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To the state secretary for
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DATUM	7 September 2017
KENMERK	CGM/170907-01
ONDERWERP	Report 'Assessment of risks to non-target organisms of the cultivation of GM crops that express one or more Bt toxins'

Dear Mrs Dijksma,

Please find attached our advisory report containing guidance on assessing the risks posed by cultivating insect-resistant genetically modified (GM) crops that produce one or more Bt toxins.

Summary:
When an application is made to cultivate a genetically modified (GM) crop, an assessment must be made of the effects this GM crop could have on non-target organisms. Non-target organisms are all the organisms present in the field, with the exception of the pest organism against which the introduced trait in the GM crop is directed. If it is plausible that an inserted gene could have an adverse effect on non-target organisms, information must be provided to permit an assessment of the possible risks to these organisms.

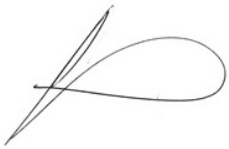
GM crops are made resistant to certain insect pests by introducing genes from the bacterium *Bacillus thuringiensis* that code for Bt toxins. The trend is now for GM crops to contain several inserted genes that code for different Bt toxins. This raises new questions for the risk assessment that has to be made on applications for the cultivation of such crops, because there may be synergistic interactions between the Bt toxins which could cause non-target organisms that are not affected by the individual toxins to be susceptible to the combined toxins.

To obtain information about any synergism between the many different Bt toxins in preparation for writing this report, COGEM carried out a literature study and organised a symposium on this topic in cooperation with a number of international sister organisations. The insights obtained from that study

and the symposium form the basis for the guidance presented by COGEM in this report for assessing the risks to non-target organisms of the cultivation of GM crops that express multiple Bt toxins.

The grounds on which COGEM has reached its conclusions and the resulting advice are set out in the enclosed report.

Yours sincerely,



Professor Sybe Schaap
Chair of COGEM

c.c. H.P. de Wijs, Head of the GMO Office
J.K.B.H. Kwisthout, Ministry of Infrastructure and the Environment

Assessment of risks to non-target organisms of the cultivation of GM crops that express one or more Bt toxins

COGEM report CGM/170907-01

Contents

1. Introduction

Agricultural crops made resistant to insect pests (target organisms) by genetic modification usually carry one or more Bt genes. These Bt genes come from the bacterium *Bacillus thuringiensis* (Bt) and code for Bt toxins that are toxic to certain insects. There are several known Bt toxins with different specificities for different species of insects. When an application is made for the cultivation of a Bt crop, an important part of the environmental risk assessment is the assessment of the possible risks to organisms other than the insect pest (non-target organisms). In recent years there has been a rapid increase in the number of genetically modified (GM) crops carrying multiple Bt genes. These plants are given several Bt genes either to confer resistance to a number of different insect pests or to obtain more effective resistance to a single insect pest.

Much research has been done on the possible effects on non-target organisms of GM crops that produce a single Bt toxin. In the past COGEM has commissioned various research projects on environmental risk assessment methods and has made recommendations on how to improve them.[1,2,3,4] COGEM has also organised a symposium on this topic with the European Food Safety Authority (EFSA).[5] The principles for the risk assessment adhered to by COGEM which are based on this research and experience are summarised in Chapter 4.

The production of multiple Bt toxins by a single GM crop raises new questions for the risk assessment. It is known that interaction between toxins can influence their activity, which means that it is possible for the activity of a Bt toxin to be altered if the GM crop expresses another Bt toxin as well. Organisms that are not adversely affected by the individual Bt toxins could then be adversely affected by a combination of Bt toxins.

There is still much debate about when the possible risks of interactions between Bt toxins to non-target organisms should be investigated, and about the research method to be employed. A range of proposals have been made from within the scientific and business communities. Some argue that research into possible interactions is always needed,[Error! Bookmark not defined.,8,6] while others contend that such studies are only needed when the GM crop produces multiple toxins that affect the same order of insects. There are also different opinions about which organisms should be included in these studies. Some say that sensitive pest organisms should be used,[6,7,8] while others are of the opinion that studies on pest organisms have little predictive value[9] and assert that the studies should be carried out on non-target organisms.[10] Also, some think that studies on non-target organisms are always necessary, others say that studies on non-target organisms have to be carried out when non-target organisms are present that belong to an insect order affected by multiple of the Bt toxins produced by the GM crop. Yet another group think that such studies are only needed when non-target organisms are present that are affected by at least one of the Bt toxins produced by the crop.[9]

The coming into force of the new national competence to restrict or prohibit the cultivation of GMOs (Directive (EU) 2015/412) could lead to a rise in the number of applications for the cultivation of GM crops in the European Union (EU). Currently there are no applications pending for the cultivation of GM crops that produce multiple Bt toxins in the EU, but any new applications will probably be for GM crops that produce multiple Bt toxins. It is important to have information about possible interactions between Bt toxins when assessing the risks to non-target organisms of such GM crops.

In anticipation of future permit applications, COGEM commissioned a study to review current knowledge about the mechanism of action of Bt toxins and about interactions between different Bt toxins.[11,12,13,14] Based on the results of these studies (summarised in Chapters 2 and 3) and an international workshop run by COGEM and sister organisations from the United Kingdom, Belgium and Spain,[15] in this report COGEM provides guidance on assessing the environmental risks of GM crops that express multiple Bt toxins.

2. Bt toxins and their mechanism of action

2.1 Mechanism of action

The bacterium *Bacillus thuringiensis* naturally produces proteins that are toxic to other organisms, particularly to insects and nematodes. These Bt proteins themselves are not directly toxic, but become toxic once they are converted to an active δ-endotoxin by proteases[a] in the midgut of sensitive insects or nematodes. Conversion to active δ-endotoxins in the midgut is an important factor in the specificity of Bt toxins. In this report the term 'Bt toxin' is used to refer to both the inactive Bt protein and the active δ-endotoxin.

The genes encoding Bt toxins in GM crops are altered in such a way that they are expressed optimally when inserted into a plant genome. The Bt toxins expressed by GM crops lack a part of the C-terminus and the N-terminus that are not necessary for the insecticidal activity, but they still have to be activated by proteases in the midgut. The amino acid sequences[b] of the Bt toxins produced by GM crops are often different from those of the toxins produced by *B. thuringiensis*. The sequences are changed to improve the expression of Bt toxins in plants, to increase toxicity in smaller quantities or to make the Bt toxin active against several insect pest species.

