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Effects of three-year cultivation of Cry1Ab-expressing Bt maize on soil microarthropod communities

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ABSTRACT

The impact of Cry1Ab-expressing Bt maize (event MON810) on non-target fauna has been a major concern since its deployment in Europe. In this paper, we have assessed the levels of Cry1Ab in rhizosphere soil samples from a Bt maize crop and evaluated the potential effects of Bt maize on soil microarthropods by a three-year trial in an experimental farm-scale field in Central Spain. The Cry1Ab toxin was detected in decaying soil organic matter (OM) from Bt maize plots up to three months after harvest, with values ranging between 0.10 and 0.18 ng Cry1Ab/mg OM, but it showed low insecticidal activity. The study focused on Acari and Collembola, the two major components of the soil microarthropod community. They accounted for 88% of the total specimens collected, and they were identified at the suborder and species level, respectively. Interestingly, Cry1Ab was detected for the first time in field collected collembolans, *Entomobrya* spp., demonstrating their exposure to the toxin. The abundance of mites and collembolans and the frequency of occurrence of the main collembolan species did not rely on the type of maize except for *Parisotoma notabilis*, more abundant and frequent in Bt maize plots. However, significant differences among years were common in both groups. Noticeably, we found higher values of species richness and diversity of collembolans in Cry1Ab-expressing Bt maize than in non-Bt plots, which could be explained under different scenarios. Our results suggest that continuous cultivation of Bt maize does not negatively affect soil microarthropods, indicating that Bt maize could be compatible with this community.

1. Introduction

Genetically modified (GM) maize plants expressing the insecticidal toxin Cry1Ab from *Bacillus thuringiensis* (Bt maize) are cultivated in Spain since 1998 to control two major lepidopteran pests: the Mediterranean corn borer, *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae) and the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Nowadays, only varieties with the event MON810 are cultivated, covering an area of 131,538 ha that represents 29% of the total surface of maize in Spain and 92% of the cultivated Bt maize in the European Union (EU) (James, 2014; http://www.magrama.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/esyrce/).
The wide acceptance of Bt maize in Spain has raised concerns about its possible environmental impact, especially in regions with high adoption rate and continued cultivation. The maize growing (including Bt maize) in the same field for several years is a common practice, being the area cultivated with maize for at least two consecutive years over 50% between 2009 and 2011. Post-market environmental monitoring field studies have been performed in Spain to assess possible effects of Cry1Ab-expressing Bt maize on non-target fauna, evidencing that non-target arthropods in maize agroecosystems are exposed to the toxin (Álvarez-Alfageme et al., 2009; Obrist et al., 2006). However, no detrimental impacts have been found on on-plant and ground-dwelling herbivores and predators inhabiting Bt maize crops (Albajes et al., 2012; de la Poza et al., 2005; Farinós et al., 2008; Pons et al., 2005). The only exception was a punctual decrease in the abundance of rove beetles detected in Bt maize in some areas and years, although laboratory studies did not evidence negative effects of Cry toxins on this group (García et al., 2010, 2012). However, effects of Bt maize on soil microarthropod communities have been poorly explored in Spain, even though they play an important role as soil

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decomposers and they are part of different trophic levels in the soil microfood-web.

The Cry1Ab toxin expressed in Bt maize can be incorporated into the soil following different routes. So, it has been reported that Cry1Ab can be released to the soil during the vegetative period through root exudates, natural wounding or senescence of root cells of Bt maize (Baumgarte and Tebbe, 2005; Saxena et al., 1999). The insecticidal toxin could also be incorporated into the soil from pollen released during tasseling (Mendelsohn et al., 2003) and other above-ground maize tissues that fell off the plant; and mainly by Bt maize residues remaining in the field after harvest (Saxena and Stotzky, 2001; Flores et al., 2005). Once incorporated, a small fraction of the toxin may bind to clay particles and humic acids, making possible its persistence in soil without losing its insecticidal activity (Icoz and Stotzky, 2008; Zwahlen et al., 2003). As a result, non-target soil fauna might be continuously exposed to the Bt toxin by ingesting the bound toxin, by feeding on living or decomposing plant parts containing the toxin or by feeding on herbivorous that have ingested the Bt plant (Álvarez-Alfageme et al., 2009; Groot and Dicke, 2002; Saxena and Stotzky, 2001).

Mites (Acari) and springtails (Collembola) are the two major components of the soil microarthropod community. They have a worldwide distribution and represent significant reservoirs of biodiversity, although their relative abundance varies depending on the environmental conditions, soil type, soil use, etc. (Álvarez et al., 2001; Coleman et al., 2004). As decomposers, soil mites (mostly oribatids and free-living astigmatids) and collembolans have been traditionally included in the group of fine comminuters, capable of reducing litter to smaller and finer particles, enhancing mineralization of nutrients (Scheu et al., 2005; Yang et al., 2012). Mites feed either on dead plant material or on soil microflora, while springtails are mostly fungivorous, although they can also feed on decomposing plant or animal residue, live plant parts, bacteria or algae (Endlweber et al., 2009; Scheu et al., 2005). Some epedaphic collembolans can also use the aerial parts of plants in certain moments of their life cycle (Frampton et al., 2001; Peterson et al., 2010), as it happens in the case of entomobryid springtails on maize (Dively, 2005). Both groups have been used as indicators of soil quality (Behan-Pelletier, 1999; van Straalen, 1998); however, collembolans can respond more rapidly than oribatids to ecosystem disturbance as they are primarily "r-selected" detritivorous organisms (Behan-Pelletier, 2003). Besides, oribatids and collembolans are significant source of food for higher trophic levels, since they are preyed upon by epigeic generalist predators (Johnston, 1999; Bilde et al., 2000; Peterson et al., 2010). Assessment of the effects of Cry1Ab using short-term laboratory and greenhouse studies has revealed no negative effects on collembolans and other microarthropods (Sims and Martin, 1997; Heckmann et al., 2006). Additionally, different field studies have been performed in Europe (Atlantic and Continental biogeographical regions) and North America to evaluate the impact of Cry-expressing crops on non-target microarthropod fauna. The composition of the soil microarthropods community was only slightly affected by Cry1Ab-expressing Bt maize, no major changes being observed (Cortet et al., 2007; Zwahlen et al., 2007). Likewise, no significant effects were reported on the abundance of individual collembolan species and on species diversity (Bitzer et al., 2005) or on the abundance of soil mites and collembolans (Al-Deeb et al., 2003) after planting Cry3Bb1-expressing Bt maize. Yet, there is little information about the incorporation of the toxin to the soil and on the impact that repeated cultivation of Bt maize may have on soil dwelling microarthropods in Mediterranean agroecological conditions (Cortet et al., 2007).

The objective of this multiyear field study is to evaluate possible effects of continuous cultivation of Bt maize on the non-target soil microarthropods community in an experimental farm-scale field in Central Spain. The study focuses on mites and collembolans, the main components of soil microarthropod fauna. We have compared their abundances between Bt and non-Bt maize fields, as well as species richness and diversity for collembolans. Additionally, we have determined the presence of Cry1Ab in two different soil fractions and assessed levels of the toxin in *Entomobrya* spp., the most abundant collembolans in soil samples.

2. Material and methods

2.1. Study area and experimental design

The study was conducted during the years 2009, 2010, 2011 and 2013 in an experimental maize field of 3.5 ha located in the province of Madrid (Central Spain). The field was situated on a loam soil, with a distribution of particle size of 43% sand, 42% silt and 15% clay, a content of organic matter of 2.96% and slightly alkaline (pH of 8.03). Other chemical properties of the soil were 0.19% of total N (Kjeldahl), 1.631% of humic acids, C/N ratio of 9.033 and soil minerals made up as follows: K-0.387, Na-0.143, Ca-23.178, Mg-1.089 (in mEq/100 g) and P-36.84, Fe-1.703, Mn-90.619, Zn-3.557, Cu-2.119 (in mg/kg) (soil samples analyzed by Innoagral, Grupo Hespérides Biotech S.L., Sevilla, Spain). Temperature and rainfall in the study area were registered weekly between 2009 and 2011 (see inline Supplementary Fig. S1).

The experimental set up was a randomized block design involving three blocks and two treatments that were the maize varieties: transgenic maize plants (DKC 6451 YG, event MON810) expressing the toxin Cry1Ab from *B. thuringiensis* (Bt maize) and its near-isogenic line DKC 6450 (non-Bt maize). The size of each plot was ~0.5 ha (80 × 60 m), with corridors of 3 m between them. The arrangement of treatments and plots was exactly the same from 2008 (one year before the beginning of the study) to 2013 to assess potential cumulative effects.

