XX.X Effects of Cotton Expressing the *Bacillus thuringiensis* var. *kurstaki* Endotoxin on Soil Microorganisms.

Katherine K. Donegan[1] and Ramon J. Seidler[2].

1 Introduction

The genetic engineering of plants has facilitated the production of agronomically-desirable crops that exhibit increased resistance to pests, herbicides, pathogens and environmental stress and enhancement of qualitative and quantitative crop traits (Gasser and Fraley 1992). Along with these many benefits, however, comes the potential for adverse ecological effects because of the often sustained expression in the genetically engineered (transgenic) plant of the engineered trait(s) and the persistence of the transgenic plant or plant residue in the environment. Consequently, we have undertaken research to evaluate the potential ecological effects of transgenic plants and their products.

Some of our research has included microcosm studies with cotton plants that are genetically engineered to produce the *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*) endotoxin (Perlak et al. 1990). Many agriculturally important plants have been engineered to produce endotoxins from different subspecies of the bacterium *Bacillus thuringiensis* (*B.t.*) (Vaeck et al. 1987; Delanney et al. 1989; Perlak et al. 1990; Lundstrum 1992; Koziel et al. 1993). The endotoxin of *Bacillus thuringiensis* var. *kurstaki* (*Btk*) has demonstrated insecticidal activity against lepidopterans (Hofte and Whiteley 1989). Although high specificity has been assumed for most *B.t.* endotoxins, their effects on non-target organisms have not been fully evaluated. Studies have been performed exposing non-target invertebrates to various *Bt*-producing bacterial strains and have demonstrated such detrimental effects as mortality and reduced fecundity (Ali et al. 1973; Tolstova et al. 1976; Molloy and Jamnback 1981; Mulla et al. 1982; James et al. 1993; Flexner et al. 1986; Miller 1990). In preliminary experiments where transgenic *Btk* cotton plants were placed in natural soils and decomposed (Pratt et al. 1993; Palm et al 1994), we discovered that the *B.t.k.* endotoxin persisted and retained its immunological and biological activity at levels similar to what has been observed with microbially-produced *Bt* endotoxins. Therefore, we considered it important to determine the impact of the *B.t.k.* endotoxin in decomposing transgenic plants on soil microorganisms because of the ubiquity of microorganisms in soil and the crucial role they play in soil processes.

Most concern about the environmental release of plants containing *Bt* endotoxins has been for the development of resistance in the target pests (Fox 1991; Johnson and Gould, 1992; USDA 1992) or for gene flow and plant invasiveness (Umbeck et al. 1991; Manasse 1992; Crawley et al. 1993;

---

[1] Dynamac Corporation and [2] U.S. Environmental Protection Agency, WED, NHERL, 200 S.W. 35th Street, Corvallis, OR, 97333 USA

Kareiva et al. 1994; Klinger and Elstrand 1994) . Some studies have considered non-target effects of the *Bt* endotoxin but have used microbial *Bt* strains rather than plants that produce *Bt* toxins (Molloy and Jamnback 1981; Flexner et al. 1986; Miller 1990). Only a few studies have used transgenic plants to assess the potential direct or indirect effects of *Bt* endotoxins on plant and soil ecosystems (Donegan et al. 1995; Donegan et al. 1996a; Donegan et al. 1996b).

In addition to the potential effects of *Bt* endotoxins produced by transgenic plants, there is the possibility that other plant characteristics may be unintentionally altered during the insertion of the transgene (Lange 1990; MacKenzie 1990; The Economist 1990; Jenkins et al. 1991; Gene Exchange 1992; Yamada 1992). These types of alterations in plant characteristics caused by genetic manipulation (e.g., changes in plant enzyme production and biomass), that are independent of expression of the inserted gene, may also produce ecological effects.

In this chapter we describe four experiments that investigated the biological and molecular changes in microbial populations following the incorporation of purified *B.t.k.* endotoxin or *B.t.k.*-producing cotton into natural soils. Microbial populations were monitored for changes in the total numbers and species composition of culturable bacteria and fungi, in the substrate utilization of the bacterial community and in the total DNA content and DNA fingerprints of the eubacteria.

2 Experimental Studies

2.1 Plant Propagation, Sample Preparation and Experimental Design

Seeds of parental and transgenic cotton were provided by the Monsanto Company (St. Louis, MO). The parental cotton line was Coker 312. The transgenic cotton lines producing *B.t.k.* endotoxin were line 81, HD-1, CryIA(b); line 247, HD-73, CryIA(c); and line 249, HD-73, CryIA(c). Cotton seeds were propagated in a microcosm and harvested when flowers began forming. Only cotton leaves were used in these experiments because studies showed they have the highest expression level of the *B.t.k.* endotoxin (Ream et al. 1992). For treatments containing plant material, parental or transgenic cotton leaves were incorporated at 1:3 by weight (leaves:soil) into a fine sandy loam soil (Wasco) that was obtained from the USDA Cotton Research Station in Shafter, CA. In Experiment 4 only, an additional soil, a clay loam soil (Panoche) that was received from the USDA Agricultural Research Station in Fresno, CA, was used. For the purified endotoxin treatments, the HD-1 or HD-73 endotoxin provided by the Monsanto Company was added at a concentration of 0.05 μg toxin/g soil; calculated as the equivalent of the endotoxin concentration in the transgenic plant treatments. Sterile water was added to all treatments to bring the soil to 45% water holding capacity. Three replicates were prepared for each treatment and sampled on days 0, 7, 14, 21 and 28 in experiments 1, 2, and 3 and on days 0, 7, 14, 28 and 56 in experiment 4.

