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Soil Microbial and Faunal Community Responses to *Bt* Maize and Insecticide in Two Soils

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ABSTRACT

The effects of maize (*Zea mays* L.), genetically modified to express the Cry1Ab protein (*Bt*), and an insecticide on soil microbial and faunal communities were assessed in a glasshouse experiment. Soil for the experiment was taken from field sites where the same maize cultivars were grown to allow comparison between results under glasshouse conditions with those from field trials. Plants were grown in contrasting sandy loam and clay loam soils, half were sprayed with a pyrethroid insecticide (deltamethrin) and soil samples taken at the five-leaf stage, flowering, and maturity. The main effect on all measured parameters was that of soil type and there were no effects of *Bt* trait or insecticide on plant growth. The *Bt* trait resulted in more soil nematodes and protozoa (amoebae), whereas insecticide application increased plant *Bt* concentration and altered nematode community structure. The only significant effects on soil microbial community structure, microarthropods, and larvae of a nontarget root-feeding Dipteran, were due to soil type and plant growth stage. The results indicate that, although there were statistically significant effects of the *Bt* trait on soil populations, they were small. The relative magnitude of the effect could best be judged by comparison with the insecticide treatment, which was representative of current best practice. The *Bt* trait had no greater effect than the insecticide treatment. Results from this glasshouse experiment were in broad agreement with conclusions from field experiments using the same plant material grown in the same soils.

The global commercial area of genetically modified (GM) plants reached 81.0 million ha in 2004 and that of GM maize expressing the insecticidal protein from *Bacillus thuringiensis* (*Bt*) was 11.2 million ha (James, 2004). Exposure of nontarget soil organisms to *Bt* protein is potentially important as the protein is expressed constitutively in all parts of the plant (Wilkinson et al., 1997), so both plant residues remaining after harvest and root exudates released during plant growth could contain *Bt* protein and be incorporated into the soil. Sims and Ream (1997) estimated that a potential maximum of 1.6 mg Cry2A protein kg^{-1} soil would result from the incorporation of *Bt* cotton residues. Persistence of the insecticidal activity of Cry1Ab protein in soil has been observed following the addition of the following: purified protein (Tapp and Stotzky, 1998); *Bt* maize leaves (Muchaonyerwa et al., 2004); or from root exudates (Saxena et al., 2002). The incorporation of *Bt* proteins in soil suggests that they might affect the biology of those soils, including nontarget organisms (Stotzky, 2004).

Risk assessment forms part of the cost–benefit analysis needed to determine whether or not to release a novel material such as a genetically modified (GM) crop (Jepson et al., 1994). It is also an obligatory part of the regulatory dossier that must be submitted to gain an authorization for placing a GM product on the market. For terrestrial ecological risk assessment, the advice of previous expert panels has been to adopt a tiered approach (i.e., laboratory, glasshouse, and field experiments) (Angle, 1994) with an emphasis on soil communities and ecosystem functioning (Jepson et al., 1994; Trevors et al., 1994). More recent panels have emphasized the value of pot and glasshouse tests, particularly as a means of testing methodology and as a prerequisite to field experiments, while also recognizing that the smaller scale tests are not predictive of outcomes in the field (Bruinsma et al., 2002).

Despite the large area of *Bt* maize and other *Bt* crops planted, there are still scientifically interesting questions to be addressed with regard to the little-known soil compartment (see for example reviews by Dunfield and Germida, 2004; Motavalli et al., 2004; Groot and Dicke, 2002; Bruinsma et al., 2003; Stotzky, 2004; O'Callaghan et al., 2005). Although maize is the most studied of the *Bt* crops, there is still a lack of continuity between environmental studies at the laboratory, glasshouse, and field scales. A notable exception being the study of Hopkins and Gregorich (2003), who studied soil Cry1Ab protein concentrations in both field and laboratory studies. Also, as the potential benefits of plants expressing the *Bt* protein include the reduced application of insecticide, it is relevant to compare effects of *Bt* maize with those of insecticides applied to conventional maize. To address these shortcomings the EU-funded ECOGEN project (www.ecogen.dk; verified 24 Jan. 2006) was initiated. Results after the first 2 yr of field trials with Cry1Ab expressing maize showed that changes to microbial and microfaunal (protozoan and nematode) communities due to the *Bt* trait were small and less than changes due to

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Abbreviations: ANOVA, analysis of variance; awcd, average well color development; *Bt*, *Bacillus thuringiensis* (specifically related to the insecticidal protein); *Bt* maize, maize genetically modified to express insecticidal protein from the bacterium *Bacillus thuringiensis*; CLPP, community level physiological profile; GM, genetically modified; PC, principal component; PLFA, phospholipid fatty acid.

different (non-*Bt*) maize cultivars and different crops (Griffiths et al., 2005). Decomposition of wheat straw in the field was unaffected by the *Bt* maize (Cortet et al., 2006). To complement these field studies, a range of laboratory and glasshouse tests were also undertaken. Results of a glasshouse experiment are reported here.