Maize was planted on 29th April, 28th April, 19th April and 13th May in 2009, 2010, 2011 and 2013, respectively, and it was harvested from late October to the end of November, depending on the weather each year. All plots were sprayed soon after sowing yearly with a preemergence herbicide of 45% acetochlor + 21.4% terbuthylazine (Harness® GTZ) at 4.5 l/ha or 33% pendimethalin (Assistan®) at 4 l/ha. In 2011 a post-emergence herbicide of 4% nicosulfuron (Bandera® 4 SC) at 1.5 l/ha was also sprayed. During harvest, maize stalk were cut ≈20–30 cm above the ground and plant residues remained on the field. The crop was grown under irrigation without crop rotation (except barley cultivation in the winter 2011–2012) and it was maintained according to local agronomical practices, excluding the use of insecticides.

2.2. Sampling of soil-dwelling microarthropods

Microarthropods were collected during three consecutive years (2009–2011) from soil cores taken with a soil auger of 50 mm internal diameter × 150 mm depth (Burkard Scientific Co. Ltd., Uxbridge, UK) between two adjacent plants of the same row in the middle of each plot, at more than 20 m from the edges. Each sample was comprised by two soil cores to a depth of 9 cm, separated by about 8 cm, lumped together in one hermetic plastic bag. The number of sampling dates throughout the year was 9, 10 and 16 in 2009, 2010 and 2011, respectively, collecting twelve samples for each sampling date (two samples per plot, separated by ≈30 m).

The microarthropods were extracted from the soil samples by Berlese–Tullgren funnels (Burkard Scientific Co. Ltd., Uxbridge, UK) with a 2 mm wire gauze. Each sample was heated for three days with an overhanging 25 W incandescent lamp to dry out the soil from above and drive the mesofauna downward to a collecting vessel filled with Scheerpeltz's solution (60% alcohol, 39% distilled

water and 1% acetic acid) attached to the base of the funnel. Microarthropods from each sample were counted and sorted using a Leica M125 stereomicroscope (Leica Microsystems S.A., Barcelona, Spain). Adult mites were identified to suborder level (Actinedida, Gamasida, Oribatida and Acaridida) (Krantz, 1978). All collembolans were identified to genus or species level (Jordana and Arbea, 1989), which required previous slide-mounted preparations. For this, each specimen was transferred to a watchglass with 25% lactic acid for 72 h and then rinsed and mounted in Hoyer's medium. Slides were examined under optical microscope (CX31, Olympus Optical Co., Tokyo, Japan) at magnifications up to ×1000.

2.3. Cry1Ab protein

Purified Cry1Ab toxin for the ELISA's standard curve and the susceptibility bioassay was supplied by J. Ferré (University of Valencia, Spain) who obtained the toxin from *Escherichia coli* cultures, strain XL1-blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F0 proAB lacIqZDM15 Tn10 (Tetr)]), transformed with the plasmid pBD140 (Bosch et al., 1994), kindly donated by Dr. R.A. de Maagd (Plant Research International B.V., Wageningen, The Netherlands).

2.4. Detection of Cry1Ab in the soil

The presence of the toxin in the soil of the experimental maize field was evaluated in 2013, the last year of the study, when a higher accumulation of toxin might be expected. Cry1Ab rates were examined in soil samples collected from one Bt and one non-Bt plot from the experimental field, at different times (36, 78 and 99 days) after maize harvest. To obtain rhizosphere soil, standing cut stalks (20–30 cm) were pulled out manually from the soil along with the roots and the surrounding soil and then stowed individually in plastic bags. Each sample consisted in the soil surrounding the roots of one stalk. Two rhizosphere soil samples per date were collected from the Bt plot, whereas one sample was collected from the non-Bt plot each date to be used as control. In the laboratory the maize roots were vigorously shaken into the bags to separate the rhizosphere soil fraction attached to the roots (Fig. 1). Afterward, samples were dried (T 6060, Heraeus instruments, Hanou, Germany) at 30 °C for 72 h and sieved through a 0.84 mm diameter mesh to disrupt soil aggregates and to remove pebbles and large tissues. The sieved rhizosphere soil (RS) was weighed and sieved again through a 0.20 mm diameter mesh to discard the smallest size fraction where the toxin is not sufficiently concentrated to be quantified (Hopkins and Gregorich, 2003) (Fig. 1). To extract the organic matter (OM), the soil fraction not discarded (size between 0.84 and 0.20 mm) was immersed in distilled water, gently moved to recover by flotation the part of the particulate OM, mainly plant debris. It was left to repose and the suspended fraction was carefully removed and placed on filter paper; this process was repeated 3 times. The OM recovered was oven dried for 48 h at 25 °C, weighed and ground with liquid nitrogen in a mortar for homogenization to facilitate the extraction of the toxin.

Levels of the Cry1Ab toxin were measured in RS and OM by double-antibody sandwich enzyme-linked immunosorbent assays (DAS ELISA), using the Agdia Bt-Cry1Ab/Cry1Ac Microtiter Plate Kit (Elkhart, IN, USA). Each sample was analyzed in duplicate. For extraction, 0.5 ml of extraction buffer (PBST: phosphate buffered saline—Tween-20), provided in the kit, was mixed with 100 mg of RS or 7.5 mg OM in 1.5-ml micro-centrifuge tubes for 30 min at 500 rpm on a orbital shaker (OS-20, Boeco, Germany), and centrifuged at 14,500 rpm for 15 min. A 100 μl aliquot of the supernatant was dispensed in ELISA wells, along with Cry1Ab standards, positive control and negative control, following the manufacturer' instructions. Standard curves were made using as calibrators different concentration solutions (0.125, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3 and 6 ng/ml) of the purified Cry1Ab protein. The limit of detection (LOD) was calculated using the following equation: $LOD = 3\sigma/S$, where σ is the standard deviation of 8 buffer-only controls and S is the slope of the calibration curve (ICH, 2005). The resulting LOD was 0.17 ng Cry1Ab/ml protein solution, so the toxin was considered as non-detected when values were below this number. Spectrophotometric measurements were conducted in a microtiter plate reader at a wavelength of 650 nm, using VersaMax™ Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA).

2.5. Insecticidal activity of the Cry1Ab toxin extracted from the soil OM

To test the insecticidal activity, the toxin was extracted using the same methodology explained in Section 2.4. In this case the OM recovered in the three dates was pooled, and three grams of the mix were vigorously homogenized in a mortar with 70 ml of PBST buffer before centrifuging. The supernatant was separated, recovering a total of 66 ml, which was lyophilized to concentrate the toxin and resuspended in 1.67 ml of sterile Milli-Q water (Milli-Q System, Millipore, France). The same procedure was followed with samples from conventional non-Bt maize, used as control. The toxin was quantified by ELISA following the procedure described in Section 2.4.

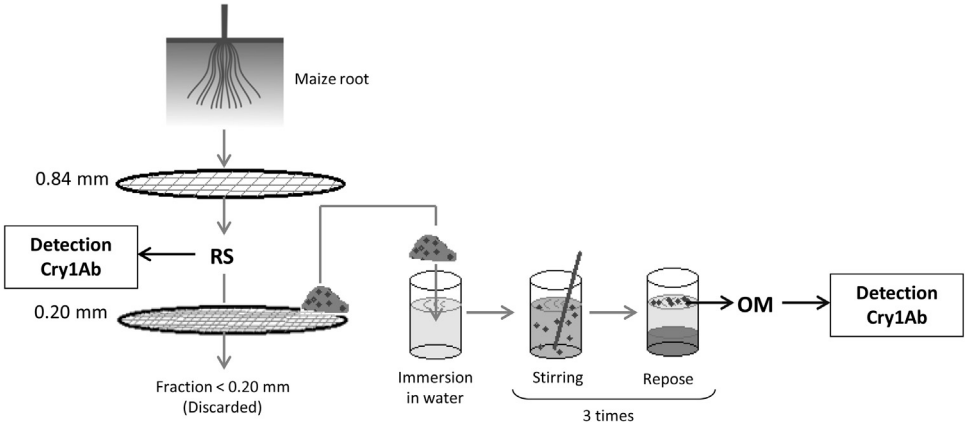

Fig. 1. Fractioning of rhizosphere soil samples and extraction of organic matter. RS = rhizosphere soil after it was sieved through a 0.84 mm diameter mesh; OM = organic matter, extracted from the soil fraction between 0.84 and 0.20 mm.

