Soil Biology & Biochemistry 91 (2015) 109–118

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Risk assessment of the cultivation of a stacked Bt-maize variety (MON89034 × MON88017) for nematode communities

Sebastian Höss [a, b, *], Nicola Reiff [b], Richard Ottermanns [c], Sibylle Pagel-Wieder [d], Anja B. Dohrmann [e], Christoph C. Tebbe [e], Walter Traunspurger [f]

[a] Institute of Biodiversity – Network (ibn), Drei-Kronen-Gasse 2, 93047 Regensburg, Germany
[b] Ecossa (Ecological Sediment and Soil Assessment), Giselastr. 6, 82319 Starnberg, Germany
[c] RWTH Aachen University, Institute for Environmental Research, Worringerweg 1, 52074 Aachen, Germany
[d] Institute of Applied Biotechnology in the Tropics at the University of Göttingen, Marie-Curie-Str. 7, 37079 Göttingen, Germany
[e] Thünen Institute of Biodiversity, Federal Research Institute for Rural Areas, Forestry and Fisheries, Bundesallee 50, 38116 Braunschweig, Germany
[f] University of Bielefeld, Animal Ecology, Konsequenz 45, 33615 Bielefeld, Germany

ARTICLE INFO

Article history:
Received 5 May 2015
Received in revised form
19 August 2015
Accepted 20 August 2015
Available online 6 September 2015

Keywords:
Stacked Bt-maize
Nematoda
Risk assessment
Field study
Cry proteins

ABSTRACT

Genetically modified Bt-maize MON89034 × MON88017 contains three different genes derived from *Bacillus thuringiensis* (Bt) which enable protection against insect pests, due to expression of three different insecticidal crystal proteins (Cry proteins), i.e., Cry1A.105 and Cry2Ab2 against the European corn borer and Cry3Bb1 against the Western corn root worm. Nematodes are important organisms in agricultural soil ecosystems, and on fields with Bt-maize cultivation they will be exposed to Cry proteins released into the soil from roots or plant residues. The objective of this study was to analyze in a field experiment the effect of Bt-maize MON89034 × MON88017 on nematodes as non-target organisms. Nematode communities from soil planted with the Bt-maize were compared to those from soil planted with the near-isogenic cultivar (with and without chemical insecticide treatment) and two conventional maize cultivars. The experimental field consisted of 40 plots in a completely randomized block design (eight plots for each treatment), which were monitored over two growing seasons (2008 and 2009) at six sampling dates for nematode diversity at the genus level in the rhizosphere soil. Physicochemical soil properties and Cry protein concentrations were also analyzed. Nematodes showed very high abundances, as well as a high diversity of taxa and functional guilds, indicating the relevance of maize fields as their habitat. Neither Bt-maize cultivation, nor insecticide treatment adversely affected abundance or community structure of nematode assemblages in field plots compared to several non-Bt cultivars including a near-isogenic cultivar. This confirmed the risk estimations based on the analyzed soil concentrations of extractable Cry protein, not exceeding 4.8 ng g^{-1} soil dry weight and thus revealing a safe toxicity-exposure ratio of >20.

1. Introduction

Over the past twenty years, transgenic crops have gained increasing importance in agriculture, and resistance to insect pests is one of the prevailing traits provided by genetic engineering (James, 2012). By inserting genes encoding for insecticidal proteins from strains of the bacterial species *Bacillus thuringiensis* (Bt), i.e. delta endotoxins (crystal proteins, Cry proteins), into the plant genome, pest resistant crops could be generated (Schnepf et al., 1998). During and after cultivation, these Cry proteins are released to agricultural soil (Baumgarte and Tebbe, 2005; Miethling-Graff et al., 2010), potentially harming non-target organisms (NTO) when exposed to Cry protein residues. Therefore, the risk on NTO, which include many organisms with soil beneficial activities must be assessed before Bt-maize can be grown commercially (Conner et al., 2003; Snow et al., 2005).

Free-living, non-parasitic nematodes are the most abundant and species-rich metazoans in soils and contribute considerably to important soil ecosystem services (Yeates, 1981; Andrassy, 1992). Nematodes have successfully occupied key positions in terrestrial

* Corresponding author. Ecossa, Giselastr. 6, 82319 Starnberg, Germany. Tel.: +49 8151 5509172; fax: +49 8151 5509173.
E-mail address: hoess@ecossa.de (S. Höss).

http://dx.doi.org/10.1016/j.soilbio.2015.08.022
0038-0717/

food webs by evolving different feeding strategies (Yeates et al., 1993), thus influencing nutrient cycling in soils (Yeates and Coleman, 1982; Ingham et al., 1985; Beare, 1997). The presence of nematodes and the structure of nematode communities are therefore important to agricultural production and sustainability (Fiscus and Neher, 2002). Thus, nematodes should be considered for the environmental risk assessment of GMOs to reach the protection goals of EU legislation regarding biodiversity and ecological functions (EFSA, 2010).

Soil nematodes are potentially exposed to Bt-toxins in soil either via the dissolved phase in the pore water or by feeding on Cry protein containing plant material (plant feeders), detritus, bacteria and fungi (bacteria and fungi feeders) or by feeding on organisms that were exposed to Cry-proteins (predatory nematodes). Moreover, strongly involved in the soil food web, nematodes are subjected to Bt-induced changes in other parts of the food web (e.g. bacteria, fungi) and are therefore good indicators of soil health (Bongers and Ferris, 1999; Ferris et al., 2001). Thus, nematodes are regarded as an important organism group when assessing GMO effects on the soil biota (Ruf et al., 2013).

The stacked maize varieties with the events MON89034 × MON88017 express three different Cry proteins which provide insecticidal activity against the European corn borer (*Ostrinia nubilalis*; Cry1A.105; Cry2Ab2) and the Western corn root worm (*Diabrotica virgifera*; Cry3Bb1). Although these insecticidal toxins should specifically act against the respective pests, there is evidence that also free-living nematodes can be harmed through a similar mode of action as insects (Höss et al., 2011, 2013). Furthermore, soil collected from fields grown with Bt-maize (Mon810) affected the reproduction of the nematode *Caenorhabditis elegans* compared to soil from plots with near-isogenic maize (Höss et al., 2008). Field studies, assessing the effects of Bt-maize on nematode communities were not always consistent for their results on impairing effects. While some field studies showed that Bt-maize affected the abundance of nematodes (event MON 810; Griffiths et al., 2005), nematode feeding type composition (Bt 176: Manachini and Lozzia, 2002; Mon863: Neher et al., 2014) or their genus composition (Mon 88017; Höss et al., 2011), other studies observed no effects (NK4640Bt: Saxena and Stotzky, 2001; MON863: Al-Deeb et al., 2003). This makes it difficult to predict on a theoretical basis the risk of the stacked maize from field studies with single events.

The simultaneous expression of several Cry proteins in one maize plant also results in a potential multi-toxin exposure for NTO, including those inhabiting soils. Exposure to Cry proteins with distinct toxic potentials is expected to cause additive, synergistic or antagonistic mixture toxicity (e.g. Li and Bouwer, 2014). A toxicity study with aqueous solutions of Cry1A.105, Cry2Ab2 and Cry3Bb1 using the nematode *C. elegans* revealed no novel or elevated risks that stacked maize events would have when compared to their single events (Höss et al., 2013). However, to knowledge of the authors, of the three expressed Cry proteins by MON89034 × MON88017, only the single maize varieties with events expressing Cry3Bb1 have been investigated in field experiments for their effects on nematode communities (e.g. Höss et al., 2011; Neher et al., 2014). In contrast, no data from field studies are available concerning effects of single maize events expressing Cry1A.105 and Cry2Ab on nematode communities.