MATERIALS AND METHODS

Soils

Soil was collected from the field sites at Foulum, Denmark and Varois, France where *Bt* maize (MON810, Cry1Ab) was being grown as part of the ECOGEN project (Griffiths et al., 2005) and transported to the Scottish Crop Research Institute. Soil was collected separately from the top 5 cm and the lower 5 to 20 cm from experimental plots growing the non-*Bt* maize cultivar Monumental, passed through a 7-mm diameter mesh to remove large debris and stored at ambient temperature and moisture until use. Soil from Foulum is a sandy loam (62.2% sand, 23.2% silt, 8.3% clay, pH_{CaCl2} 5.6) containing 6.4% organic matter and that from Varois is a clay loam (20% sand, 41% silt, 30% clay, pH_{CaCl2} 7.1) containing 4.8% organic matter. We used 22.7-cm diameter plastic pots filled with 5.5 kg mixed lower soil, to which granular N–P–K fertilizer (22–4–14) (equivalent to 100 kg N ha^{-1}) was added, then a final layer of 500 g mixed topsoil added. Water content in the pots was maintained at a matric potential of −10 kPa, which equated to a gravimetric water content of 29.9% for the Foulum soil and 26.2% for the Varois soil. Soil temperature and water content were monitored by moisture probes and thermocouples inserted in single pots of each soil–maize combination (these were additional replicate pots not used for sampling) and attached to a data logger to record daily fluctuations in soil water content and temperature.

Plant Growth

Pots were assigned to a treatment and randomized within a temperature controlled, containment glasshouse set to give a 16-h light period (20°C, with supplementary lighting activated at light levels <100 W m^{-2}) and an 8-h dark period (15°C). The treatments in a fully factorial design were two soils (Foulum and Varois) × two maize lines (*Bt* and non-*Bt*) × two insecticide treatments (with and without) × three plant growth stages (five-leaf, flowering, and maturity), with five replicate pots per treatment, giving a total of 120 pots. One week after filling the pots with soil, they were sown with two seeds of either MEB307*Bt* (a Mon 810 *Bt* variety expressing the Cry1Ab protein, from Monsanto) or Monumental (a registered conventional variety near-isogenic to MEB307*Bt* but without the *Bt* trait, from Monsanto). Pots were watered to constant weight three times per week with tap water. A top-dressing of 100 mL liquid N–P–K fertilizer (16–5–32) (equivalent to 80 kg N ha^{-1}) was added to all pots assigned to the final sample (at maturity) after 83 d growth.

Insecticide Application

After 39 d growth, when the plants had five leaves, and 87 d growth, during flowering, half the pots were treated with insecticide (Decis from Bayer CropScience, Cambridge, UK, which is a pyrethroid insecticide containing 2.5% w/w deltamethrin with xylene, toluene, ethyl, and propyl benzenes). The recommended application rate of 200 mL ha^{-1} equates to 2.5 μL $plant^{-1}$ given a typical sowing of 80 000 seeds ha^{-1}. An aqueous solution of Decis was prepared at 0.45 mL L^{-1} and 5.5 mL (2.5 μL Decis) sprayed directly onto the soil surface. Pots not receiving Decis were sprayed with the same volume of water. Spraying took place 24 h before sampling.

Sampling and Analysis

Five replicate pots of each treatment were sampled at the five-leaf stage (after 40 d of growth), at flowering (88 d of growth), and at maturity (123 d of growth). The plant was carefully removed from the pot and soil shaken from the roots. Plants were separated into leaves, stems, cob (at maturity only), and roots (roots were further washed to remove all adhering soil), dried at 50°C, weighed, and milled through a 0.2-mm mesh for analysis of C, N, and Cry1Ab protein content (see below). Carbon and N were measured (as CO_2 and N_2) following combustion using a Europa Scientific (Crewe, UK) ANCA-SL sample converter, using a Europa Scientific 20-20 mass spectrometer.

Soil was mixed carefully and used for analysis, with subsamples being frozen at −80°C, for later phospholipid fatty acid (PLFA) and Cry1Ab analysis, or at −20°C for the later determination of residual insecticidal activity toward nontarget soil insects.