Although it is known that Bt toxins kill sensitive insects or nematodes, the exact mechanism of action has not yet been described. There are various theories.[11] According to one of these theories, Bt toxins bind to receptors on the cell membrane of midgut cells, where they induce pore formation which makes the membrane permeable and causes the cell to die. . According to another theory, the Bt toxin bound to the cell membrane causes the cell to form bulges or protrusions (called 'blebbing'), swell up and die.

2.2 Bt toxin groups

Based on the structure of the protein, Bt toxins can be classified into a number of groups. These include the 'three-domain Cry', 'Bin', 'Mtx', 'Vip' and 'Cyt' groups.

The names of Bt toxins are based not on the structure of the proteins, but on whether the toxin is present in the spore or produced during the growth phase of the bacterium, or on the function of the protein. This means that the names of Bt toxins are not always consistent with the groups based on the structure of the proteins. Three of the Bt toxin groups mentioned above (three-domain Cry, Bin and Mtx) are all called Cry proteins because they are present in the form of protein crystals in the spores of *B. thuringiensis*.

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[a] Enzymes that break down proteins.

[b] Proteins consist of amino acids in a sequence determined by the DNA in the cell.

In contrast to the Cry proteins, the Cyt proteins all belong to the same protein structure group. They are produced in the spores of the bacterium like the Cry proteins and are cytolytic – they cause cells to burst. The Vip proteins also have a similar protein structure and are produced in the growth phase of *B. thuringiensis* (vegetative induced proteins).

Most of the Bt proteins produced by GM crops belong to the group of three-domain Cry proteins. Only a few GM crops produce Bin (i.e. Cry35) and Vip proteins.[16]

2.3 Specificity of three-domain Cry proteins

In the past, Bt toxins were thought to be toxic to specific groups of insects. On this basis, the three-domain Cry proteins were divided into a number of subgroups:

- Cry1 proteins toxic to butterflies and moths (Lepidoptera);
- Cry3 proteins toxic to beetles (Coleoptera);
- Cry4 proteins toxic to flies and mosquitos (Diptera);
- Cry2 proteins toxic to butterflies, moths and beetles (Lepidoptera and Coleoptera).

In recent years, though, it has become clear that Bt toxins can also be toxic to groups other than those listed above. A third of the Bt toxins whose effects on several orders of insects have been investigated, have been shown to be toxic to insects from other orders as well (although to a lesser degree) or even toxic to organisms that belong to another phylum (i.e. Nematoda).[17,18] This means that the earlier assumptions about specificity are not correct and the toxins may have wider activity spectra than was previously thought.

2.4 Conclusions

- In sensitive insects and nematodes, Bt toxins bind to receptors on the cell membrane of gut cells, causing the gut cells to die off, which in turn causes the death of the insect or nematode.
- Too little is known about the mechanism of action of Bt toxins to be able to predict whether or not Bt toxins can influence each other's activity.
- Bt toxins can be classified into groups based on their protein structure (including the three-domain Cry, Bin, Vip, Mtx and Cyt protein groups).
- The names of Bt toxins are not related to their protein structure.
- Bt toxins can be toxic to several taxonomic groups, more specifically to various orders of insects and/or nematodes.

3. Interactions between Bt toxins

3.1 Possibilities for interactions in mixtures

When organisms are exposed to a mixture of substances, these substances may influence each other's activity. If a substance has an effect on an organism, the effect of this substance may be magnified by the presence of another substance that does not itself affect the organism (e.g. 1 + 0 > 1). This is called potentiation.

If each of two substances has an effect on its own, a mixture of these substances may have an additive, synergistic or antagonistic effect. An additive effect is when the combined effect of the mixture is equal to the sum of the effects of the individual substances (1 + 1 = 2). The expected additive effect serves as a reference to determine whether or not there are any interactions. The difference between the expected (additive) effect and the observed effect is expressed by the synergy factor. A synergy factor of 1 indicates an additive effect. When the effect is greater than the additive effect (e.g. 1 + 1 > 2), there is synergy. The synergy factor is then greater than 1. When the effect is less than the additive effect (e.g. 1 + 1 < 2), this is called antagonism. The synergy factor then lies between 0 and 1.

A more extensive explanation of additivity, antagonism and synergism and a discussion of how these phenomena are investigated can be found in the COGEM research reports CGM 2014-2 and CGM 2014-05.[11,12]

3.2 Possible consequences for the risk assessment of interactions between Bt toxins

Most studies into the possible effects on non-target organisms carried out for the assessment of permit applications are performed on a single BT toxin. When the GM crop contains various Bt toxins and these Bt toxins enhance each other's toxicity (synergism), non-target organisms may be affected at lower concentrations than expected from studies on individual Bt toxins. If there is a synergistic effect, the risk to non-target organisms may be underestimated.

3.3 Research into interactions between Bt toxins

Various studies have been done on the effects of mixtures of Bt toxins. Almost all these studies have been carried out on sensitive pest organisms and all but a few involved mixtures of Bt toxins active against species from the same order of insects. In most cases the effects were investigated using the method described by Tabashnik in which Bt toxins are mixed in certain proportions, always keeping the total quantity of Bt toxins in the mixture the same.[19]

3.4 Interactions between different groups of Bt toxin

Information in the available literature indicates that Bt toxins can influence each other's activity. For almost all the Bt toxin protein groups there are known cases of mixtures of Bt toxins in which the toxins influence each other's activity.[11,12,6]

A study commissioned by COGEM shows that about half of the experiments on the effects of a mixture of Bt toxins described in the literature (53%) provided evidence of synergism. The degree of synergy varied from weak (a synergy factor between 2 and 10) to very strong (a synergy factor of more than 50). In 18% of the experiments the synergy factor was higher than 10. In a small number of experiments (7%) there was an antagonistic effect (a synergy factor lower than 0.5).[11] Measured synergy factors between 0.5 and 2 were generally found not to differ enough from an additive effect to have a biologically significant effect.[20]

As almost all the experiments were on sensitive pest organisms, no conclusions can be drawn about the effects of Bt toxin mixtures on non-target organisms. Some of the experiments were carried out on pest organisms which had become resistant to one of the Bt toxins in the mixture. Most of the very high synergy factors (>50) were found in experiments with these resistant pest organisms.[11] These high synergy factors are probably a consequence of the resistance to one Bt toxin being counteracted by the other Bt toxin in the mixture, causing a return to the original sensitivity of the insect to the first Bt toxin. It is therefore highly questionable whether these high synergy factors are indicative of situations in which the insects have not previously acquired any resistance.