Four experiments with the following combinations of treatments and bioassays were conducted:

EXPERIMENT	TREATMENTS	ASSAYS
1 and 2	Soil only (Wasco sandy loam)	Bacterial populations
	+Purified HD-73 toxin	Fungal populations
	+Parental cotton	ELISA
	+Transgenic 249 cotton	
3	Soil only (Wasco sandy loam)	Bacterial populations
	+Purified HD-1 toxin	Fungal populations
	+Parental cotton	ELISA
	+Parental cotton+HD-1 toxin	
	+Transgenic 81 cotton	
4	Soil only (Wasco sandy loam)	Bacterial populations
	+Purified HD-73 toxin	Fungal populations
	+Parental cotton	Amoebae populations
	+Parental cotton+HD-73 toxin	Ciliate populations
	+Transgenic 247 cotton	Flagellate populations
	+Transgenic 249 cotton	Target insect bioassay
	Soil Only (Panoche clay loam)	Biolog/Bacterial community
	+Purified HD-73 toxin	Biolog/Species ID
	+Parental cotton	ELISA
	+Parental cotton+HD-73 toxin	Total DNA content
	+Transgenic cotton 247	DNA fingerprints
	+Transgenic cotton 249	

2.2 Determination of endotoxin concentration

Endotoxin concentrations in the samples were determined immunologically by enzyme-linked immunoabsorbent assay (ELISA) according to Palm et al. (1994) and by determining bioactivity using bioassays with *Heliothis virescens* according to Pratt et al. (1993).

2.3 Determination of microbial populations, bacterial species composition and DNA fingerprints.

Population levels of total culturable bacteria and fungi were determined by plating samples on selective media. Numbers of protozoa (amoebae, ciliates and flagellates), performed only in experiment 4, were determined at the Microbial Biomass Service at Oregon State University, Corvallis, OR, USA according to the method of Darbyshire et al. (1974).

In experiment 4, for the parental, transgenic 247 and transgenic 249 treatments in Wasco and Panoche soil on sample days 0, 14 and 56, bacterial species identification was performed. Bacterial colonies were subcultured, Gram stained and identified biochemically based on substrate utilization

of 95 carbon sources in Biolog Gram negative or Gram positive microtiter plates (Biolog 1992). In all of the experiments, fungal colonies were examined visually for qualitative morphological differences.

In experiment 4, the parental, transgenic 247 and transgenic 249 cotton treatments in Wasco soil were evaluated for differences in bacterial community composition. Diluted soil samples that were used for the total bacteria and fungi plating were also placed in Biolog Gram negative microtiter plates to determine bacterial community utilization of 95 carbon sources (Garland and Mills 1991).

In experiment 4, 3 replicate samples of Wasco and Panoche soil treatments were analyzed for total DNA content. The method of Porteous et al. (1994) was used for DNA extraction, polymerase chain reaction (PCR) amplification and DNA fingerprinting.

2.4 Statistical analyses

Total bacterial, fungal and protozoan population levels were analyzed in SAS with ANOVA and repeated measures analysis to determine significant ($p < 0.05$) differences among treatments. The Log CFU/g means of the treatments were compared within each sample day with Tukey's Studentized Range Test (SAS Institute 1989).

Principal components analysis (PCA) was used to analyze the bacterial community substrate utilization assays for each sample day in order to distinguish differences in patterns of carbon source utilization among the parental cotton treatment and the transgenic 247 and 249 cotton treatments (Garland and Mills 1991).

3 Results

3.1 Enzyme-linked, insect, and protozoan assays

Both immunological and biological activity of the *B.t.k.* toxin persisted in the purified toxin treatments and in the transgenic plant treatments. Purified *B.t.k.* HD-1 and HD-73 toxins and *B.t.k.* toxin in the transgenic lines 81 and 279 cotton plants were determined by ELISA to persist at detectable levels (the ELISA detection limit is 0.5 ng toxin/g soil) for up to 28 days in experiments 1, 2 and 3. In experiment 4, the purified *B.t.k.* toxin was detectable by ELISA up to 28 days and the purified toxin + parental plant, and the *B.t.k.* toxin in the transgenic 247 and 249 cotton plants, were detectable by ELISA up to 56 days in the Wasco soil. In experiment 4, the purified *B.t.k.* toxin and the *B.t.k.* toxin in the transgenic 247 and 249 cotton plants were detectable by ELISA up to 28 days and the purified toxin + parental cotton plant was detectable by ELISA up to 56 days in the Panoche soil. The target insect bioassays (performed only in experiment 4) indicated biological activity of the *B.t.k.* toxin remained up to 28 days in the purified *B.t.k.* toxin treatment and in the transgenic 247 and 249 plant treatments (day 56 samples were not assayed) in the Wasco soil and in the Panoche soil. Further discussion of the persistence of the immunological and biological activity of the *B.t.k.* toxins are provided in publications on ELISA studies (Palm et al. 1994) and target insect bioassay studies (Pratt et al 1993).

No significant differences occurred on any sample day in the number of amoebae, ciliates or flagellates per g dry weight of soil among the transgenic 247, transgenic 249 and parental cotton

treatments for the protozoan assays that were performed in experiment 4

3.2 Culturable bacteria and fungi counts

Results were compared among treatments without plant material (with the soil only treatment as the control) and among treatments with plant material (with the soil+parental cotton treatment as the control). This was done because the addition of the cotton leaves, whether they were parental or transgenic, nearly always caused a significant increase in bacterial and fungal numbers. This effect was due to the nutrients from the added cotton leaves promoting microbial growth; plant material added to soil often results in high microbial counts (Broder and Wagner 1988; Parr and Papendick 1978).