Gravimetric water content was determined at 105°C.

Nematodes were extracted from ca. 20 g fresh soil from each sample using a modified Whitehead and Hemming tray technique (Whitehead and Hemming, 1965), in which the soil was spread over two-ply paper tissue supported on a 12-cm diameter section of pipe with a base of 1 mm aperture nylon mesh. The soil was supported in a filter funnel with enough tap water to cover the surface (Brown and Boag, 1988) and nematodes collected after 48 h, heat-killed for 2 min at 60°C, and preserved in 4% formaldehyde. Total nematode numbers were counted under low-power microscopy, then further processed through glycerol and mounted on a glass slide for identification at higher magnification.

Total numbers of protozoa (i.e., active and encysted forms) were estimated by a most probable number technique (Darbyshire et al., 1974) in which 5 g soil were dispersed in 50 mL Neffs Modified Amoeba Saline (NMAS; Page, 1976) on a roller bed for 20 min. Four 100-μL aliquots were added to flat-bottomed microtiter plates and diluted threefold in 50-μL sterile nutrient broth (Oxoid) in NMAS at 1:9 (v/v). The microtiter plates were incubated at 15°C and the presence of flagellates, ciliates, and amoebae recorded after 7, 14, and 21 d. Numbers were calculated according to Hurley and Roscoe (1983) and biomass calculated using approximate weights (Griffiths and Caul, 1993).

Micro-arthropods were extracted from 100 g soil, over a 5-d period, using a Tullgren funnel apparatus (Burkard Manufacturing Co. Ltd., Rickmansworth, UK) and preserved in 70% ethanol. Total micro-arthropod numbers were counted under low-power microscopy.

Soil–saline suspension remaining from the protozoan measurement was used to determine the community-level physiological profile (CLPP; Garland and Mills, 1991). The suspension was further diluted in sterile NMAS to give an absorbance of 0.4 at 595 nm and 150 μL inoculated into each well of a Biolog GN2 plate (Oxoid). The absorbance of each well at 595 nm was read initially and after incubation for 3, 4, and 5 d at 15°C.

Total lipids were extracted from the frozen (−80°C) aliquots (2 g) of soil with an extractant of citrate buffer, methanol, and chloroform (Nielsen and Petersen, 2000). Phospholipid fatty acids (PLFA) were then separated by solid phase extraction, converted to methyl esters by mild alkaline transesterification and analyzed by gas chromatography using a low polarity column (Frostegård et al., 1991).

To determine residual insecticidal activity of *Bt* protein or *Bt* maize residues on a nontarget soil pest, frozen soil (−20°C) from the mature stage was thawed, mixed with sand (750 g soil and 250 g sand, to improve drainage) and 2 g granular N–P–K fertilizer (22–4–14), then placed in a plastic pot and planted with a pregerminated swede (*Brassica napus* L. var. Magres) seedling. The seedlings were incubated in a temperature-controlled glasshouse maintained at 20°C for 16 h while additional lighting was provided and 15°C with no additional lighting for 3 wk. At this stage 20 eggs (1–2 d old) of the cabbage root fly (*Delia radicum* L.), obtained from laboratory culture, were added to the base of each plant (Birch, 1988). After a further 3 wk of incubation the soil and roots of each plant were gently shaken in a bucket of water and larvae and pupae of the root fly collected on a 1-mm aperture sieve.

Cry1Ab protein in the milled plant material was determined using an enzyme-linked immunosorbent assay (ELISA) kit (PathoScreen EnviroLogix QuantiPlate kit for *Bt* Cry1Ab/1Ac protein, EnviroLogix, Portland, OR) to quantify the Cry1Ab protein. For extraction, 0.5 mL of the manufacturer's extraction/dilution buffer was mixed with 100 mg milled plant material in 2-mL micro-centrifuge tubes, vortexed, and centrifuged at 825 × *g* for 1 min. A 100-μL aliquot of the supernatant was dispensed in ELISA wells for measurement along with a dilution series of lyophilized positive controls and a negative control supplied with the kit. The test procedure described by the manufacturers was followed and optical densities of the wells were measured on a plate reader at 450 nm. The *Bt* protein in the soil samples was quantified using the same ELISA Envirologix kit used for the plant material. A 1-ml volume of extraction–dilution buffer was added to 0.5 g of soil in a 2-mL micro-centrifuge tube, vortexed, and centrifuged as above. A 100-μL aliquot of supernatant was pipetted into ELISA kit wells and *Bt* protein detected and quantified by following recommended kit test procedures. To calibrate recovery of *Bt* protein from the soils, soil samples were spiked with known amounts of the *Bt* standards supplied with the kit and extracted as above.

