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Environmental risk assessment of proteins expressed by genetically modified plants

Applicability of standard tests used for chemical pesticides

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This investigation has been performed by order and for the account of VROM, the Netherlands Ministry of Housing, Spatial planning and the Environment, within the framework of Genetically Modified Organisms

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Abstract

Environmental risk assessment of proteins expressed by genetically modified plants
Applicability of standard tests used for chemical pesticides

Various crops have been genetically modified in such a way that they are able to produce proteins which provide resistance to attack from insects or fungi. However, it is also possible that these proteins have undesirable effects on other organisms, such as birds, fish, algae and bees. A recent study carried out by the RIVM (National Institute for Public Health and the Environment), by order of the Ministry of Housing, Spatial Planning and the Environment (VROM), has found that standard tests used to assess the undesirable effects of chemical crop protection agents can also be applied to assess the risks of such proteins. The RIVM has also developed templates for determining whether specific standard tests are suitable for the testing of genetically modified plants.

The overall study focused on three case studies. The first case study was on the enzyme chitinase, which is produced by genetically modified sugar beet. Chitinase is an enzyme that can break down chitin, an essential component of the cell wall of insects and many fungi. The second case study was on GNA lectin, an insecticidal lectin produced by genetically modified potato plants, which has a negative effect on insects and fungi. The third case focused on the enzyme EPSP synthase, which renders genetically modified rape insensitive to the herbicide glyphosate, thus allowing the selective destruction of weeds.

The templates have been developed in such a way that they can also be used for other proteins produced by genetically modified plants. The possibility that proteins are excreted continuously by the genetically modified plant, in contrast to chemical pesticides that are sprayed onto the plant only once of several times, will have to be considered in the study design. Continuous excretion may have long-term effects on several organisms in the soil or on the plant.

Key words: genetically modified plants, proteins, chitinase, GNA lectin, EPSP synthase, data requirements, chemical crop protection agents, standard tests, 91/414/EC

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Rapport in het kort

Milieurisicobeoordeling van eiwitten geproduceerd door genetisch gemodificeerde planten
Toepasbaarheid van standaard testen voor chemische bestrijdingsmiddelen

Een groep (consumptie)gewassen is zodanig genetisch gemodificeerd dat ze eiwitten produceren die insecten of schimmels bestrijden. Ze kunnen echter ook ongewenste effecten veroorzaken bij organismen, zoals vogels, vissen, algen en bijen. Uit onderzoek blijkt dat standaardtesten om ongewenste effecten van chemische gewasbeschermingsmiddelen te beoordelen, bruikbaar kunnen zijn om de risico's van dergelijke eiwitten te beoordelen. Het RIVM heeft dit onderzoek in opdracht van het ministerie van VROM uitgevoerd. Het instituut heeft bovendien templates ontwikkeld waarmee kan worden onderzocht of de standaardtesten geschikt zijn voor het testen van eiwit-producerende genetisch gemodificeerde planten.

Voor het onderzoek zijn drie casussen gebruikt. Het betreft het enzym chitinase, dat wordt geproduceerd door genetisch gemodificeerde suikerbiet. Chitinase breekt chitine af, de bouwsteen van insecten en schimmels. Het GNA-lectine, dat een schadelijke werking heeft op insecten en schimmels, en wordt geproduceerd door genetisch gemodificeerde aardappel; en het enzym EPSP synthase, dat genetisch gemodificeerde koolzaad ongevoelig maakt voor het onkruidbestrijdingsmiddel glyfosaat terwijl het onkruid hiermee wordt bestreden.

De templates zijn zodanig opgesteld dat ze ook kunnen worden gebruikt voor andere eiwitten die door genetische gemodificeerde planten kunnen worden geproduceerd. Bij de testen moet er rekening mee worden gehouden dat de eiwitten mogelijk continu worden uitgescheiden door de genetisch gemodificeerde plant, in tegenstelling tot chemische bestrijdingsmiddelen waarmee gewassen slechts een of meerdere keren worden bespoten. Continue uitscheiding kan mogelijk op lange termijn effect hebben op diverse organismen in de bodem of op de plant.