A soil microcosm study, using a mixture of the Cry-proteins expressed by MON89034 × MON88017, caused significant deleterious effects on nematode communities at nominal concentrations of 1 μg g^{-1} soil dry weight (Höss et al., 2014). Using literature data for typical Cry protein concentrations extractable from soil, a risk assessment revealed a toxicity-exposure ratio (TER) of >20, which should be protective for nematode communities in the field (Höss et al., 2014). However, this risk assessment for nematode communities was based on nominal and not on measured protein concentrations of Cry1A.105, Cry2Ab2 and Cry3Bb1, which might have been considerably lower than the aimed concentrations. An overestimation of Cry protein concentration might have led to an underestimation of the realistic risk in the field. Moreover, even at very low concentrations of Cry3Bb1, which should be protective for nematodes according to results of toxicity studies, the *Diabrotica*-resistant Mon88017 exhibited subtle but significant effects on the nematode genus composition compared to the near-isogenic and conventional maize cultivars (Höss et al., 2011).

Therefore, the aim of this study was to assess the risk of the stacked Bt-maize MON89034 × MON88017 cultivation for soil nematodes under field conditions. In a field experiment, 40 plots were cultivated with Bt-maize (MON89034 × MON88017; 8 plots), the near-isogenic cultivar (DKC5143; 8 plots) and two conventional cultivars (Benicia, DKC4250; 8 plots each) over a period of two years. Additional eight plots grown with the near-isogenic cultivar were treated with the soil insecticide Tefluthrin. Nematodes were sampled from soil near the rooting zone and analyzed for their community structure on three sampling occasions per year, accompanied by analysis of soil properties and Cry protein soil concentrations. It was hypothesized that nematode community structure is not affected by Bt-maize, as long as extractable soil concentrations Cry-protein stay below the threshold concentrations for nematodes revealed by an earlier study (Höss et al., 2014).

2. Materials and methods

2.1. Maize cultivars and field design

Bt-maize MON89034 × MON88017 (Monsanto Company, St. Louis, MO, USA), the genetically modified maize event used in the field experiment, expresses three insecticidal Bt-toxins (Cry1A.105; Cry2Ab2; Cry3Bb1), due to genomic insertion of the following genes: the synthetic cry1A.105 showing analogies with three Bt-genes (cry1Ac, cry1Ab, cry1F) from two different *B. thuringiensis* strains (*kurstaki* and *aizawai*), cry2Ab2 of *B. thuringiensis* ssp. *kurstaki*, and cry3Bb1 gene of *B. thuringiensis* ssp. *kumamotoensis*. Moreover, the stacked event expresses the glyphosate resistance protein CP4 EPSPS, encoded by the cp4 epsps gene of *Agrobacterium* sp. strain CP4. By expressing Cry1A.105, Cry2Ab2 and Cry3Bb1, the plant is protected against the European corn borer (*O. nubilalis*, Lepidoptera: Crambidae) and the western corn rootworm (*Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae). The event was in the genetic background of the variety DKC5143.

The field site was located in Braunschweig (Germany) within the area of the Thünen institute (52.29252°N, 10.45174°E). In the preceding years of this experiment, the field had been planted with grass (2006) and silo maize (Gavott; 2007). Within an area of approximately 6.7 ha (including surrounding crop), five different treatments (four maize lines; one line with and without insecticidal treatment) were arranged in a randomized complete block design (RCBD) with eight replicates each. The soil was characterized as a Lessivé soil type (according to the Food and Agriculture Organization: Luvisol) and showed a mineral nitrogen (Nmin) content of 2.0–4.4 mg kg^{-1} (Albers, 2013). To account for heterogeneities in soil properties within the field, replicate plots were grouped in two different blocks, each containing four replicates of each treatment. Besides Bt-maize MON89034 × MON88017 (Bt; Monsanto Co.) and its near-isogenic (non-Bt) variety DKC5143 (Monsanto Co.), two conventional maize varieties, i.e., Benicia (Pioneer Hi- Bred, Johnston, IA, USA) and DKC4250 (Monsanto Co.), were used for this field study. For the near-isogenic cultivar, 16 plots were seeded, with eight plots being treated with the pyrethroid soil insecticide Tefluthrin (Force 1.5 G, Syngenta). Tefluthrin was applied as a

granule insecticide in the seed furrow with sowing (13.3 kg ha^{-1}). Individual plots measured 42 × 30 m (0.126 ha) and contained 40 rows of maize with 75 cm distance between them and 15 cm between individual plants. The plots were aligned in five parallel rows of eight plots each, with a 3-m-wide clearance between neighboring rows for easy access. The experimental field was surrounded by a 3-m clearance strip, which was bordered by a 10-m-wide perimeter of a surrounding crop (DKC4250). The experiment was performed during three successive years, and the location of the plots with their respective maize lines remained unchanged. The field was seeded in June 2008, May 2009, and May 2010, respectively.

2.2. Samples for nematode community analysis

Soil for the analysis of nematode communities was sampled near the rooting zone in 2008 and 2009 at three sampling dates each (May/June: after sowing; August: bloom; October: after harvest) using 2-cm (interior diameters) corers. In each of the 40 field plots, sub-samples were collected from six randomly selected points (total sampled area per plot: 18.8 cm^2). Only the upper 20 cm of the soil were included in the analysis, resulting in a total sample volume of approx. 380 cm^3 soil. In the laboratory, all sub-samples collected from each plot were combined and thoroughly mixed. Aliquots of 18 (±3 sd) cm^3 were treated with formalin (4% v/v) for fixation and stained with rose Bengal for better recovery of the nematodes. For each aliquot, the exact volume was noted, so that nematode individual numbers could be calculated based on cm^3.

2.3. Analysis of soil properties

Selected soil properties were analyzed for each plot by collecting samples from the topsoil (0–20 cm) according to Höss et al. (2011). Field-moistened samples were air-dried and sieved through a 2-mm mesh, and the particle size distribution determined by removing organic matter from the air-dried samples with H_2O_2 (30%). Complete dispersion of the samples was achieved by pre-treatment with sodium pyrophosphate (0.04 M $Na_4P_2O_7$). Clay fractions (<2 μm) and silt fractions (2–63 μm) were distinguished by sedimentation using the pipette method (Kilmer and Alexander, 1949). The sand fraction (63 μm–2 mm) was obtained by wet sieving. The pH of the air-dried samples was measured potentiometrically at a soil to solution ratio of 1:25 using 0.01 M $CaCl_2$. The amount of organic carbon was measured by dry combustion in oxygen using a CNS-analyzer (CNS-2000, Leco, St. Joseph, MI, USA).