The insecticidal activity of the toxin recovered in the OM was tested against a laboratory population of the susceptible species *O. nubilalis* following the procedure described in Farinós et al. (2004), with some modifications. The bioassay was performed in "Bio-Ba-128" plastic trays (Color-Dec Italy, Capezzano Pianore, Italy). About 0.5 ml of artificial diet was dispensed in each well. Once solidified, 50 μl of the extracts of the Bt or non-Bt (control) OM were dispensed on the surface of the diet. A final concentration of 1.1 ng Cry1Ab/cm^2 (according to our ELISA estimation) was applied in the bioassay. After drying, two larvae <24 h were placed per well using a fine brush and covered with a breathing adhesive cover "Bio-Cv-16" (Color-Dec Italy, Capezzano Pianore, Italy). The number of larvae tested were 62 and 32 in the Bt and non-Bt (control) treatments, respectively. The trays were incubated in rearing chambers at 25 ± 1 °C, 70 ± 5% relative humidity and total darkness. After 7 days of exposure mortality was recorded, considering dead larvae those not showing any reaction when they were gently pushed.

A parallel bioassay to estimate the expected mortality at 1.1 ng Cry1Ab/cm^2 was carried out using purified Cry1Ab toxin and the same laboratory population of *O. nubilalis*, following the methodology explained in Farinós et al. (2004). Three replicates were performed, with 32 larvae at each concentration (0.25, 0.5, 1, 2, 4, 8, 16 and 32 ng Cry1Ab/cm^2) and 64 for the control. The log-dose/probit mortality model yielded the regression line $y = 1.3x + 4.95$ ($\chi^2 = 85.5$; d.f. = 22) and an expected mortality after seven days of 49.5% at 1.1 ng Cry1Ab/cm^2.

2.6. Exposure of Entomobrya spp. to the Cry1Ab toxin

Cry1Ab levels were measured in specimens of *Entomobrya* spp. collected from the husk leaves of maize ears. These species are the most abundant epedaphic collembolans found in soil samples (see Section 3), but are also found on maize plants at the end of the maize season. Ten maize ears from a Bt maize plot and five from a non-Bt maize plot were taken on October 2013 from the experimental field, shortly before harvesting. Ears were snapped off by hand and put into plastic bags. In the laboratory, the ears were dissected by removing husk leaves one by one, and live collembolans were captured with an insect aspirator and immediately frozen at −20 °C. They were then placed in Petri dishes over dry ice and examined using a stereomicroscope to select only *Entomobrya* specimens. Collembolans picked from Bt maize ears were randomly divided into three groups containing 150–200 specimens, each of which was considered a replicate, whereas a sample with 200 specimens collected from non-Bt maize ears was used as control. Cry1Ab levels were determined by ELISA following the same protocol as described above, but in this case 0.3 ml of PBST was mixed with the collembolans for extraction and centrifuged at 12,000 rpm for 5 min.

2.7. Statistical analysis

A Generalized Estimating Equations model (GEE) was used to determine the effects of Bt maize cultivation on different population parameters of soil-dwelling microarthropods: abundance, species richness, species diversity and frequency of occurrence. The GEE procedure is an extension of the generalized linear model that allows the analysis of repeated measurements whether or not the data follow a normal distribution. The *sampling date* each year was entered as a repeated-measure factor. In our analysis, the factors *treatment* (Bt and non-Bt), *year* (2009, 2010 and 2011) and *block* were used as fixed factors and the interactions *treatment* × *year* and *treatment* × *block* were also analyzed. In addition, the factor *sampling date* nested within *year* was analyzed, since the number of sampling dates varied from year to year. Differences in the abundances of soil mites and springtails between Bt and non-Bt plots were analyzed assuming Poisson distributions, being the dependent variables the average number of mites, collembolans, mite suborders and collembolan species per sample. In the case of collembolans, species richness and two diversity indices (Shannon and Simpson) were calculated, based on the number of individuals from each species per soil sample, and differences in these indices were analyzed assuming a normal distribution. Differences in the frequency of occurrence of the seven predominant collembolan species between samples of Bt and non-Bt maize plots was also analyzed by GEE. For that purpose, the data of abundances of these species in each sample were transformed to a binomial distribution in which the value 1 represented the presence of the species in the sample and the value 0 absence. For all analyses, when a significant interaction between the factors *year* and *treatment* was found, data were

Table 1
Main microarthropods found in Bt and non-Bt maize during three consecutive years. Data represent total percentages of individuals collected in the soil samples per type of maize and year.

	Groups of microarthropods		Proportion of specimens (%)					
			2009		2010		2011	
			Bt	non-Bt	Bt	non-Bt	Bt	non-Bt
Arachnida	Acari		76.19	71.70	71.88	76.02	71.05	70.72
	Araneae		0.12	0.08	0.23	0.18	0.18	0.11
	Collembola		17.03	22.07	17.66	12.29	12.12	10.45
	Coleoptera	Staphylinidae	2.07	1.83	1.98	1.99	1.51	1.40
		Carabidae	0	0	0	0.06	0.23	0.07
		Other	0.52	0.12	0.17	0.48	0.35	0.39
Hexapoda	Diptera (larvae)	Chironomidae	1.04	0.56	1.39	1.57	9.53	9.85
		Other	0.52	0.95	1.39	2.65	1.06	1.72
	Psocoptera		0.20	0.12	0.12	0.06	0	0.04
	Thysanoptera		0.16	0.40	0.23	0.30	0.28	0.60
	Homoptera	Cicadellidae	0.48	0.56	1.22	1.20	0.73	0.49
	Hymenoptera	Formicidae	0	0	0.06	0.18	0.20	0.32
Myriapoda	Diplopoda	Julidae	0.24	0.32	1.92	1.02	1.08	2.49
		Polydesmidae	0.44	0.32	0.46	0.42	0.03	0
	Pauropoda		0.36	0.48	0.35	0.42	0.83	0.81
	Symphyla		0.56	0.40	0.29	0.72	0.55	0.35
	Chilopoda	Scolopendromorpha	0.08	0.12	0.64	0.30	0	0
		Lithobiomorpha	0	0	0	0.12	0.28	0.21

analyzed pooling the data of the three years to retain the total available degrees of freedom and improve the error estimates. Statistical analyses were performed using SPSS software (IBM© SPSS© Statistics, Version 20, 2011).

The susceptibility of the lepidopteran species *O. nubilalis* to Cry1Ab was determined by means of larval mortality data. A log-dose/response regression line was calculated for purified Cry1Ab by probit analysis using the program Polo-Plus (LeOra Software©, Version 1.0, 2002–2015). Differences between observed larval mortality in soil-extracted Cry1Ab at 1.1 ng Cry1Ab/cm^2 and expected mortality at this concentration, estimated using purified Cry1Ab, were analyzed by Pearson's χ^2 test, and the Yates's correction for 1 d.f. was applied. All the data of variability showed in the text refer to the standard errors of the means. A significance level of $P < 0.05$ was used for all tests.

3. Results

3.1. Composition of the microarthropod community

A total of 15,235 microarthropods belonging to different taxonomic groups were collected through 2009, 2010 and 2011. The mean total number of microarthropods per soil sample was different each year, having the samples of 2009 a higher density (48.64 ± 4.41) than those of 2010 and 2011 (28.53 ± 2.26 and 36.06 ± 2.73, respectively). Considering the three years together, the majority of arthropods were mites (Acari, 73%) and springtails (Collembola, 15%), both maintaining similar rates throughout the study (Table 1). To a lesser extent, other microarthropods were also regularly found in soil samples from Bt and non-Bt maize fields. Dipterans, mostly chironomid larvae (Diptera: Chironomidae), accounted for an average of 6.21% of the total of specimens collected, although with variable numbers among years. Myriapods (2.73% of the total) were represented by the four classes, comprising individuals with mainly detritivore feeding habits (Diplopoda, Pauropoda and Symphyla) and other primarily predators (Chilopoda). The following group in abundance was Coleoptera (2.16% of the total collected), mainly composed of larvae and adults of rove beetles (Coleoptera: Staphylinidae) and ground beetles (Coleoptera: Carabidae). Other minority taxonomic groups that did not exceed 1% of the total number of captures were spiders (Araneae), psocids (Psocoptera), thrips (Thysanoptera), leafhoppers (Homoptera: Cicadellidae) and ants (Hymenoptera: Formicidae).

