

From: Jennings, Henry
Sent: Wednesday, November 14, 2007 9:06 AM
To: Schlein, Paul B
Subject: FW: More data on the effects of Bt corn on aquatic invertebrates

Importance: High

Attachments: Nontarget PNASv1-1_gpd.doc

-----Original Message-----

From: Andrei Alyokhin [mailto:Andrei_Alyokhin@umit.maine.edu]
Sent: Tuesday, November 13, 2007 2:07 PM
To: Hicks, Lebel; Jennings, Henry; jjemison@umext.maine.edu; titus@dialmaine.com; esideman@mofga.org
Subject: More data on the effects of Bt corn on aquatic invertebrates
Importance: High

Hello,

Attached is the manuscript that Galen Dively and his collaborators are putting together. It is worth circulating among Board members and other interested parties before the public hearing.

Andrei

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----- Original Message -----

Andrei,

Attached is the manuscript for PNAS. There is still a few missing citations to add but the results and discussion are in the final form.

I would appreciate your help in getting or presenting these results to the pesticide board.

Galen

Andrei Alyokhin wrote:

>>Galen,

>>

>>Thank you for your phone call. It was nice talking to you yesterday. From the spreadsheet, you have assayed 12 populations (I thought that the last one collected did not produce eggs, but it did). Therefore, you need to invoice me for \$6,000.

>>
>>Andrei
>>
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1 For: PNAS

2 Classification: Biological Sciences/Environmental Sciences

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5 Non-Target Effects of Transgenic Corn Debris in Streams

6

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19 Author contributions: P.D.J, W.O.L., G.P.D., and C.M.S designed research; P.D.J,

20 W.O.L., G.P.D., and C.M.S performed research; P.D.J and G.P.D. analyzed data; and

21 P.D.J. wrote the paper.

22 **Abstract**

23 Insecticidal proteins from *Bacillus thuringiensis* expressed in genetically modified corn
24 are biologically active on only one order of insects. Most studies have shown no
25 significant effect of these toxins on the terrestrial non-target organisms but few have
26 examined the potential non-target effects in the aquatic environment. In laboratory
27 feeding trials, we show altered growth in three aquatic shredders common in headwater
28 streams in Maryland exposed to corn tissue containing the protein Cry1Ab, but not to
29 tissue containing two proteins Cry1Ab+Cry3Bb. This inconsistency suggests that other
30 factors beyond the Cry proteins are responsible for the growth responses. Our
31 measurements also indicate significant differences among hybrid families, which can
32 confound non-target studies if true non-*Bt* isolines are not included. We also show rapid
33 degradation and non-detectable bioactivity of Cry1Ab protein in corn tissue exposed after
34 two weeks to the elements in a terrestrial or aquatic environment through laboratory
35 bioassays using the European corn borer (*Ostrinia nubilalis*). Our overall findings do not
36 provide convincing evidence for risk to aquatic non-target shredders due to the
37 expression of Cry1Ab in senesced corn plant tissue.

38

39 Key words: *Bacillus thuringiensis* (Bt), genetically modified crops, non-target

40

41 **Introduction**

42 Genetically engineered corn (*Zea mays* L.) transformed with a gene from the
43 bacterium *Bacillus thuringiensis* (*Bt*) was first introduced in 1996. In 2007, 49% of all
44 U.S. corn was planted in insect resistant *Bt* hybrids (NASS 2007). Beyond a potential
45 reduction in insecticide use, the advantage of *Bt* transgenic crops over conventional
46 insecticides is their high specificity, such that potential toxic effects on non-target insects
47 should be minimal or nonexistent (Betz et al. 2000, MacIntosh et al. 1990, Ostlie et al.
48 1997, Schuler et al. 1999). However, like any insect control technology, transgenic crops
49 may present a risk to non-target organisms, particularly beneficial insects that are
50 taxonomically similar to the target pests (Obrycki et al. 2001).

51 Most types of *Bt* proteins expressed in transgenic crops are biologically active on
52 only one order of insects with susceptibility determined by specific receptors in the
53 membranous lining of the midgut epithelial cells (Shelton et al. 2002). Previous studies
54 have evaluated the potential impact of the transgenic plants expressing the *Bt* Cry toxins
55 on nontarget phytophagous and beneficial species. Most of these studies have shown no
56 significant effect of these toxins on the nontarget arthropods (Sims 1995, Donegan et
57 al. 1996, Dogan et al. 1996, Yu et al. 1997, Pilcher et al. 1997, Orr and Landis 1997,
58 Riddick and Barbosa 1998, Riddick et al. 1998, Lozzia 1999, Lozzia et al. 1999, Zwahlen
59 et al. 2000, Reed et al. 2001, Moar et al. 2002, Naranjo and Ellsworth 2002, Candolfi et
60 al. 2003, Fitt and Wilson, 2003, Dively et al. 2004, Sisterson et al. 2004, Dively 2005,
61 Bhatti et al. 2005, Daly and Buntin 2005, Head et al. 2005, Naranjo 2005, Torres and
62 Ruberson 2005, Rose and Dively 2007, but see Hilbeck et al. 1998 and Romeis et al.
63 2004), suggesting only direct impact on targeted pest species which are selectively

64 susceptible to the mode of action of the expressed *Bt* lepidopteran-specific Cry1Ab
65 proteins.