2.4. Extraction and quantification of Cry proteins in soil samples

The Cry1A.105 and Cry2Ab2 proteins were quantified using ELISA systems kindly provided by Monsanto (St. Louis, Mo) and Cry3Bb1 was quantified with the Cry3Bb1 ELISA system from Agdia (Elkhart, Indiana, USA), all following protocols of the suppliers. The extractions of the Cry-proteins from soil were conducted as described elsewhere (Miethling-Graff et al., 2010). Briefly, three parallels of one g wet weight soil of each sample were separately mixed each with three ml of PBST buffer (137 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 10 mM KH_2PO_4, 0.5% Tween-20, pH 7.4) and homogenized by vortexing. The soil suspensions were then centrifuged at 12,000 × *g* and 4 °C for 30 min. The individual 1 ml volumes of each of the three parallels were combined and concentrated 17-fold by ultrafiltration (Amicon® Ultra-4 Centrifugal Filter Units Merck Chemicals GmbH, Schwalbach, Germany). Higher sensitivity of the Cry3Bb1 ELISA was achieved by extending the recommended 1-h incubation period of the Cry3Bb1-enzyme-conjugate to 17 h at 4 °C in the dark. For quantification of the Cry-proteins, positive controls supplied by the manufacturers were diluted with PBST buffer to reach final concentrations between 0.1 and 7 ng Cry-protein ml^{-1}, respectively. These dilutions were used for setting up calibration curves. This ELISA quantification method has previously been evaluated in other studies (Nguyen et al., 2008).

2.5. Nematode isolation and identification

Nematodes were separated from soil particles using the centrifugal-flotation method, modified from Higgins and Thiel (1988). Soil samples were mixed with a colloidal silica suspension (Ludox TM 50; Sigma–Aldrich, Munich, Germany) adjusted to a density of 1.13 g cm^{-3} with deionized water. After centrifugation for 15 min at 800 × *g*, the supernatant was filtered through a 10-μm-mesh gauze filter, which retained all nematodes. The extraction steps were repeated three times, with <10% of the total number of extracted nematodes found in the third extraction step. The retained organisms were rinsed into Petri dishes and then counted using a dissecting microscope (25–40 × magnification). From each sample, approximately 50 nematodes were transferred and prepared in glycerol (Seinhorst, 1959), with 11,972 nematodes microscopically determined to the genus level (500–1125 × magnification; Diaplan, DIC).

2.6. Nematode community analysis

Principal Response Curves (PRCs) were calculated to analyze the effects of the various maize cultivars (Bt, near-isogenic + tefluthrin, DKC4250, Benicia) on the nematode community composition compared to the near-isogenic maize (DKC5143), using CANOCO for Windows 4.55 (Biometris – Plant Research International, Wageningen, The Netherlands, 1997–2006). PRC technique uses a multivariate ordination method based on a redundancy analysis (RDA; Van den Brink and Ter Braak, 1999) to compare the responses of the nematode communities in the various maize cultivars with those in the control (DKC5143) over time. Interactions between treatment and time (sampling date) were used as the explanatory variables, and time served as co-variable. A linear combination of variables (changes of ln (2x + 1)-transformed relative abundance) was calculated to determine the deviation of each assemblage sampled from the treatment plots from control (DKC5143) assemblages at each sampling date expressed as the first principal component of the variance explained by treatment differences in time (c_{dt}). PRCs were derived by plotting c_{dt} against time. With the accompanying species scores (b_k), PRCs can be interpreted at the genus level. The absolute value indicates the relative weight within the linear PRC axis (magnitude of deviation from control) and the sign represents the direction of change. The statistical significance of the effects of the explanatory variables on the community composition was tested with Monte-Carlo permutation tests by permuting the entire time series based on the RDA from which the PRC has been derived (Van den Brink and Ter Braak, 1999).

Nematode taxa were ranked along a colonizer-persister (c-p) scale of 1–5 according to Bongers and Bongers (1998). The Maturity Index was calculated using the equation of Bongers (1990):

$$MI = \sum_{i=1}^{n} (f_i \times v_i) \quad (1)$$

where f_i is the fraction of species *i* in a sample and v_i is the *c-p*-value of species *i*.

Additionally, nematode taxa were classified in different feeding types according to Yeates et al. (1993): (1) plant feeders (PF), (2)

hyphal feeders (HF), (3) bacterial feeders (BF), (4) predators (Pre), (5) omnivorous nematodes (mainly dorylaims), (6) unselective detritus feeders and (7) algae feeders. Based on the *c-p* and feeding type classification, nematode taxa were also categorized in functional guilds according to Ferris et al. (2001): Ba_n, Fu_n, Ca_n, Om_n = bacterial feeders, hyphal feeders, predators, and omnivores, with n = *c-p*-value, respectively. The following indices were calculated to describe the enrichment and structure conditions as well as the predominant decomposition channels in the soil food webs:

Enrichment index:

$$EI = 100 \times \frac{e}{e+b} \quad (2)$$

Structure index:

$$SI = 100 \times \frac{s}{s+b} \quad (3)$$

where $b = (Ba_2 + Fu_2) \times 0.8$, $e = Ba_1 \times 3.2 + Fu_2 \times 0.8$, and $s = Ca_2 \times 0.8 + (Ba_3 + Ca_3 + Fu_3 + Om_3) \times 1.8 + (Ba_4 + Ca_4 + Fu_4 + Om_4) \times 3.2 + (Ba_5 + Ca_5 + Fu_5 + Om_5) \times 5$.

Channel index:

$$CI = 100 \times \frac{Fu_2 \times 0.8}{Ba_1 \times 3.2 + Fu_2 \times 0.8} \quad (4)$$

2.7. Statistical analysis

To test for effects of the treatments, different generalized linear models (GLM) were fitted to the various measured parameters: soil properties, Cry protein concentrations, nematode community measures, such as nematode abundances, community maturity (MI), feeding types (PF, BF, HF, Pre) and community indices (EI, SI, CI) following the recommendations in Semenov et al. (2013). Calculations were done with the R statistical language (R Core Team, 2014). Block and treatment as well as year and season were used as crossed, fixed factors. Treatment with the near-isogenic maize was always used as the control treatment.

Count data, such as the nematode abundance were tested for over-dispersion with the function dispersion test from package AER (Kleiber and Zeileis, 2008). Variables without over-dispersion were modeled using a Poisson model with log-link function, while for variables with over-dispersion a negative binomial model (function glm.nb from package MASS (Venables and Ripley, 2002) with log-link function was preferred. Soil Cry-protein concentrations (Cry1.105, Cry3Bb1, Total Cry), organic content and pH as well as, maturity index (MI) and channel index (CI) were modeled using a Gaussian model with log-link function. Proportional data (in the range 0,1), like soil sand, silt and clay content as well as feeding type ratios (PF, HF, BF, Pre) and community indices (SI, EI) were modeled with a beta-distribution (function betareg from package betareg; Cribari-Neto and Zeileis, 2010) after dividing by 100.

For all analyses nested GLMs with increasing complexity were compared (x ~ 1, x ~ treatment, x ~ treatment*block, x ~ treatment*block + year*season). For Cry-protein concentrations 'treatment' was not used as parameter, as only Bt-plots were analyzed for Cry-proteins. For environmental variables year and season were not used as only data from the first year were available. Models were compared by analysis of deviance for the different nested model candidates performed using function anova.glm (R Core Team, 2014) or a likelihood ratio test (function lrtest from package lmtest; Zeileis and Hothorn, 2002). The best model for our purpose was selected by use of Akaike's information criterion AIC (function AIC; R Core Team, 2014) based on the penalized likelihood. The goodness of fit for the finally selected model was tested using a Chi^2-test on the model residual deviances (1 − pchisq(-res.deviance, df)). All GLM model types, link functions, the best resulting model and significant effects are reported in Table 1. For number of genera, data of all eight replicate plots of a treatment were pooled to achieve the maximal number of identified nematodes (n = 400) per treatment, because the number of identified nematodes per replicate (n = 50), were considered too low to get robust values. However, due to the loss of the replicate information, no statistical analysis could be performed.