- *Three-domain Cry proteins*

  Most of the published studies are experiments with mixtures of two proteins from the three-domain Cry protein group. The literature contains reports of experiments on a total of 83 different combinations of pest organisms and mixtures of three-domain Cry proteins (effective against the same order of insects) designed to investigate whether or not the three-domain Cry proteins influence each other's activity.[11,8,21,22] The results of these experiments indicate that there is a large variation in the degree to which the different three-domain Cry proteins can enhance (synergism) or weaken (antagonism) each other's activity. A synergy factor higher than 10 was reported for four organism/mixture combinations. The synergy factor lay between 5 and 10 for five combinations.[11] The highest reported synergy factor was 26.[23]

  Besides these studies with mixtures of two three-domain Cry proteins, a small number of experiments have been done with a mixture of three three-domain Cry proteins. Depending on the organism studied, these experiments found evidence of an additive, slightly synergistic or slightly antagonistic effect (synergy factors between 0.4 and 5.1).[19,24]

- *Cyt proteins*

The literature contains descriptions of a number of experiments with a mixture of a three-domain Cry protein and a Cyt protein. In 5 of the 16 different organism/mixture combinations examined, a synergy factor higher than 5 was observed, 3 of which were above 10. The highest reported synergy factor was 33.[22,25] Besides experiments with a mixture of one three-domain Cry protein and one Cyt protein, a few experiments have been done on the effect of a mixture of two three-domain Cry proteins and one Cyt protein. In these experiments a slight synergistic effect was observed (a synergy factor of 2.6).[26,27] A mixture of four three-domain Cry proteins and one Cyt protein gave a synergy factor of 4.1.[22]

- *Vip proteins*
  Several studies have been published on the effect of a Vip protein mixed with another Bt toxin. Of the 18 investigated combinations of a pest organism and a mixture of a Vip and a three-domain Cry protein, 7 produced an antagonistic effect. In 4 of the investigated combinations there were indications of a synergistic effect. In 2 of the combinations a synergy factor higher than 5 was found. The highest reported synergy factor was 14.3.[28] Synergy was also indicated for a mixture of a Vip protein and a Cyt protein, with a maximum synergy factor of 4.3.[29]

  Besides experiments with a mixture of one three-domain Cry protein and one Vip protein, a few experiments have been done on the effect of a mixture of two three-domain Cry proteins and one Vip protein. In these experiments no antagonistic or synergistic effects were observed.[8,30]

- *Mtx proteins*
  Most of the published experiments on mixtures of Bt toxins containing at least one Mtx protein were carried out on resistant pest organisms. These experiments suggest that Mtx proteins can considerably enhance the toxicity of other Bt toxins. The maximum synergy factor found in tests on sensitive organisms was 120. The maximum synergy factor found in tests on resistant pest organisms was 135.[11,31]

- *Bin proteins*
  Bin proteins are Bt toxins that display little or no toxicity on their own, but are toxic in combination with another Bt protein. As the two proteins are always found together in the bacterium, the actual toxin can be considered to consist of two proteins. Just a few Bin proteins are known. Because Bin proteins are in themselves not toxic or only slightly toxic, they all display a strong synergistic effect when they are combined with the other protein in the conjugate pair.

3.5 Effects of mixtures of Bt toxins active against different insect orders

In the experiments described above, the Bt toxins in the mixtures were active against the same order of insects. GM crops can produce a mix of Bt toxins that have a primary effect on different

orders of insects. Just five studies on such mixtures of Bt toxins have investigated possible interactions between the toxins.[12] In four of these studies a three-domain Cry protein active against Lepidoptera was combined with a three-domain Cry protein active against Coleoptera. In another study experiments were done in which two three-domain Cry proteins active against Coleoptera were combined with two three-domain Cry proteins and one Vip protein active against Lepidoptera.[8] No interactions were observed in any of these experiments.

Although there are no known cases in which three-domain Cry proteins that are toxic to insects from different orders influence each other's activity, the limited number of studies means that it cannot be concluded with certainty that no such interactions between three-domain Cry proteins are possible.

3.6 Effect of mixtures of Bt toxins on different organisms

A number of publications describe the effects of the same mixture of Bt toxins (including mixtures of three-domain Cry proteins, Mtx proteins, and three-domain Cry proteins with a Vip protein[8]) on various sensitive insect species (mostly pests). The effect of the Cry1Ab–Cry1Ac combination on various Lepidoptera species has been investigated and found to be antagonistic for the gypsy moth (*Lymantria dispar*), additive for the cotton bollworm (*Helicoverpa armigera*) and the silkworm (*Bombyx mori*), and synergistic for the spotted stem borer (*Chilo partellus*).[12,21] The way the Bt toxins in the Cry1Aa–Cry1Ac combination influenced each other's activity was also found to differ depending on the species.[12,21] This was also the case for combinations of a three-domain Cry and a Vip protein (Cry1C–Vip3Aa).[28] The same mixture can therefore have an additive effect on one organism, but an antagonistic or synergistic effect on another.[9,12,21]

3.7 Conclusions

- Almost all the research into the effects of mixtures of Bt toxins has been carried out on sensitive pest organisms. Little research has been done on the effects of mixtures of Bt toxins on non-target organisms.
- In almost all Bt toxin protein groups (the three-domain Cry, Vip, Bin, Mtx and Cyt proteins groups) there are reported cases of mixtures of Bt toxins in which at least some of the toxins influence each other's activity.
- When Bin proteins are combined with their conjugate partner the activity of both proteins is enhanced.
- The proteins in mixtures of three-domain Cry proteins, mixtures of three-domain Cry proteins with Cyt, Vip or Mtx proteins, and mixtures of Mtx proteins can influence each other's activity if they are active against the same order of insects. Synergistic (enhancement) effects have been reported more often than antagonistic (weakening) effects.
- Little research has been done on mixtures of Bt toxins active against different orders of insects. In the studies that have been done no interaction was observed.

- Proteins in the same mixture can have a synergistic effect, antagonistic effect or no effect on each other's activity depending on the organism concerned.