66 To our knowledge, only one published study has examined the potential non-
67 target effects of the plant incorporated *Bt* δ -endotoxins in the aquatic environment (Rosi-
68 Marshall et al. 2007). Given the intimate association between the ecology of first order
69 streams and land use, the likelihood of transgenic plant-tissue being delivered via wind
70 and water action to these streams is high, although, has yet to be quantified over broad
71 spatial scales (but see Stone et al., 2005, Rosi-Marshall et al. 2007). Ecologists are
72 becoming increasingly aware of the implications of the cross-ecosystem movement of
73 organisms and resources, and the subsequent cascading effects the presence of such
74 allochthonous subsidies can have on food web dynamics. Corn, being the greatest in
75 terms of individual biomass of the transgenic crops, may be the most abundant input of
76 crop detritus to those streams draining agricultural fields.

77 We selected four aquatic shredders common in headwater streams in Maryland
78 during and following the corn harvest as test organisms for this study. Two of these non-
79 target taxa were trichopterans: *Lepidostoma* sp, and *Pycnopsyche* sp. Because of the
80 close phylogenetic relationship between Trichoptera (caddisflies) and Lepidoptera
81 (Wiggins 1995), the caddisflies may be especially impacted by the input of tissue
82 containing lepidopteran-specific *Bt* Cry1Ab protein into streams. Caddisflies are one of
83 the dominant groups within streams, serve important trophic roles including
84 decomposition of allochthonous inputs of vegetation, and are susceptible to changes in
85 water quality (Allan 1995, Wiggins 1995). Rosi-Marshall et al. (2007) found two
86 trichopteran species to be susceptible to Cry1Ab-containing corn tissue, although several

87 other studies have shown no effect of other sources of *Bt* on trichopteran (Merritt and
88 Wipfli 1991 as *Bti*, Eidt 1985 as Thuricide).

89 Our third test species was in the order Diptera (*Tipula* sp.) and was selected
90 because of the high volume of detritus that these larvae process during their maturation.
91 The fourth test organism selected was a crustacean, the aquatic isopod *Caecidotia*
92 *communis* due to its ubiquity and high numbers in agricultural headwater streams in
93 Maryland. While only the trichopteran were expected to respond to the Cry1Ab toxin,
94 high doses of *Bt* applied under laboratory conditions demonstrated a decrease in detritus
95 decomposition (Kreutzweiser et al. 1993), and thus we included a wider range of non-
96 target taxa.

97 Beyond the selection of non-target test subjects, an important consideration in our
98 proposed exposure scenario is the environmental degradation of the senesced corn tissue
99 and the bioactivity of the Cry proteins. Several studies have examined the degradation
100 rates of the *Bt* Cry proteins associated with a soil matrix (Palm et al. 1994, Palm et al.
101 1996, Saxena and Stotzky 2000, Tapp and Stotzky 1998, Zwahlen et al. 2003) and found
102 low levels of bioactive Cry proteins many months after tissue senescence. However, the
103 aquatic environment provides a more rigorous environment for decomposition with the
104 addition of constant physical abrasion and ultraviolet light to the components found in the
105 soil environment. We included Cry1Ab bioassays, using the European corn borer,
106 *Ostrinia nubilalis* (the target organism in corn for this pesticide) in this study to evaluate
107 the longevity of the bioactive Cry1Ab protein in corn tissue under field conditions.

108 Thus the purpose of this study was twofold: 1) to assess the duration of Cry1Ab
109 protein bioactivity in senesced corn tissue subjected to terrestrial and aquatic field

110 conditions, and 2) to assess possible non-target effects of senesced and microbially
111 conditioned transgenic corn tissue to aquatic invertebrates in Maryland. Specifically, we
112 chose common aquatic invertebrates most likely to be exposed to post-harvest corn tissue
113 debris due to their functional role in allochthonous organic matter processing. The use of
114 a hybrid expressing two stacked *Bt* traits in addition to the single trait, was expected to
115 act as a replicate of the Cry1Ab only treatment, in addition to screening the selected taxa
116 for potential Cry3Bb susceptibility.

117 The hypotheses tested in this study were that 1) the toxicity of Cry1Ab proteins to
118 ECB would decrease over time under field conditions, 2) the Cry1Ab isoline would cause
119 negative lethal or sublethal effects in the non-target aquatic invertebrates compared with
120 the control isoline, 3) the stacked Cry1Ab plus Cry3Bb isoline would cause similar or
121 additional lethal or sublethal effects compared with the single Cry1Ab trait, and 4) the
122 aquatic invertebrates more closely related to the target organism would exhibit the greater
123 negative effects in response to the Cry1Ab trait.