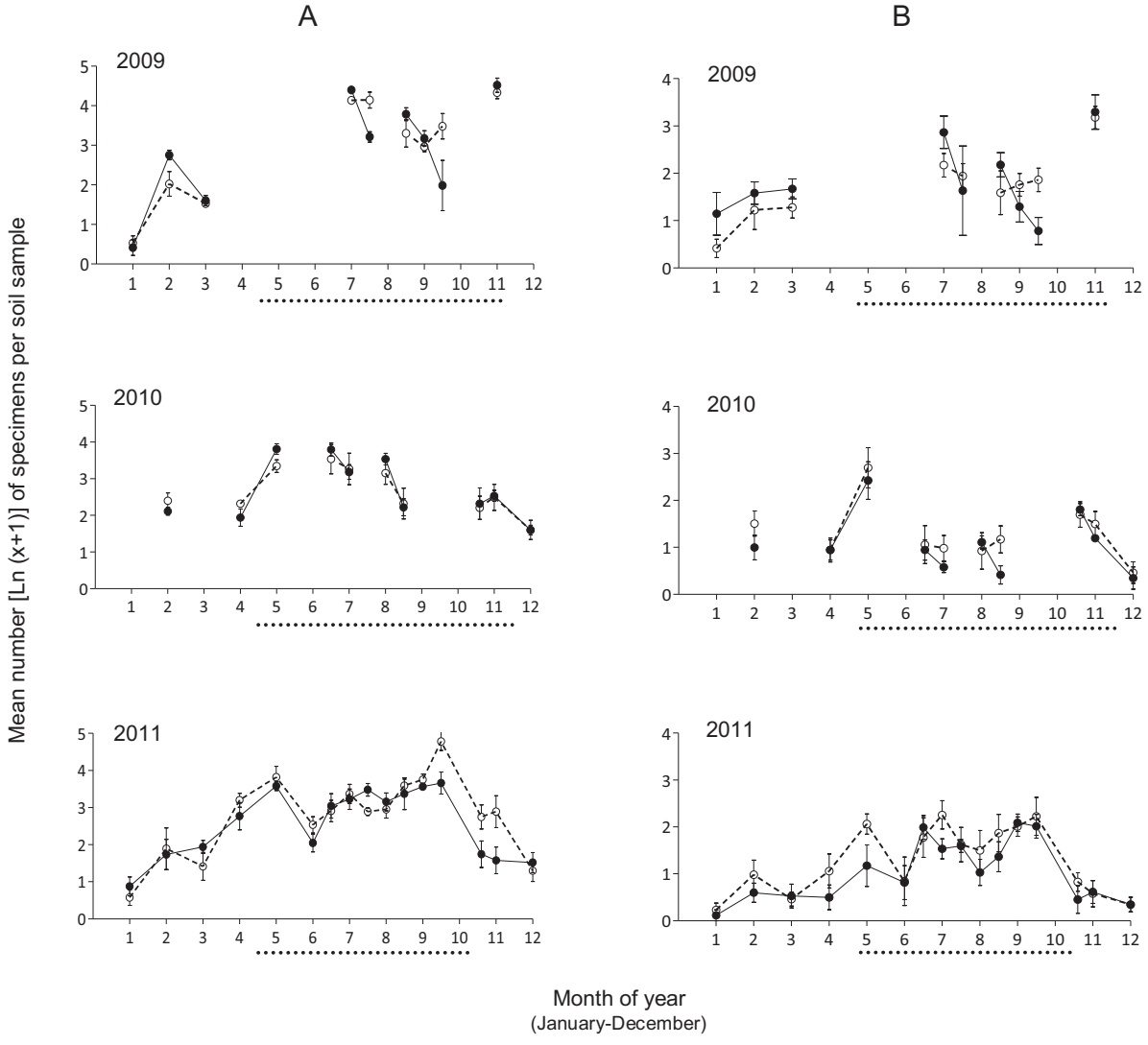

Fig. 2. Abundance of soil mites (A) and collembolans (B) collected in Bt maize (—○—) and non Bt maize (—●—) plots during the three years of study. Data are expressed as mean number [Ln (x+1)] of specimens (±S.E.) per soil sample. The dotted line (••••) every year represents the period in which the crop is in the experimental field.

3.2. Abundance, population dynamics and community structure of soil mites in Bt and non-Bt maize

Acari was the most abundant taxon in soil samples, with a total of 11,057 specimens collected. The majority (94%) could be identified at the suborder level, being the main suborders represented in the samples: Oribatida (45%), Actinedida (37%), Gamasida (10%) and Acaridida (1%). Dynamics of the total number of soil mites was similar through the study, and total abundances did not show significant differences among years and between Bt and non-Bt plots (Fig. 2A, Table 2). The factor *year* significantly affected the abundances of the suborders Oribatida, Gamasida and Actinedida, whereas significant differences between treatments were only found in Actinedida (Table 2), mainly due to the high values observed in Bt plots in one sampling date of 2011 (data not shown). Acaridida showed very stable values of abundance regardless of the treatment and sampling year. In no case significant interactions between the two factors analyzed were found in soil mites.

3.3. Abundance, population dynamics and species composition of springtails in Bt and non-Bt maize

Collembolans were the second group in abundance in soil samples of Bt and non-Bt maize fields. The four suborders (Entomobryomorpha, Poduromorpha, Neelipleona and Symphypleona) were represented in the samples, making a total of 2271 specimens that were identified at species level. Twenty-five species were recorded, most of them belonging to the families Entomobryidae and Isotomidae (Entomobryomorpha) (Table 3). Seven species (*Entomobrya schoetti* Stach, *Parisotoma notabilis* (Schäffer), *Ceratophysella gibbosa* (Bagnall), *Entomobrya lanuginosa* Nicolet, *Isotomurus palustris* (Müller), *Isotomodes productus* (Axelson) and *Cryptopygus thermophilus* (Axelson)) accounted for 85% of the total specimens recorded, from which the first two, *E. schoetti* and *P. notabilis*, were clearly predominant (25.28% and 21.44% of the total, respectively).

Analysis of the collembolan abundance showed that there was a significant interaction between the factors *year* and *treatment* when the total springtails were considered and for the species *E. schoetti* and *E. lanuginosa*, so variations in abundance between Bt and non-Bt plots were analyzed pooling the data of the three years. No differences in the abundance of total springtails (Wald $\chi^2 = 0.64$, $P = 0.43$), *E. schoetti* (Wald $\chi^2 = 0.08$, $P = 0.77$) and *E. lanuginosa* (Wald $\chi^2 = 1.90$, $P = 0.17$) were detected between treatments. In the remaining five species the abundances were significantly influenced by the year, but only in the case of *P. notabilis* the factor *treatment* also presented a significant effect, being more abundant in Bt maize (Fig. 2B, Table 4). Species richness varied depending on both the sampling year and the type of maize, with significant higher values in Bt plots than in non-Bt plots (Table 4). Likewise, species diversity, analyzed by Simpson and Shannon indices, showed significant differences between both treatments, also showing higher values in Bt plots than in non-Bt ones, whereas no differences were detected among years (Table 4).

Significant differences in the frequencies of occurrence were detected among years for the seven main species, but only *P. notabilis* presented differences between treatments, being more frequently found in Bt than in non-Bt plots (Table 5). From these species, *P. notabilis*, *E. schoetti*, *C. gibbosa*, *I. productus* and *E. lanuginosa* were found in both types of maize plots in the three years. The occurrence of *I. palustris*, one of the most common species in 2009 (28% and 38% of samples of Bt and non-Bt plots, respectively) decreased considerably in 2010 (to 5% in both Bt and non-Bt plots) and it was not present in 2011. In contrast, *C. termophilus* was absent in 2009 and increased gradually the frequency of occurrence in 2010 and 2011 in both types of maize fields. The presence of the rest of collembolan species in soil samples was uneven depending on the year (data not shown).

3.4. Detection of Cry1Ab and insecticidal activity

The Cry1Ab toxin was detected in OM samples coming from Bt maize plots at 36, 78 and 99 days after harvest, with values ranging between 0.10 and 0.18 ng Cry1Ab/mg of OM. On the contrary, it was not detected in the OM from non-Bt plots or in any of the samples of the RS fraction, either from Bt or from non-Bt plots (Table 6). The OM recovered from rhizosphere soil samples represented in average the 0.22 ± 0.03 and 0.22 ± 0.06% of the dry weight of RS in Bt and non-Bt samples, respectively.

The insecticidal activity of the Cry1Ab extracted from the OM of Bt plots was tested at 1.1 ng/cm^2 against neonates of *O. nubilalis*. Larval mortality after seven days was 12.8% (after Abbott's correction for control mortality), which resulted significantly different from the expected mortality at this concentration (49.5%) predicted from the bioassay with purified Cry1Ab toxin (Pearson's $\chi^2 = 52$; $P < 0.05$).