The addition of purified *B.t.k.* toxin never caused a detectable effect on microbial populations. No significant differences in bacterial and fungal population levels were observed between the soil only treatment and the purified HD-1 toxin treatment or purified HD-73 toxin treatment on any of the sample days in all four experiments.

In contrast, the addition of two of the three lines of transgenic cotton caused detectable, and often significant, changes in microbial population levels. In experiment 1, bacterial population levels on sample days 7, 14 and 21 and fungal population levels on sample days 7 and 14 were significantly higher in the transgenic 249 cotton treatment than in the parental cotton treatment (Figure 1). In experiment 2, bacterial population levels on sample days 7 and 14 and fungal population levels on sample days 7, 14, 21 and 28 were significantly higher in the transgenic 249 cotton treatment than in the parental cotton treatment (Figure 2). In experiment 3, there were no significant differences in bacterial or fungal population levels on any sample days between the transgenic 81 cotton treatment and the parental cotton treatment, or the parental cotton treatment + purified HD-1 toxin treatment. In the experiment 4 treatments with Wasco soil, bacterial populations in the transgenic 247 cotton treatment were significantly higher than in the parental cotton+purified HD-73 toxin treatment on sample days 7, 14 and 28. The transgenic 249 cotton treatment was significantly higher than the parental cotton treatment in bacterial population levels on sample day 56. The transgenic 249 cotton treatment was also significantly higher than the parental cotton+purified HD-73 toxin treatment in bacterial population levels on sample days 7 and 56 and in fungal population levels on sample days 7 and 14. For the treatments in experiment 4 with Panoche soil, bacterial populations in the transgenic 247 cotton treatment were significantly higher than those in the parental cotton treatment on sample day 7 .

3.3 Bacterial species identification

Identification of subcultures from the plates used for the bacterial counts in experiment 4 indicated that differences in bacterial species composition developed among the transgenic 247 cotton treatment, transgenic 249 cotton treatment and the parental cotton treatment over the course of the experiment in the Wasco soil and to a lesser extent in the Panoche soil. Differences among the treatments in bacterial species composition were not observed at the start of the experiment; on sample day 0, all colonies subcultured from the parental, transgenic 247 and transgenic 249 cotton treatments in Wasco or Panoche soil were Gram positive and there were few differences in genus or

species composition (colonies were mainly *Actinomycete* or *Bacillus* spp. in the Wasco soil and mainly *Streptococcus* or *Bacillus* spp. in the Panoche soil).

The greatest differences in Gram identification and species composition in the Wasco soil were observed on sample day 14. The parental cotton treatment had a larger number of Gram positive bacteria (34%) than the 247 transgenic treatment (0%) and the 249 transgenic treatment (4%), and had mainly *Bacillus* spp (25% of the identified Gram positive colonies), *Enterobacter cloacae B* (25% of the identified Gram negative colonies) and *Enterobacter* spp (25% of the identified Gram negative colonies). In the transgenic 247 cotton treatment, the predominant species were *E. cloacae B* (23% of the identified Gram negative colonies) and *Pseudomonas corrugata* (35% of the identified Gram negative colonies) and in the transgenic 249 cotton treatment *Enterobacter asburiae* (86% of the identified Gram negative colonies) was the dominant species. On sample day 56 in the Wasco soil, bacterial composition was most similar between the parental and transgenic 249 cotton treatments; both had a predominance of Gram positive bacteria (64% for the parental treatment and 75% for the transgenic 249 treatment) that were mainly *Bacillus* spp. (36% for the parental treatment and 39% for the transgenic 249 treatment of the identified Gram positive colonies) and also relatively high numbers of *Pseudomonas diminuta* (23% for the parental treatment and 8% for the transgenic 249 treatment of the identified Gram negative colonies). In contrast, in the transgenic 247 cotton treatment on day 56, the majority of colonies were Gram negative and were identified as *Alcaligenes denitrificans* (20% of the identified Gram negative colonies) and *P. corrugata* (25% of the identified Gram negative colonies).

In the Panoche soil on sample day 14, the parental, transgenic 247 and transgenic 249 cotton treatments were similar and had a fairly equal distribution of Gram positive and Gram negative bacteria and mainly *Bacillus* spp. and *E. asburiae* colonies. On sample day 56, there was a predominance of Gram positive bacteria in the Panoche soil, particularly for the parental (69%) and transgenic 249 (78%) cotton treatments. The most prevalent species in Panoche soil on sample day 56 in the parental treatment were *Bacillus* spp. (19% of the identified Gram positive colonies) whereas the transgenic 247 and 249 cotton treatments did not generally have predominant species, in part because a high number (62%) of the Gram positive colonies in the transgenic 249 cotton treatment could not be identified.

Based on fungal colony morphology, differences were also observed in fungal species composition between the parental and transgenic cotton treatments on a few of the sample days in experiments 1 and 2. For example, in experiment 2 on sample days 21 and 28, all the fungal colonies isolated from the transgenic 249 cotton treatment were *Mucor* sp. whereas only a few *Mucor* sp. were isolated from the parental cotton treatment.

3.4 Bacterial community metabolic substrate utilization

Differences in bacterial species composition were also observed in the Biolog assays for substrate utilization measured for the diluted soil samples. The Principal Components Analysis of these assay results indicated differences in microbial usage of metabolic substrates among the parental, transgenic 247 and transgenic 249 cotton treatments on sample days 7 and 14. Differences in utilization of the amino acids L-asparagine, L-aspartic acid and L-glutamic acid on sample days 7 and 14 were important in the separation of the parental and transgenic 247 and 249 treatments as evidenced by their large contribution to the PCA score. No other single metabolic substrates were as

consistently used to separate the treatments. The utilization of these 3 amino acids was significantly higher in the transgenic 247 and 249 treatments than in the parental treatment on sample days 7 and 14.