124 **Results**

125 ***Ostrinia nubilalis* Bioassay Results**

126 To obtain a robust estimate of Cry1Ab protein activity in senesced leaves prior to
127 environmental exposure, leaf tissue from three isolines (non-Bt, Cry1Ab expressing,
128 Cry1Ab+Cry3Bb expressing) from three hybrid families (1, 2,3) grown in three field
129 blocks (A,B,C) was used in an initial replicated set of diet incorporated feeding bioassays
130 using *O. nubilalis*. The presence of the insecticidal Cry1Ab protein (isoline) was a
131 significant main effect (Table 1) in determining larval weight gain. *Ostrinia nubilalis*
132 larvae gained significantly more mass when feeding upon the non-*Bt* corn tissue

133 compared with tissue expressing Cry1Ab or Cry1Ab+Cry3Bb (Table 2). While a
134 significant interaction was detected between field block and hybrid (Table 1), the larval
135 weight gain was not significantly different between any block hybrid combinations with
136 the exception of larvae feeding on hybrid 3 from block A (15.24 ± 1.65 mg) gaining more
137 weight than larvae feeding on hybrid 1 from block A (7.04 ± 1.73 mg). No significant
138 difference in larval weight gain was detected between field blocks, hybrid families or
139 interactions between field block, hybrid family, and isolate.

140 To test the hypothesis that the Cry proteins remain biologically active in corn
141 leaves after harvest in the environment, we used the same diet-incorporated feeding
142 bioassay in a time-course study. Leaf tissue from all three hybrid families (9 isolines)
143 from field block A was placed in mesh bags and set in a riparian terrestrial habitat and a
144 headwater-stream aquatic habitat. Tissue samples were collected from the two habitats at
145 two week intervals after harvest. The environmental degradation of the Cry1Ab protein
146 in the terrestrial riparian environment occurred very rapidly, with differences in larval
147 weight gain attributable only to hybrid family (Table 1). No significant differences were
148 found in larval weight gain across isolines, or according to the duration of environmental
149 exposure. Similarly, tissue exposed to the aquatic environment did not cause any
150 significant differences in larval weight gain across any of the main effects: hybrid family,
151 isolate, or duration of environmental exposure (Table 1). These results indicate that
152 Cry1Ab protein bioactivity was not detectable in any of the tissue after only two weeks of
153 exposure to the elements in the terrestrial riparian or aquatic environments.

154 **Non-Target Organism Bioassays**

155 We did not find any significant differences between the treatments in lethal or
156 sublethal metrics for *Lepidostoma* larvae (Table 3), including initial head width, final
157 head width, change in head width, or final dry weight. No differences in survival were
158 detected between treatments using a probit analysis (DF = 2, Wald Chi-sqr = 0.0373, p =
159 0.9815).

160 The average final dry mass of the *Pycnopsyche* larvae were significantly different
161 across treatments (Table 3). While we could not obtain an initial wet or dry mass for the
162 larvae without extracting them from their cases, we did measure the head capsule width
163 at the start of the experiment, and found no significant difference across treatments
164 (Figure 1). Final head capsule width was also not significantly different across
165 treatments, and there was no significant difference in the change in head capsule width.
166 We also did not find a significant difference in larval survival across the treatments.
167 It appears that pycnopsyche larvae are molting at roughly the same rate in the three
168 treatments (head capsule width remains the same), however, the larvae in the stacked
169 treatment were significantly heavier than in the control or Cry1Ab treatments, indicating a
170 difference in growth.

171 We did not find any significant difference between treatments in the initial wet
172 mass of the *Tipula* sp. larvae (Table 3) indicating standardization across treatments.
173 After a month of feeding on corn tissue, however, we found that the larvae in the Cry1Ab
174 treatment had the lowest change in mass compared with the control and stacked
175 treatments (Figure 2). We could not use the tipulid survival data in an ANOVA, due to a
176 violation of the assumption of normality (since each experimental unit contained only one

177 larva). A probit analysis indicated that there was no significant difference in survival
178 across the three treatments (2 df, Wald Chi-sqr = 2.3336, p = 0.3114).

179 We did not find any significant difference between treatments in the initial length
180 of the isopod larvae. We did, however find significant differences in every other
181 parameter that we measured, including final mass, change in length, and even survival
182 (Table 3). In agreement with the *Tipula* sp. results, the Cry1Ab treatment exposed
183 isopods had significantly shorter length, less mass, and lower percent survival (Figure 3)
184 compared with isopods exposed to the non-*Bt* isoline and the Cry1Ab + Cry3Bb stacked
185 treatments.

186 **Discussion**

187 The scenario that we have considered in this set of experiments is the movement
188 of senesced corn leaf tissue from field to agricultural headwater stream following harvest.
189 When leaf debris enters the stream it is colonized by fungi and bacteria, and consumers
190 are adapted for shredding the leaf material and feeding on the matrix of leaf components
191 and associated microbes (Arsuffi and Suberkropp 1985, 1988). Through the enzymatic
192 degradation of the leaf material and physical impact of the running water, the Cry1Ab
193 proteins will degrade over time. Our goal was to determine if the Cry1Ab protein
194 remains active after entering streams, and to determine potential lethal or sublethal
195 impacts on local shredders.

196 The degradation of the Cry1Ab protein in the environment is an integral
197 component of the exposure analysis in the assessment of risk to non-target organisms
198 from plant incorporated protectants (PIPs) in genetically modified crop tissue. The results
199 from our bioassays using the European corn borer verified the bioactivity of the Cry1Ab

200 proteins in senesced corn prior to environmental exposure. The subsequent exposure of
201 the senesced leaf tissue to environmental conditions in a terrestrial or aquatic
202 environment eliminated any detectable bioactivity against the European corn borer after
203 only two weeks. This is especially relevant because all of the corn tissue used in the non-
204 target bioassays had been “conditioned” in stream water for two weeks prior to
205 incorporation in the bioassay. This suggests that any differences in the growth and
206 survival of the non-target taxa are not likely due to exposure to the Cry-proteins.