The Cry1Ab toxin was detected in the three samples of *Entomobrya* spp. collembolans collected on husk leaves of Bt maize plants (0.136 ± 0.006 ng Cry1Ab/mg of collembolan), whereas no Cry1Ab toxin was detected in those captured on non-Bt maize plants.

4. Discussion

The potential impacts of Cry-expressing Bt maize on soil organisms depend, at least in part, on the persistence of the insecticidal toxin and its biological activity in the soil. In this study we demonstrate that the Cry1Ab toxin expressed in MON810 maize is detected in the partially decomposed organic

Table 2
Abundance of soil mites collected in soil samples of Bt and non-Bt maize during three consecutive years.

	Abundance per soil sample (mean ± S.E.)[a]						GEE results[b]		
	2009		2010		2011		Year	Treatment	Year × Treatment
	Bt	Non-Bt	Bt	Non-Bt	Bt	Non-Bt	Wald χ^2 (*P*-value) (d.f. = 2)	Wald χ^2 (*P*-value) (d.f. = 1)	Wald χ^2 (*P*-value) (d.f. = 2)
Total mites	35.37 ± 4.69	36.12 ± 5.31	20.62 ± 2.67	21.03 ± 2.55	29.43 ± 4.01	21.01 ± 2.21	3.68 (0.16)	2.13 (0.14)	2.75 (0.25)
Oribatida	16.09 ± 2.50	16.76 ± 2.69	7.60 ± 1.20	7.02 ± 0.97	14.18 ± 1.93	11.20 ± 1.67	15.35 (<0.00)*	1.72 (0.19)	1.02 (0.60)
Actinedida	14.87 ± 2.28	14.68 ± 2.60	9.57 ± 1.91	8.43 ± 1.67	10.16 ± 2.42	4.96 ± 0.93	18.88 (<0.00)*	8.54 (<0.00)*	5.71 (0.06)
Gamasida	2.70 ± 0.56	2.04 ± 0.40	2.07 ± 0.35	2.83 ± 0.47	3.19 ± 0.47	3.00 ± 0.48	11.11 (<0.00)*	0.00 (0.99)	4.74 (0.09)
Acaridida	0.33 ± 0.12	0.44 ± 0.20	0.37 ± 0.22	0.33 ± 0.10	0.49 ± 0.25	0.12 ± 0.05	4.79 (0.09)	1.85 (0.17)	3.99 (0.14)

[a] The number of samples were 54 (2009, Bt plots), 50 (2009, non-Bt plots), 60 (2010, Bt and non-Bt plots) and 96 (2011, Bt and non-Bt plots).
[b] Generalized Estimating Equations analysis. The results of the factors *block*,*sampling date* nested within *year* and the interaction *block* × *treatment* are not shown.
* Significant differences ($P < 0.05$).

Table 3
List of the collembolan species collected in soil samples of Bt and non-Bt maize plots from 2009 to 2011.

Order	Family	Species	Proportion (%)
Entomobryomorpha	Entomobryidae	*Entomobrya schoetti*	25.28
		Entomobrya lanuginosa	6.25
		Entomobrya sp1	0.40
		Entomobrya sp2	1.32
		Pseudosinella imparipunctata	2.07
		Pseudosinella templadoi	1.41
		Sinella coeca	0.97
		Lepidocyrtus lusitanicus	0.22
		Willowsia platini	0.04
		Orchesella quinquefasciata	0.04
	Isotomidae	*Parisotoma notabilis*	21.44
		Isotomurus palustris	6.12
		Isotomodes productus	4.98
		Cryptopygus thermophilus	4.45
		Folsomia checae	1.54
		Proisotoma sp.	1.37
		Pseudanurophorus isotoma	0.13
		Folsomides parvulus	0.13
	Cyphoderidae	*Cyphoderus bidentaculatus*	1.94
Poduromorpha	Hypogastruridae	*Ceratophysella gibbosa*	16.03
	Onychiuridae	*Mesaphorura macrochaeta*	1.98
Neelipleona	Neeliidae	*Megalothorax minimus*	0.79
		Neelus sp.	0.04
Symphypleona	Sminthurididae	*Sphaeridia pumilis*	0.35
	Arrhopalitidae	*Arrhopalites microphthalmus*	0.18
Not identified			0.53

Table 4
Abundance, species richness and diversity indices of springtails collected in soil samples of Bt and non-Bt maize during three consecutive years.

	Data per soil sample (mean ± S.E.)[a]						GEE results[b]		
	2009		2010		2011		Year	Treatment	Year × Treatment
	Bt	Non-Bt	Bt	Non-Bt	Bt	Non-Bt	Wald χ^2 (*P*-value) (d.f. = 2)[c]	Wald χ^2 (*P*-value) (d.f. = 1)	Wald χ^2 (*P*-value) (d.f. = 2)[c]
Abundance									
Total springtails	7.91 ± 1.33	11.12 ± 2.06	5.07 ± 1.35	3.40 ± 0.89	5.02 ± 0.68	3.10 ± 0.38	#	#	16.09 (<0.00)*
E. schoetti	1.70 ± 0.62	3.30 ± 1.52	0.72 ± 0.25	0.33 ± 0.09	1.61 ± 0.42	1.03 ± 0.24	#	#	11.75 (<0.00)*
E. lanuginosa	0.96 ± 0.38	0.36 ± 0.20	0.30 ± 0.11	0.52 ± 0.18	0.17 ± 0.07	0.07 ± 0.03	#	#	14.65 (<0.00)*
P. notabilis	0.94 ± 0.27	1.10 ± 0.43	1.72 ± 0.59	1.00 ± 0.36	1.35 ± 0.27	0.92 ± 0.23	15.63 (<0.00)*	6.52 (0.01)*	2.55 (0.28)
I. palustris	1.00 ± 0.32	1.54 ± 0.41	0.05 ± 0.03	0.08 ± 0.05	0	0	34.02 (<0.00)*	1.14 (0.29)	0.04 (0.83)
I. productus	0.44 ± 0.23	0.66 ± 0.22	0.15 ± 0.06	0.08 ± 0.04	0.28 ± 0.09	0.16 ± 0.06	10.42 (0.01)*	1.31 (0.25)	3.58 (0.17)
C. thermophilus[d]	0	0	0.05 ± 0.03	0.12 ± 0.06	0.75 ± 0.29	0.20 ± 0.06	5.23 (0.02)*	0.01 (0.91)	–
C. gibbosa	1.15 ± 0.52	2.86 ± 0.74	1.30 ± 0.74	0.90 ± 0.59	0.16 ± 0.10	0.12 ± 0.06	45.02 (<0.00)*	0.40 (0.53)	3.36 (0.19)
Species richness	2.48 ± 0.25	2.16 ± 0.24	1.72 ± 0.16	1.37 ± 0.14	1.66 ± 0.15	1.35 ± 0.13	14.03 (<0.00)*	9.27 (<0.00)*	0.06 (0.97)
Diversity indices									
Simpson	0.35 ± 0.04	0.24 ± 0.04	0.33 ± 0.04	0.22 ± 0.03	0.29 ± 0.03	0.28 ± 0.03	2.55 (0.28)	10.46 (<0.00)*	2.74 (0.25)
Shannon	0.63 ± 0.04	0.45 ± 0.07	0.53 ± 0.06	0.34 ± 0.06	0.49 ± 0.06	0.46 ± 0.06	4.80 (0.09)	9.80 (<0.00)*	2.00 (0.37)

[a] The number of samples were 54 (2009, Bt plots), 50 (2009, non-Bt plots), 60 (2010, Bt and non-Bt plots) and 96 (2011, Bt and non-Bt plots).
[b] Generalized Estimating Equations analysis. The results of the factors *block*, *sampling date* nested within *year* and the interaction *block* × *treatment* are not shown.
[c] Except for *I. palustris* y *C. thermophilus*, with d.f. = 1.
[d] In this species the interaction *year* × *treatment* could not be computed.
[#] Results of the statistical analysis for the actors *year* and *treatment* have been omitted in the Table when their interaction was significant. In this case, data were reanalyzed pooling the data of the three years. No significant differences were detected between treatments in total springtails (Wald $\chi^2 = 0.64$, $P = 0.43$); *E. schoetti* (Wald $\chi^2 = 0.08$, $P = 0.77$) and *E. lanuginosa* (Wald $\chi^2 = 1.90$, $P = 0.17$).
[*] Significant differences ($P < 0.05$).

matter (OM) extracted from the rhizosphere soil (RS) for at least three months after maize harvest. The presence and persistence of Cry1Ab in decaying residues from Bt maize have been evidenced under different agronomical conditions (Baumgarte and Tebbe, 2005; Muchaonyerwa et al., 2004; Hopkins and Gregorich, 2003; Zwahlen et al., 2003). However, other studies have shown that the levels of Cry1Ab incorporated into the soil from Bt maize residues decreased drastically throughout the post-harvest period (Daudu et al., 2009; Zurbrügg et al., 2010). Temperature has been suggested as having a major influence on decomposition, most probably due to the correlation between microbial activity and temperature (Zurbrügg et al., 2010). Our results show that toxin levels did not

Table 5
Frequency of occurrence of the seven predominant collembolan species collected in soil samples of Bt and non-Bt maize during three consecutive years.

