Semi-field and field studies for assessing impacts on non-target organisms: their use to date in regulatory risk assessment

Alan Raybould, Product Safety, Syngenta
Overview of NTO field testing for transgenic crop ERA

● Testing for harmful effects of the intended trait
  - General concepts in a tiered assessment
  - Options: higher tier laboratory studies → large-scale field trials
  - Design, analysis, common problems

● Other considerations
  - Risk communication and risk perception
  - Criticisms of tiered risk assessment for transgenic crops
  - Biosafety research

● Conclusions
General concepts in tiered risk assessment

Tier 1 – laboratory

Tier 2 – laboratory

Tier 3 – outdoor microcosm

Tier 4 – field study
Rationale for tiered testing for pesticides

- Decreasing likelihood of an adverse effect
- Intermittent exposure
- Reduced bioavailability
- Ecological compensation
- Immigration

Over-estimation of effect in use
A tiered system for assessing the risk of genetically modified plants to non-target organisms

Monica GARCIA-ALONSO\textsuperscript{1}, Erik JACOBS\textsuperscript{2}, Alan RAYBOULD\textsuperscript{1}, Thomas E. NICKSON\textsuperscript{3}, Peter SOWIG\textsuperscript{4}, Hilde WILLEKENS\textsuperscript{5}, Pier VAN DER KOUWE\textsuperscript{6}, Raymond LAYTON\textsuperscript{7}, Firoz AMJEE\textsuperscript{8}, Angel M. FUENTES\textsuperscript{9} and Francesca TECALLA\textsuperscript{2}\textsuperscript{10}

\textsuperscript{1} Syngenta, Jealott’s Hill International Research Centre, Bracknell, RG42 6EY, United Kingdom
\textsuperscript{2} Monsanto Europe, Avenue de Tervueren 270 – 272, B-1150 Brussels, Belgium
\textsuperscript{3} Monsanto Company, 800 N. Lindbergh Blvd, St. Louis, MO 63141, USA
\textsuperscript{4} Bayer CropScience, Industriepark Höchst H871, D-65926 Frankfurt am Main, Germany
\textsuperscript{5} Syngenta International AG, Avenue Louise 240, Box 4, B-1050 Brussels, Belgium
\textsuperscript{6} Bayer CropScience, Rue Jean-Marie Leclair 16, CP 106, F-69266 Lyon Cedex 09, France
\textsuperscript{7} Pioneer Hi-Bred International, 7300 NW 62nd Avenue, PO Box 1004, Johnston IA 50131-1004, USA
\textsuperscript{8} Pioneer Overseas Corporation, Avenue des Arts 44, B-1040 Brussels, Belgium

Figure 3. Decision tree for tiered safety testing of GM plants to non-target organisms (Tiers 1 to 4).
A comparison with efficacy testing

Start at lowest tier and stop if no adverse effect on the pest is observed

Laboratory (≡ tier 1)

Glasshouse (≡ tier 2)

Fields (≡ tier 4)
Higher tier study design

- The experimental design depends on the purpose of the study!
- A study triggered by an adverse effect in a laboratory experiment will be different from a study to “confirm” inference of negligible risk
- Suppose an adverse effect is seen in a laboratory study
- Don’t panic and start a large field study!
  - Confirm the effect, especially if it is unexpected
  - Repeat the study
  - Artefact of experimental design?
- If the effect is real, what is the risk?
  - Characterise the effect
  - NOAEC → LC$_{50}$
  - Compare with refined exposure estimates for the groups at risk
  - e.g., LC$_{50}$/EEC ≥ 5 may be acceptable
## Expression study

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Highest mean at either site at any developmental stage</th>
<th>Highest mean across sites at any developmental stage</th>
<th>Growth stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>56.56 μg Vip3Aa20/g (NE)</td>
<td>50.41 μg Vip3Aa20/g</td>
<td>Seed maturity</td>
</tr>
<tr>
<td>Kernels (grain)</td>
<td>30.90 μg Vip3Aa20/g (IL)</td>
<td>29.81 μg Vip3Aa20/g</td>
<td>Seed maturity</td>
</tr>
<tr>
<td>Roots</td>
<td>6.20 μg Vip3Aa20/g (IL)</td>
<td>5.29 μg Vip3Aa20/g</td>
<td>Seed maturity</td>
</tr>
<tr>
<td>Pollen</td>
<td>47.85 μg Vip3Aa20/g (NE)</td>
<td>43.21 μg Vip3Aa20/g</td>
<td>Anthesis</td>
</tr>
<tr>
<td>Whole plants</td>
<td>24.62 μg Vip3Aa20/g (IL)</td>
<td>17.35 μg Vip3Aa20/g</td>
<td>Seed maturity</td>
</tr>
<tr>
<td>Vip3Aa20 per hectare</td>
<td>1481 g Vip3Aa20/ha (IL)</td>
<td>1128 g Vip3Aa20/ha (IL)</td>
<td>Seed maturity</td>
</tr>
</tbody>
</table>
Refining exposure