3.5 Total DNA content and eubacterial DNA fingerprints

Differences among the treatments in bacterial population levels and species composition were also indicated by the DNA extractions and fingerprints that were performed in experiment 4. As observed with the total bacterial and fungal counts, the addition of plant material caused an increase in microbial levels; two to five times more total DNA was extracted from the treatments containing parental or transgenic plant material than from the treatments containing soil only or soil+purified HD-73 toxin in both the Panoche and the Wasco soils on sample days 7, 14, 28 and 56. For the Wasco soil on sample days 7, 14 and 28, the highest DNA content was measured in the transgenic 247 cotton treatment. For example, on day 28 the A_{260} readings indicated the following DNA recoveries: 20 to 35 μg DNA per g of the soil or soil+purified HD-73 toxin treatments; 50 to 65 μg DNA per g of the soil+parental cotton or soil+parental cotton+purified HD-73 toxin treatments; 50 to 75 μg DNA per g of the soil+transgenic 249 treatment; and 80 to 100 μg DNA per g of the soil+transgenic 247 cotton treatment.

Differences among treatments in the composition of microbial populations were indicated by eubacterial DNA fingerprint patterns (restriction endonuclease digested fragment patterns) of amplified rDNAs from Wasco soil containing parental, transgenic 247 and transgenic 249 cotton on sample days 14 and 56. Fingerprint patterns for the treatments varied in the size or length of fragments, the number, intensity, or absence of bands and the reoccurring presence of a series of fragments in the fingerprint patterns. For example, on sample day 14 the fragment patterns for the parental treatment showed intense bands at 600 - 800 bp which were not observed in the fingerprints of either transgenic treatment. On sample days 14 and 56, the transgenic 247 treatment fingerprints contained a unique series of 4 intense bands between 150 - 300 bp. In addition, the fingerprint for the transgenic 249 treatment on sample day 56 had bright 650 bp fragment bands present that were not observed in the fingerprints for the other treatments.

4. Discussion

The addition of purified *B.t.k.* endotoxin to natural soil did not have any measurable effects on the indigenous soil microorganisms. In all of the experiments, no changes were observed in the levels of culturable, aerobic soil bacteria, fungi or protozoa (effects on protozoa were evaluated only for HD-73) when purified *B.t.k.* HD-1 or HD-73 toxin was added to natural soils. In contrast, the increase in culturable, aerobic bacterial and fungal populations that occurred when leaves of cotton were added to soil was significantly higher in the transgenic cotton treatments relative to the parental cotton treatment. This significant stimulation in microbial populations from the transgenic cotton, however, was only observed with the addition of the 247 and 249 lines, but not the 81 line, of the transgenic *B.t.k.*-producing cotton plants. The increase in microbial populations observed with the plate counts from the addition of cotton leaves was also confirmed with the bulk DNA extractions performed in experiment 4; the amounts of DNA extracted in the treatments with parental or transgenic plant

leaves increased as total microbial plate counts increased. For example, the greatest amount of DNA extracted and the highest bacterial counts coincided; they both occurred in the transgenic 247 treatment on sample day 28 in the Wasco soil in experiment 4.

Qualitative changes in the soil microbial community were also observed with the addition of the 247 and 249 lines of transgenic *B.t.k.* cotton to Wasco or Panoche soil. The metabolic substrate utilization assays and species identifications of subcultures performed in experiment 4 indicated differences in microbial species composition among the treatments. For example, the community substrate utilization assays indicated major differences on sample days 7 and 14 among the parental, transgenic 247 and transgenic 249 cotton treatments in the type and level of substrates utilized; there was significantly greater utilization of asparagine, aspartic acid and glutamic acid in the transgenic 247 and 249 cotton treatments than in the parental cotton treatment even though the population levels of the treatments were not significantly different. Interestingly, these substrates are important intermediates, along with glutamine (which is not included as a substrate in the Biolog plates), in nitrogen assimilation reactions. Species identifications of subcultures from plates used for the total bacterial populations in experiment 4 similarly showed the greatest differences between the parental treatment and the transgenic 247 and 249 treatments on sample day 14 (species identifications were not done for sample day 7). DNA fingerprints of amplified rDNAs also reflected qualitative differences among the treatments in eubacterial population. Distinct differences in fragment molecular weight and number and location of bands were observed among the Wasco soil parental, transgenic 247 and transgenic 249 treatments in experiment 4. These differences between the parental treatment and the transgenic treatments were pronounced on sample day 14, as occurred with the substrate utilization measurements and species identifications.

It is somewhat surprising that the line 81 transgenic cotton plant did not cause the same impact on microbial populations that was observed with the line 247 and line 249 transgenic cotton plants. The transgenic cotton lines produce endotoxins (HD-1 for line 81 and HD-73 for lines 247 and 249) that are coded for by genes from closely related, bacterial strains and the HD-1 and HD-73 endotoxins share 86% amino acid identity. It seems unlikely that the small difference in endotoxins between the transgenic plant line 81 and the lines 247 and 249 would account for the difference in effects from the transgenic plants because the purified endotoxins did not differ in their effects and neither had any detectable impacts on microbial populations.