	Frequency of occurrence (% of samples)[a]						GEE results[b]		
	2009		2010		2011		Year	Treatment	Year × Treatment
	Bt	Non-Bt	Bt	Non-Bt	Bt	Non-Bt	Wald χ^2 (*P*-value) (d.f. = 2)[c]	Wald χ^2 (*P*-value) (d.f. = 1)	Wald χ^2 (*P*-value) (d.f. = 2)[c]
E. schoetti	35.19	24.00	23.33	23.33	32.29	28.13	143.17 (<0.00)*	2.29 (0.13)	0.60 (0.74)
E. lanuginosa	16.67	10.00	15.00	18.33	8.33	5.21	195.90 (<0.00)*	2.29 (0.13)	3.28 (0.19)
P. notabilis	29.63	24.00	41.67	31.67	36.46	26.04	747.38 (<0.00)*	8.94 (<0.00)*	0.29 (0.87)
I. palustris	27.78	38.00	5.00	5.00	0	0	705.33 (<0.00)*	2.47 (0.12)	0.28 (0.60)
I. productus	18.52	28.00	11.67	6.67	13.54	9.38	398.19 (<0.00)*	1.53 (0.22)	4.20 (0.12)
C. thermophilus[d]	0	0	5.00	8.33	19.79	13.54	–	–	–
C. gibbosa	20.37	32.00	21.67	15.00	7.29	7.29	100.36 (<0.00)*	1.82 (0.18)	3.93 (0.14)

[a] The number of samples were 54 (2009, Bt plots), 50 (2009, non-Bt plots), 60 (2010, Bt and non-Bt plots) and 96 (2011, Bt and non-Bt plots).
[b] Generalized Estimating Equations analysis. The results of the factors *block*, *sampling date* nested within *year* and the interaction *block* × *treatment* are not shown.
[c] Except for *I. palustris* y *C. thermophilus*, with d.f. = 1.
[d] In this species the analysis was not computed due to an insufficient sample size.
* Significant differences ($P < 0.05$).

Table 6
Presence of Cry1Ab in rhizosphere soil samples collected from Bt and non-Bt plots after maize harvest. Data are means ± S.E.

Time after maize harvest	Type of maize (n° of samples)	Cry1Ab in RS[a] (ng Cry1Ab/mg RS)	Cry1Ab in OM[b] (ng Cry1Ab/mg OM)
36 days	Bt (2)	n.d.	0.11 ± 0.01
	Non-Bt (1)	n.d.	n.d.
78 days	Bt (2)	n.d.	0.18 ± 0.02
	Non-Bt (1)	n.d.	n.d.
99 days	Bt (2)	n.d.	0.10 ± 0.01
	Non-Bt (1)	n.d.	n.d.

n.d.: non detected.
[a] RS: soil obtained after sieving rhizosphere soil samples through 0.84 mm diameter mesh to discard the coarse fraction.
[b] OM: part of the particulate organic matter obtained after sieving rhizosphere soil samples through 0.84 and 0.02 mm diameter mesh to discard the coarse and the fine fractions, and then separated by flotation.

follow a decay tendency over time (36, 78 and 99 days after harvest), indicating that exposure of detritivore soil organisms to Cry1Ab could be persistent also in temperate climates as is the case of southern Europe. The maintenance of the Bt-toxin level in the period of time analyzed herein is not surprising, since the OM fraction analyzed contains partially decomposed plant remains, i.e. mainly root pieces. They will pass to a more decomposed stage (inaccessible to our method), but the Cry1Ab toxin will remain present due to the input of new pieces. However, when the toxicity of the Cry1Ab extracted from the OM recovered from rhizosphere soil samples was tested against neonate larvae of *O. nubilalis*, the mortality obtained (12.8%) was lower than expected (~50.0%, according to our bioassay with purified Cry1Ab). Lutz et al. (2005) showed that the antibody used in the ELISA kit employed in our study reacts also with degraded fragments of the Cry1Ab protein of approximately 17 and 34 kDa size. Thus, the reduced toxicity observed in the toxin extracted from the OM could be because the ELISA kit is quantifying the intact insecticidal toxin as well as the degradation products of the toxin without insecticidal activity, which could have resulted in an overestimation of the concentration of the toxin used in the bioassay. Zurbrügg et al. (2010) also showed in a field experiment with litterbags that the toxicity of Bt maize leaf residues was lost 4 months after harvest, even though the Cry1Ab toxin was still detected. In the light of all these points, our results suggest that the Cry1Ab toxin expressed in MON810 is incorporated to the soil and can persist in the OM for months though we cannot discriminate if all the toxin remains in an active form.

The Cry1Ab toxin has been successfully quantified in particle-size fractions of soil >2 mm, probably because they contained relatively large pieces of organic matter from Bt maize residues, whereas only traces were detected in soil fractions <2 mm, with more fragmented residues of organic matter (Hopkins and Gregorich, 2003; Gruber et al., 2012). Likewise, we could not detect the Cry1Ab toxin when analyzing samples of rhizosphere soil from Bt maize plots (RS fraction <0.84 mm), being necessary to extract the partially decomposed organic matter (fragments of OM comprised between 0.84 and 0.2 mm) to detect the toxin. The discrepancy relative to the detection of Cry1Ab in OM vs. RS samples could be explained by the fact that the OM extracted represents in average the 0.22% of the dry weights of RS in Bt and non-Bt samples. On the basis of the Cry1Ab levels recovered in the OM, we have estimated values between 0.18 and 0.48 ng Cry1Ab g^{-1} RS, which are under the minimum amount required to be detected in the ELISA (0.85 ng Cry1Ab g^{-1} RS). Other factor that may have contributed to the failure to quantify the toxin in RS could be the strong binding of Cry1Ab to clay minerals and humic acids, impeding its extraction. Accordingly, Gruber et al. (2012) reported a strong correlation between the recovery of Cry1Ab protein from soils fortified with known amounts of Cry1Ab protein and the clay content of the soils assessed. The approach for the detection of Cry1Ab in soil samples proposed in this study is a simple method with which the partially decomposed fragments of OM comprised between 0.84 and 0.2 mm are concentrated from a rhizosphere soil sample, enabling the standardization of the quantification of the toxin in this fraction.

There are no reports of the detection of Cry toxins in microarthropod fauna, probably due to the difficulty to obtain a large number of live specimens of the same species that must be immediately identified and frozen to avoid the metabolization and/or excretion of the toxin. In this study we report for the first time that collembolans are exposed to Cry1Ab toxin from maize plants in the field. Thus, the Cry1Ab toxin could be quantified in specimens of the epedaphic *Entomobrya* spp., the main

collembolan genus in our soil samples, collected from the husk leaves of ears, where they were very abundant at the end of the maize season. These species have been described mainly as saprophagous and fungal feeders, so their presence in mature maize plants just prior to harvest might be either to exploit the decaying organic matter and fungi accumulated among maize foliage in the final stage of development of the crop or to avoid adverse environmental conditions (Frampton et al., 2001). Collembolans, and specifically *Entomobrya* spp., seem to be an important source of food for spiders (McNabb et al., 2001; Peterson et al., 2010) and for epigeic generalist predators in agricultural soils, such as carabids (Bilde et al., 2000). Thus, our results indicate that in field conditions *Entomobrya* spp. could be intermediaries so that toxin could reach other trophic levels. Actually, exposure to Cry toxin from Bt crops has been demonstrated in field-collected soil generalist, such as the carabid *Poecilus cupreus* L. (Álvarez-Alfageme et al., 2009) or the epigeic spider *Pardosa occidentalis* Simon (unpublished results) coming from Cry1Ab-expressing Bt maize fields from central Spain. We cannot conclude if the acquisition of toxin by *Entomobrya* spp. happens while they are in the soil, when they climb maize plants or in both situations, but their position in the trophic web and the possibility to occupy different spatial niches could entail the relocation of the toxin from one to another.