Table 5 Summary of estimated environmental concentrations (EECs) of Vip3Aa20 via MIR162 maize

<table>
<thead>
<tr>
<th>NTO group</th>
<th>Worst-case exposure to Vip3A20</th>
<th>Conservative exposure to Vip3Aa20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Route</td>
<td>Concentration or dose</td>
</tr>
<tr>
<td>Foliar arthropods</td>
<td>Diet 100% MIR162 leaves</td>
<td>56.56 µg/g leaves</td>
</tr>
<tr>
<td>Soil-dwelling invertebrates</td>
<td>Diet 100% MIR162 roots</td>
<td>6.20 µg/g roots</td>
</tr>
<tr>
<td>Pollinators</td>
<td>Diet 100% MIR162 pollen</td>
<td>47.85 µg/g pollen</td>
</tr>
<tr>
<td>Wild birds</td>
<td>Diet 100% MIR162 grain</td>
<td>10.82 mg/kg body weight</td>
</tr>
<tr>
<td>Wild mammals</td>
<td>Diet 100% MIR162 grain</td>
<td>10.20 mg/kg body weight</td>
</tr>
<tr>
<td>Aquatic invertebrates</td>
<td>US EPA GENELEC model scenario</td>
<td>74.05 µg/L water</td>
</tr>
<tr>
<td>Fish</td>
<td>Feed from 100% MIR162 grain</td>
<td>9.27 µg/feed</td>
</tr>
</tbody>
</table>
Higher tier study design

- If the risk is unacceptable, consider higher tier laboratory studies
- The main options are exposure *via* plant material instead of microbial test substance, exposure *via* prey, or both
- Examples from regulatory studies
  - Exposure of pollinators and aquatic organisms *via* transgenic pollen
  - Exposure of soil organisms *via* lyophilised leaves in soil
- Often done this way owing to problems exposing to purified protein
- Protein study guidelines are relevant: endpoints, controls, power etc.
- Some regulatory systems (e.g., the EU) require tests using plant material even when protein studies have shown no adverse effect
  - Could be seen as an additional feeding study (to rodent and chicken studies) to detect unintended harmful effects of transformation
  - But its real purpose is not explicit
Higher tier study design

- Potential to use exposure *via* prey

![Transgenic Res (2011) 20:467–479
DOI 10.1007/s11248-010-9430-5](Transgenic Res (2011) 20:467–479
DOI 10.1007/s11248-010-9430-5)

**Laboratory toxicity studies demonstrate no adverse effects of Cry1Ab and Cry3Bb1 to larvae of *Adalia bipunctata* (Coleoptera: Coccinellidae): the importance of study design**

Fernando Álvarez-Alfageme · Franz Bigler · Jörg Romeis

- Not yet used in regulatory dossiers
  - Not triggered
  - Not validated
  - More difficult to interpret – direct or indirect effect?
Higher tier study design

- If the risk is still unacceptable, consider semi-field studies
  - Enclosures or cages in the field

- Realistic, but controlled exposure; realistic, but controlled behaviour
  - e.g., degradation of toxin, foraging behaviour, reproduction
  - e.g., limited choice of diet, no immigration or emigration
Higher tier study design

- Semi-field studies have been used rarely in ecological risk assessment of transgenic crops
  - Not triggered
  - Field studies preferred for reasons of risk communication

- An exception is semi-field studies of bees
  - Exposure of bee larvae is difficult in the lab (until recently)
  - Validated semi-field methods from IGR pesticide studies
  - Bee hives exposed to microbial test substance and positive control (IGR) dissolved in sucrose solution
  - Worker bees feed the sucrose solution to larvae
  - Larval development is the test endpoint
Semi-field study of bees

Study timed to minimise availability of other sources of food
Semi-field study of bees

![Image of bees on honeycomb]

**FIG. 1.** Illustration of the stages of brood development.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Brood development assessments a)</th>
<th>Mean area of frame sides of brood per replicate before and after treatment b)</th>
<th>Mean number of dead bees collected post-treatment per hive c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells originally containing eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>% mortality</td>
<td>% CM b)</td>
<td>% mortality</td>
</tr>
<tr>
<td>Control</td>
<td>25.5</td>
<td>-</td>
<td>16.8</td>
</tr>
<tr>
<td>eCry3.1Ab</td>
<td>38.2</td>
<td>17.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Dimilin Flo</td>
<td>99.8 ***</td>
<td>99.7</td>
<td>100 ***</td>
</tr>
</tbody>
</table>

a) Stage of brood development: eggs, larvae, pupae, adults
b) % mortality: percentage of brood mortality
   % CM: percentage of colony mortality
c) Mean area and mean number of dead bees calculated per replicate and per hive.
Field studies