Key words: genetische gemodificeerde planten, eiwitten, chitinase, GNA lectin, EPSP synthase, data vereisten, chemische gewasbeschermingsmiddelen, standaard testen, 91/414/EC

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Summary

Similar active substances can be assessed in different frameworks. The question is whether the test methodologies used in one framework can also be used in the other framework.

There are specific data requirements for the evaluation and environmental risk assessment of genetically modified plants and the proteins that they express. However, there is no requirement to use standardised tests to test non-target effects of these GM plant produced proteins on birds, mammals, aquatic organisms, bees, other non-target insects, earthworms and soil micro-organisms. This is in contrast to the evaluation and risk assessment of chemical plant protection products for crop protection. The objective of this report is therefore to evaluate which standardised tests that are part of the safety testing of chemical and microbial products may be useful for the environmental safety assessment of GM plants.

The similarity between proteins expressed by GM plants and chemical plant protection products containing these proteins such as chitinases is evident. Products containing these proteins may have an insecticidal or fungicidal mode of action and need to be evaluated in the framework of chemical crop protection products. It is assumed that proteins expressed by GM plants have the same direct insecticidal or fungicidal mode of action. The stability of sprayed proteins of the proteins outside the plant may be short and should be taken into consideration. In this report an inventory is made of the data requirements for fate and behaviour and ecotoxicology that are asked for chemical control agents. The standard tests that are available to fulfil these data requirements are also collected.

In first instance, general templates have been developed in which the data requirements and their tests are incorporated. In this general template there is as yet no focus on a specific protein or a specific GM plant. Following this approach, all possible data requirements were dealt with. All possible plant stages identified in sugar beet, potato and oilseed rape were also used for the imaginary plant. The advantage of this approach is that the general template can be used for any protein/GM plant combination in the future. In the next step, the templates were specified for three cases: 1) chitinase expressed by GM sugar beet, 2) GNA lectin expressed by GM potato and 3) EPSP synthase expressed by GM oilseed rape.

The suitability of the tests necessary for the evaluation of the proteins in the sprayed product is evaluated for the GM plants. Many can be used but the main problems faced in all tests are that the concentrations of the protein in plant tissues and soil (after leakage or excretion) are unknown, making it difficult to determine test concentrations. Furthermore proteins expressed by GM plants are assumed to be expressed during its complete lifespan and the protein may also be present in crop residues on the field after harvest. The fact that there is a possibility that the protein is present in plant tissues and in the soil during and after the life span of the crop indicates that the exposure to non-target organisms may be chronic. Fate and ecological tests therefore would need adaptation to be able to evaluate chronic effects.

The outcome of this report can be used for further investigation/selection of tests and adaptations thereof that could be applicable for the environmental safety assessment of GM plants expressing proteins with an insecticidal or fungicidal mode of action.

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1 Introduction

This study investigates whether data requirements for proteins being ´crop protection agents of natural origin´ and their tests can be used for the environmental risk assessment and authorisation of genetically modified plants (GM plants) expressing proteins.
This question is of concern as the different frameworks of chemical/biological crop protection products and genetically modified organisms do not normally communicate at the level of data requirements and tests, but useful information may be adapted from the one into the other framework. In this study an attempt is made to investigate whether some studies used in the risk assessment of chemical pesticides can also be used in the risk assessment of GM plants that express proteins.

The questions to be answered are:

1. Which tests should be performed with proteins expressed by the GM plants, when the protein would be regarded as a chemical or biological crop protection agent or a crop protection agent of natural origin?
2. Are these tests applicable to the evaluation of proteins expressed by GM plants?

For each of these data requirements the rationale was given for why they should be considered and how the tests are interpreted. Thereafter, it was evaluated whether this approach could be used for GM plants in the same way. It was considered important to evaluate the rationale (s this idea feasible for the evaluation of a GM plant?) and the test method (is the test useful for the evaluation of a GM plant or should it be replaced by another test?).

