt concentration in MON810 is derived from two years in which the plants were grown on three and four locations. However, cultivation approval is given for the whole of the EU (including new bioregions that when the field trials were conducted in 1994/95, were not even part of the EU).

Standardise the methods

There is no standardised method for determining the Bt content of MON810 in particular or for Bt crops in general. The data from different working groups that have for example undertaken research for risk assessments is therefore only comparable to a limited degree.

There are also no details available for the ELISA protocol that Monsanto used to determine the Bt levels as part of the cultivation approval. To obtain a cultivation approval in the EU, applicants need to provide a qualitative test protocol for identifying the presence of the GE organism, but they do not need to provide a quantitative protocol for determining the Bt concentration. However, for a reliable risk assessment and post-market monitoring of Bt crops, it is essential to have a standardised method for determining Bt concentrations.

There are differences not only in the actual test protocol (ELISA), but already in the question as to what should be measured. In general, the Bt content is given as µg Bt toxin/g plant material (or ng Bt/ mg), often for the fresh weight of the plant material. However, the fresh weight depends on the water content of the plant and on abiotic factors, so fresh weight concentrations are not easily comparable. In other studies the Bt content is given per dry weight, and some studies attempt to correlate Bt concentrations in fresh material through the dry weight of the plant.[38] Other studies calculate the Bt content in relation to the amount of plant material that target or non-target organisms consume. They therefore give the Bt concentration per cm² leaf surface.[39]

More than a decade after the first commercial approval of MON810 and other Bt plants in the EU, there is still no adequate method for determining the amount of Bt toxin released by plants into the

[36] Nguyen 2004, Jehle 2005, Nguyen & Jehle 2007

[37] Monsanto 2002

[38] Nguyen 2004.

[39] For example Dutten et al., 2005 or Manachini, B. et al. 2006.

soil, even though the question how long Bt toxin remains in the soil and whether these levels are relevant for non-target organisms has been repeatedly discussed. (At the moment the recovery rate of Bt toxin from the soil is below 40%.[40])

At the moment it is part of EU approval procedure that the applicant should provide a method for identifying the GE organism, for example to detect contamination. However, it is also essential to request a standardised method for determining the transgenic gene expression. In addition, the scientific question of what would be the best point of reference for determining Bt concentrations needs to be answered.

Publish existing data

Presumably in a handful of studies, the individual Bt content of MON810 must have been determined in order to provide summarised data (of mean averages and standard deviations). It is essential that these original data are published for it to be possible to estimate how variable the Bt expression of MON810 actually is. In this context, the data from the German BMBF project (2001-2004), in which Nguyen & Jehle (2007) observed differences over three years as well as significant differences between the two locations studied, are highly relevant. Data or samples from the follow-up projects that are currently taking place would also be highly useful.

Of special interest would be data from Monsanto. These must be considered as essential data for the risk assessment of EU Regulation 2001/18, and therefore need to be made publicly available.

Is MON810 actually effective against pests, or is the Bt toxin more potent than expected?

We observed such low Bt contents that we have to wonder whether these plants actually were (or would have been) able to control ECB infestations. The answer to this question has far-reaching implications.

Hypothesis 1: Low Bt concentrations fail to control ECB larvae or only do so incompletely

In general, it is assumed that MON810 plants effectively control ECB even if a few individual larvae can still be found in the fields. There are no reports that MON810 failed to control an ECB infestation in Germany in 2006. On the other hand, nor is there any comprehensive monitoring on whether the plants in the field and under commercial cultivation actually had the desired effect. Reports from farmers who grow MON810 are difficult to evaluate, if only because ECB infestation does not happen consistently or regularly.[41] In addition, the early summer of 2006 was so hot and dry in Brandenburg that the maize plants were extremely dry and whole fields did not grow properly. Some fields were harvested much earlier then usual so that it is rather difficult to say whether the MON810 plants in Brandenburg actually effectively controlled ECB infestation.

It is therefore possible that the very low Bt concentrations that we observed (especially in Brandenburg) were not sufficiently able to control ECB larvae. In Borken, where we measured very low Bt contents in July/August, ECB larvae are not present at all. If such low Bt contents are not effective against the target organism, then one also has to expect lesser effects on non-target organisms. For MON810 cultivation, this would mean that there are (unstable) GE crops in the field that might have only a limited effect on the target pest, and lesser effects on non-target organisms. This would make the entire cultivation of MON810 pointless, as the only reason to grow MON810 is Bt production and control of ECB larvae.