207 The interpretation of the non-target bioassay results is less straightforward. Three
208 of the four taxa tested showed significant sublethal growth effects when exposed to one
209 of the Cry-protein containing treatment tissues. The only test species that did not respond
210 significantly was *Lepidostoma* sp. This contrasts with recent results from Rosi-Marshall
211 et al. (2007), who showed significantly lower length measurements and higher mortality
212 in Bt treatments vs non-Bt treatments using *L. liba*. The non-significant growth
213 inhibition observed in three of the four test species in our study occurred in the Cry1Ab
214 treatment, but no similar decrease was observed in the Cry1Ab + Cry3Bb stacked
215 treatment. The second trichopteran species tested (*Pycnopsyche* sp.) exhibited a
216 significant response, although it was an increase in growth in the stacked treatment
217 compared with the control isoline. This positive effect suggests that *Pycnopsyche* was
218 not adversely affected by the Cry1Ab, Cry3Bb toxins, or any other tissue differences
219 between the treatments. The positive effects also suggest that a difference exists between
220 the treatments beneficial to this trichopteran. Findlay et al. (1996) found a decrease in
221 decomposition rate in cottonwood leaves that were previously damaged by mites or

222 ozone, suggesting that the protection afforded by the *Bt* Cry proteins may increase the
223 quality of the tissue as a resource to non-susceptible aquatic shredders.

224 The sublethal negative growth response exhibited by *Tipula* sp. to the Cry1Ab
225 treatment compared with the non-*Bt* isolate was surprising due to the taxonomical
226 specificity of the Cry proteins. Equally unexpected for the same reason was the
227 significant lethal and sublethal negative responses by *Caecidotia communis* to the
228 Cry1Ab treatment. This disagrees with the lack of a response in two terrestrial isopod
229 species to purified Cry1Ab protein reported by Clark et al. (2006). These negative
230 responses to the Cry1Ab treatment, however, were tempered by the lack of a difference
231 between the Cry1Ab + Cry3Bb treatment compared with the non-*Bt* isolate in both
232 species. This lack of response in the stacked *Bt* isolate suggests that another factor
233 beyond the Cry proteins is responsible for the negative responses.

234 The increasing rarity of true non-*Bt* isolines will make it increasingly difficult to
235 untangle any existing causal relationships in similar results. Our results indicate a need
236 for an aquatic artificial diet that would allow the partition and testing of individual tissue
237 components, as well as the direct delivery of the Cry proteins to aquatic non-target
238 organisms in a Tier 1 test. Our results also show that significant differences exist among
239 hybrid families, which can confound non-target studies if true non-*Bt* isolines are not
240 included. With regards to potential differences in the tissue used in these experiments,
241 chemical analyses indicated significant differences in Nitrogen and NDF (Lignin,
242 Cellulose, and Hemicellulose) between hybrid families, but not between any isolines
243 within a hybrid (cite Swan MS).

244 While our results indicate that it is difficult to link Cry1Ab with the negative
245 growth responses of common aquatic shredders, isolating the causal factor will require
246 additional and careful research. Further experiments will also require a suitable aquatic
247 non-target test organism. While each of the test species used were easily acquired and
248 relatively robust, none possess all of the characteristics needed to be a standard test
249 organism. The trichopterans had the greatest taxonomic specificity required for testing a
250 PIP like *Bt* Cry1Ab, but their winged adult stage would likely require complex rearing
251 facilities, in addition to a careful consideration of the developmental chronology and
252 geographic distribution of the many potential trichopterans species. The dipteran used
253 lacked both the taxonomic specificity and an adult aquatic life stage. The isopod life
254 history (viviparous, hemimetabolous development, aquatic adult stage) lent itself the
255 most to the requirements of our bioassays, but their relationship with the target species is
256 distant, not even sharing the same taxonomic class.

257 Several considerations in the extrapolation of our results to existing populations in
258 agricultural headwater streams include the presence of riparian buffers or filter strips in
259 many production areas, the degradation in Cry1Ab bioactivity over time observed in our
260 ECB bioassays, and the lack of food choice in our non-target organism bioassays. USDA
261 conservation buffer programs and scientific evidence regarding water quality and
262 nitrogen removal are encouraging the implementation of land adjacent to streams
263 designed to buffer waterways from agricultural impact (Mayer et al. 2005). These
264 buffers would also likely decrease the input of corn tissue into headwater streams
265 bordering agricultural production. Degradation of the Cry proteins may occur before
266 tissue enters the stream. Swan et al. (submitted) found that after an initial pulse of tissue

267 attributable to corn harvesting activity, most corn tissue input into a stream from an
268 adjacent corn field occurred months after harvest during storm events. Finally, once in
269 the stream, the tissue containing bioactive Cry proteins may be avoided by the shredders.
270 To our knowledge, no choice studies have been performed with genetically engineered
271 corn and aquatic consumers.