Statistical Analysis

Data were analyzed using standard analysis of variance (ANOVA) and multivariate procedures with GenStat eighth edition (VSN International, 2005) and presented as means with an associated least significant difference (LSD, at the 5% level) and degrees of freedom (df), using as factors: plant growth stage, soil type, plant type, and insecticide application. Protozoan biomass and soil *Bt* protein concentrations were transformed (natural logarithm) before analysis and the de-transformed means are presented in the text. The time-course profiles of the CLPP data were analyzed from the area under the color development profile (Hackett and Griffiths, 1997). The average well color development (awcd) of the CLPP data was also calculated (Garland and Mills, 1991) and analyzed by ANOVA. The results of the CLPP time-course and the PLFA profile were analyzed by principal component (PC) analysis and the resulting PC scores analyzed by ANOVA. Canonical variate analysis was used to identify linear combinations of CLPP or PLFA scores that best discriminated between factor levels.

RESULTS

Soil Water, Temperature, Nitrogen, Plant Growth, and *Bt*

The maize plants were significantly ($p < 0.001$) heavier when grown in the silt-rich Foulum soil than in the clay-rich Varois soil (Table 1), but within soil type there were no effects of *Bt* or insecticide on plant weight. The %C and %N of the plant material only differed between the growth stages and soil types, being greater in the Foulum soil than the Varois soil (data not shown), with no significant effects of the *Bt* trait or insecticide.

Table 1. Dry weights (g) of maize growing in Foulum or Varois soil under glasshouse conditions at the five-leaf, flowering, and mature stages, as described in the text. Data are means of five replicates. Means are detransformed from $\log_e$ transformed data where indicated, meaned over *Bt* trait and insecticide treatments as there were no significant effects of these factors.

		Plant biomass				
Soil	Stage	Leaves	Stem	Roots	Grain	Total
		g				
Foulum	five-leaf	2.5	nd†	1.0	nd	3.5
	flower	18.8	31.4	19.3	nd	70.0
	mature	15.5	38.6	21.3	36.5	113.2
Varois	five-leaf	1.0	nd	0.9	nd	1.9
	flower	12.7	10.8	9.3	nd	33.0
	mature	11.8	26.9	19.7	30.8	88.7
LSD‡		0.12§	0.06§	0.17§	1.5	0.12§

† Not determined as structures not yet present.
‡ Least significant difference, $P < 0.05$.
§ LSD of $\log_e$ transformed data, data in table are detransformed means.

Soil temperature was the same regardless of treatment with average daily fluctuations between 12 and 24°C. Volumetric soil water content varied between watering intervals, from 30 to 13% as maximum and minimum during the experiment.

Cry1Ab protein was detectable in the MEB307*Bt* plant material but was not significantly greater than zero in the near-isogenic Monumental material. In the leaves there were significant effects of soil type ($p < 0.001$), with a greater concentration in Varois- than Foulum-grown plants; soil × time interactions ($p < 0.001$), as *Bt* protein increased between the five-leaf and flowering stages of plants grown in both soils, but there was a further significant increase at the mature stage only in plants grown in the Varois soil (Table 2); and also soil × insecticide interactions ($p < 0.05$) with the application of Decis increasing leaf *Bt* protein concentrations in Foulum but not Varois soil (Table 2). In the roots there were soil × time × insecticide interactions ($p < 0.01$), with an overall decline in *Bt* protein concentration with plant growth stage and an overall increase with the application of Decis (Table 2). The *Bt* protein concentration in the stems was significantly ($p < 0.001$) greater in the Varois-grown plants but there were no significant differences in the grain (Table 2). The *Bt* protein concentration in the soil was significantly higher in Varois than Foulum soil ($p < 0.002$, Table 2), whereas in both soils there was significantly more Cry1Ab protein at the mature stage of the plants than at the five-leaf or flowering stages ($p < 0.001$, Table 2). There were no effects of insecticide application on the amounts of *Bt* protein detected in soil.

Nematodes

Nematode abundance increased significantly ($p <$ 0.001) between the five-leaf (mean of 19.2 g^{-1}) and

Table 2. The *Bt* protein concentration of maize (mg Cry1Ab kg^{-1} plant material) growing in Foulum or Varois soil types under glasshouse conditions either without (none) or with (Decis) insecticide, at the five-leaf, flowering, and mature stages, and in the soil used to grow the maize (μg Cry1Ab kg^{-1} dry soil). Data are means of five replicates. The *Bt* toxin concentrations only given for MEB307*Bt* as concentrations in Monumental were not significantly different from zero.