One possible explanation for the lack of effects on the total microbial population levels from the addition of the purified HD-1 and HD-73 *B.t.k.* toxins and the transgenic cotton line 81 is that there were characteristics of the transgenic 247 and 249 cotton plants, other than production of the *B.t.k.* toxin, that impacted soil microorganisms. In most of the experiments, the stimulatory effects of the transgenic 247 and 249 cotton plants on bacterial and fungal populations were short term, suggesting that the transgenic plants may have decomposed faster than the parental plants and thus more rapidly provided nutrients for microbial growth. This potential increase in decomposition rate of the transgenic 247 and 249 cotton plants may have resulted from genetic manipulation of the plants. Unanticipated changes in plant quality occurring from insertion of genes have been documented. In several laboratory and field studies there have been unintentional changes in plant characteristics due to genetic manipulation and position effects from site(s) of insertion (Gene Exchange 1992). In addition, there may be genes used to confer a specific trait but which are actually pleiotropic and change several plant traits (Fitzpatrick 1993). Finally, the practice of tissue culturing, used for both traditional and genetically engineered plant breeding, frequently produces plant aberrations, some of

which may not be detected during screening for efficacy of the added trait(s).

These sorts of risk assessment studies for transgenic plants need to be performed under a variety of environmental conditions. The greater quantitative and qualitative impacts of the transgenic plant lines in the Wasco soil as compared to the Panoche soil and the higher recovery of DNA from the Wasco soil than the Panoche soil demonstrate the importance of one environmental variable, soil type. The clay and organic matter content of the Panoche soil is higher than that of the Wasco soil and probably resulted in greater binding of the transgenic plant material, making it less available to exert an impact on the soil microorganisms (Stotzky 1986). In past ELISA studies, we have obtained much higher recovery of the purified toxin and the transgenic plant toxin from the Wasco soil than from the Panoche soil (Palm et al. 1996). Similarly, target insect bioassays with *Heliothis virescens* have shown higher bioactivity of the same quantity of purified toxin or transgenic plant toxin when measured in the Wasco soil relative to the Panoche soil (Pratt et al. 1993).

In these studies with *B.t.k.* producing transgenic cotton plants, we have demonstrated both quantitative and qualitative changes in exposed soil microorganisms. The quantitative effects were generally transient and not what are typically considered detrimental (i.e, population levels were stimulated rather than depressed). The qualitative effects of apparent changes in the microbial species composition, however, has a potential to impact soil processes and may be of ecological significance. These results are valid only for the methods and test conditions used and additional research is necessary to determine their scope, extent and significance.

In the continuing debate about the degree and types of risks posed by the environmental release of transgenic plants, the argument has been made that transgenic plants pose no more risk than the transgenic compounds they produce and that plants should not be evaluated based on their having originated from genetic engineering. We believe our results challenge this argument; two of the transgenic *B.t.k.*-producing cotton lines impacted soil microorganisms yet no effects were observed from the purified *B.t.k.* endotoxins. We suggest that the risk assessment of transgenic plants should address and monitor for potential ecological effects that may result from changes in plant characteristics other than expression of the inserted gene(s).

Acknowledgments

The authors thank Dr. Roy Fuchs, Sharon Berberich, Joel Ream and Bill Schuler of the Monsanto Company, St. Louis, MO, USA, for providing the cotton seeds, purified toxin and technical information. The authors would like to acknowledge the excellent technical and scientific support provided for this study by Valerie Fieland, Lisa Ganio, Curt Palm, Arlene Porteous, Grahame Pratt, Lynn Rogers and Debbie Schaller.

References

Ali AUDD, Adellatif MA, Bakry NM, El-Sawaf SK (1973) Studies on biological control of the greater wax moth, *Galleria mellonella*. 1. Susceptibility of wax moth larvae and adult honeybee workers to *Bacillus thuringiensis*. J. Agric. Res. 12: 117-123

Biolog MicroStation System Manual, Release 3.01. (1992) Biolog, Inc., Hayward, CA.

Broder MW, Wagner GH (1988) Microbial colonization and decomposition of corn, wheat, and soybean residue. Soil Sci. Soc. AM. J. 52: 112-117

Crawley, HJ, Hails RS, Rees M, Kohn D, Baxton J (1993) Ecology of transgenic oilseed rape in natural habitats. Nature 363: 620-623

Delannay X, LaVallee BJ, Proksch RK, Fuchs RL, Sims SR, Greenplate JT, Marrone PG, Dodson RB, Augustine JJ, Layton JG, Fischhoff DA (1989) Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. *kurstaki* insect control protein. Bio/Technology 7: 1265-1269

Darbyshire JF, Wheatley RE, Greaves MP, Inkson RH (1974) A rapid micromethod for estimating bacterial and protozoan populations in soil. Ecology 61: 764-771

Donegan KK, Palm CJ, Fieland VJ, Porteous LA, Ganio LM, Schaller DL, Bucao LQ, Seidler RJ (1995) Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. Appl. Soil. Ecol. 2: 111-124

Donegan KK, Schaller DL, Stone JK, Ganio LM, Reed G, Hamm PB, Seidler RJ (1996a) Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the *Bacillus thuringiensis* var. *tenebrionis* endotoxin. Transgenic Research 5: 25-35

Donegan KK, Seidler RJ, Fieland VJ, Schaller DL, Palm CJ, Ganio LM, Cardwell DM, Steinberger Y (1996b) Decomposition of genetically engineered tobacco under field conditions: Persistence of the proteinase inhibitor I product and effects on soil microbial respiration and protozoa, nematode and microarthropod populations. Submitted to J. of Appl. Ecol.