272 We have demonstrated a rapid decrease in activity with environmental exposure
273 of the senesced corn tissue, resulting in target larval growth inhibition declining rapidly
274 within two weeks. Degradation notwithstanding, we observed lethal and sublethal effects
275 in three of our four non-target test species, due to exposure to tissue from the Cry1Ab
276 containing isolate, but not supported by exposure to tissue from the Cry1Ab + Cry3Bb
277 isolate. Taken as a whole, our results do not provide evidence for risk to aquatic non-
278 target shredders due to the expression of Cry1Ab in transgenic plant tissue.

279 **Methods**

280 We used plants from three hybrid families (1, 2, 3) with different background
281 genetics, each with the same maturation time (115 days) and well adapted for the north-
282 eastern growing region. Within each hybrid family we used three isolines, all of which
283 contained the Roundup Ready gene (NK603): 1) a non-expressing isolate, 2) an isolate
284 expressing the Cry1Ab gene (Mon810 “YieldGard”), and 3) an isolate stacked with both
285 Cry1Ab and Cry3Bb1 genes (Mon810 and Mon863 “YieldGard Plus”). For hybrid
286 families 1 and 2, the designation was an experimental number for each isolate. For
287 hybrid family 3, the isolate names were: Dekalb DKC 63-80 (non-Bt), Dekalb DKC 63-
288 81 (Cry1Ab expressing), and Dekalb DKC 63-74 (Cry1Ab + Cry3Bb expressing).

289 The corn was grown in three randomized blocks (A, B, C) in field plots at the
290 University of Maryland's Western Maryland Research & Education Center near
291 Keedysville until plant senescence. Blocks were employed to ensure that plot-specific
292 environmental factors did not confound our treatments. All plots were cultivated on no-
293 till or minimum-till Hagerstown Silt Loam (pH 7, P-58, K -76) with a fertilizer
294 application of 144.8 kg of nitrogen ha⁻¹ plus 26.7 kg of sulfur ha⁻¹ (38.0-0-0-7) in the first
295 week of April 2005. Planting dates were in the first week of May 2005 with a JD
296 vacuum planter in 76 cm rows. Pesticides included Lumax (7 L ha⁻¹), Simazin (2.3 L ha⁻¹)
297 ¹), 2,4D (0.87 L ha⁻¹), and Credit Exta (2.3 L ha⁻¹). Rain accumulation (cm) by month
298 was as follows: April (7.85), May (5.44), June (16.46), July (6.20), August (2.79), and
299 September (16.92). Leaves from all nodes were removed from the senesced plants when
300 grain moisture reached 22% in late September and air dried under greenhouse conditions
301 to remove surface moisture.

302 ***Ostrinia nubilalis* Bioassays**

303 For the time-course study, leaf tissue from all three hybrids (9 isolines) from field
304 block A, was cut into sections of no more than 10 cm, and placed in mesh bags in packets
305 of approximately 5 g. The bags were set in a riparian terrestrial habitat and in a
306 headwater-stream, aquatic habitat. Three bags of each hybrid was collected at each
307 sampling time, and remaining leaf tissue was removed from bags and dried in a freeze
308 dryer. The lyophilized material was ground in a tissue grinder using a 1-mm sieve plate
309 and kept at -80°C until used. The *Bt* and non-*Bt* tissue of each hybrid group sampled
310 directly from the corn plots served as positive and negative controls during all bioassays.

311 To test for Cry1Ab activity, European corn borer (*Ostrinia nubilalis* Hübner) was
312 used as the sensitive indicator. Eggs were purchased from a commercial laboratory and
313 shipped overnight, usually 5-7 days before each assay. Eggs were incubated in a growth
314 chamber under temperature regimes manipulated to schedule a supply of test larvae of
315 approximately the same size for bioassay.

316 A meridic diet for *O. nubilalis* (Southland Products, Lake Village, AR) was
317 mixed with the lyophilized leaf powder at a concentration of 6 g of per liter of diet.
318 Initial range-finding assays determined that this concentration of senesced *Bt* leaf tissue
319 at harvest resulted in 40-60% growth inhibition of 1st instars after seven days of feeding.
320 Each bioassay included a replicate series of diet incorporated with the leaf powder from
321 the three isolines of one hybrid group collected at 2, 4, 6, and 8 weeks from terrestrial and
322 aquatic sites. For each diet treatment, cohorts of 32 pairs of neonates were reared
323 individually in 97.5 ml Solo® plastic cups containing 15 ml of diet.

324 After seven days, each cup was examined to record larval survival, instar stage,
325 and larval weight. Surviving larvae of each cohort were pooled and weighed together.
326 The average weight gain per larva was calculated by dividing the pooled weight by the
327 number of larvae in the cohort minus the average initial weight and used in our analysis
328 of larval growth.

329 **Non Target Bioassays**

330 Bioassays of four test organisms were performed in 250 ml aerated Erlenmeyer
331 flasks filled to the neck with a 50:50 mixture of stream water and deionized water. Water
332 lost to evaporation was replaced daily with deionized water. All assays were performed
333 in a walk-in growth chamber maintained at 14°C on a 16:8 L:D cycle. Twenty replicates

334 of each test organism were exposed to the leaf tissue of each isolate from hybrid 1 grown
335 in field block A. All corn tissue was “conditioned” for two weeks before bioassay
336 initiation in 20:80 filtered stream water to deionized water to ensure bacterial and fungal
337 growth on the leaf tissue (Bird and Kaushik 1985) and encourage shredder feeding. All
338 organisms were collected from headwater streams located at the University of
339 Maryland’s Central Maryland Research & Education Center - Clarksville farm between
340 July and September of 2006.