			Plant structure				
Soil type	Insecticide	Stage	Leaves	Roots	Stem	Grain	Soil
			mg kg^{-1}				μg kg^{-1}
Foulum	none	five-leaf	1.36	0.99	nd†	nd	2.87
		flower	7.89	3.37	1.96	nd	5.80
		mature	8.51	0.74	1.55	0.142	15.80
	Decis	five-leaf	4.53	7.46	nd	nd	2.02
		flower	8.98	4.46	2.31	nd	8.23
		mature	9.08	4.64	1.48	0.117	10.11
Varois	none	five-leaf	2.75	1.94	nd	nd	0.99
		flower	8.19	3.27	3.47	nd	8.76
		mature	13.28	3.95	2.00	0.05	42.86
	Decis	five-leaf	2.73	6.85	nd	nd	1.99
		flower	8.19	3.78	4.88	nd	13.72
		mature	13.53	2.78	2.69	0.08	33.38
LSD‡			1.79	1.32	1.77	0.22	0.865§

† Not determined as structures not yet present.
‡ Least significant difference, $P < 0.05$.
§ The *Bt* protein values in soil were transformed ($\log_e$) for analysis; data in table are detransformed means, LSD of $\log_e$ transformed data.

flowering stages (mean of 34.0 g^{-1}) and was significantly different ($p < 0.001$) between the soil types, with more nematodes in the Foulum soil at all plant growth stages. There was an overall effect of *Bt*, when data from both soils were pooled, with a mean of 29.7 nematodes g^{-1} dry soil under the *Bt* maize and 26.1 g^{-1} under conventional maize ($p < 0.01$). When the nematode community at the mature stage was analyzed (Table 3), there were differences in the proportions of bacterial-feeders, with more in Varois than Foulum soil ($p < 0.01$) and fewer in soil treated with insecticide than without insecticide ($p < 0.01$); omnivores, with more in Varois than Foulum soil ($p < 0.01$) and fewer under *Bt* than non-*Bt* maize ($p < 0.01$); and plant feeders, with more in Foulum than Varois soil ($p < 0.001$) and more in soil treated with insecticide than without insecticide ($p < 0.05$). There were significant differences in Principal Component 1 (PC1) due to soil ($p < 0.001$) and in PC 2 a significant insecticide × plant interaction ($p < 0.05$) (Fig. 1) in that for the non-*Bt* Monumental variety the nematode community was altered by the application of insecticide. Differences between soils were largely due to an increased proportion of *Acrobeloides* and *Pratylenchus* in the Varois soil, whereas differences due to the application of insecticide to Monumental were largely due to reduced proportions of *Pratylenchus* and Rhabditidae and increased proportions of Helicotylenchidae.

Table 3. Mean abundance (g^{-1} dry soil) and percentage composition of bacterial- (BF), fungal- (FF), omnivore (OM), and plant-feeding (PF) nematodes under mature maize, *Bt*, or non-*Bt* with or without insecticide, growing in Foulum or Varois soil under glasshouse conditions as described in the text. Data are means of five replicates.

				% Composition			
Soil	Maize	Insecticide	Abundance	BF	FF	OM	PF
			g^{-1}	%			
Foulum	Monumental	none	32.2	42.2	18.6	6.4	32.3
	MEB307*Bt*		42.2	50.4	16.5	7.2	25.0
	Monumental	Decis	37.8	36.9	18.1	12.6	31.2
	MEB307*Bt*		41.6	36.8	23.7	7.9	30.5
Varois	Monumental	none	22.4	52.0	16.1	14.3	12.9
	MEB307*Bt*		21.9	55.4	17.3	10.7	10.8
	Monumental	Decis	22.9	41.9	16.1	15.3	21.4
	MEB307*Bt*		23.2	48.5	20.9	8.5	16.7
LSD†			9.4	7.6	ns‡	3.5	6.6

† Least significant difference, $P < 0.05$.
‡ No significant differences between means.

Protozoa

Protozoa differed significantly ($p < 0.001$) between the soils, such that Varois soil had greater amoebal, ciliate, and total protozoan biomass than Foulum soil (Table 4) but a lesser biomass of flagellates. There was a significant effect ($p < 0.05$) of the *Bt* trait on amoebal and total protozoan biomass in Foulum soil at the five-leaf stage, when the biomass under *Bt* maize was significantly greater than that under non-*Bt* maize (Table 4). There were no significant effects of insecticide application on protozoa.