[40] Baumgarte & Tebbe 2004

[41] Plans of state authorities to monitor *Ostrinia nubilas* infestation in 2006 in Brandenburg were unfortunately not put into practice.

Production of low Bt levels without complete ECB control would also mean that there is no effective resistance management. In fact, low and sub-lethal Bt concentrations would even assist the development of resistance against Bt toxins in pests.

The chance of insect resistance developing is even higher if one takes differences in susceptibility of ECB individuals to Bt toxins into account. Preliminary results of a Europe-wide project show that the baseline susceptibility of ECB (*Ostrinia nubilalis*) populations can differ in individual European countries.[42] It is remarkable that even though the variation in susceptibility observed was rather high but still close to the reference strain from a German lab, the Spanish populations appeared to be much more susceptible. This raises the general question of whether Bt contents found to be sufficient in other countries or for other ECB populations are also high enough in other cultivation areas to guarantee the 'High dose/refuge' strategy of Insect Resistance Management.

MON810 plants with Bt content too low to control the target organism would also have consequences for risk assessment studies conducted using these plants. This will be further evaluated later (see below).

Hypothesis 2: Even low Bt levels in MON810 control ECB larvae.

If MON810 still successfully controls ECB despite the low Bt content as we observed, then this would mean that the Cry1Ab toxin produced by the MON810 plant in the form taken up by the ECB larvae is considerably more toxic than expected. This would also mean that the MON810 Cry1Ab toxin is more toxic than the one produced by GE bacteria and used as an isolated toxin for a number of non-target studies.

The toxins generally described as 'Cry1Ab' are in fact at least four different proteins. First of all there is the bacterial Bt toxin Cry1Ab that is naturally produced by *Bacillus thuringiensis* ssp. *kurstaki*. This protein has a size of 130 kDa and is sprayed in combination with other natural Bt toxins as a natural pesticide in organic agriculture. It only becomes an active toxin when it is degraded to a 60-65 kDa protein by specific enzymes in the gut of Lepidoptera.

Several Bt crops are genetically engineered to produced different, smaller forms of the Bt protein. MON810 produces an N-terminal fragment of 92 kDa, Bt176 maize another that is 65 kDa in size. These transgenic plant Bt toxins are smaller in order to anticipate activation by insect enzymes. The Bt toxin of GE plants is therefore fundamentally different from the natural Bt protein.[43]

Risk assessment studies with non-target organisms however often do not use GE plant material containing the Bt toxin, but yet another Bt toxin, one produced by GE *E.coli* bacteria. The GE bacteria have been engineered to produce Bt toxins of different sizes. The BMBF project in Germany used GE *E.coli* that produce a 130 kDa protein which was then artificially degraded and thereby activated using an enzyme (trypsin). This resulted in 60-65 kDa toxins that are different from the 92 kDa toxin in MON810.

For the sake of the risk assessment studies, it is assumed that these trypsinated, isolated GE bacterial toxin are identical to the different GE plant toxins - if not in size, then at least in effect. This assumption however needs to be questioned.

Newer studies show that lower Bt toxin levels cannot necessarily be equated with lesser effects when the Bt toxin comes from different Bt crops. In the BMBF project mentioned, the development of Sciaridae larvae was significantly delayed after feeding on MON810 pollen, but this effect could not be observed when they fed on Bt176 pollen, even though the Bt toxin content in Bt176 pollen is about 30 times higher than that of MON810 pollen.[44] A similar effect could be found in studies on the effect of Bt maize on monarch butterflies in the US. While early studies could not confirm acute toxicity of Bt176, long-term studies showed considerable negative effects of MON810 and Bt11

[42] Manachini, B. et al. 2006.

[43] Hilbeck, A. & Schmidt, J.E.U. 2006.

[44] www.biosicherheit.de/de/sicherheitsforschung/14.doku.hmtl

maize on the larvae. These Bt maize events have much lower Bt concentrations in pollen than Bt176, and it was therefore assumed that there would hardly be any effect from MON810.[45]

If the MON810 plants that we sampled with their very low Bt contents still controlled ECB larvae sufficiently, this would then question the assumed toxicity of the MON810 Cry1Ab toxin. The plant Bt toxin would then be more potent then expected.

Risk assessment studies?

The explanatory power of risk assessment studies depends on the effectiveness and the amount of Bt toxin in the plants used.

If our second hypothesis is correct and MON810 is more potent than expected, then studies conducted with GE bacterial Bt toxin are not sufficiently meaningful. All risk assessment studies would then have to be conducted with plant material instead.