4. Guidance for the assessment of effects on non-target organisms

When an application is made for the cultivation of a GM crop, COGEM assesses the possible effects on non-target organisms. The Commission has previously formulated the following principles for this assessment.[1,2,4]

4.1 Research is only needed when adverse effects are possible

Investigating potential effects on non-target organisms is only necessary when it is plausible that expression of the inserted gene could have an adverse effect on non-target organisms.[2] This is the case, for example, for GM crops that produce Bt toxins. Conversely, research into effects on non-target organisms is not necessary for GM crops with herbicide tolerances.[c]

4.2 The activity spectrum must be determined

For GM crops that produce a Bt toxin, the activity spectrum of that toxin must be determined. Bt toxins can affect various orders of insects.[9,12,17,18,6] The possibility of a Bt toxin affecting organisms that belong to a different taxonomic group from the target pest insect cannot therefore be ruled out. To obtain information about the activity spectrum of a Bt toxin, its effect must be investigated using organisms from different taxonomic groups, such as insects (Coleoptera, Neuroptera, Hemiptera, Hymenoptera, Lepidoptera, Diptera, etc.) springtails (Collembola), spiders (Arachnida) and nematodes (Nematoda).

4.3 The first step is laboratory research

The first step in obtaining information for use in assessing the possible risks to non-target organisms is to carry out laboratory experiments. These laboratory experiments test various non-target organisms to determine whether or not they are adversely affected by expression of the gene inserted into the GM crop.

4.3.1 Selection of non-target organisms

The non-target organisms to be investigated must be as representative as possible of all non-target organisms that may be exposed to the GM crop in the field. As there are numerous types of non-target organisms that may be present on a crop, it is impossible to study the possible effects on every non-target organism that could be exposed to the GM crop. A number of representative species must therefore be selected.[32] COGEM has defined a number of detailed criteria for selecting suitable non-target organisms.

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[c] In these crops a gene has been inserted that makes the plant resistant to certain herbicides. The gene product has no influence on the survival of insects.

- A minimum of five species of non-target organisms must be tested in the laboratory to determine whether or not they are adversely affected by the GM crop. To do this they are exposed to plant material from the GM crop or to purified protein extracted from the GM crop. If the latter is not possible, protein produced by microorganisms may be used, as long as certain conditions are met.
- The non-target organisms must be from different ecological groups: predators, parasitoids (ichneumon wasps), pollinators and detritivores.[2,33]
- One of the non-target organisms investigated must belong to the insect order against which the protein is active.[34] If the protein is active against several insect orders, non-target organisms from all these insect orders must be investigated.
- If there is a reason to expect that a rare or endangered species will be affected, possible effects on this species must also be investigated.[2]
- The tested non-target organisms must be found on the crop in the Netherlands or Europe, or belong to the same genus as these non-target organisms.[4,35]
- In addition, the species investigated must satisfy one or more of the following criteria:[2]
    - they may be highly sensitive to the protein *or* may be exposed to high concentrations of the protein if the crop is cultivated;
    - they are ecologically or economically important;
    - they are representative of larger groups.

4.3.2 Exposure of non-target organisms

During the laboratory experiments the non-target organisms must be exposed to at least two different quantities of Bt toxins so that the dose dependency can be determined.[36] One of these quantities must be equal to the maximum amount of the Bt toxin the non-target organism could be exposed to in the field.[32,36] To determine the maximum amount of toxin the non-target organisms could be exposed to in the field, it is first necessary to establish how the non-target organism will be exposed to the Bt toxin and the likely degree of exposure.

In the test, the non-target organism must also be exposed to a larger amount of the Bt toxin (10 times the maximum environmental exposure concentration). This safety margin is necessary because the non-target organism studied may be less sensitive to the Bt toxin than other non-target organisms present in the field. It is also possible that under certain circumstances the GM crop will produce more Bt toxin, because the expression of Bt toxins is partly dependent on growing conditions and the stage of development of the plant.[41]

COGEM points out that the maximum concentration of toxin the non-target organism may be exposed to is not always the same as the maximum concentration of the Bt toxin in the plant material (e.g. leaves and pollen). If a Bt toxin accumulates in prey species, predators in the field may be exposed to higher concentrations. COGEM is of the opinion that the possible accumulation of Bt toxins should be taken into account when determining the maximum

environmental exposure concentration.[37] This requires knowledge of the degree of accumulation of Bt toxins, or research to obtain this information, when identifying any effects on predators and parasitoids.

Ideally, in the laboratory experiments the non-target organisms should be exposed to plant material from the crop concerned. Also, the insects should be exposed to different quantities of the toxins, but in practice it is often not possible to obtain plant material containing sufficient quantities of toxin to investigate the effect of exposure to 10 times the expected maximum environmental exposure concentration. Moreover, many non-target organisms, such as predators and parasitoids, are indirectly exposed to Bt toxins in the field via their prey or hosts and eat little or no plant material themselves. Partly for this reason it is preferable to expose these non-target organisms to purified Bt toxins extracted from plant material. However, purifying proteins extracted from plant material is often not possible or problematic, and in such cases it will not be possible to obtain sufficient quantities of plant-produced Bt toxin for use in the laboratory experiments. As an alternative, sufficient quantities of Bt toxin can be obtained by expressing the gene in a bacterium. Although Bt toxins produced by bacteria may be slightly different from the Bt toxin produced by the GM crop plant (for example because of differences in protein folding or glycolisation), if it can be demonstrated beyond reasonable doubt that the biological activity of the protein is equivalent to that of the protein produced by the GM crop, COGEM considers it acceptable to use purified protein extracted from bacteria.[38,39,40]

4.3.3 Quality standards for laboratory research

The laboratory experiments must also meet the following quality standards:

- Experiments must include a positive and a negative control. The mortality in the negative control group must be lower than 15%.[39]
- The concentration of the Bt toxin under investigation must be verified.[36]
- The activity of the Bt toxin must be demonstrated[41] and must not decline sharply during the experiment.[36]
- It must be demonstrated that the Bt toxin is taken up by the non-target organism.[41,36]
- The study must include a sufficient number of replications. To detect biologically significant effects, in most cases it is sufficient to demonstrate an effect magnitude of 30–50% with a statistical power of at least 80%.[42]
- A correct statistical analysis must be performed and the grounds for selecting the method of statistical analysis employed must be explained. In addition, the p-value, the size of the observed effect, the size of the effect tested for in the experiment, the number of replications, the averages for the different groups, the standard deviation and the confidence interval must be given.[39,43]

4.3.4 Research into population growth rate

Laboratory experiments measure the lethality of a Bt toxin, but other (sublethal) factors important for the survival of a species are often overlooked.[6] Sublethal effects that reduce the fitness of an organism can eventually have major consequences for the survival of organisms and populations. Sublethal effects include a reduction in weight, changes in behaviour, a reduction in the number of offspring, changing generation time, etc. The growth rate of a population is therefore a better and ecologically more relevant indicator of effects on non-target organisms.[44]

4.4 No effect: no follow-up study needed

If no effect is detected in the laboratory study using 10 times the expected maximum environmental exposure concentration on non-target organisms that meet the stated criteria, COGEM is of the opinion that no further studies are needed. The 10 times safety margin is based on current scientific understanding of the differences in the sensitivity of organisms and on knowledge about the feasibility of carrying out experiments on exposure to large quantities of proteins.