3. Results

3.1. Physico-chemical soil properties and concentrations of the Cry protein

Soil from the different plots of the experimental field showed pH values of 5.8–6.1, organic matter contents of 2–5% and sand, silt and clay fractions ranging from 42 to 67%, 27–49% and 5–10%, respectively. Replicate field plots showed no significant differences in regard to their soil properties between the two blocks of the RCBD, or between the various maize treatments (Table 1), indicating homogeneous conditions at the field site. Thus, effects of the measured soil properties on the nematode communities, masking the treatment effects, were not expected.

Concentrations of extractable Cry proteins in rhizosphere soil of the Bt-plots were very low, compared to the expected amounts inside the roots, with mean concentrations for Cry1A.105 and Cry3Bb1 ranging from 0.34 to 0.67 and 0.14–0.99 ng g^{-1} soil dry weight (dw), respectively, depending on the sampling date (Table 2). Concentrations of Cry2Ab2 were consistently below the limit of detection (0.07 ng g^{-1} dw; Table 2). Peak concentrations reached 1.66 and 3.16 ng g^{-1} soil dw for Cry1A.105 and Cry3Bb1, respectively (Table 2). Concentrations of Cry1A.105 and Cry3Bb1 were higher during flowering (August) than after harvest (October). A significant effect on Cry-concentrations was observed for the season (August vs. October, Table 1). No increase of extractable Cry protein concentrations from soils, suggesting an accumulation, over the three years, was observed (Table 2).

Based on the maximum total Cry protein concentrations of 4.82 ng g^{-1} soil dw (August 2009; Table 2) and a "no observed effect concentration" for nematode communities reported from a microcosm study ($NOEC_{Community}$: 100 ng g^{-1} soil dw; for a mixture of Cry1A.105, Cry2Ab2 and Cry3Bb1; Höss et al., 2014), a toxicity-exposure ratio (TER) value of 21 was calculated. Using the mean value for the sum of all three Cry proteins over time (1.00 ng g^{-1} soil dw) the calculated TER was even higher (100).

3.2. Nematode communities

With numbers ranging from 27 to 140 individuals (ind) cm^{-3}, nematodes showed high abundances at the field site. Average nematode abundances for the various treatment plots ranged from 56 to 98 ind cm^{-3} (DKC5143), 60–101 ind cm^{-3} (DKC5143 + tefluthrin), 57–97 ind cm^{-3} (Bt), 61–84 ind cm^{-3} (DKC4250), 53–86 ind cm^{-3} (Benicia), depending on the sampling date (Table 3), with relatively small variances within the treatments (coefficient of variance (CV): 10–27%). No significant treatment effects (Bt, DKC4250, Benicia, DKC5143 + tefluthrin) against the control (DKC5143) were found. Nevertheless, strong temporal variability was observed for year, season and the interaction of year and season (Table 1).

Table 1
Model types, link functions, the best resulting model and significant effects for fitted GLMs.

Dependent variable[a]	Error distribution	Link function	GLM in R syntax for best model[b]	p < 0.05[c]
Cry1A.105	Gaussian	Log	glm(Cry1A.105~block + year*season)	season
Cry3Bb1	Gaussian	Log	glm(Cry3Bb1~block + year*season)	
TotalCry	Gaussian	Log	glm(TotalCry ~ block + year*season)	season
Organic matter (humus)	Gaussian	Log	glm(humus ~ block*treatment)	
pH	Gaussian	Log	glm(pH ~ block*treatment)	
Sand	Beta	Logit	betareg(Sand ~ treatment*block)	
Silt	Beta	Logit	betareg(Silt ~ treatment*block)	
Clay	Beta	Logit	betareg(Clay ~ treatment*block)	
Abundance	Negative binomial	Log	glm.nb(Abundance ~ block*treatment + year*season)	year, season, year:season
PF (%)	Beta	Logit	betareg((PF/100)~treatment*block + year*season)	DKC4250, Benicia, block, year, DKC4250:block, Benicia:block
HF (%)	Beta	Logit	betareg((HF/100)~treatment*block + year*season)	year
BF (%)	Beta	Logit	betareg((BF/100)~treatment*block + year*season)	season
Pre (%)	Beta	Logit	betareg((Pre/100)~treatment*block + year*season)	year, season, year:season
MI	Gaussian	Log	glm(MI ~ block*treatment + year*season)	year, season, year:season
SI	Beta	Logit	betareg(SI/100~treatment*block + year*season)	year, season, block, year:season
EI	Beta	Logit	betareg(EI/100~treatment*block + year*season)	year, season, year:season
CI	Gaussian	Log	glm(CI ~ treatment*block)	DKC5143 + tefluthrin:block

[a] Cry1.105, Cry3Bb1, TotalCry: soil concentrations of the respective Cry proteins, PF = plant feeder, HF = hyphal feeder, BF = bacterial feeder, Pre = predator; MI = Maturity Index, SI = Structure Index, EI = Enrichment Index, CI = Channel Index.
[b] glm = Gaussian GLM with logarithmic link function, betareg = Beta distribution GLM with logit link function, glm.nb = GLM based on negative binomial distribution with logarithmic link function; percentage values were divided by 100 (/100).
[c] DKC4250, Benicia: conventional maize cultivars; DKC5143 = near-isogenic maize (non-Bt) cultivar.

Table 2
Cry-protein concentrations (mean ± standard deviation: sd; maximum: Max) in soil of plots grown with Bt-maize (MON89034 × MON88017), sampled at various sampling dates.

Date	Cry1A.105		Cry2Ab2[a]		Cry3Bb1		Total Cry[b]	
	Mean ± sd	Max	Mean ± sd	Max	Mean ± sd	Max	Mean ± sd	Max
	ng g^{-1} soil dry weight							
August 08[c]	0.47 ± 0.25	1.00	<0.07 ± 0.05	<0.07	0.28 ± 0.11	0.47	0.78 ± 0.32	1.30
August 09[c]	0.65 ± 0.44	1.66	<0.07 ± 0.05	<0.07	0.99 ± 0.94	3.16	1.68 ± 1.38	4.85
October 09[c]	0.34 ± 0.10	0.47	<0.07 ± 0.05	<0.07	0.14 ± 0.16	0.52	0.52 ± 0.24	1.02
August 10[d]	0.67 ± 0.06	0.73	<0.07 ± 0.05	<0.07	0.40 ± 0.07	0.50	1.10 ± 0.13	1.26

[a] Soil concentrations below limit of detection (LOD = 0.07 ± 0.05).
[b] For calculating the total sum of Cry protein concentrations, values below LOD were included as 0.5 × LOD.
[c] Mean calculated for n = 8.
[d] Mean calculated for n = 5.

In total, 11,972 nematodes were identified to genus level. Of these, 132 different genera were identified in all 240 analyzed soil samples (Table S1). The numbers of genera in combined samples of the 8 replicated plots of one treatment (i.e. within 400 identified individuals) ranged from 37 to 54.