However, our data also show that the Bt content in plants of the same variety, in the same field and on the same day can vary considerably. If a risk assessment study with plant material shows no negative effect, but if the Bt content of this material has not been determined, then the results of this study cannot sufficiently show that there are no adverse effects, because it simply has not been proven that the test organisms actually consumed the assumed amount of Bt toxin in their diet. Without a sufficient level of determination, it might simply have been that organisms not showing any effect simply fed on a Bt plant with a Bt level as low as 0.1 µg Bt/ g fw.

Inconsistent results

Adverse effects on non-target organisms and in feeding studies or changes in the composition of GE plants are repeatedly discarded because the observed effects are not consistent, for example because they were not recorded every year or at each location. However, the assumption that any 'valid' adverse effect would consistently be observed at nearly all locations or years or for nearly all test organisms is based on the assumption that Bt production is also consistent.

Our data show that there was no such consistent Bt production - at least not in the fields and on the times that we sampled. But without uniform plants, one cannot expect a consistent effect.

Consistent results can only be expected for those adverse effects that are caused by very low Bt levels. In all other cases, the inconsistent results that have been observed in a number of studies could also be caused by the varying amounts of Bt toxin that the test organisms received.

Conclusions

- The Bt concentrations in the Bt plants that we sampled and analysed do not tally with the Bt content stated in the documentation used for the approval for commercial growing of MON810. They are much lower then the means given by Monsanto as part of the summarised data, and they show a very high variation in the field. The majority of MON810 plants only produce low amounts of Bt toxin. Monsanto's original data on the Bt content of MON810 cultivated in Europe have not been published.

- MON810 plants show high variation in the field. There is clearly a lack of published data on this issue. This variation could be caused by environmental as well as epigenetic factors, but there are next to no studies of environmental effects on Bt plants or other GE plants.

- There is no standardised test protocol for determining the Bt content of Bt plants. The comparability of the results of different studies is therefore limited, and the Bt content of commercially available Bt maize varieties cannot be expressly determined.

- It is unclear whether the MON810 plants that we analysed were effective against ECB larvae, or whether the Bt toxin in MON810 might be much more potent than assumed, so that even

[45] Dively, G.P. et al. 2004.

such small amounts would have been toxic. If MON810 with low Bt content only incompletely controls the target organism, or even not at all, then the concept of Insect Resistance Management is flawed.

•All risk assessment studies on non-target organisms in which the Bt content or the effectiveness of the toxin has not been determined are meaningless, as it is unclear how much Bt toxin the test organisms actually received.

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Annex 1: ELISA Protocol

Double Antibody Sandwich ELISA Procedure (DAS-ELISA)

Ecostrat, Ökologische Technologiefolgenabschätzung und Umweltberatung: Hottingerstrasse 32, CH-8032 Zürich, Schweiz, Tel.: +41 (0)44 / 430 30 60, Fax: +41 (0) 44 / 430 30 61, Email: ecostrat@ecostrat.ch

Materials used

Material	Supplier
Universal extraction bags 12x14 cm	BIOREBA, Switzerland (www.bioreba.com)
Plant homogeniser (mounted to an electric drill)	BIOREBA, Switzerland (www.bioreba.com)
Nunc Immuno Plates MaxiSorp F95	BIOREBA, Switzerland (www.bioreba.com)
Polyclonal antibodies: Bt-Cry1Ab IgG and conjugate	BIOREBA, Switzerland (www.bioreba.com)
Lyophilized Cry1Ab-toxin	Dr. M. Pusztai-Carey, Case Western Reserve University, Cleveland, Ohio
PBST extraction buffer and washing solution	agdia, USA (www.agdia.com)
Mμlti®-reaction tubes (low binding)	Carl Roth, Germany (www.carl-roth.de)

Sample extraction

•Corn leaves were taken from the freezer (-20°C) just prior to analysis. Any dirt on the leaves was washed of with tapwater, and leaves dried with a paper towel.

•For sample extraction, approximately 200 mg leaf material was cut off from the tip of the leaf, weighed, and placed in a universal extraction bag (BIOREBA, Switzerland).

•3 ml of PBST was added to the leaf material and homogenised with the BIOREBA homogenizser mounted to an electric drill.

•1.75 ml homogenate was recovered from each extraction bag and pipetted into a 2 ml reaction tube. Tubes were centrifuged for 10 minutes at 12,000 RPM. The supernatant was transferred into a 1.5 ml reaction tube and used for the analyses.