As stated earlier, numerous different non-target organisms are present in the field, many of which may be more sensitive to the Bt toxin than the non-target organism used in the laboratory tests. If the non-target organism studied in the experiments is found to be unaffected by exposure to the safety margin of 10 times the expected maximum environmental exposure concentration, COGEM considers this to be sufficient evidence to assume that the GM crop will not pose a risk to other non-target organisms that have not been studied. However, COGEM is also aware that the 10 times safety margin is an arbitrary figure and therefore points out the need for general surveillance. [d]

4.4.1 Non-specific field trials unsuitable for the environmental risk assessment

In addition to laboratory research, field trials with a GM crop can be essential for investigating the possible effects in practical situations. If effects are found in the laboratory studies, specific tests will be needed to determine whether or not the adverse effect also occurs in field conditions and what the magnitude of the effect is. The EFSA states that in order to identify any unanticipated effects of a GM crop, the environmental risk assessment must include field trials.[45] Field trials that are not based on a hypothesis about the possible specific effect are considered to be non-specific field trials. Research commissioned by COGEM has shown that non-specific field trials are only able to detect very large effects.[46] Any such effects of insect-resistant crops will have already been detected in the previous laboratory studies. COGEM is therefore of the opinion that the

[d] General surveillance is compulsory in the European Union in a number of situations, including the cultivation of GM crops, to monitor for the occurrence of unanticipated adverse effects.

non-specific field trials that are now carried out for each application provide little or no useful information for the risk assessment and can therefore be dispensed with.

4.5 Effect detected: second step is field trials

If the laboratory study shows that the GM plant material or the Bt toxin has an effect on one or more non-target organisms, this is evidence that these and similar non-target organisms in the field could be affected. To test for this, it is first necessary to identify the concentrations needed to cause an effect, then to investigate whether or not the effect occurs in the field[44] and whether or not other non-target organisms in the same group are also affected. Conditions in the field (temperature, exposure, etc.) are variable and will differ from the standardised conditions in the laboratory. Moreover, in the field the non-target organism is part of an ecosystem and serves as food for other organisms and so it is possible that an effect detected in the laboratory will have no consequences for the population of non-target organisms.

4.5.1 Field trials must be adapted to the non-target organism concerned

To be able to detect an effect and establish a reliable causality, field trials should be held where sufficient numbers of non-target organisms are present. As almost all non-target organisms species are highly mobile, are sometimes present in the field only in small numbers, or exist naturally in highly fluctuating populations, the design and method of the field trial should be adapted to the non-target organism under investigation to ensure that sufficient numbers of non-target organisms can be found to enable effects to be identified.

4.5.2. Field trials with the GM crop

To determine whether or not non-target organisms in the field are affected by the GM crop, it is important that the field trial is carried out with the GM crop for which the application has been made. If another GM crop is used that also possesses other traits or expresses other substances, any effects may be masked or intensified. Field trials should therefore be carried out on the relevant GM line and not on older or hybrid lines.[40,39] Exceptions to this are only possible when the traits that could have an effect on non-target organisms are fully consistent with those of the GM crop under investigation. This may be the case, for example, when a GM crop produces the same Bt toxins in the same quantities *and* any other introduced traits they may have (e.g. herbicide tolerance) do not have an effect on non-target organisms and do not influence the activity of the Bt toxins.

4.5.3 Field trials in European cultivation areas under conventional agronomic practices

Field trials should be carried out in relevant agricultural areas in Europe. There are differences between the species of non-target organisms present in Europe and the non-target organisms present outside Europe (e.g. in North or South America). For this reason, COGEM considers that field trials carried out in other areas are unsuitable for assessing the possible risks to European

non-target organisms.[47] The conditions under which field trials are held must also be equivalent to those in conventional agronomic practices. For example, field trials must be held in the correct growing season, because otherwise the non-target organisms (or relevant life stages of these organisms) that are normally present in the field may not be present during the trial.[40,48]

4.5.4 Rationale for the statistical analysis

The grounds for choosing the method of statistical analysis employed must be explained. When a relatively small number of organisms are observed, this should be taken into account in the statistical analysis.[49]

4.6 Preference for experimental studies

Some non-target organisms (such as butterflies and bees) are found in field trials in such small numbers that it is usually impossible to detect any effects on these species. In view of this, models (e.g. theoretical exposure analyses) are sometimes used to estimate the effects on these non-target organisms.[50] As models are often based partially on assumptions, estimates and data derived from other organisms (extrapolation), errors can occur.[51] COGEM therefore has a strong preference for the use of experimental data, for example obtained from laboratory studies, when assessing the possible effects on non-target organisms. Models should only be used if they have been validated and there are sufficient reliable experimental data available to provide input to the model.

5. Guidance for the environmental risk assessment of GM crops that express multiple Bt toxins

5.1 Accounting for possible interactions

In addition to the points described in the guidance contained in Chapter 4, the environmental risk assessment for GM crops that produce multiple Bt toxins must also take account of possible interactions between the Bt toxins and their effects on non-target organisms.

As described in Chapter 3, when Bt toxins active against the same order of insects are mixed in certain combinations (three-domain Cry proteins, three-domain Cry proteins in combination with Cyt, Vip or Mtx proteins, Bin proteins and mixtures of Mtx proteins) they can influence each other's activity. There are no known cases of Bt toxins active against different insect orders influencing each other's activity, but because few studies have been done on such combinations the possibility of Bt toxins active against different orders of insects influencing each other's activity cannot be ruled out.

Possible interactions between Bt toxins should be considered in the risk assessment. However, based on current knowledge it is not possible to predict in advance whether or not Bt toxins in a certain combination will influence each other's activity. This has to be investigated experimentally. A complicating factor is that the same mixture of Bt toxins can either enhance or weaken each other's effects depending on the organism concerned. However, in practice it is not feasible to investigate the effects of a certain combination on all the many possible non-target organisms, and so a method has to be found to obtain as much relevant and representative information as possible. The various options and their limitations are set out below.