Micro- and Macro-Arthropods

Collembolan abundance remained very low throughout the experiment, with an overall mean density of 0.004 individuals g^{-1}. Mites increased significantly ($p < 0.001$) in number between each plant growth stage, but showed no significant differences due to soil type, insecticide, or *Bt* trait (Table 4). Microarthropods were unaffected by insecticide application.

Tests with the cabbage root fly in soil sampled at the mature stage of maize growth indicated no effect of *Bt* or Decis on development of the larvae, but a significant ($p < 0.01$) effect of soil type on larval growth. Although an equivalent number of pupae were recovered from each soil type, their average weight in Foulum, 1.58 mg, was heavier than in Varois, 1.49 mg (LSD 0.07, 35 df).

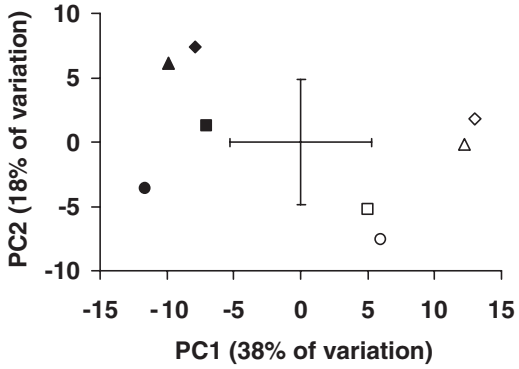

Fig. 1. Principal component (PC) plot of the nematode communities under maize at the mature stage. Data points represent the mean of five replicates; bars are the least significant difference ($p < 0.05$). Symbols for Foulum soil are open and for Varois soil are filled, and are: *Bt* maize = diamond; non-*Bt* maize = triangle; *Bt* maize + insecticide = square; non-*Bt* maize + insecticide = circle.

Table 4. Biomass (μg kg^{-1}) of amoebae, flagellates, ciliates, and total protozoa, and the abundance of mites and Collembola at the five-leaf, flowering, and mature stages of maize, *Bt*, or non-*Bt*, growing in Foulum or Varois soil. Data are means of five replicates and are meaned over insecticide treatment as no significant effects of insecticide appplication.

Soil	Maize	Stage	Amoebae	Flagellates	Ciliates	Protozoa	Mites	Collembola
			μg kg^{-1}				*n*	
Foulum	Monumental	five-leaf	234	169	27	501	0.0	0.1
		flower	189	188	26	455	5.1	0.0
		mature	604	224	10	921	55.7	0.0
	MEB307*Bt*	five-leaf	531	226	54	853	0.1	0.1
		flower	266	205	18	590	8.8	0.2
		mature	378	242	15	735	37.0	0.0
Varois	Monumental	five-leaf	1346	66	118	1638	0.3	0.0
		flower	1415	98	229	1868	2.4	0.0
		mature	1144	83	144	1613	17.8	0.1
	MEB307*Bt*	five-leaf	906	64	98	1133	0.3	0.0
		flower	1087	98	132	1473	3.9	0.1
		mature	1474	90	302	2034	18.4	0.0
LSD†			0.66	0.64	1.14	0.53	1.63	1.84

† Least significant difference, $P < 0.05$ of $\log_e$ transformed data, data in table are detransformed means.

Phospholipid Fatty Acid

Principal component analysis revealed that the first PC accounted for 48% of the variation and showed two main groups, basically a division between the two soil types, Varois and Foulum. ANOVA of the first PC also showed significant differences ($p < 0.001$) for the plant growth stages, with the five-leaf stage having significantly larger values than the flowering and mature stages. Canonical variate analysis confirmed that the most significant difference was between the soils. There were no significant effects of either insecticide or *Bt* trait, nor any interaction. The total amount of extracted phospholipid-P, an indicator of microbial biomass, was significantly ($p < 0.001$) different between soils, with greater amounts in Foulum than Varois soil (61.4 and 52.0 μmol P kg^{-1}, respectively, LSD 3.6.), and between the five-leaf, flowering, and mature plant growth stages (47.6, 61.0, and 61.6 μmol P kg^{-1}, respectively, LSD 4.4). There were no significant differences in PLFA-P as a result of *Bt* trait or insecticide application.