The trophic structure of the communities was dominated by bacterial feeders (BF: average proportion: 38–66%), followed by plant feeders (PF: average proportion: 13–30%), predators (Pre: average proportion: 2–20%) and hyphal feeders (HF: average proportion: 2–7%), respectively. In the course the two growing seasons (May/June to October), the trophic structure shifted slightly towards higher percentages of predators and lower percentages of bacterial feeders. This effect was more pronounced in the first compared to the second year of maize cultivation (2008; Table 3). For different factors a significant effect on trophic structure was found for the feeding types, however, no significant Bt effect could be observed (PF, HF, BF and Pre; Table 1).

A trait based categorization of the nematodes according to Bongers (1990) revealed for both years a clear decrease of relative abundances of *c-p* 1 taxa (the so-called enrichment opportunists) in course of the growing season (2008: from 26 to 32% in June to 15–19% in October; 2009: 30–38% in May to 12–19% in October). Proportions of *c-p* 2 taxa (the so-called general opportunists) only decreased in 2008 within the experiment from 21 to 27% in June to 12–15% in October, while in 2009 *c-p* 2 taxa showed relatively low relative abundances throughout the growing season (11–17%). In contrast, *c-p* 3–5 taxa (the so-called persisters) increased during the growing season in relative abundances in both years (2008 from 13 to 15% in June to 35–44% in October; 2009: 29–32% on May to 36–49% in October). This shift in life history strategists was also reflected in the Maturity Index (MI), showing increasing values within the experiments of both years (2008: from 1.93 to 2.03 in June to 2.91–3.13 in October; 2009: 2.34–2.50 in May to 2.96–3.31 in October). However, treatment induced changes of the MI were not observed, but a strong seasonal and inter-annual trend was found (Table 1).

Merging trophic and trait based classifications, the nematodes were further categorized according to their functional guilds, allowing to obtain information on the enrichment and structure status of the soil nematode community. In the faunal ordination according to Ferris et al. (2001), in which EI is plotted against SI, all data points were located in quadrant B (SI and EI > 50%; Table 3; Fig. 1), representing N-enriched, lowly to moderate disturbed conditions. It is obvious, that during the first year the SI increased in all treatments, while for the EI almost no variation occurred during the two growing seasons (Fig. 1). Although for SI and EI

Table 3
Univariate measures determined for nematode communities sampled from field plots cultivated with various maize cultivars at six sampling dates within two years (2008 and 2009).

Date/Treatment[a]	Measures for nematode communities[b]								
	Ind cm^{-3}	MI	%PF	%HF	%BF	%Pre	EI	SI	CI
10.6.08									
DKC4250	65.9 ± 29.9	1.93 ± 0.21	19.9 ± 7.1	1.5 ± 0.9	66.3 ± 9.3	1.8 ± 1.8	83.9 ± 9.0	67.9 ± 15.4	0.9 ± 0.8
Benicia	53.2 ± 18.2	1.99 ± 0.32	27.7 ± 11.8	2.3 ± 1.7	58.1 ± 11.4	2.5 ± 2.3	83.0 ± 8.3	70.8 ± 5.9	1.4 ± 1.2
Bt	66.9 ± 17.6	1.86 ± 0.20	21.9 ± 10.6	1.8 ± 2.2	64.5 ± 11.3	3.4 ± 3.7	79.7 ± 5.0	60.7 ± 12.4	1.9 ± 2.5
DKC5143	55.6 ± 28.1	2.03 ± 0.28	25.1 ± 8.4	2.0 ± 1.9	58.7 ± 9.5	1.5 ± 1.4	82.7 ± 3.7	68.9 ± 12.7	1.9 ± 1.8
DKC5143 + T	59.6 ± 23.5	1.99 ± 0.28	25.4 ± 10.3	3.0 ± 2.1	60.2 ± 10.1	1.5 ± 2.3	78.6 ± 8.6	62.7 ± 13.0	3.2 ± 2.7
11.8.08									
DKC4250	79.8 ± 13.4	2.71 ± 0.26	15.5 ± 5.0	1.8 ± 2.0	58.9 ± 8.8	11.0 ± 5.6	79.4 ± 10.1	89.1 ± 3.3	1.8 ± 1.8
Benicia	77.0 ± 26.1	2.54 ± 0.28	17.1 ± 4.4	1.8 ± 2.3	61.3 ± 7.1	8.3 ± 3.7	85.6 ± 6.9	88.2 ± 4.8	1.5 ± 2.3
Bt	97.5 ± 20.6	2.54 ± 0.29	24.7 ± 9.7	3.5 ± 1.4	50.6 ± 6.8	12.4 ± 4.4	77.8 ± 13.0	83.7 ± 6.2	5.0 ± 4.5
DKC5143	86.0 ± 8.2	2.48 ± 0.30	20.9 ± 5.0	3.3 ± 1.9	56.0 ± 4.7	10.3 ± 4.1	79.8 ± 10.3	83.4 ± 5.5	3.1 ± 1.8
DKC5143 + T	73.5 ± 10.0	2.71 ± 0.23	21.4 ± 5.4	1.5 ± 3.0	54.3 ± 5.4	11.9 ± 6.0	87.6 ± 7.6	92.1 ± 3.5	0.8 ± 1.2
20.10.08									
DKC4250	73.6 ± 12.9	3.11 ± 0.36	27.7 ± 7.4	2.9 ± 2.4	47.1 ± 11.4	13.2 ± 6.2	79.9 ± 8.1	92.7 ± 4.1	1.3 ± 1.9
Benicia	71.3 ± 11.1	2.91 ± 0.15	19.1 ± 6.2	3.7 ± 2.8	51.2 ± 7.5	13.7 ± 6.6	81.5 ± 7.2	90.8 ± 2.9	3.2 ± 3.1
Bt	78.1 ± 23.1	3.03 ± 0.44	24.9 ± 10.3	3.7 ± 2.5	46.8 ± 7.1	13.4 ± 4.6	72.9 ± 17.8	90.6 ± 5.3	3.7 ± 3.6
DKC5143	81.6 ± 10.1	3.13 ± 0.25	25.2 ± 8.7	4.0 ± 2.3	47.7 ± 11.3	15.7 ± 5.5	79.4 ± 7.7	93.2 ± 1.7	3.4 ± 3.0
DKC5143 + T	80.4 ± 13.6	2.95 ± 0.33	30.4 ± 13.3	3.7 ± 2.2	44.4 ± 11.2	11.3 ± 4.8	76.5 ± 11.0	90. 6 ± 1.9	3.4 ± 4.4
23.5.09									
DKC4250	84.1 ± 8.8	2.46 ± 0.17	15.7 ± 5.5	3.3 ± 2.6	59.2 ± 6.8	8.5 ± 1.8	90.3 ± 7.1	91.4 ± 4.3	1.5 ± 1.1
Benicia	85.7 ± 13.4	2.50 ± 0.26	16.6 ± 6.6	7.3 ± 8.7	54.3 ± 7.7	10.4 ± 3.6	86.6 ± 7.8	88.0 ± 8.2	5.6 ± 7.6
Bt	85.9 ± 20.0	2.50 ± 0.38	21.2 ± 5.4	3.5 ± 2.7	54.6 ± 6.4	9.5 ± 3.5	90.3 ± 6.6	91.5 ± 4.5	1.6 ± 2.4
DKC5143	97.7 ± 19.8	2.42 ± 0.37	15.0 ± 4.4	5.3 ± 5.0	57.7 ± 10.6	12.8 ± 8.5	89.8 ± 4.5	88.7 ± 5.1	3.0 ± 3.8
DKC5143 + T	100.7 ± 21.6	2.34 ± 0.31	13.3 ± 7.6	2.3 ± 2.3	62.8 ± 5.9	11.5 ± 5.8	91.2 ± 5.3	91.6 ± 2.8	0.9 ± 1.4
24.8.09									
DKC4250	61.4 ± 12.0	2.87 ± 0.42	10.8 ± 5.3	3.0 ± 3.2	54.3 ± 10.8	17.8 ± 5.4	84.0 ± 7.9	91.7 ± 3.8	2.8 ± 2.6
Benicia	59.1 ± 10.0	2.92 ± 0.41	12.5 ± 8.2	6.3 ± 5.4	47.1 ± 9.3	18.1 ± 5.4	83.6 ± 6.5	91.9 ± 3.0	8.9 ± 8.3
Bt	56.6 ± 6.2	2.77 ± 0.35	16.0 ± 8.7	3.2 ± 2.8	51.3 ± 7.7	16.9 ± 4.1	85.6 ± 8.1	91.3 ± 4.7	3.0 ± 3.3
DKC5143	67.3 ± 11.1	2.91 ± 0.24	13.2 ± 9.1	2.0 ± 2.1	54.4 ± 9.5	14.2 ± 5.0	80.0 ± 6.7	91.0 ± 1.8	1.9 ± 2.8
DKC5143 + T	69.5 ± 11.7	2.96 ± 0.18	16.8 ± 13.0	6.0 ± 2.1	46.5 ± 12.9	19.3 ± 3.7	81.8 ± 6.5	91.3 ± 2.5	7.2 ± 4.7
13.10.09									
DKC4250	81.9 ± 14.5	3.03 ± 0.27	21.8 ± 14.4	7.3 ± 4.5	42.0 ± 11.4	18.5 ± 3.8	80.8 ± 8.9	93.4 ± 2.3	6.1 ± 3.3
Benicia	79.7 ± 18.2	3.18 ± 0.30	23.6 ± 9.5	5.2 ± 2.1	37.8 ± 6.2	15.6 ± 6.6	75.4 ± 10.3	92.9 ± 2.8	9.0 ± 6.9
Bt	57.7 ± 15.5	3.22 ± 0.41	21.5 ± 10.3	2.3 ± 1.3	43.5 ± 9.9	19.0 ± 7.0	79.5 ± 12.3	92.8 ± 4.7	3.9 ± 6.3
DKC5143	74.8 ± 19.2	2.96 ± 0.40	25.7 ± 13.5	2.7 ± 1.0	44.9 ± 10.8	16.5 ± 6.7	72.3 ± 13.3	88.6 ± 4.5	5.2 ± 5.7
DKC5143 + T	84.6 ± 20.5	3.31 ± 0.36	17.9 ± 14.4	3.5 ± 4.2	46.7 ± 16.8	20.2 ± 6.1	81.5 ± 8.2	94.3 ± 3.3	3.7 ± 5.8