Fitzpatrick T (1993) Pleiotropic gene found in barley plant. Genetic Engineering News 13(5), pp. 1 and 22

Flexner JL, Lighthart B, Croft BA (1986) The effects of microbial pesticides on non-target, beneficial arthropods. Agric., Ecosystems, Environ. 16: 203-254

Fox, J (1991) *Bt* resistance prompts early planning. Bio/Technology 9: 1319

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source. Appl. Environ. Microbiol 57: 2351-2359

Gasser CS, Fraley RT (1992) Transgenic Crops. Scientific American June 1992: 62-69

Hofte H, Whitely HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 242-255

James RJ, Miller JC, Lighthart B (1993) *Bacillus thuringiensis* var. *kurstaki* affects a beneficial insect, the cinnabar moth (Lepidoptera: Arctiidae). J. of Econ. Entom. 86: 334-339

Jenkins JN, Parrott WL, McCarty JC Jr., Barton KA, Unbeck PF (1991) Field test of transgenic cottons containing a B.t. gene. Dept. of Information Services, Division of Agriculture, Forestry and Veterinary Medicine. MAFES Technical Bulletin #174, Jan. 1991

Johnson, MT, Gould, F (1992) Interaction of genetically engineered host plant resistance and natural enemies of *Heliothis virescens* (Lepidoptera: Noctuidae) in tobacco. Environ. Entomol. 21: 586-597

Kareiva P, Morris W, Jacobi CM (1994) Studying and managing the risk of cross-fertilization between transgenic crops and wild relatives. Mol. Ecol. 3: 15-21

Klinger T, Ellstrand NC (1994) Engineered genes in wild populations: fitness of weed-crop hybrids of *Raphanus sativus*. Ecolog. Applic. 4: 117-120

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola SV (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology 11: 194-200

Lange P (1990) The German experience gained with field testing of genetically modified plants. Federal Ministry for Research and Technology, Bonn, Germany.

Lundstrum L (1992) Monsanto develops beetle resistant plants - plots show remarkable control. Potato Grower of Idaho 36

MacKenzie D (1990) Jumping genes confound German scientists. New Scientist. Dec. 15, 199.

Manasse RS (1992) Ecological risks of transgenic plants: Effects of spatial dispersion on gene flow. Ecolog. Applic. 2: 431-438

Miller JC (1990) Field assessment of the effects of microbial pest control agents on nontarget Lepidoptera. Amer. Entomol. 36: 135-139

Molloy D, Jamnback H (1981) Field evaluation of *Bacillus thuringiensis* var. *israelensis* as a black fly biocontrol agent and its effect on nontarget stream insects. J. Econ. Entomol. 74: 314-318

Mulla MS, Federichi BA, Darwazeh HA (1982) Larvicidal effect of *Bacillus thuringiensis* serotype H-14 against stagnant-water mosquitoes and its effect on nontarget organisms. Environ. Entom. 11: 788-795

Palm CJ, Donegan KK, Harris DL, Seidler RJ (1994) Quantitation in soil of *Bacillus thuringiensis* var. *kurstaki* delta-endotoxin from transgenic plants. Molecular Ecology 3: 145-15.

Palm CJ, Schaller DL, Donegan KK, Seidler RJ (1996) Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. *kurstaki* endotoxin. Canadian J. Of Microbiol. (Submitted)

Parr JF, Papendick RI (1978) Factors affecting the decomposition of crop residues by microorganisms. In Oschwald, WR (ed). Crop Residue Management Systems. American Society of Agronomy, Madison, WI., pp. 101-129

Perlak FJ, Deaton RW, Armstrong TA, Fuchs RL, Sims SR, Greenplate JT, Fischhoff DA (1990) Insect resistant cotton plants. Bio/Technology 8: 939-943

Porteous LA, Armstrong JA, Seidler RJ, Watrud LS (1994) An effective method to extract DNAs from environmental samples for polymerase chain reaction amplification and DNA fingerprint analysis. Current Microbiology 29: 301-307

Pratt GE, Royce LA, Croft BA (1993) Measurement of toxicity of soils following incorporation of plant residues engineered with *Bacillus thuringiensis* v. *kurstaki* endotoxin, using a *Heliothis virescens* growth bioassay, Proc Fifth Investigators Meeting for EPA's Environmental Release of Biotechnology Research ProGram, College Park, Maryland 1992

Ream JE, Berberich SA, Sims SR, Rogan GJ, Fuchs RL (1992) *In Planta* distribution and environmental fate of insect resistant cotton proteins. Supplement to Plant Physiology. 99(1): 80

SAS Institute (1989) SAS/STAT User's Guide, Version 6, 4th ed., Vol. 2, SAS Institute, Cary, N.C. 846pp.

Stotzky G (1986) Influence of soil mineral colloids on metabolic processes, growth, adhesion and ecology of microbes and viruses. In: Huang PM, Schnitzer M (eds), Interactions of soil mineral with natural organics and microbes. Soil Science Society of America, Madison, Wisconsin, pp 305-428

The tomatoes of the tree of knowledge. (1990) The Economist. July 14, 1990

Tolstova YS, Ionova ZA (1976) Toxicity of pesticides to TrichoGramma. Zasch. Rast. (Moscow) 9: 21

Vaeck M, Reynaerts A, Hofte H, Jansens S, De Beuckeleer M, Dean C, Zabeau M, Van Montagu M, Leemans J (1987) Transgenic plants protected from insect attack. Nature 328: 33-37

Umbeck PF, Barton KA, Nordheim EV, McCarty JC, Parrot WL, Jenkins JN (1991) Degree of pollen dispersal by insects from a field test of genetically engineered cotton. J. Econ. Entom. 84: 1943-1950

Unexpected results in transgenic organisms (1992) The Gene Exchange 3(3): 6-7

USDA-CRS and USDA-ARS (1992) Scientific evaluation of the potential for pest resistance to the *Bacillus thuringiensis* (Bt) delta-endotoxins. A Conference to Explore Resistance Management Strategies, Beltsville, MD, January 21-23, 1992

Yamada K (1992) Genetic vegomatics splice and dice with weird results. Wall Street Journal. April 13, 1992

Fig. 1. Numbers of total bacteria (**A**) and fungi (**B**) in experiment 1, averaged from duplicate plates of 3 treatment replicates per sample day, of soil alone, soil+purified HD-73 toxin, soil+parental cotton and soil+transgenic 249 cotton. Significant ($P < 0.05$) differences among treatments were determined with Tukey's Studentized Range Test. The minimum significant differences in log values calculated for bacterial populations were 0.42 (day 0), 0.29 (day 7), 0.95 (day 14), 0.42 (day 21). 0.66 (day 28) and for fungal populations were 0.44 (day 0), 0.14 (day 7), 0.19 (day 14), 0.28 (day 21), 0.34 (day 28).