•To determine dry weight, approximately 500 mg was cut off the same part of the leaf used in the analyses as per the extraction, and was then weighed and dried in an oven at 45°C until no further weight loss could be recorded (usually after two days).

ELISA Protocol

In general, *Bacillus thuringiensis* toxin in corn leaf samples was quantified by ELISA as described by Gugerli (1979 and 1986) and Zwahlen et al. (2003).

1. Coating of 1 ELISA plates (96 wells)

1.15.5 µl undiluted IgG was added to 25 ml coating buffer (carbonate: 50 mmol/l, NaN_3: 3 mmol/l, pH 9.6) and mixed thoroughly

2.220 µl of IgG coating solution was pipetted into each well

3.ELISA plate was covered with Parafilm® and incubated at 30°C for 4h30min.

2. Wash-off of coating buffer

1.A coating buffer was poured into a waste container

2.The plate was rinsed twice with H_2O deion

3.The plate – held upside down – was tapped firmly on a paper towel to remove the remaining drops of water from the wells.

3. Preparation of reference curve and samples

The reference curve using 100 mg/ml Cry1Ab standard solution (made from the lyophilised Cry1Ab toxin) was made in pooled homogenate from non-Bt corn leaves processed the same way as the Bt corn leaves (see 'Sample extraction' above). The following Cry1Ab concentrations were used: 0.1 µg/ml, 0.05 µg/ml, 0.02 µg/ml, 0.01 µg/ml, 0.005 µg/ml, 0.002 µg/ml, 0.001 µg/ml, 0.0005 µg/ml, 0.0002 µg/ml, 0.0001 µg/ml, and 0.00001 µg/ml.

1.200 µl/well of each sample homogenate and reference curve concentration was pippeted onto the plate in duplicates

2.The plate was covered with Parafilm® and incubated at 10°C for 16 h.

4. Wash-off of sample homogenate

1.After incubation, sample homogenates were poured into a waste container

2.The plate was rinsed four times with H_2O deion using a 12 channel pipette

3.The plate – held upside down – was tapped firmly on a paper towel to remove the remaining drops of water from the wells.

5. Preparation of the conjugate solution

The conjugate solution was prepared according to the following recipe:

0.05 g	bovine serum albumine
0.5 g	polyvinylpyrrolidone (PVP)
0.005 g	$MgCl_2$
12.5 µl	tween 20
25 ml	PBS (phosphate: 10 mmol/l, NaCl: 137 mmol/, KCl: 2.7 mmol/l, NaN_3: 3 mmol/l, pH 7.4)
16.5 µl	AP-labeled IgG (conjugated antibody)

6. Addition of the conjugate

1.200 µl of conjugate solution was pipetted into each well

2.The plate was covered with Parafilm® and incubated at 30°C for 5h30min.

7. Wash-off of conjugate solution

1.After incubation, the conjugate solution was poured into a waste container

2.The plate was rinsed 7 times with PBST wash buffer using a 12 channel pipette

3.After that, each well was filled with PBST and allowed to sit for at least a minute

4.The plate – held upside down – was tapped firmly on a paper towel to remove the remaining drops of buffer from the wells.

8. Addition of the substrate solution

1.The substrate solution was prepared as follows: 25 mg of 4-nitrophenyl phosphate diluted in 25 ml substrate buffer (DEA: 1 mol/l, NaN_3: 3 mmol/l, pH 9.8) and shaken for three minutes

2.Each well was filled with 200 µl substrate solution using a 12 channel pipette and the starting time was recorded.

9.Plate measurement

1.After color development, the plates were read on a Dynatch MR5000 microplate reader computer controlled by MikroWin 2000 software (Mikrotek Laborsysteme, Overath, Germany)

2.Obtained OD (optical density) values were analysed and Bt concentrations determined using the "four parameter" algorithm of the curve fit function of the MikroWin 2000 software.

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Annex 2: Descriptive statistics

Table 5: Tabulated fresh weight (fw) and dry weight(dw) by sampling month (month fieldsample) and field/country