No negative effects of Bt maize on the soil microarthropod community were found in this multiyear field study. An examination of dynamics of mites and collembolans, the main soil microarthropods, showed similar patterns of abundance within a year, regardless of the type of maize except for *P. notabilis*, that was more abundant in Bt maize. However, significant differences in abundance among years were common in mite suborders and in collembolan species, indicating that abiotic factors rather than the use of GM maize could affect the populations. Similarly, the frequency of occurrence of collembolan species did not rely on the type of maize, except in the case of *P. notabilis*. This species was also detected in higher numbers in Bt maize expressing Cry3Bb1 than in non-Bt maize in one of the sampling dates (Bitzer et al., 2005). It was remarkable the progressive increase in the frequency of occurrence of *C. thermophilus*, absent in 2009, and the contrary case in *I. palustris*, not present in 2011. Our results also indicate that the exposure to toxin by different ways did not affect species living at different depths. In fact, among the dominant species of collembolans we found species associated with epedaphic, hemiedaphic or euedaphic ecological niches, being so distributed through different soil profiles. This is the case of the two predominant ones, which normally occur in different soil levels: *E. schoetti* is epedaphic, whereas the cosmopolitan *P. notabilis* is hemiedaphic (Álvarez et al., 2001). Interestingly, a field study carried out in the same experimental plots as our work, showed that the cultivation of Bt maize during a four-year period (2008–2011) did not change the maize rhizobacterial communities with respect to those of the non-Bt maize plots (Barriuso et al., 2012), so mites and collembolans feeding on them should neither be affected by this reason. On the whole, findings of the present field study suggest that the differences in abundance observed in the groups or species considered in more detail derive more from seasonal or environmental factors. Some field experiments carried out in different agroecological conditions have concluded that the effects of Bt maize expressing Cry1Ab on microarthropod fauna were comparable to effects produced by other common agricultural practices, such as insecticide treatments or the use of different non-Bt maize varieties (Candolfi et al., 2004; Cortet et al., 2007). Likewise, a 2-years field trial with Cry3Bb1-expressing Bt maize detected only few differences in the abundance of some springtail species when compared with the isoline maize (Bitzer et al., 2005). However, in the case of this protein it has been reported that its degradation in the soil is faster than that of Cry1Ab, so exposure level of soil microarthropods to Cry3Bb1 is likely to be low and transitory (Xue et al., 2014; Zurbrügg et al., 2010). Additionally, laboratory studies have been performed in controlled conditions with the collembolans *Folsomia candida* (Wilem) and *Xenylla grisea* Axelson, tipically used as test organisms for estimating the effects of different environmental pollutants on non-target soil arthropods. They showed that neither of the two species were adversely affected when they fed on diet treated with different purified insecticidal Cry toxins (Sims and Martin, 1997), nor survival and fitness of *F. candida* were affected after feeding on leaves of Cry1Ab-expressing Bt maize (Clark and Coats, 2006).

Noticeably, we found higher values of species richness and diversity of collembolans in Cry1Ab-expressing Bt maize than in non-Bt plots, in contrast to the absence of changes in collembolan diversity observed by Priestley and Brownbridge (2009) in an experiment using the same types of maize. The increase in species richness and diversity of collembolans and in the abundance of *P. notabilis* that we observe could be explained under different scenarios. Firstly, by the higher lignin content that some authors have observed in Bt maize compared to levels in their corresponding isogenic lines (Flores et al., 2005; Poerschmann et al., 2005), which could contribute to the accumulation of organic matter on the topsoil and consequently to the formation of a number of micro-environments that could be exploited by different species. Besides, higher lignin content has been related with a higher resistance of Bt maize to decay (Stotzky, 2004). However, other field studies using different methodologies concluded that the decomposition rates of Bt and non-Bt tissues were the same (Daudu et al., 2009; Lehman et al., 2010), so the differential decomposition and lignin content in Bt maize compared to its isogenic line remains unclear. Secondly, certain changes in the agroecosystem might modify the trophic connections between predators and prey populations, producing variations in soil communities. For example, the cultivation of Bt maize in Central Spain evidenced in some years a decrease in the number of rove-beetles, which are potential predators of soil microarthropods (de la Poza et al., 2005; Farinós et al., 2008). Further laboratory investigation under controlled conditions would be needed to explore possible alterations in trophic linkages due to the cultivation of Bt maize.

In summary, continuous cultivation of MON810 maize did not negatively affect the community structure of soil decomposers studied. The toxin was detected in a fraction of rhizosphere soil organic matter up to three months after maize harvest, as well as in the epedaphic collembolans of the genus *Entomobrya* collected on maize plants. However, its concentration in an active form resulted very low. This study complements a series of long term field reports carried out in Spain to assess the potential effects of MON810 maize cultivation on non-target arthropods, in which we have not found large shifts with respect to those of conventional maize, providing evidence that Bt maize could be compatible with the arthropod fauna present in the crop.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2015.09.007.

References

Al-Deeb, M.A., Wilde, G.E., Blair, J.M., Todd, T.C., 2003. Effect of Bt corn for corn rootworm control on nontarget soil microarthropods and nematodes. Environ. Entomol. 32, 859–865.

Albajes, R., Farinós, G.P., Pérez-Hedo, M., de la Poza, M., Lumbierres, B., Ortego, F., Pons, X., Castañera, P., 2012. Post-market environmental monitoring of Bt maize in Spain: non-target effects of varieties derived from the event MON810 on predatory fauna. Spanish J. Agric. Res. 10, 977–985.

Álvarez, T., Frampton, G.K., Goulson, D., 2001. Epigeic Collembola in winter wheat under organic, integrated and conventional farm management regimes. Agric. Ecosyst. Environ. 83, 95–110.

Álvarez-Alfageme, F., Ortego, F., Castañera, P., 2009. Bt maize fed-prey mediated effect on fitness and digestive physiology of the ground predator *Poecilus cupreus* L. (Coleoptera: Carabidae). J. Insect Physiol. 55, 143–149.

Baumgarte, S., Tebbe, C.C., 2005. Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. Mol. Ecol. 14, 2539–2551.

Barriuso, J., Valverde, J.R., Mellado, R.P., 2012. Effect of Cry1Ab protein on rhizobacterial communities of Bt-maize over a tour-year cultivation period. PLoS One 7, e35481. doi:http://dx.doi.org/10.1371/journal.pone.0035481.

Behan-Pelletier, V.M., 1999. Oribatid mite biodiversity in agroecosystems: role as bioindicators. Agric. Ecosyst. Environ. 74, 411–423.

Behan-Pelletier, V.M., 2003. Acari and Collembola biodiversity in Canadian agricultural soils. Can. J. Soil Sci. 83, 279–288.

Bilde, T., Axelsen, J.A., Toft, S., 2000. The value of Collembola from agricultural soils as food for a generalist predator. J. Appl. Ecol. 37, 672–683.

Bitzer, R.J., Rice, M.E., Pilcher, C.D., Pilcher, C.L., Lam, W.K.F., 2005. Biodiversity and community structure of epedaphic and euedaphic springtails (Collembola) in transgenic rootworm Bt corn. Environ. Entomol. 34, 1346–1376.

Bosch, D., Schipper, B., van der Kleij, H., de Maagd, R.A., Stiekema, W.J., 1994. Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. Nat. Biotechnol. 12, 915–918.

Candolfi, M.P., Brown, K., Grimm, C., Reber, B., Schmidli, H., 2004. A faunistic approach to assess potential side-effects of genetically modified Bt-corn on non-target arthropods under field conditions. Biocontrol Sci. Technol. 14, 129–170.

Clark, B.W., Coats, J.R., 2006. Subacute effects of CrylAb Bt corn litter on the earthworm *Eisenia fetida* and the springtail *Folsomia candida*. Environ. Entomol. 35, 1121–1129.

Coleman, D., Crossley, D., Hendrix, P., 2004. Fundamentals of Soil Ecology, second ed. Elsevier Ademic. Press, San Diego, CA.

Cortet, J., Griffiths, B.S., Bohanec, M., Demsar, D., Andersen, M.N., Caul, S., Birch, A.N. E., Pernin, C., Tabone, E., de Vaufleury, A., Ke, X., Krogh, P.H., 2007. Evaluation of effects of transgenic Bt maize on microarthropods in a European multi-site experiment. Pedobiologia 51, 207–218.