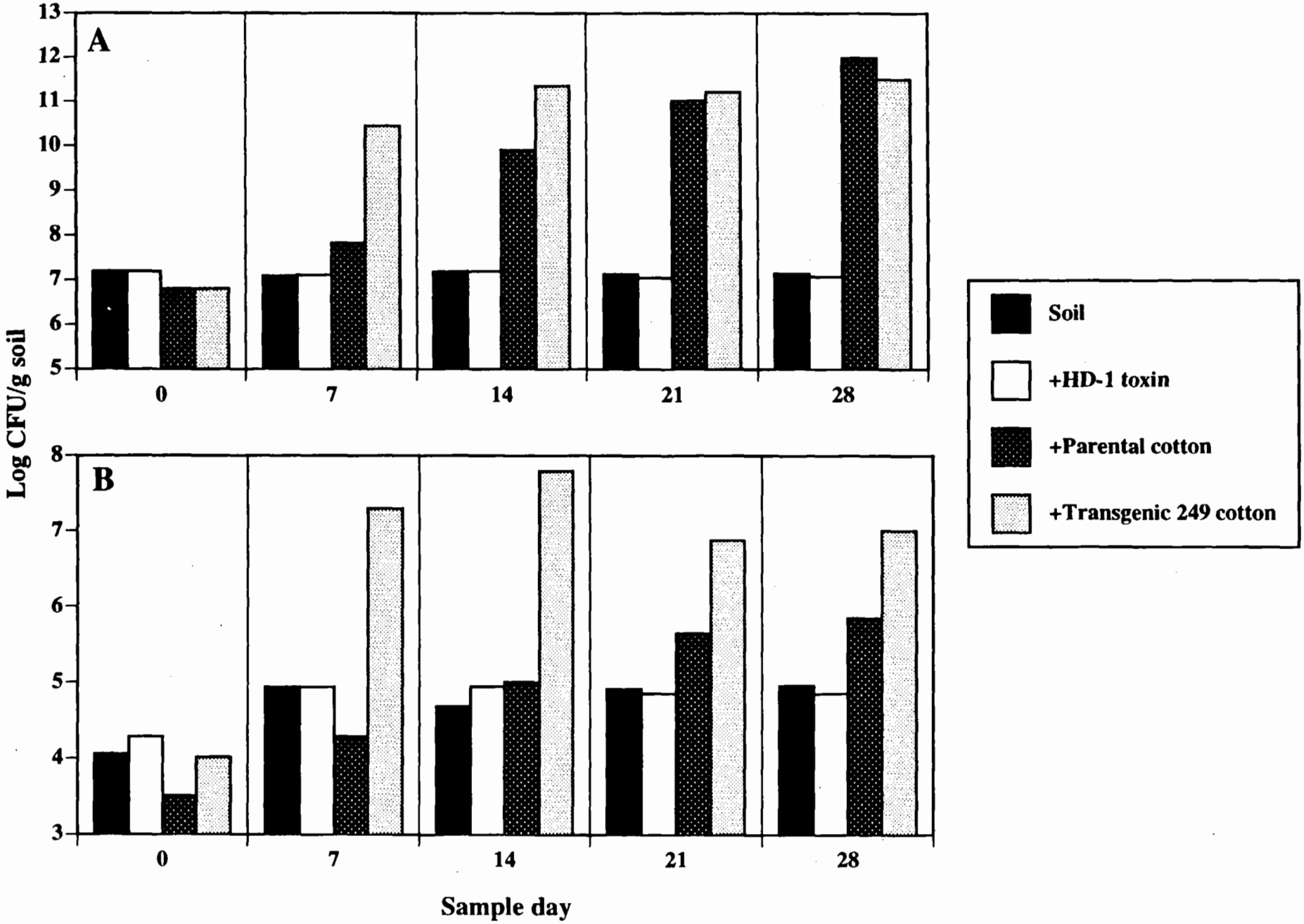
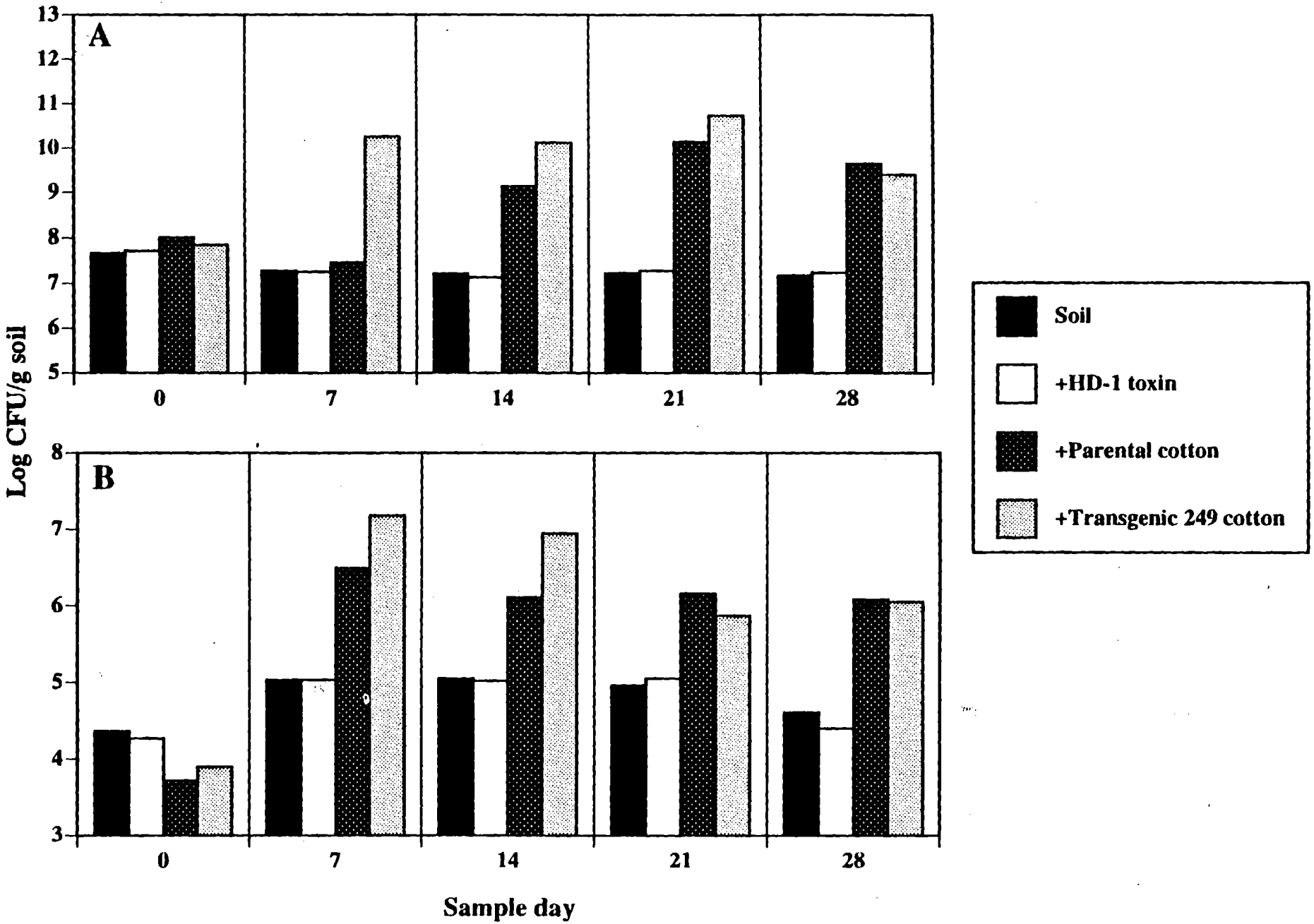