- For transgenic crops, no NTO field study has been triggered because of adverse effects in lower tier studies
- If a study were triggered, there is much experience of field studies of pesticides to call on for experimental designs
  - Earthworms
  - Bees
  - Non-target arthropods
- These studies are large and expensive
  - You need a very good reason to do them
  - Other methods have been exhausted
  - Risk management is not possible or commercially unattractive
  - The product solves an important problem
- Not suitable for confirmation of lower tier risk assessment
Field studies

- Although none has been triggered under tiered assessment, many field studies of the effects of transgenic crops on non-target organisms have been conducted
  - Often as part of a regulatory risk assessment
  - Sometimes as a condition of registration
  - Academic research
- The initial reason for field studies was probably uncertainty about the applicability of tiered assessment to transgenic crops
- Results show that tiered risk assessment of transgenic crops is conservative
  - Effects are predictable from the intended effect of the trait
  - Parasitoids of the target, pests taxonomically related to the target
  - Laboratory studies over-estimate effects
Field studies

A Meta-Analysis of Effects of Bt Cotton and Maize on Nontarget Invertebrates

Michelle Marvier,¹,² Chanel McCready,¹ James Regetz,² Peter Kareiva¹,³

Although scores of environmental groups have raised concerns about transgenic crops, scientific reviews and risk assessments have revealed limited evidence of the effects of transgenic crops on nontarget organisms. To address this gap, we pooled data from independent studies to construct a database for nontarget invertebrates in nontransgenic and Bt cotton and maize fields. Our findings reveal that the overall mean abundance of nontarget invertebrates was significantly lower in Bt compared with non-Bt control fields, certain nontarget invertebrates were more abundant in Bt cotton than in non-Bt fields, and Bt maize had a minor impact on nontarget invertebrates relative to conventional maize. These results suggest that lower abundance of nontarget invertebrates in Bt crops is a consistent and widespread phenomenon, furthering calls for expansion of monitoring and research on the effects of Bt crops on nontarget invertebrates.

Extrapolating non-target risk of Bt crops from laboratory to field

Jian J. Duan, Jonathan G. Lundgren, Steve Naranjo and Michelle Marvier

Biol. Lett. published online 9 September 2009
doi: 10.1098/rsbl.2009.0612
Current position of regulatory field studies for NTOs

● Many regulatory authorities still require NTO field studies as part of regulatory dossiers even though there are no effects in low tier studies

● Why?
  - Inflexible regulatory requirements: case-by-case = do the same for every crop, not start from existing knowledge
  - *Ad hoc* regulatory requirements and lack of clear decision-making
  - Risk communication

● Lack of a trigger presents difficulties for experimental design
  - What to measure?
  - How many treatments?
  - Size of experiment?
  - Interpretation of effects?
Regulatory field study design

● What to measure?
  - NTOs that are likely to be abundant?
  - NTOs that are valued for aesthetics or function?
  - NTOs that are easy to catch?
  - NTOs that are easy to identify?
  - NTOs that are related to the target pest?
  - NTOs with low dispersal ability?
  - Non-target pests?

● How many treatments?
  - Transgenic and near-isoline; what about reference varieties?
  - Positive control?
  - Avoid areas whether the target pest occurs, simulate effect of trait?
  - Use herbicides on transgenic HT crops?
Regulatory field study design

● Size of experiment?
  - How many sites?
  - How many replicates, how many samples within each replicate?
  - How many years?
  - How often and when to sample?
  - Size of plots?
  - Separation of treatments?

● Interpretation?
  - Power to detect effects?
  - Direct or indirect effects of the transgenic crop?

● What do (relatively) small NTO field trials add to risk analysis that are not, or could not be, provided by agronomic field trial data?
  - Additional “NTO plots” in agronomic field trials is a possibility
Criticisms of tiered assessment of transgenic crops

- Field studies, other than for risk communication, are difficult to justify if tiered assessment is valid for transgenic crops
- Criticism of tiered testing comes mainly from ecology
- Field studies are needed regardless of absence of effects in the lab

1. Tiered assessment fails to consider the transgenic crop, only the trait
   - False: potential for harmful unintended effects is tested
   - False: even regulations triggered by production of a pesticide must consider the means by which the pesticide is produced

2. Tiered assessment fails to account for ecological complexity

3. Laboratory studies are insufficiently sensitive
   - Subtle sublethal effects may be important ecologically
Ecological complexity

- Increasing likelihood of an adverse effect
- Intermittent exposure
- Reduced bioavailability
- Environmental interactions
- Immigration
- Under-estimation of effect in use
Ecological complexity: confusion between research and ERA?