341 For the trichopterans, five larvae of *Lepidostoma* sp. and three larvae of
342 *Pycnopsyche* sp. were randomly picked for each replicate and photographed for digital
343 measurement of initial head capsule width. Larval length was not used as a metric due to
344 the tendency of the larvae to not reclaim their cases once extracted in pilot studies, with
345 changes in their behavior ensuing in the bioassays. Larvae were placed in flasks with 10g
346 (ww) of leaf tissue placed in each flask after being photographed for head capsule width
347 measurement. Bioassays were conducted for 30 days, after which survival was recorded.
348 Larvae were photographed to digitally measure final head capsule width, removed from
349 their case, and dried at 60°C for 24 hrs. Average dry mass was recorded for each
350 replicate. Head capsule width measurements were made using ImageJ 1.36b (National
351 Institutes of Health, USA).

352 For *Tipula* sp., one randomly picked larva was used in each replicate. Initial wet
353 mass was recorded and larvae were placed in flasks with 10g (ww) of leaf tissue were
354 placed in each flask. The bioassays were conducted for 30 days, after which larval
355 survival and wet mass were recorded for each larva. At the termination of the bioassay,
356 larvae were dried at 60°C for 24 hrs and dry mass was recorded and used in a wet-dry

357 regression to calculate initial dry mass ($DW = 0.0676WW$, $r^2=0.81$, $n = 47$, range =
358 0.077-0.533g).

359 For the isopod, gravid adult females (*Caecidotia communis*) were held in a beaker
360 with stream water and leaf debris and checked daily for newly emerged first instar larvae.
361 Replicates of five randomly collected instars were measured for individual length and
362 placed in flasks with 5g (ww) of leaf tissue. After 30 days, individual length and average
363 mass of each larva were recorded, and all larvae for each replicate were dried at 60°C for
364 24 hrs to determine average dry mass.

365 **Statistical Analyses**

366 All statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary,
367 NC). The *O. nubilalis* bioassay results were divided into three groups: 1) assays
368 performed with tissue prior to environmental exposure (time = 0), 2) assays performed
369 using tissue exposed to the terrestrial environment, and 3) assays performed using tissue
370 exposed to the aquatic environment. Each of these groups were analyzed in turn with a
371 three-way ANOVA (proc mixed) blocked by bioassay replication to evaluate differences
372 between hybrid, time, and isoline treatments. Post-hoc differences were determined
373 using a Tukey-Kramer adjustment with $\alpha= 0.05$. Data compliance with model
374 assumptions of normality and variance heterogeneity were checked using Shapiro-Wilk's
375 test and by using rank correlation between absolute residuals and predicted values,
376 respectively (proc univariate). All survival results were arcsin square-root transformed
377 prior to analysis. A probit analysis (proc probit) was used to evaluate differences in
378 survival between treatments for *Lepidostoma* and *Tipula* due to a violation of the
379 assumption of normality in the ANOVA model.

380 **Literature cited**

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384

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544 Table 1. Factors determining corn tissue bioactivity in A) prior to environmental
545 exposure B) terrestrial conditions and C) aquatic conditions.

546

547 Table 2. Differences in mean *O. nubilalis* weight gain (mg) in a 7 day bioassay due to A)
548 three treatments of senesced corn tissue in artificial diet prior to environmental exposure:
549 Non-*Bt* isoline, Cry1Ab-expressing variety, Cry1Ab+Cry3Bb-expressing variety and B)
550 three hybrid families of senesced corn tissue exposed for 2-8 weeks to terrestrial riparian
551 environmental conditions.

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553 Table 3. ANOVA results for non-target bioassays.

554

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558

559 Figure 1. Final dry mass of *Pycnopsyche* sp. exposed for 30 days to three treatments of
560 senesced corn tissue: Non-*Bt* isoline, Cry1Ab-expressing variety, Cry1Ab+Cry3Bb-
561 expressing variety.

562

563 Figure 2. Evidence of sublethal growth inhibition in *Tipula* sp. exposed for 30 days to
564 three treatments of senesced corn tissue: Non-*Bt* isoline, Cry1Ab-expressing variety,
565 Cry1Ab+Cry3Bb-expressing variety.

566

567 Figure 3. Results from sublethal and lethal metrics of effects in *Caecidotea communis*
568 exposed for 30 days to three treatments of senesced corn tissue: Non-*Bt* isoline, Cry1Ab-
569 expressing variety, Cry1Ab+Cry3Bb-expressing variety. A) Change in length (final –
570 initial), B) Final average mass, C) Percent survival.

571

Table 1.