Option 1: Test for interactions in sensitive pest organisms

The approach currently taken for most applications is to run tests on sensitive organisms, usually pests, to detect interactions between Bt toxins produced by the GM crop. The assumption is that it is easier to detect a change in the activity of the Bt toxins if the organism is already known to be affected by at least one of the Bt toxins. Follow-up studies into the effect of the combination of Bt toxins on non-target organisms are then only necessary if an interaction effect has been observed in the tests on the sensitive organisms. If that is the case, tests are carried out to determine the effect of the combined Bt toxins on the standard set of non-target organisms.

In this option, the results of experiments on sensitive pest organisms are used to draw conclusions about the occurrence or not of interaction effects in other, non-target organisms that are not sensitive to the individual Bt toxins. The way in which Bt toxins influence each other's activity may vary from organism to organism, which means that studies on sensitive pest organisms will have little predictive value regarding the occurrence of interactions in other, non-target organisms.

Option 2: Test for interactions in the standard set of non-target organisms
Laboratory tests for interactions between Bt toxins that result in changes in their activity can also be performed on the standard set of at least five non-target organisms. The standard set of five non-target organisms consist of organisms from different orders. To date, interactions have only been observed in mixtures of three-domain Cry proteins that are active against insects from the same order. Effects of Bt protein mixtures on insects from other orders would appear to be exceptional.[9] It is therefore doubtful that including insects from other orders than those known to be affected by the Bt toxins concerned in studies on non-target organisms would serve any useful purpose. COGEM is of the opinion that the likelihood of combinations of Bt proteins interacting and having an effect on tested insects from other orders can be considered to be very small to negligible.

Option 3: Test for interactions in a specific group of non-target organisms
Because interactions have only been observed in organisms belonging to the insect order against which several Bt toxins in the mixture are active, the most likely outcome is an enhanced effect on non-target organisms belonging to the same order. The third option, therefore, is to focus the investigation on these non-target organisms.

The first step in this option is to determine the full activity spectrum of the various Bt toxins produced by the GM crop, adhering to the condition described in section 4.2 above. The next step is to determine whether there are multiple Bt toxins that have an effect on insects from the same order. It must then be established if any non-target organisms from this insect order are present in the field or its immediate surroundings. To do this, use can be made of the database of arthropods found on various crops compiled for the EFSA.[52,53] If such non-target organisms are present, studies must be done to establish whether or not these non-target organisms are affected by a mixture of Bt toxins produced by the GM crop that are active against this insect order (see also 5.2.1). If so, the size of the effect must be determined to establish whether or not it is relevant under field conditions. If no non-target organisms from the relevant insect order are found in the GM crop field or the immediate surroundings, no further studies are needed.

Most of the available scientific data are from studies carried out on combinations of three-domain Cry proteins. Less research has been done on the occurrence of interactions in mixtures containing Bt toxins from other Bt protein groups. There are indications that interactions in such mixtures may occur more frequently. This suggests that although this option would appear to provide enough information to be able to estimate the risks posed by GM crops containing various three-domain Cry proteins, there remain doubts as to whether or not this is the case for GM crops containing other Bt toxins.

Option 4: Modelling worst case scenarios

A fourth option is to model a worst case scenario of the effect of a combination of Bt toxins on a non-target organism. Such a model is based on the maximum expression level in the GM crop reported thus far, a theoretical maximum exposure to the various Bt toxins (taking account of the Bt concentration in plant material or prey species and of the intake by the relevant non-target organism), a maximum enhancement factor and an extremely sensitive non-target organism. This worst case effect can then be taken into account in the risk assessment. A disadvantage of theoretical analyses and models is that they involve assumptions and estimates and use data on other organisms (extrapolation), all of which can introduce errors into the calculations.

5.1.1 COGEM advice: Study the effects of possible interactions on species from the same order

The previous section shows that there are several options for investigating the possible effects, but that all these options have their limitations. None of them can provide conclusive evidence that adverse effects on non-target organisms will or will not occur as a result of any synergism between the Bt toxins produced by the GM crop. However, this limitation is inherent in environmental risk assessments. Even in studies of the possible effects of a single introduced Bt toxin, the method employed for testing at least five different non-target organisms provides no guarantee of detecting all possible adverse effects.

A research method is needed that maximises the probability of detecting possible adverse effects, that is sufficiently representative and indicative for the situation in the field, and that is practical and feasible to implement.

Given these requirements and the advantages and disadvantages of the various options, COGEM's advice for assessing a GM crop that produces multiple three-domain Cry proteins is to use the approach described in Option 3, in which the tests are restricted to a certain group of non-target organisms.

Any additional risks could also be evaluated by modelling worst case scenarios (Option 4). COGEM points out that models should only be used if the inputs consist of good quality experimental data. However, in many cases such data would seem to be lacking and so it is important that wide safety margins are used. In the scientific literature just one combination of Bt toxins has been reported with a synergy factor of 33. Based on current knowledge, COGEM therefore advises that calculations of the worst case scenario should use a minimum safety factor of 35 times the expected concentration in order to fully account for any synergistic effects.

Synergism can be expected to occur more often in mixtures with other three-domain Cry proteins, but less research has been done on such mixtures, which makes it more difficult to determine when any interactions could occur. For GM crops that produce multiple Bt toxins including at least

one Bt toxin that does not belong to the three-domain Cry proteins, COGEM advises studying the effect of the mixture on the standard set of non-target organisms (Option 1).

5.2 Additional points to consider

5.2.1 Studies of interactions at the 10 times expected environmental exposure concentration

If tests with a mixture of Bt toxins are necessary, the non-target organism must be exposed to a mixture of Bt toxins at 10 times the maximum expected environmental exposure concentration. The proportions of Bt toxins in the mixture must be equivalent to proportions of Bt toxins the non-target organism will be exposed to in the environment.

Although the literature contains reports of situations in which interactions did or did not occur depending on the proportions of Bt toxins in the mixture, it is not feasible in practice to conduct studies using a range of different proportions of Bt toxins because the number of test combinations increases exponentially. COGEM therefore endorses the conventional toxicological approach used for studies on the effects of Bt toxins, in which two different concentrations are tested (the in-field exposure concentration and a 10 times higher concentration).

5.2.2 Sometimes the risks of subcombinations should also be assessed

COGEM notes that the offspring of a GM crop may contain a different combination of Bt toxins because traits can segregate in the next generation. This is a complicating factor when assessing the possible effects on non-target organisms, because each possible combination of Bt toxins has to be tested to establish if it could have an effect on non-target organisms. The need to assess the risks of subcombinations depends on the particular GM crop. For example, if a GM crop produces three different Bt toxins (A, B and C) that are all active against the same insect order, but some offspring in the next generation possess only two of these Bt toxins, the possible combinations of two Bt toxins (A + B, A + C, B + C) will have to be tested.