[a] Cultivation with DKC4250 and Benicia (conventional cultivars), Bt: MON89034 × MON88017; DKC5143 (near-isogenic); DKC5143 + T: DKC5143 with tefluthrin treatment.
[b] Mean ± standard deviation (n = 8) for individuals per cubic centimeter (ind cm^{-3}), Maturity Index (MI), plant feeder (%PF), hyphal feeder (%HF), bacterial feeder (%BF), predator (%Pre); Enrichment Index (EI); Structure Index (SI); Channel Index (CI).

strong seasonal and inter-annual effects as well as an effect of block on SI were found, no treatment related effects were observed (Table 1). The low channel index (CI) values (<10; Table 3) indicate bacterial decomposition pathways dominated in the soil. Only for the interaction term of DKC5143 + tefluthrin and block a significant effect on the channel index was observed (Table 1).

The most dominant nematode genera identified in this study were the bacterial feeding genera Rhabditoidea gen. 9, *Alaimus*, *Acrobeles* and *Eucephalobus*, the predator *Aporcelaimellus*, the hyphal feeder *Filenchus* and the plant feeding genera *Bitylenchus* and *Pratylenchus*, respectively (total mean relative abundance > 5% or maximal relative abundance > 20%; see Supplementary material: Table S1). The relative abundances of these genera in the various treatments are shown in Fig. 2. Multivariate analysis (i.e. PRC) of nematode genus composition in the differently treated plots revealed no significant relation between genus composition and treatment ($p = 0.102$; $F = 3.019$). Principle response curves of the treatments meandered closely to the control line (representing canonical coefficients of DKC5143 plots), indicating only small differences among the various treatments (Fig. 3), with the first axis of the PRC explaining 13% of the species–treatment interaction (sum of all canonical eigenvalues: 0.091; eigen values of first a axis: 0.012). Of the total variance in genus composition, 17.3 and 9.2% could be explained by the variables time and treatment, respectively. Deviations of principle response curves of the first axis from the near-isogenic maize mainly occurred in August of 2008 and 2009. However, these showed opposite directions for the two successive years. Species scores indicate subtle increases or decreases of relative abundances of the hyphal feeder *Filenchus*, the predator Qudsianematidae gen. 1 and the plant feeders *Pratylenchus*, *Aglenchus* and *Helicotylenchus* in August 2008 or August 2009, respectively.

4. Discussion

In this study, no differences were detected in the nematode communities in field plots cultivated with stacked Bt-maize, as compared to non-GM varieties, including a near-isogen cultivar. Moreover, nematode communities could not be distinguished between the various non-Bt cultivars (DKC5143, Benicia, DKC4250), which minimized the probability that large among-cultivar variability masked effects of the specific GMO trait. However, the nematode communities in the 40 field plots changed during the

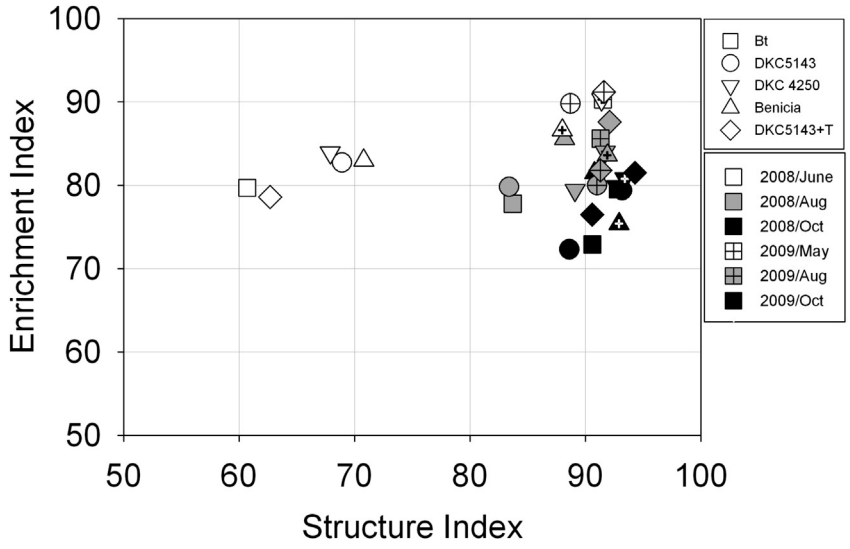

Fig. 1. Faunal profile representing the structure and enrichment conditions of the soil food web for plots grown with various maize cultivars: Bt-maize Mon89034 × Mon88017 (Bt), its near-isogenic line DKC5143, DKC5143 with insecticidal treatment (DKC5143 + T), and the two conventional hybrids DKC4250 and Benicia; symbols represent average values for eight plots (n = 8); symbol shapes and color refer to maize cultivars and sampling date, respectively.