A. Effect	NDF	DDF	F value	Pr>F
Field Block	2	49.8	0.63	0.535
Hybrid Family	2	51.4	0.50	0.607
Block*Hybrid	4	50	4.05	0.006
Isoline	2	51	19.01	<0.001
Block*Isoline	4	49.7	0.72	0.581
Hybrid*Isoline	4	50.9	0.48	0.752
Block*Hybrid*Isoline	8	49.5	0.86	0.555
B. Effect	NDF	DDF	F value	Pr>F
Hybrid	2	70	3.19	0.047
Week	3	70	0.83	0.483
Hybrid*Week	6	70	0.13	0.992
Isoline	2	70	1.91	0.156
Hybrid*Isoline	4	70	1.79	0.140
Week*Isoline	6	70	0.13	0.993
Hybrid*Week*Isoline	12	70	0.09	1.0
C. Effect	NDF	DDF	F value	Pr>F
Hybrid	2	54	0.57	0.567
Week	3	54	0.39	0.759
Hybrid*Week	6	54	0.24	0.960
Isoline	2	54	1.41	0.253
Hybrid*Isoline	4	54	1.71	0.161

Week*Isoline	6	54	0.09	0.997
Hybrid*Week*Isoline	11	54	0.28	0.987

574 Table 2.
575

		Mean Weight					
A) Isoline	Gain (mg)	Std. Error	DF	t value	Pr > t	Group	
Non- <i>Bt</i>	15.22	2.87	1	5.29	0.119	A	
Cry1Ab	9.34	2.87	1	3.26	0.190	B	
Cry1Ab+Cry3Bb	7.70	2.9	1	2.65	0.229	B	
B) Hybrid		Mean Weight					
Family	Gain (mg)	Std. Error	DF	t value	Pr > t	Group	
1	15.78	1.48	71	3.04	0.003	A	
2	12.39	0.86	71	1.22	0.228	B	
3	15.30	1.15	71	2.89	0.005	A	

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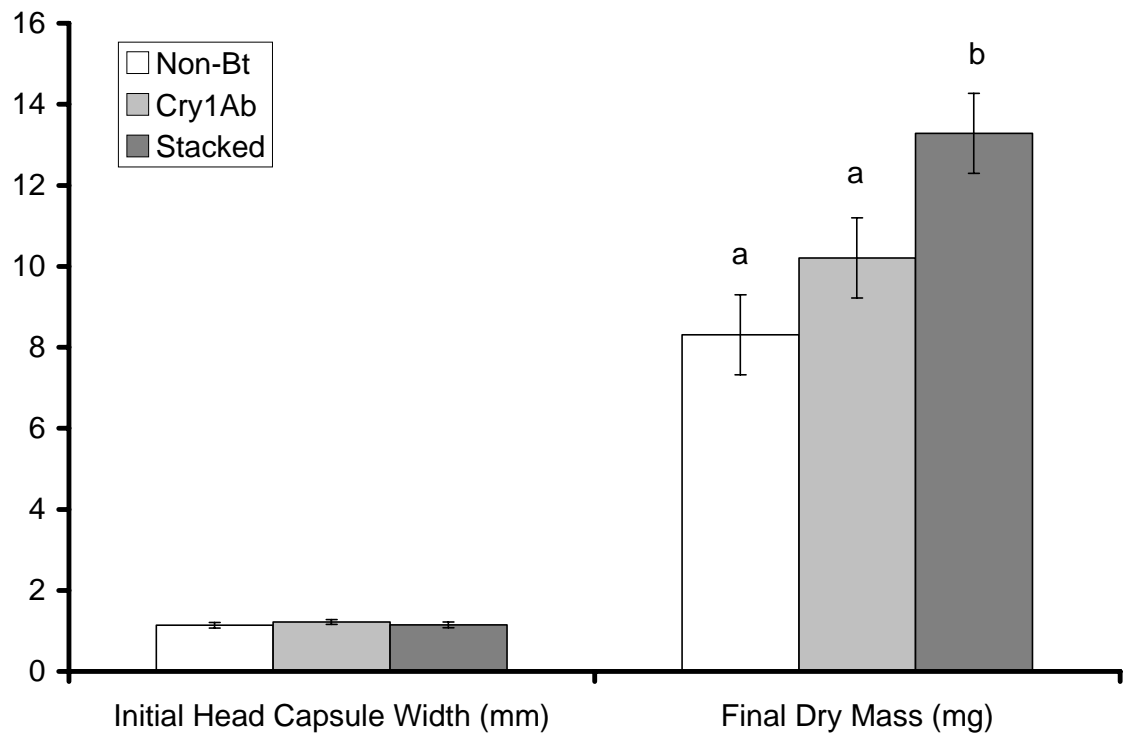
Table 3.

Test organism	Variable	NDF, DDF	F-value	p-value
<i>Lepidostoma</i> sp.	Initial head width	2, 45	0.06	0.945
	Final head width	2, 45	0.05	0.956
	Head change	2, 44	0.34	0.716
	Final dry mass	2, 45	1.73	0.189
	Survival*	--	--	--
<i>Pycnopsyche</i> sp.	Initial head width	2, 57	0.44	0.646
	Final head width	2, 51	0.32	0.726
	Head change	2, 51	1.10	0.339
	Final dry mass	2, 51	6.42	0.003
	Survival	2, 57	2.24	0.116
<i>Tipula</i> sp.	Initial mass	2, 56	0.66	0.521
	Mass change	2, 45	3.93	0.027
	Percent Increase	2, 45	1.68	0.197
	Survival*	--	--	--
<i>Caecidotia communis</i>	Initial length	2, 45	0.52	0.599
	Length change	2, 36	9.97	< 0.001
	Final mass	2, 35	4.71	0.016
	Survival	2, 45	3.71	0.032