5.2.3 Always take possible additive effects into account

COGEM points out that when assessing GM crops that produce multiple Bt toxins, possible additive effects must always be taken into account. An additive effect can cause an adverse effect on an organism even though the concentration of each of the individual toxins is too low to have an effect on its own. This needs to be accounted for in the design of the experiment, for example by using sufficiently high concentrations of each of the toxins, or the mixture of toxins, in the tests.

5.3 General surveillance is a necessary complementary measure

COGEM is of the opinion that the approach recommended above is suitable for assessing the risks to non-target organisms. However, the occurrence of interactions between Bt toxins depends on

the species concerned and as it is impossible to test all non-target organisms, there will always be a possibility that the Bt toxins will enhance each other's activity in one of the many non-target organisms not included in the tests.

Monitoring for unanticipated effects of the GM crop (called general surveillance) is compulsory when a GM crop is approved for use in the EU.[54] General surveillance can pick up on interactions between Bt toxins that lead to substantial unexpected adverse effects on non-target organisms. General surveillance is therefore an important and necessary tool for detecting effects resulting from unexpected synergistic interactions.

6. Summary of the key principles for assessing risks to non-target organisms

- The possible adverse effects of a GM crop on non-target organisms only need to be investigated when the action of the inserted gene gives reason to suppose that such effects could occur.

- The first step in such an investigation is to conduct laboratory experiments in which a selection of non-target organisms are exposed to the GM crop (or the purified protein).
- Further research is only necessary if an effect is detected in these experiments. In such cases, follow-up laboratory experiments should be done to determine the concentrations at which the effect occurs. In addition, European field trials must be held to establish whether or not the GM crop affects the same or similar non-target organisms in the field.
- Non-specific field trials, such as those that are currently carried out when applications are made for the cultivation of GM crops in order to detect any unanticipated effects on non-target organisms, cannot detect small effects. COGEM is therefore of the opinion that such field trials serve no useful purpose.

- In addition, the risk assessment of GM crops that produce multiple Bt toxins should take account of possible interactions between the Bt proteins.
- Because in mixtures of three-domain Cry proteins such interactions have so far only been observed between three-domain Cry proteins that are active against the same insect order, additional studies are only necessary when the GM crop contains multiple three-domain Cry proteins that have an effect on insects from the same order. In such cases, it is necessary to establish if any non-target organisms from this insect order are present in the field or its immediate surroundings. It must then be determined whether or not these non-target organisms are affected by a mixture of all the three-domain Cry proteins that are active against this insect order. If the results are positive, the size of the effect must be determined to establish whether or not it is relevant under field conditions.
- Synergism can be expected to occur more often in mixtures containing Bt proteins that do not belong to the three-domain Cry protein group, but less research has been done on such mixtures and so it is more difficult to determine when any interactions could occur.
- For GM crops that produce multiple Bt toxins including at least one Bt toxin that does not belong to the three-domain Cry proteins, the effect of the mixture of Bt toxins must be tested on the standard set of five non-target organisms.

- COGEM is of the opinion that the risks to non-target organisms of GM crops that produce multiple Bt toxins can be assessed with sufficient certainty by following the guidance for the risk assessment presented in this report.

- Laboratory studies and field trials can never provide conclusive evidence that non-target organisms will or will not be adversely affected by Bt toxins produced by a GM crop as a result of synergistic or other effects. COGEM therefore underlines the importance of general surveillance as a complementary measure to ensure environmental safety.

References

1. COGEM (2009). Letter of advice on standardising laboratory tests on non-target organisms. COGEM advice CGM/090217-02 [In Dutch]
2. COGEM (2005). Guidelines on selecting non-target organisms for the risk assessment of the introduction on the market of genetically modified crops. COGEM advice CGM/051020-01
3. Charleston DS & Dicke M (2009). Designing experimental protocols to investigate the impact of GM crops on non target arthropods. COGEM onderzoeksrapport CGM 2008-01
4. COGEM (2016). Advisory letter on the 'Representativity of non-target organisms'. COGEM advice CGM/161012-02 [in Dutch]
5. European Food Safety Authority and the Netherlands Commission on Genetic Modification (2013) Event Report, International scientific Workshop “Non-Target Organisms and Genetically Modified Crops: Assessing the effects of Bt proteins” (29-30 November 2012, Amsterdam, the Netherlands) EFSA. Supporting Publications 2013:EN-484
6. EPA (2009). Position paper on scientific issues associated with the data required to register plant-incorporated protectants submitted to the FIFRA scientific advisory panel for review and comment. www.regulations.gov/document?D=EPA-HQ-OPP-2008-0835-0003 (visited: July 13th, 2017)
7. Raybould A *et al.* (2012). Ecological risk assessments for transgenic crops with combined insect-resistance traits: the example of Bt11xMIR604 maize. J Appl Entomol. 136: 27-37
8. Graser G et al. (2017). A general approach to test for interaction among mixtures of insecticidal proteins which target different orders of insect pests. J Insect Sci. 17(2): 39; 1-12
9 . De Schrijver A *et al.* (2015). Relevance of Bt toxin interaction studies for environmental risk assessment of genetically modified crops. Plant Biotechnol J . doi: 10.1111/pbi.12406
10. Hilbeck A & Otto M (2015). Specificity and combinatorial effects of *Bacillus thuringiensis* Cry toxins in the context of GMO environmental risk assessment. Front Environ Sci. 3:71 doi: 10.3389/fenvs.2015. 00071
11. Van der Hoeven N (2014). *Bacillus thuringiensis* toxins: Their mode of action and the potential interaction between them. COGEM research report CGM 2014-02
12. De Schrijver A *et al.* (2014). Can interactions between *Bt* proteins be predicted and how should effects on non-target organisms of GM crops with multiple Bt proteins be assessed? COGEM research report CGM 2014-05
13. COGEM (2014). Aanbieding onderzoeksrapport ‘*Bacillus thuringiensis* toxins: their mode of action and the potential for interaction between them’. Signalerende aanbiedingsbrief CGM/141014-01 [in Dutch]
14. COGEM (2014). Aanbieding onderzoeksrapport ‘Can interactions between Bt proteins be predicted?’. Signalerende aanbiedingsbrief CGM/141203-01 [in Dutch]
15. ACRE, CNB, WIV-ISP & COGEM (2014). Event report International scientific workshop ‘Stacked Bt genes: assessment of effects on non-target organisms’. COGEM event report CGM/141217-01
16. International Service for the Acquisition of Agri-biotech Applications (ISAAA). GM Approval Database. http://www.isaaa.org/gmapprovaldatabase/
17. Van Frankenhuyzen K (2009). Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J Invertebr Pathol. 101: 1-16
18. Van Frankenhuyzen K (2013) Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. J Invertebr Pathol. 114: 76-85
19. Tabashnik BE (1992). Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl Environ Microbiol. 58(10): 3343-3346
20. Cedergreen N (2014). Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9(5): e96580