- Ecology has been suspicious of laboratory [controlled] testing:
  Laboratory studies may...“magnify incidental or trivial factors...indeed, laboratory experiments can likely show some effect of any factor by using sufficiently extreme conditions. Laboratory studies are effective in isolating the response to a factor but the response may not be ecologically relevant”


- May lead to suspicion of the sufficiency lab tests for risk assessment
- The reasons for suspicion of laboratory testing in ecological research should lead to support for the HQ approach
  - Laboratory tests over-estimate effects
Over-estimating effects in laboratory studies

- Recognised in research where the presence of an effect is of interest
- Hypothesis that a plant chemical influences the amount of herbivory

- Effect in lab, corroborates hypothesis – but test in the field because effect may be absent or unimportant because of other factors
- No effect in lab, reject hypothesis – no field testing, because effect is unlikely to appear because of other factors
Over-estimating effects in laboratory studies

- Less well recognised in ERA where the absence of an effect is of interest
- Hypothesis that transgenic protein does not harm NTOs in the field

- No effect in lab – stop testing (for protein toxicity)
- Effect in the lab – evaluate whether harmful in the field
- Same conclusions as in research
  - Nothing special about transgenic crop x environment interactions
Ecological complexity: insensitivity of lab studies?

- Small effects might have large consequences
- What size of effect is important in the lab?
- What type of effect is important in the field – structure or function?

- Ecosystem simplified by grouping individuals by function, not by species
- Ecosystem dynamics emerge from interactions among individuals
- The crop is a unique functional type
- Crop management is a disturbance to growth, survival, fecundity etc.
Predicting ecosystem effects from laboratory data

- Simple system: crop, lacewings and aphids
  - Management (e.g., crop is IR) affects lacewings, aphids unaffected
  - Adverse effect is loss of biological control of aphids
  - Also examined effect of adding a second predator – a ladybird
- Biological control of aphids has a significant effect on crop yield
Examine “what if” there were adverse effects on lacewings
Mitigation by complexity (redundancy)

Same simulations, but with a second aphid predator in the system
Conclusions from modelling

● Need a severe reduction in survival before even large sublethal adverse effects result in significant harm to function
  - If no mortality in a laboratory study, function is protected
  - Not essential to measure sublethal effects unless there is significant (and high) mortality
● Biological control is very resilient to adverse effects on lacewings when a second predator is present
  - Complexity mitigates effects
● Model can be used in a tiered manner
  - The simplest system is likely to be worst-case

● Caron-Lormier et al., Ecological Modelling 220: 1935-1949; 222: 1163-1173
● Raybould et al. Journal of Agricultural and Food Chemistry DOI: 10.1021/jf1042079
The role of ecological research on transgenic crops

- What role should ecological research on transgenic crops play in ERA?
  - Ecological research on transgenic crops often not necessary for ERA
  - Could use transgenic crops to develop and test ecological theory
  - New theory can provide new tools for ERA

- Transgenic crops are used to test fundamental theories in plant science
  - Pathology, physiology, molecular biology, genetics, epigenetics...

- There is much “ecological research” on transgenic crops, but rarely, if ever, are fundamental theories in plant ecology tested
  - Regulatory problems?
  - No ecological theory to test?
  - Theory is not seen as relevant?
  - The transgenic crop is the end not the means?
The role of ecological research on transgenic crops

● A typical hypothesis in ecological research on transgenic crops
  - Transgenic HT crops will have “no effect on farmland biodiversity compared with a conventional cropping system”
  - No definition of harm, no theory behind the hypothesis

● A better hypothesis
  - “Direct effects to the arthropod community exposed to the stacked Bt hybrid would be less than those caused by the pyrethroid insecticide treatment”
  - Approaches a definition of harm, a theory behind the hypothesis (?)

● Effects are explained *post hoc*, not compared with predictions from theory
  - Natural history not science
The role of ecological research on transgenic crops

● Ecological research on transgenic crops tends to fall between two stools

● Does not test risk hypotheses
  - No definition of harm
  - Perhaps trying to avoid being subjective

● Does not test ecological theory
  - Attempts to be relevant may distract from opportunities to test theory
  - All data about transgenic are interesting and relevant per se
  - Fill that bucket!

● Predictions are not interesting or relevant

● Testing new theory may ultimately be more useful to ERA than a study that may appear immediately relevant
An example of how theory could be used

**Fundamental ecological theory** → **Risk assessment tool**
Conclusions

● Current regulatory NTO field studies are problematic
  - Not triggered by tiered assessment
  - Risk communication maybe a good reason, but leads to serious uncertainty about study design

● “Ecological complexity” not a good argument for requiring field studies
  - Lab studies over-estimate effects in ERA as in ecological research
  - Functional redundancy

● Support and exploit fundamental ecological research
  - Must be truly theoretical – hypotheses are not *ad hoc*

● “Biosafety research” is often unhelpful
  - Often doesn’t clarify risk assessment or advance ecological theory