			fw				dw		
Month fieldsample	country	field	N	Mean	Std Dev	CV	N	Mean	Std Dev
05.2006	Brandenburg	LEB	6	0.35	0.23	65.04	6	2.03	1.43
		PRA	6	0.00	0.00	71.84	6	0.02	0.02
		SEL	6	0.63	0.43	69.09	6	3.41	1.90
06.2006	Bavaria	FIN	19	1.98	2.20	110.85	19	7.12	6.18
		POI	13	088	0.84	95.19	13	2.95	1.93
	Brandenburg	LEB	12	0.19	0.20	106.94	12	0.87	0.98
		NTR	12	0.52	0.48	92.61	12	1.81	2.02
		PRA	8	0.24	0.53	220.07	8	0.86	1.78
		SEL	18	0.53	0.37	70.77	18	2.17	1.64
	Spain	AL1a	12	0.95	0.63	65.75	12	3.01	1.62
		AL1b	13	1.56	2.52	161.67	13	4.58	6.59
		BE1	15	1.29	1.73	133.54	15	3.10	3.24
		BE2	14	1.13	0.86	76.25	14	4.42	2.94
		BE3	15	1.39	2.09	150.42	15	4.45	6.34
07.2006	Bavaria	FIN	27	1.46	1.30	89.47	27	5.22	4.74
		POI	19	2.08	2.35	112.76	19	6.86	7.26
	Brandenburg	LEB	11	0.73	1.11	152.31	11	2.54	3.83
		NTR	13	1.24	0.92	74.40	13	3.61	2.49
		PRA	11	0.72	0.91	126.23	11	2.43	3.38
		SEL	19	1.03	0.99	95.93	19	3.57	3.35
	Spanien	AL1a	6	0.63	0.54	85.55	6	1.92	1.61
		AL1b	6	0.51	0.50	97.25	6	0.96	1.09
		BE1	6	0.82	0.80	98.14	6	2.31	2.37
		BE2	6	0.74	0.55	73.59	6	2.52	1.91
		BE3	5	0.39	0.49	125.56	5	0.85	1.13
	Borken	BOR1,1	3	0.71	0.33	46.18	3	2.48	1.19
		BOR1,2	3	1.15	0.55	48.21	3	4.53	2.28
		BOR1,6	3	1.02	0.44	42.78	3	3.67	1.51
		BOR19,10	3	1.34	0.51	37.71	3	4.58	1.78
		BOR31,4	3	1.49	0.29	19.71	3	5.59	1.27
		BOR31,6	3	1.30	0.85	65.28	3	4.01	2.57
		BOR35,3	3	1.29	0.67	52.14	3	4.52	2.39
		BOR35,7	3	0.91	0.30	33.35	3	3.54	1.06
		BOR35,9	3	1.18	0.58	49.48	3	3.94	1.87
		BOR7,1	3	0.81	0.77	94.54	3	2.74	2.49
		BOR7,11	3	0.51	0.14	28.46	3	1.88	0.59
		BOR7,3	3	1.15	0.90	78.46	3	3.91	2.97
08.2006	Bayern	FIN	9	1.38	1.79	130.20	9	4.34	5.23
		POI	9	2.87	3.71	129.65	9	9.09	10.95
	Borken	BOR1,1	6	1.07	0.90	83.95	6	3.60	3.07
		BOR1,2	6	1.53	0.76	49.82	5	5.67	2.76
		BOR1,6	6	0.33	0.37	113.26	6	1.07	1.19
		BOR19,10	6	1.21	0.63	52.33	6	4.18	2.08
		BOR2,1	10	1.85	1.36	73.77	10	6.13	4.87
		BOR31,4	6	1.23	0.70	56.80	6	4.52	2.63
		BOR31,6	6	0.49	0.16	31.86	6	1.69	0.54
		BOR35,3	6	0.96	0.64	65.95	6	3.37	2.19

Month fieldsample	country	field	fw N	fw Mean	fw Std Dev	fw CV	dw N	dw Mean	dw Std Dev
		BOR35,7	9	1.16	0.47	40.70	9	4.31	1.73
		BOR35,9	6	0.71	0.32	45.43	6	2.44	1.09
		BOR7,1	6	0.56	0.31	54.83	6	1.95	1.10
		BOR7,11	18	0.71	0.51	72.02	17	2.26	1.70
		BOR7,3	9	0.96	0.56	58.00	9	3.29	1.86
	Brandenburg	LEB	15	2.53	2.73	108.05	15	8.12	8.66
		NTR	15	1.54	2.02	131.00	15	4.01	4.67
		PRA	15	1.33	1.16	87.34	15	3.90	3.65
		SEL	15	1.71	2.25	131.86	15	4.78	6.31
	Spanien	AL1a	9	0.93	0.81	87.29	9	2.01	1.45
		AL1b	7	2.11	2.29	108.45	7	3.71	3.42
		BE1	6	2.37	2.83	119.36	6	3.94	4.62
		BE2	7	2.70	3.93	145.60	7	4.35	4.94
		BE3	5	2.05	3.00	146.42	5	4.58	7.09
09.2006	Bayern	FIN	14	3.58	2.64	73.80	14	9.30	6.75
		POI	6	2.31	2.07	89.54	6	5.91	5.60
	Brandenburg	LEB	10	1.74	2.07	118.62	10	5.03	6.46
		NTR	3	0.74	0.28	37.80	3	2.36	1.32
		PRA	3	1.46	0.50	34.30	3	3.77	0.98
		SEL	9	1.11	0.43	38.64	9	2.52	1.03
	Spanien	AL1a	3	0.51	0.45	88.85	3	0.74	0.64
		AL1b	4	4.50	8.47	188.30	4	5.88	10.89
		BE1	3	0.13	0.11	85.67	3	0.14	0.12
		BE2	3	1.11	0.24	22.01	3	1.64	0.34
		BE3	3	0.16	0.04	21.95	3	0.18	0.04
10.2006	Spanien	AL1a	3	0.12	0.07	61.05	3	0.14	0.08
		AL1b	3	0.11	0.08	72.70	3	0.12	0.08
		BE1	3	0.03	0.01	37.10	3	0.05	0.02
		BE2	3	0.08	0.05	61.29	3	0.08	0.05