Community Level Physiological Profile

The area under the profile of awcd, an indication of microbial activity, was analyzed using ANOVA. There was a significant interaction between the soil and plant growth stage ($p < 0.001$), but no significant effect of insecticide or *Bt* trait. There was a greater awcd in Foulum than Varois soil at the five-leaf stage (3.01 and 2.75, respectively, LSD 0.13), no difference between Foulum and Varois at the flowering stage (2.61 and 2.49 respectively) and a greater awcd in Varois than Foulum soil at the mature stage (2.81 and 2.61, respectively, LSD 0.13).

Principal component analysis of the CLPP data did not show the same strong grouping as the PLFA data, with the first and second principal components accounting for just 15.7 and 12.3% of the variance, respectively. ANOVA of the first PC showed a highly significant interaction between soil type and plant growth stage ($p < 0.001$), while ANOVA of the second PC showed a highly significant effect of soil type ($p < 0.001$). Canonical variates were found that significantly discriminated between the soils ($p < 0.001$) and between the harvest times ($p < 0.001$), and also between combinations of these factors, but there were no significant discrimination for either insecticide or *Bt* trait, nor any interaction.

DISCUSSION

Bt Effects on Soil Biology

The effect of the *Bt* trait was relatively small compared with the effects of soil type, plant growth stage, and insecticide application. There was an overall increase in nematode numbers under *Bt* maize, from 26 to 30 nematodes g^{-1}, but no significant effect at any individual plant growth stage or in any particular soil type. Saxena and Stotzky (2001) similarly found no effect of *Bt* maize (Cry1Ab) on nematodes at a single harvest date. That a difference due to the *Bt* trait was observed only when all the data were pooled indicates that the effect was weak. There was a reduction in the contribution of omnivorous nematodes to the nematode community structure, from 12% under non-*Bt* maize to 9% under *Bt* maize, similar to the difference between soil types. Although statistically significant, this 3% reduction in the proportion of omnivorous nematodes is unlikely to have any ecological significance, and is far less than the 20% increase in fungivorous nematodes and 30% decrease in phytophagous nematodes observed with Cry1Ac-expressing oilseed rape under field conditions (Manachini et al., 2004).

Amoebae were the only protozoa to show a *Bt* effect, but only at the five-leaf stage when there was an increase in amoebal biomass under *Bt* maize from 233 to 528 μg kg^{-1}. Again the only comparable glasshouse study (Saxena and Stotzky, 2001) found no effect of the *Bt* trait on protozoa, but they reported only total protozoan populations that correspond more closely to our total protozoan biomass. Soil mite populations increased throughout the experiment with no significant effect of the *Bt* trait. This is entirely consistent with the laboratory investigations of Yu et al. (1997), who found no toxic effects of plant residues containing *Bt* protein (Cry1Ab) on a test mite species (*Oppia nites*). Although populations of soil Collembola did not reproduce in our glasshouse experiment, probably due to fluctuating soil water content

as they are more sensitive to soil humidity than the mites (Vannier, 1970), Sims and Martin (1997) saw no toxic effects of a dietary supplement of 200 mg kg^{-1} Cry1Ab protein on two Collembolan species. The concentration of *Bt* protein tested by Sims and Martin (1997) was far in excess of the concentration measured in our harvested plant samples, which does support the general conclusion that soil micro-arthropods are not affected by the *Bt* trait. Our soil exposure test with the nontarget dipteran *Delia radicum* again indicated that there were no deleterious effects of growing maize (*Bt* protein, exudates, or plant residues) with the *Bt* trait on this nontarget, root feeding dipteran. This agrees with the findings of Candolfi et al. (2004), who observed no deleterious effects of *Bt* maize on nontarget arthropods in the field.

Both the CLPP and PLFA patterns indicated that there were no detectable differences attributable to the *Bt* trait on soil microbial community structure. Blackwood and Buyer (2004) in a similar pot study of *Bt* maize also found no effects on PLFA profiles due to the *Bt* trait, but significant differences due to soil type, whereas there were *Bt* effects on the CLPP. There was an interaction between *Bt* protein and soil type on the CLPP in the Blackwood and Buyer (2004) study, but we did not detect a similar effect despite using two contrasting soil types and sampling at three plant growth stages. A field study in which the rhizosphere bacterial community structure of maize cultivars, including one expressing Cry1Ab, were compared found specific *Bt* effects in 1 yr out of 3, but the effect was less than that of other environmental variables (Baumgarte and Tebbe, 2005).

This study, for practical reasons, was only able to study the two maize lines (i.e., the *Bt* maize and its near-isogenic control) but the inclusion of more non-*Bt* lines would provide more information on the natural variation not associated with the *Bt* trait.