21. Li H & Bouwer G (2014). Evaluation of the synergistic activities of *Bacillus thuringiensis* Cry proteins against *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Invertebr Pathol. 121: 7-13
22. Monnerat R *et al.* (2014). Synergistic activity of *Bacillus thuringiensis* toxins against *Simulium* spp. larvae. J Invertebr Pathol. 121: 70-73
23. Chakrabati SK *et al.* (1998). Synergistic effect of Cry1Ac and Cry1F δ-endotoxins of *Bacillus thuringiensis* on cotton bollworm, *Helicoverpa armigera*. Curr Sci. 75(7): 663-664
24. Poncet S *et al.* (1995). Evaluation of synergistic interactions among CryIVA, CryIVB and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J Invertebr Pathol. 66: 131-135
25. Promdonkoy B *et al.* (2005). Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism against *Aedes aegypti* and *Culex quinquefaciatus* larvae. FEMS Microbiol Lett. 252: 121-126
26. Fernández-Luna MT *et al.* (2010). Single concentration tests show synergism among *Bacillus thuringiensis* subsp. *israelensis* toxins against the malaria vector mosquito *Anopheles albimanus*. J Invertebr Pathol. 104: 231-233
27. Khasdan V *et al.* (2001). Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. Environ Microbiol. 3(12): 798-806
28. Nunes Lemes AR *et al.* (2014). Synergism and antagonism between *Bacillus thuringiensis* Vip3A and Cry1 proteins in *Heliothis virescens, Diatraea saccharalis* and *Spodoptera frugiperda.* PLoS One 9(10): e107196
29. Yu X *et al.* (2012). Co-expression and synergism analysis of Vip3Aa29 and Cyt2Aa3 insecticidal proteins from *Bacillus thuringiensis*. Curr Microbiol. 64(4): 326-331
30. Levine SL *et al.* (2016). Assessing the potential for interaction between the insecticidal activity of two genetically engineered cotton events combined by conventional breeding: an example with COT102 x MON15985. Regul Toxicol Pharmacol. 29: 35-41
31. Wirth MC *et al.* (2014). Mtx toxins from *Lynsinibacillus sphaericus* enhance mosquitocidal Cry-toxin activity and suppress Cry-resistance in *Culex quinquefasciatus*. J Invertebr Pathol. 115: 62-67
32. EFSA Panel on Genetically Modified Organisms (2010). Scientific Opinion on the assessment of potential impacts of genetically modified plants on non-target organisms. EFSA Journal 8(11): 1877. doi:10.2903/j.efsa.2010.1877.
33. COGEM (2012). Additional information concerning the application for cultivation of MON89034xNK603 maize. COGEM advice CGM/120327-01
34. COGEM (2008). Cultivation of genetically modified maize line MON88017. COGEM advice CGM/081112-02
35. COGEM (2016). Advisory letter on the 'Representativity of non-target organisms'. Advisory letter CGM/161012-02
36. COGEM (2015). Testing impacts of toxic compounds from transgenic crops on non-target arthropods in tier-1 studies: exposure and response. Aanbiedingsbrief CGM/151217-01 [in Dutch]
37. COGEM (2013). Additional advisory report on cultivation of maize line 59122. COGEM advice CGM/130507-01
38. COGEM (2011). Additional information concerning the application for cultivation of MON89034xNK603 maize. COGEM advice CGM/110803-01
39. COGEM (2009). Cultivation of maize line MON89034xNK603. COGEM advice CGM/091208-01
40. COGEM (2011). Cultivation of insect resistant maize MIR604. COGEM advice CGM/110404-01
41. De Schrijver A *et al.* (2016). Quality of laboratory studies assessing effects of *Bt*-proteins on non-target organisms: minimal criteria for acceptability. Transgenic Res. 25: 395-411
42. COGEM (2008). Cultivation of genetically modified maize line 59122. COGEM advice CGM/080207-02
43. COGEM (2015). Statistiek bij de risicobeoordeling van gg-gewassen. Signalerende brief CGM/151209-01 [in Dutch]

44. COGEM (2009). Letter of advice on standardising laboratory tests on non-target organisms. CGM/090217-02
45. EFSA (2010). Scientific opinion 'Guidance on the environmental risk assessment of genetically modified plants' EFSA Journal 2010: 8(11): 1879
46. Booij CJH (2014). Ecological and experimental constraints for field trials to study potential effects of transgenic Bt-crops on non-target insects and spiders. COGEM research report CGM 2014-06
47. COGEM (2011). Advies betreffende de Europese bindende richtlijn voor de milieurisicobeoordeling van genetisch gemodificeerde planten. COGEM advies CGM/110307-01 [in Dutch]
48. COGEM (2007). Cultivation of genetically modified maize line 1507x59122. COGEM advice CGM/071119-02
49. COGEM (2012). Aanbieding onderzoeksrapport 'The use of statistical tools in field testing for effects of GM plants on non-target organisms'. Signalerende aanbiedingsbrief CGM/121127-01 [in Dutch]
50. Perry JN *et al.* (2010). A mathematical model of exposure of non-target Lepidoptera to *Bt*-maize pollen expressing Cry1Ab within Europe. Proc Biol Sci. 277(1686): 1417-1425
51. COGEM (2011). Additional advice on cultivation of maize MON88017. COGEM advice CGM/111209-01
52. Meissle M *et al.* (2012). Establishing a database of bio-ecological information on non-target arthropod species to support the environmental risk assessment of genetically modified crops in the EU. Supporting publications 2012: EN-334 European Food Safety Authority (EFSA)
53. Riedel J *et al.* (2016). Update and expansion of the database of bio-ecological information on non-target arthropod species established to support the environmental risk assessment of genetically modified crops in the EU. EFSA supporting publication 2016: EN-956
54. Directive 2001/18/EC of the European Parliament and of the Council of 12 march 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities L 106/1-38