Annex 3: Figures: Bt content in MON810 leaves

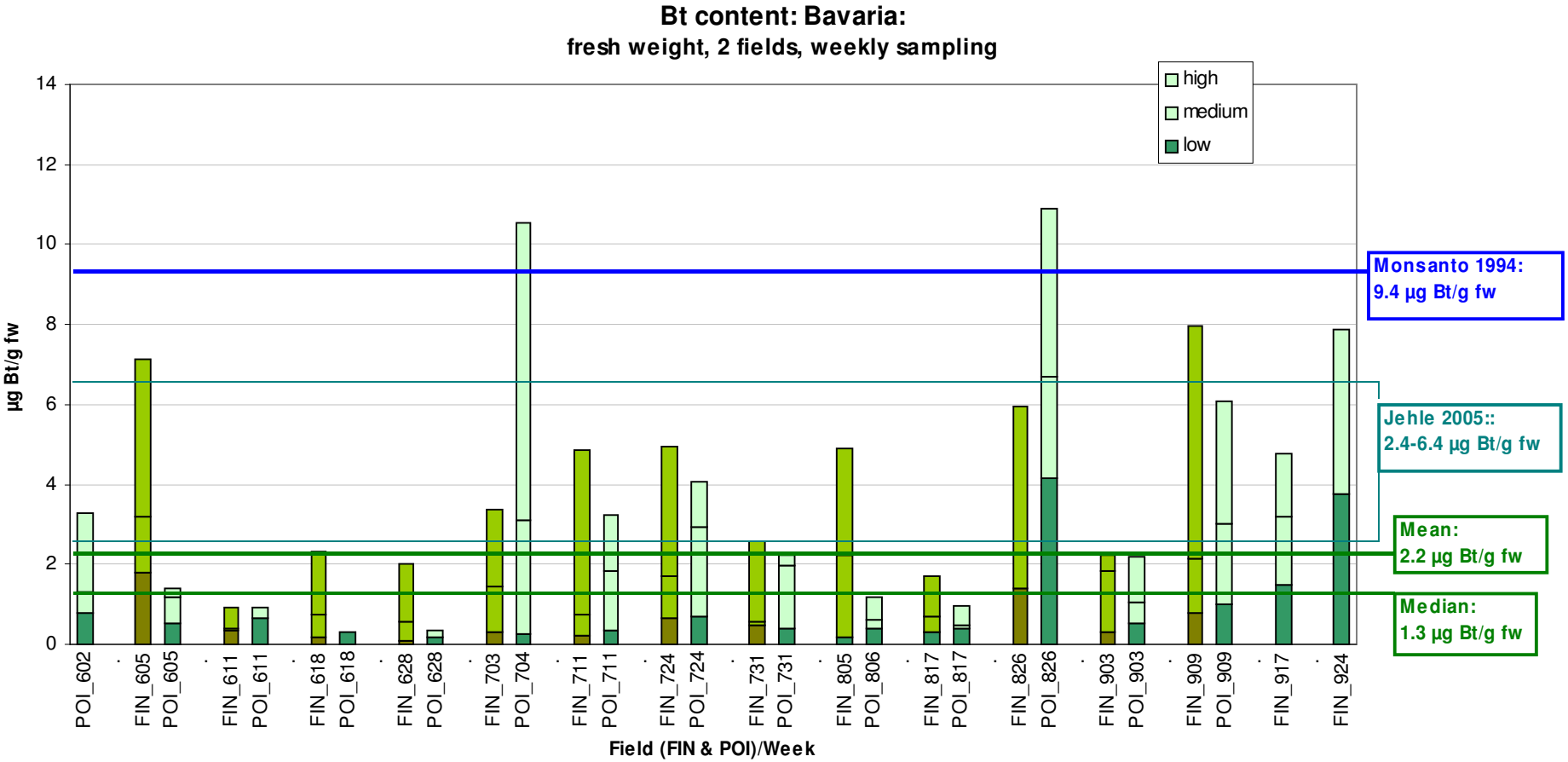

Fig. 3: B concnetration of MON80 leaves, Bavaria. Each bar gives the Bt contents of 3 plants on one sampling event on one of two locations. The values are stacked so that the lower line represents