A
B
Log CFU/g soil
Sample day
0
7
14
21
28
Soil
+HD-1 toxin
+Parental cotton
+Transgenic 249 cotton

Fig. 2. Numbers of total bacteria (**A**) and fungi (**B**) in experiment 2, averaged from duplicate plates of 3 treatment replicates per sample day, of soil alone, soil+purified HD-73 toxin, soil+parental cotton and soil+transgenic 249 cotton. Significant ($P<0.05$) differences among treatments were determined with Tukey's Studentized Range Test. The minimum significant differences in log values calculated for bacterial populations were 0.23 (day 0), 1.40 (day 7), 0.87 (day 14), 1.07 (day 21), 0.40 (day 28) and for fungal populations were 0.51 (day 0), 0.91 (day 7), 0.79 (day 14), 0.36 (day 21), 0.32 (day 28).


A
B
Log CFU/g soil
Sample day
0
7
14
21
28
Soil
+HD-1 toxin
+Parental cotton
+Transgenic 249 cotton

NHEERL-COR-2028A

TECHNICAL REPORT DATA
(Please read instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPI
EPA/600/A-96/095		

4. TITLE AND SUBTITLE	5. REPORT DATE
Effects of cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin on soil microorganisms	6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
K. K. Donegan[1], R. J. Seidler[2]	

9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
[1]Dynamac Corporation, Corvallis, OR [2]U.S. Environmental Protection Agency, NHEERL, Corvallis, OR	11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
US EPA ENVIRONMENTAL RESEARCH LABORATORY 200 SW 35th Street Corvallis, OR 97333	Book chapter
	14. SPONSORING AGENCY CODE EPA/600/02

15. SUPPLEMENTARY NOTES
1996. To be published as a book chapter in "Biotechnology in Agriculture and Forestry"

16. ABSTRACT

Many agriculturally important plants have been engineered to produce endotoxins from different subspecies of the bacterium *Bacillus thuringiensis* (*B.t.*). The endotoxin *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*) has demonstrated insecticidal activity against lepidopterans. Although high specificity has been assumed for most *B.t.* endotoxins, their effects on non-target organisms have not been fully evaluated. This chapter describes experiments that investigated the biological and molecular changes in microbial populations following the incorporation of purified *B.t.k.* endotoxin or *B.t.k.*-producing cotton into natural soils. Microbial populations were monitored for changes in the total numbers and species composition of culturable bacteria and fungi, in the substrate utilization of the bacterial community and in the total DNA content and DNA fingerprints of the eubacteria.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
sediments, ecological risk assessment		

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (*This Report*)	21. NO. OF PAGES
		18
	20. SECURITY CLASS (*This page*)	22. PRICE