Effects of Insecticide

An effect of the insecticide Decis was observed on nematode community structure. The toxicology of the deltamethrins (the class of compound that includes Decis) on soil biota has received scant attention, although they are known to be highly toxic for aquatic macroinvertbrates (Arbjörk, 2004). Tu (1980) observed short-lived and minor effects of pyrethroid insecticides on microbial populations and activities in soil. In a field study of the effects of *Bt* maize (Cry3Bb) and the insecticide tefluthrin on soil microbes, Devare et al. (2004) recorded a reduced soil respiration rate under the insecticide treatment, but no effects of the *Bt* trait. The observation that plants treated with insecticide had greater concentrations of the *Bt* toxin than untreated plants in some instances may warrant further investigation. The mechanism for an effect of the insecticide on the expression of Cry1Ab is unclear, especially as three of the four instances were at the five-leaf stage only 1 d after the soil had been treated with insecticide. The fact that the soil itself was sprayed, rather than the plant, further complicates an understanding of the processes involved but some component of the Decis (e.g., active ingredient or solubilizers) may be inducing *Bt* protein production, confounding ELISA readings, or enabling a more thorough extraction of the *Bt* protein from the soil.

Comparison with Field Data

Data from the field sites where the soil for this glasshouse study was collected showed an overall reduction in nematode abundance under *Bt* maize (Griffiths et al., 2005) rather than the increase observed in this glasshouse experiment. Protozoa, particularly at the Varois site, were also reduced, albeit transiently, under *Bt* maize compared with non-*Bt* maize in the field (Griffiths et al., 2005), while *Bt* effects seen in this pot experiment were only observed in the Foulum soil and were for an increase in numbers. The reason for the opposite trend in the field data (negative effects of *Bt* maize) and this study (positive effects of *Bt* maize) is unclear. These differences may be due to the fact that plants grown in the glasshouse do experience different environmental conditions from the field and that this might affect interactions between plants and soil organisms. The differences in nematode and protozoan populations between the two soil types in this study were generally similar to those observed in the field (Griffiths et al., 2005). The *Bt* expression appeared to be somewhat reduced in this pot experiment. Mature, field grown maize plants at the Foulum site had concentrations of approximately 10 mg kg^{-1} Cry1Ab protein in each of the 2 yr sampled (Griffiths et al., 2005), but only the concentration of leaves grown in Foulum soil in this experiment approached that value. Conversely our measurements of *Bt* protein from the soil were slightly higher than other published concentrations from field trials. Baumgarte and Tebbe (2005) and Hopkins and Gregorich (2003) detected concentrations up to 10 μg kg^{-1}, while we were detecting three to four times this amount, particularly in the Varois soil. The fact that the plants were grown in pots and probably had a higher density of roots than would be expected in the field might have accounted for the increase.

CONCLUSIONS

The overall conclusion is that although there are effects of the *Bt* trait on soil microbial and faunal communities, they are relatively small compared with effects of soil type (field site) and maybe confounded by natural variation between different maize lines. The relative magnitude of the effect can best be judged by comparison with the insecticide treatment, which is applied as current best practice. The effects of the *Bt* trait were no greater than those of the insecticide treatment.

Although in this case the glasshouse experiment reached essentially the same conclusions as the field experiments (Griffiths et al., 2005), we would agree with earlier recommendations that tiered approach (i.e., laboratory, glasshouse, and field experiments in sequence) be adopted (Angle, 1994) as glasshouse tests are not predictive of the outcome in the field (Bruinsma et al., 2002).

We wanted to look at nontarget effects at different plant growth stages, particularly as the plant flowered

and matured, because this would extend the range of observations over those made during early growth in other studies (Blackwood and Buyer, 2004; Saxena and Stotzky, 2001). This in itself created practical problems related to watering the plants. As the plants grew they obviously required watering at increasingly frequent intervals, which resulted in far greater fluctuations in soil water content than would be expected in the field situation. This emphasized the compromise to be made between the flexibility of a glasshouse experiment (more treatments, sampling times, and soil parameters can be studied than from field plots) and the fact that conditions in a glasshouse can never be the same as in the field. As an example of the benefits of the tiered approach (i.e., coupled laboratory, glasshouse, and field experiments) (Angle, 1994), our observations on the effects of insecticide compared with the effects of the *Bt* trait will now be included in future field samples at the ECOGEN sites and laboratory experiments will be undertaken to examine the positive response of nematodes and protozoa to *Bt* maize. The future inclusion of more maize lines in similar experiments would improve both prediction power and the quality of the interpretation, should the resources be available.

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