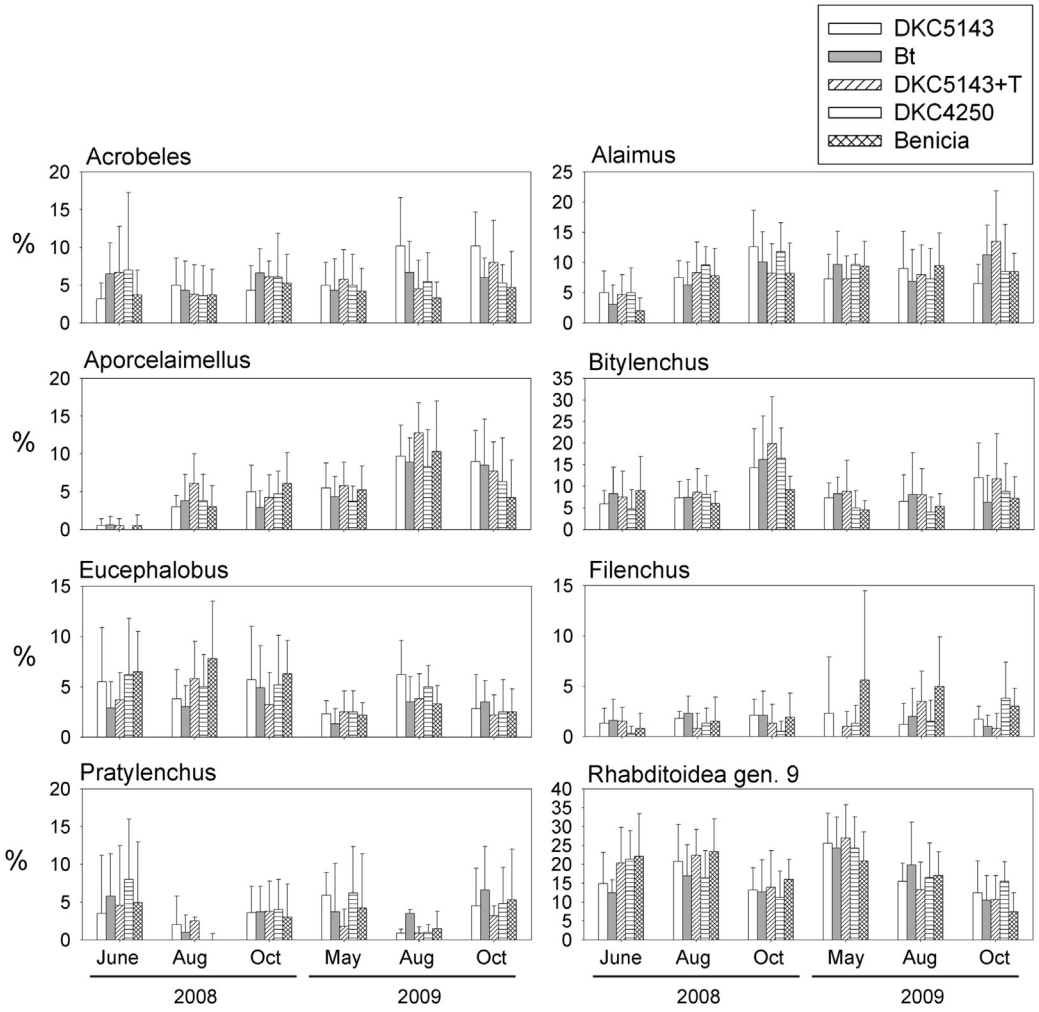

Fig. 2. Relative abundance (mean ± standard deviation; n = 8) of dominant genera (total mean relative abundance > 5% or maximal relative abundance > 20%; see Supplementary material: Table S1) in plots planted with Bt-maize Mon89034 × Mon88017 (Bt), its near-isogenic line DKC5143, DKC5143 with insecticidal treatment (DKC5143 + T), and the two conventional hybrids DKC4250 and Benicia at six sampling dates.

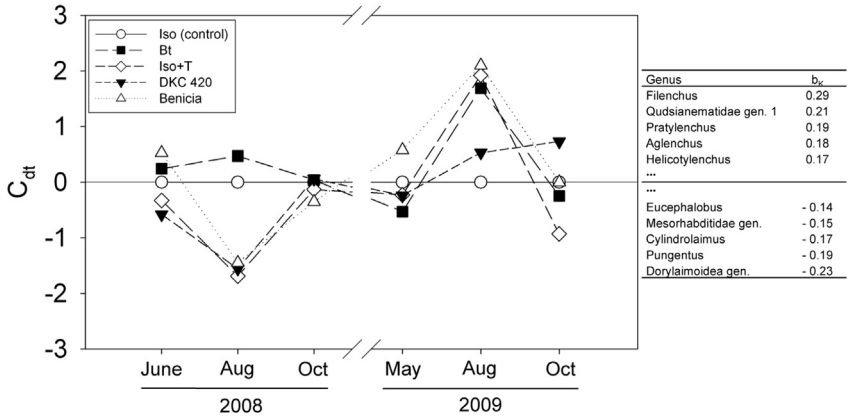

Fig. 3. Principle Response Curves (PRC) generated from relative abundances of nematode genera ($\ln(2x+1)$-transformed) from soils of experimental field plots planted with Bt-maize Mon89034 × Mon88017 (Bt), its near-isogenic line DKC5143 (control: horizontal line at y = 0), DKC5143 with insecticidal treatment (DKC5143 + T), and the two conventional hybrids DKC4250 and Benicia at six sampling dates; b_k: species score for 10 taxa with highest or lowest values, respectively; sum of all canonical eigenvalues: 0.092; eigenvalues of first axis: 0.012.

season in both cultivation periods, indicated by increasing maturity and dominance of predatory nematodes.

The very high nematode abundances of up to 140 ind cm^{-3} and the high structural and functional diversity of the nematode communities found in the rhizosphere soil confirm the high ecological relevance of nematodes in the investigated maize fields. In terms of functional guilds, the nematode communities showed a typical composition for perennial crops, representing N-enriched, lowly to moderate disturbed conditions (quadrat B in faunal ordination according to Ferris et al., 2001). High EI and low CI values indicate bacterial dominated decomposition pathways, which is comparable to the conditions in other studies (Höss et al., 2011; Neher et al., 2014).