the lowest Bt content measured on that day, while the top end of the bar gives the maximum Bt content of that day. The second inner line shows the Bt content of the third plant.

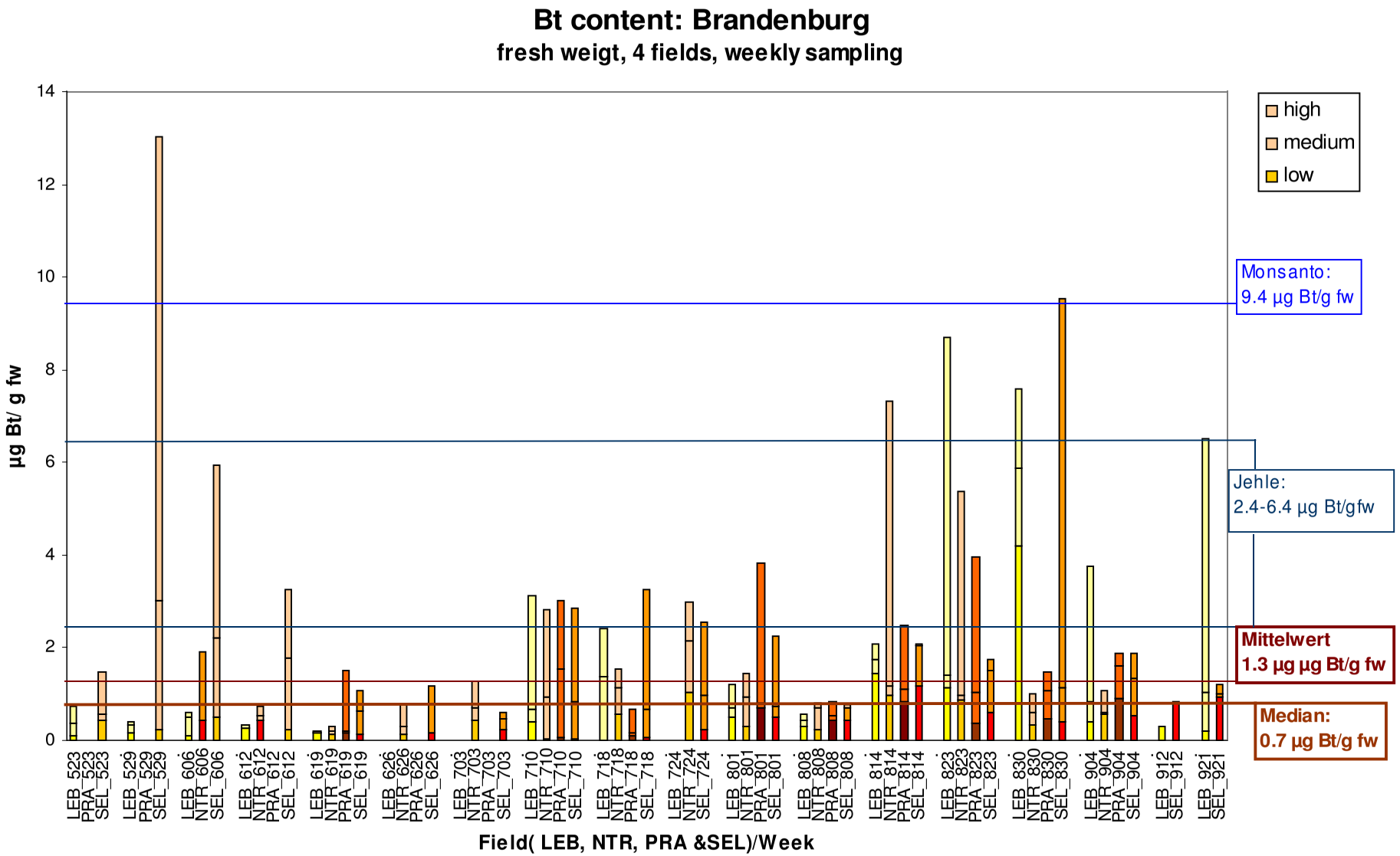

Fig. 4: Bt concentration in MON810 leaves, Brandenburg.