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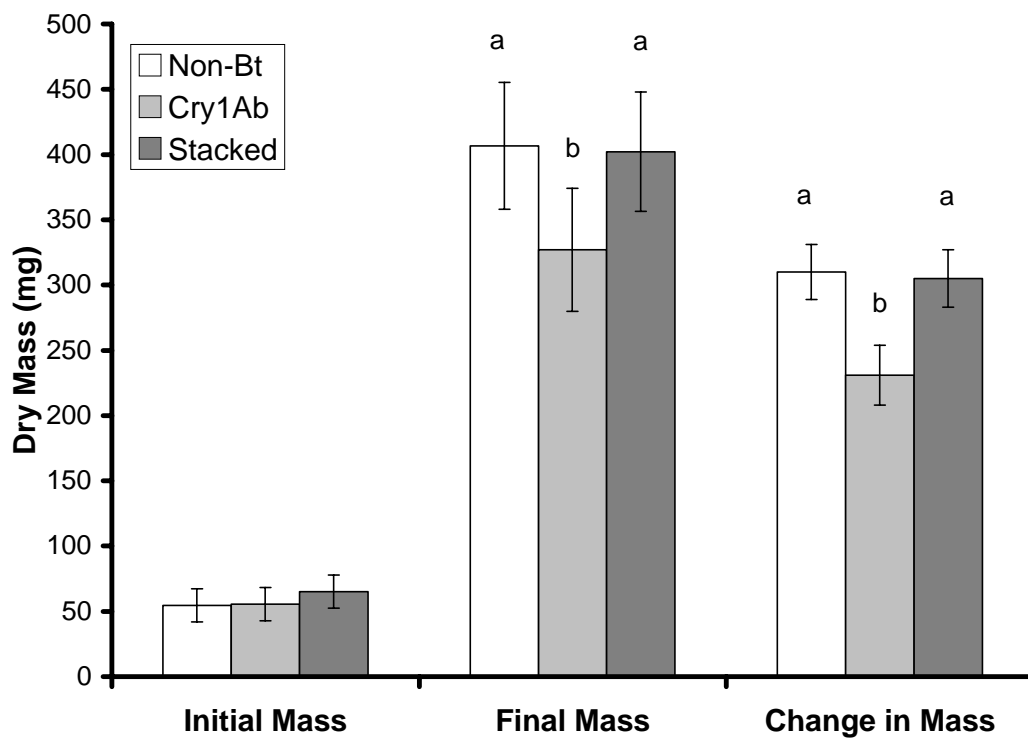
* ANOVA model assumptions were violated, a probit analysis was conducted

581 Figure 1.
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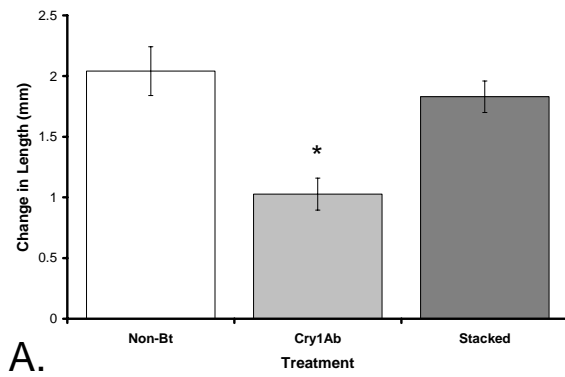
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585 Figure 2.
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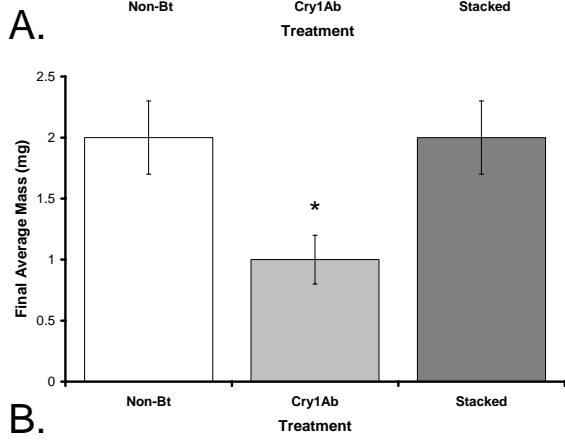


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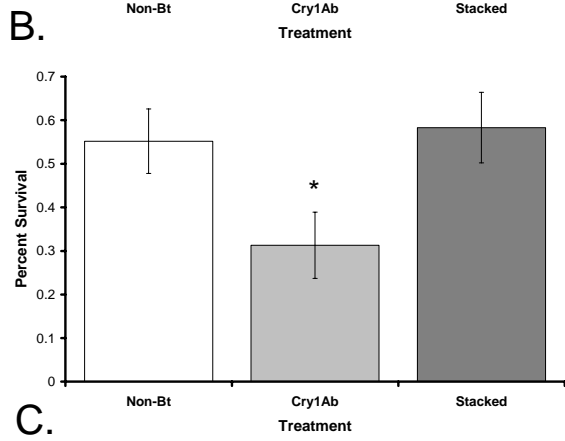
588 Figure 3.
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