Based on the $NOEC_{Community}$ of 100 ng g^{-1} soil dw derived for nematode communities in a microcosm study with a mixture of Cry1A.105, Cry2Ab2 and Cry3Bb1 (Höss et al., 2014) and measured maximal Cry protein concentrations in the soil, a TER of >20 could be calculated. Therefore, no direct toxicity of Cry proteins in the soil was expected. However, the $NOEC_{Community}$ was based on nominal concentration (Höss et al., 2014), thus implying some uncertainty, as real soil concentrations might have been lower than the nominal concentrations. Moreover, in a field experiment with the *Diabrotica*-resistant MON88017, subtle but significant effects on the nematode genus composition were observed in plots of Bt-maize compared to the near-isogenic and conventional maize cultivars, in spite of the very low Cry3Bb1 concentrations detected in the soil (Höss et al., 2011). Therefore, it was important to confirm the low risk of MON89034 × MON88017 suggested by the microcosm derived TER in a field experiment. Other studies of the same stacked Bt-maize found no effects on honey bees (Hendriksma et al., 2011, 2013), bacterial endophytes (Prischl et al., 2012), rhizosphere bacterial communities (Dohrmann et al., 2013) and for the straw decomposition and the involved microbial communities (Becker et al., 2014). Pollen of MON89034 × MON88017 inhibited feeding activity of non-target butterfly larvae at densities of 200, 300 and 400 grains cm^{-2} (Schuppener et al., 2012). Based on measured densities of Bt-pollen on host plants of the butterfly larvae, however, revealed a negligible risk for this species.

Effects of Bt-maize on nematode communities were already investigated in several field studies using events resistant to the European corn borer expressing Cry1Ab (Saxena and Stotzky, 2001; Manachini and Lozzia, 2002; Griffiths et al., 2005, 2006, 2007) or to the Western corn root worm expressing Cry3Bb1 (Al-Deeb et al., 2003; Höss et al., 2011; Neher et al., 2014). However, these studies were not consistent in terms of the results on impairing effects of Bt-maize. Generally, only subtle changes could be found for nematode communities that were exposed to Bt-maize. Griffiths et al. (2005) observed significantly lower numbers of nematodes in soil grown with MEB307Bt (a MON810 variety expressing Cry1Ab) compared to the near-isogenic cultivar in different types of soil, although the community structure differed considerably between these soil types. However, effects of Bt-maize could not be observed on nematode genus composition. Glass house experiments using the same soils as Griffiths et al. (2005) showed, that nematode communities differed to a larger extent between different non-Bt cultivars and different soil types than between Bt and non-Bt treatment (Griffiths et al., 2006, 2007). However, there were slight but significant effects of Bt-treatment on the trophic structure, with lower proportions of omnivore nematodes in Bt-compared to isogenic maize (Griffiths et al., 2006). Manachini and Lozzia (2002) found no effects of Cry1Ab expressing event Bt 176 on nematode abundances and diversity. Nevertheless, in one of eight experimental sites, fungivorous nematodes dominated in soil cultivated with Bt-maize (Novartis) while soil with the isogenic line (Tempra) was clearly dominated by bacterivorous nematodes. For coleopteran specific Cry3Bb1, previous studies found significant Bt-induced changes of nematode genus composition in a field experiment with MON88017 compared to the near-isogenic and one additional conventional cultivar (Höss et al., 2011). Interestingly, this genus shift among bacterial feeding nematodes had no consequences for the functional diversity of the nematode community. For *Diabrotica*-resistant MON863, only marginal effects on nematode communities occurred in field experiments in USA (Al-Deeb et al., 2003; Neher et al., 2014).

The results of these studies suggest that quantity and quality of effects of Bt-maize (or their released Cry proteins) on nematode communities were dependent on various confounding factors related to the field sites, making it difficult to compare the various studies. Important factors might be physico-chemical soil properties that are able to influence the bioavailability of the Cry proteins. Cry proteins, entering soil via root exudation or plant decomposition, bind to mineral (e.g. Madliger et al., 2011) and organic surfaces (e.g. Crecchio and Stotzky, 1998), resulting in reduced dissolved concentrations in the soil pore water. As nematodes are mainly exposed to chemicals via the aqueous phase (with the exception of plant feeders that suck out the content of plant cells), binding capacity of the soil for Cry proteins might be an important factor governing their bioavailability for the nematodes. In aqueous solution, where

maximal bioavailability can be assumed, insecticidal Cry proteins, i.e. Cry1A.105 and Cry2Ab, are able to intoxicate nematodes by a similar mode of action as in insects (Höss et al., 2013). Even in soils, purified Cry proteins (Cry1A.105, Cry2Ab2, Cry3Bb1) exhibited impairing effects on nematode communities in microcosms if total concentrations reached certain threshold concentrations (1 μg g^{-1} dw; Höss et al., 2014). Thus the missing effect in the present study (and probably also in other studies) should be explained by too low Cry protein concentrations rather than by the strict pest specificity of the toxins (Neher et al., 2014). However, Cry protein concentrations in soils were rarely reported along with the biological effect data. In a glasshouse experiment, Griffiths et al. (2006) reported maximum soil concentrations of Cry1Ab of 16 and 43 ng g^{-1} soil dw, depending on the type of soil, which was considerably higher than observed in the present study (Table 2), however, apparently still too low to induce toxic effects on nematodes.

In the present study, the plots with the near-isogenic cultivar treated with the pyrethroid insecticide tefluthrin (DKC5143 + tefluthin; 13 kg ha^{-1}) showed similar nematode communities compared to the non-treated variants. In other studies slight effects of tefluthrin (5 kg ha^{-1}) on both the trophic diversity (increased proportions of predatory nematodes) and maturity of soil nematode communities in maize fields (isogenic line) were detected, but effects on the MI could not unequivocally be attributed to the insecticide treatment (Neher et al., 2014). In the presence of the insecticide deltamethrin, however, the proportion of bacterial to plant feeding nematodes declined (Griffiths et al., 2006). Thus, the trophic structure of nematodes communities might be a good indicator of pesticide effects, reflecting changes in food web conditions in agricultural soils.

This field experiment demonstrated that cultivation of the stacked Bt-maize variety MON89034 × MON88017 did not affect nematode communities in rhizosphere soil. Maximal total soil concentrations of the three Cry proteins, expressed by the stacked Bt maize (Cry1A.105, Cry2Ab2, Cry3Bb1), showed a maximum below 5 ng g^{-1} soil dw resulting in a safe TER of >20. Thus, it could be confirmed that no effects of Cry proteins on nematode communities can be expected if field concentrations stay below the $NOEC_{Community}$ revealed in a microcosms study with soil nematodes (Höss et al., 2014). Moreover, with a field study not only direct toxicity of Cry proteins, but also indirect effects related to the whole genetically modified plant under realistic field conditions (e.g. above and below ground food web effects) on the nematode community could be considered. Together with previous studies on effects of the Cry proteins released by MON89034 × MON88017 on nematodes (Höss et al., 2013, 2014), this study confirms an acceptable ecological risk of cultivation of MON89034 × MON88017 on nematode communities in agricultural soils.

Acknowledgments

This study was funded by the German Ministry of Education and Research (BMBF; grant: 0315215). We thank the Monsanto Company for providing MON89034 × MON88017 maize seeds. Many thanks to Michael Faupel and Hanna Hermes who helped collecting the soil from the experimental field and Stefanie Gehner for sorting and mounting nematodes for taxonomic identification. Moreover, we are grateful to two anonymous reviewers whose comments helped to improve the quality of this paper.

Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.08.022.

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