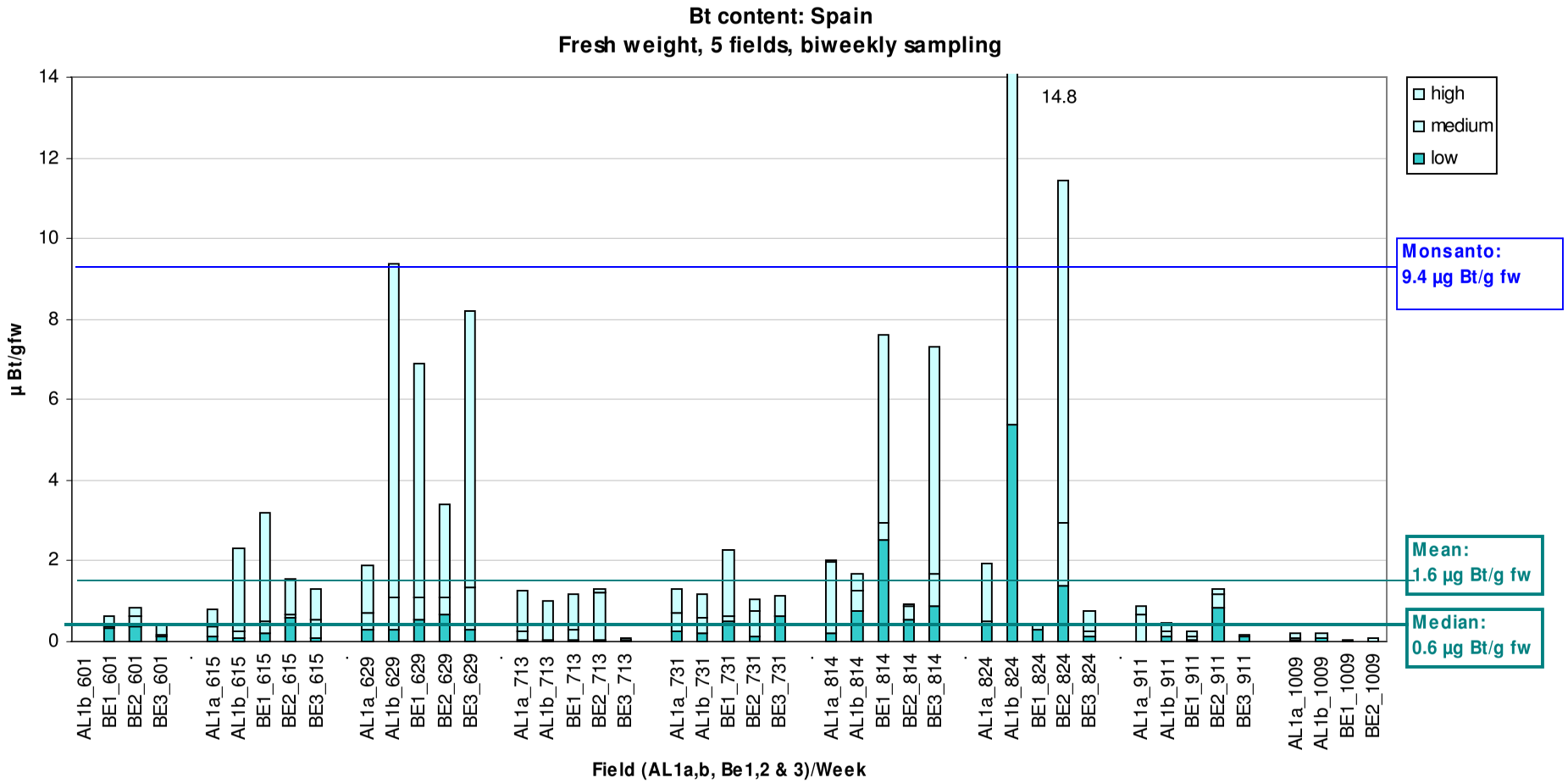

Fig. 5: Bt concentration in MON810 leaves, Spanien 2006.