Daudu, C.K., Muchaonyerwa, P., Mnkeni, P.N.S., 2009. Litterbag decomposition of genetically modified maize residues and their constituent *Bacillus thuringiensis* protein (Cry1Ab) under field conditions in the central region of the Eastern Cape, South Africa. Agric. Ecosyst. Environ. 134, 153–158.

de la Poza, M., Pons, X., Farinós, G.P., López, C., Ortego, F., Eizaguirre, M., Castañera, P., Albajes, R., 2005. Impact of farm-scale Bt maize on abundance of predatory arthropods in Spain. Crop Prot. 24, 677–684.

Dively, G.P., 2005. Impact of Transgenic VIP3A x Cry1Ab Lepidopteran-resistant field corn on the nontarget arthropod community. Environ. Entomol. 34, 1267–1291.

Endlweber, K., Ruess, L., Scheu, S., 2009. Collembola switch diet in presence of plant roots thereby functioning as herbivores. Soil Biol. Biochem. 41, 1151–1154.

Farinós, G.P., de la Poza, M., Hernández-Crespo, P., Ortego, F., Castañera, P., 2004. Resistance monitoring of field populations of the corn borers *Sesamia nonagrioides* and *Ostrinia nubilalis* after 5 years of Bt maize cultivation in Spain. Entomol. Exp. Appl. 110, 23–30.

Farinós, G.P., de la Poza, M., Hernández-Crespo, P., Ortego, F., Castañera, P., 2008. Diversity and seasonal phenology of aboveground arthropods in conventional and transgenic maize crops in Central Spain. Biol. Control 44, 362–371.

Flores, S., Saxena, D., Stotzky, G., 2005. Transgenic Bt plants decompose less in soil than non-Bt plants. Soil Biol. Biochem. 37, 1073–1082.

Frampton, G.K., Van den Brink, P.J., Wratten, S.D., 2001. Diel activity patterns in an arable collembolan community. Appl. Soil Ecol. 17, 63–80.

García, M., Ortego, F., Castañera, P., Farinós, G.P., 2010. Effects of exposure to the toxin Cry1Ab through Bt maize fed-prey on the performance and digestive physiology of the predatory rove beetle *Atheta coriaria*. Biol. Control 55, 225–233.

García, M., Ortego, F., Castañera, P., Farinós, G.P., 2012. Assessment of prey-mediated effects of the coleopteran-specific toxin Cry3Bb1 on the generalist predator *Atheta coriaria* (Coleoptera: Staphylinidae). Bull. Entomol. Res. 102, 293–302.

Groot, A.T., Dicke, M., 2002. Insect-resistant transgenic plants in a multi-trophic context. Plant J. 31, 387–406.

Gruber, H., Paul, V., Meyer, H.H.D., Mueller, M., 2012. Determination of insecticidal Cry1Ab protein in soil collected in the final growing seasons of a nine-year field trial of Bt-maize MON810. Transgenic Res. 21, 77–88.

Heckmann, L.H., Griffiths, B.S., Caul, S., Thompson, J., Pusztai-Carey, M., Moar, W.J., Andersen, M.N., Krogh, P.H., 2006. Consequences for *Protaphorura armata* (Collembola: Onychiuridae) following exposure to genetically modified *Bacillus thuringiensis* (Bt) maize and non-Bt maize. Environ. Pollut. 142, 212–216.

Hopkins, D.W., Gregorich, E.G., 2003. Detection and decay of the Bt endotoxin in soil from a field trial with genetically modified maize. Eur. J. Soil Sci. 54, 793–800.

ICH, (2005). Harmonised tripartite guideline: validation of analytical procedures: Text and methodology Q2 (R1), in International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html (accessed 9 January 2015).

Icoz, I., Stotzky, G., 2008. Fate and effects of insect-resistant Bt crops in soil ecosystems. Soil Biol. Biochem. 40, 559–586.

James, C. (2014). Global Status of Commercialized Biotech/GM Crops: 2014. ISAAA Brief No. 49. ISAAA: Ithaca, NY.

Johnston, J.M., 1999. The contribution of microarthropods to aboveground food webs: a review and model of belowground transfer in a coniferous forest. Am. Midl. Nat. 143, 226–238.

Jordana, R., Arbea, J.I., 1989. Clave de identificación de los géneros de Colémbolos de España (Insecta:Collembola). Ser. Zool. 19, 1–16.

Krantz, G.W., 1978. A Manual of Acarology, second ed. Oregon State University Book Stores Inc., Corvallis.

Lehman, R.M., Osborne, S.L., Prischmann-Voldseth, D.A., Rosentrater, K.A., 2010. Insect-damaged corn stalks decompose at rates similar to Bt-protected, non-damaged corn stalks. Plant Soil 333, 481–490.

Lutz, B., Wiedemann, S., Einspanier, R., Mayer, J., Albrecht, C., 2005. Degradation of Cry1Ab protein from genetically modified maize in the bovine gastrointestinal tract. J. Agric. Food Chem. 53, 1453–1456.

McNabb, D.M., Halaj, J., Wise, D.H., 2001. Inferring trophic positions of generalist predators and their linkage to the detrital food web in agroecosystems: a stable isotope analysis. Pedobiologia 45, 289–297.

Mendelsohn, M., Kough, J., Vaituzis, Z., Matthews, K., 2003. Are Bt crops safe? Nature Biotechnol. 21, 1003–1009.

Muchaonyerwa, P., Waladde, S., Nyamugafata, P., Mpepereki, S., Ristori, G.G., 2004. Persistence and impact on microorganisms of *Bacillus thuringiensis* proteins in some Zimbabwean soils. Plant Soil 266, 41–46.

Obrist, L.B., Dutton, A., Albajes, R., Bigler, F., 2006. Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. Ecol. Entomol. 31, 143–154.

Peterson, J.A., Romero, S.A., Harwood, J.D., 2010. Pollen interception by linyphiid spiders in a corn agroecosystem: implications for dietary diversification and risk-assessment. Arthropod-Plant Interact. 4, 207–217.

Poerschmann, J., Gathmann, A., Augustin, J., Langer, U., Gorecki, T., 2005. Molecular composition of leaves and stems of genetically modified Bt and near-isogenic non-Bt maize—characterization of lignin patterns. J. Environ. Qual. 34, 1508–1518.

Pons, X., Lumbierres, B., López, C., Albajes, R., 2005. Abundance of non-target pests in transgenic Bt-maize: a farm scale study. Eur. J. Entomol. 102, 73–79.

Priestley, A.L., Brownbridge, M., 2009. Field trials to evaluate effects of Bt-transgenic silage corn expressing the Cry1Ab insecticidal toxin on non-target soil arthropods in northern New England, USA. Transgenic Res. 18, 425–443.

Saxena, D., Flores, S., Stotzky, G., 1999. Transgenic plants—insecticidal toxin in root exudates from Bt corn. Nature 402 480–480.

Saxena, D., Stotzky, G., 2001. *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. Soil Biol. Biochem. 33, 1225–1230.

Scheu, S., Ruess, L., Bonkowski, M., 2005. Interactions between microorganisms and soil micro- and mesofauna. In: Buscot, F., Varma, A. (Eds.), Microorganisms in Soils: Roles in Genesis and Functions. Springer-Verlag, Berlin Heidelberg.

Sims, S.R., Martin, J.W., 1997. Effect of the *Bacillus thuringiensis* insecticidal proteins CryIA(b), CryIA(c), CryIIA, and CryIIIA on *Folsomia candida* and *Xenylla grisea* (Insecta: Collembola). Pedobiologia 41, 412–416.

Stotzky, G., 2004. Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. Plant Soil 266, 77–89.

van Straalen, N.M., 1998. Evaluation of bioindicator systems derived from soil arthropod communities. Appl. Soil Ecol. 9, 429–437.

Xue, K., Diaz, B.R., Thies, J.E., 2014. Stability of Cry3Bb1 protein in soils and its degradation in transgenic corn residues. Soil Biol. Biochem. 76, 119–126.

Yang, X., Yang, Z., Warren, M.W., Chen, J., 2012. Mechanical fragmentation enhances the contribution of Collembola to leaf litter decomposition. Eur. J. Soil Biol. 53, 23–31.

Zurbrügg, C., Hönemann, L., Meissle, M., Romeis, J., Nentwig, W., 2010. Decomposition dynamics and structural plant components of genetically modified Bt maize leaves do not differ from leaves of conventional hybrids. Transgenic Res. 19, 257–267.

Zwahlen, C., Hilbeck, A., Gugerli, P., Nentwig, W., 2003. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. Mol. Ecol. 12, 765–775.

Zwahlen, C., Hilbeck, A., Nentwig, W., 2007. Field decomposition of transgenic Bt maize residue and the impact on non-target soil invertebrates. Plant Soil 300, 245–257.