The study was set up by designing a general template in which a non-described crop protection agent of natural origin was used to identify the data requirements formulated under 91/414/EC[1] (European Commission) and possible tests to fill in these data requirements. These data requirements and tests were transposed to an imaginary GM plant expressing a non-described gene (a gene expressing a protein that has a similar function as a crop protection agent of natural origin). The considerations for the imaginary GM plant also included all possible plant phases that are relevant in the risk assessment. This template formed the basis for specific comparisons that were made between, for instance, chitinase used as a crop protection agent of natural origin and a GM plant expressing chitinase.

1.1 The three cases

The report of Mensink (2006) preceded this study, summarising data requirements and the type of tests necessary for the risk evaluation of crop protection agents of natural origin. Mensink (2006) described three cases (chitinase, GNA lectin and EPSP synthase) (Appendix 1). The elaboration of these three cases is being pursued from the point where Mensink (2006) arrived. For this report, it was evaluated what would be the data requirements for the proteins as part of the GM plant in which they are expressed. In this approach, the way the proteins are produced in plant tissues and released from the plant during its cultivation will also be considered. The proteins are assumed to be present in the plant and excreted actively or leaking passively from dead plant parts. When applied as crop

[1] The Commission Directive 91/414 has been replaced by Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/ EEC (European Commission 2009). This Regulation will be operational in June 2011.

protection agent of natural origin they are, for the purpose of this study, assumed to be sprayed with a conventional beamer (see Appendix 1).

1.2 Environmental data requirements

It is described by Mensink (2006) that the environmental data requirements of the 4th-stage re-evaluation (refer to list of abbreviations) could be used for chitinase and GNA lectin as more or less comparable substances are present on the list of the 4th stage (see Appendix 2). Substances on this list need a lighter package of data requirements as they are expected to be of lesser concern. Formally, the active substances chitinase and GNA lectin should be evaluated as chemicals under 91/414/EC. For EPSP synthase, more or less similar substances are not found on the list of the 4th stage. This was foreseen, as EPSP synthase cannot be regarded as a (bio)pesticide. EPSP synthase does not protect the plant against insects of pathogenic fungi, like GNA lectin and chitinase, but it is a mutation of an enzyme. As a consequence, the plant is not sensitive to the herbicide glyphosate.

The data requirements of the 4th stage are based on those of 91/414/EC for chemicals, but for the fulfilment of the data requirements it is allowed to use data from the open literature as well as existing risk evaluations. In this report, the data requirements of 91/414/EC for chemicals will be used for all three cases, bearing in mind that the fulfilment of the data requirements for the proteins applied as crop protection agents probably need to be less stringent than for chemical substances. The data requirements according to Directive 2001/36/EC for micro-organisms (European Commission 2001) cannot be used, as the protein is not a living micro-organism. Therefore, the data requirements of micro-organisms were not further considered in the templates.

Table 1 summarises in detail the data requirements according to Directive 91/414/EC for chemicals.

Table 1. Data requirements for crop protection agents of natural origin according to Directive 91/414/EC for chemicals

EC section no	General data requirements
7	**Fate and behaviour in the environment**
7.1	*Fate and behaviour in soil*
7.1.1	Rate and route of degradation (to 90 per cent degradation), including identification of the processes involved and identification of metabolites and breakdown products in at least three soil types under appropriate conditions.
7.1.2	Adsorption and desorption in at least three soil types and where relevant, adsorption and desorption of metabolites and breakdown products.
7.1.3	Mobility in at least three soil types and where relevant, mobility of metabolites and breakdown products.
7.1.4	Extent and nature of bound residues.
7.2	*Fate and behaviour in water and air*
7.2.1.	Rate and route of degradation in aquatic systems – biodegradation, hydrolysis, photolysis (as far as not covered by point 2.8), including identification of metabolites and breakdown products.
7.2.2	Adsorption and desorption in water (sedimentation) and where relevant, adsorption and desorption of metabolites and breakdown products.
7.2.3	Rate and route of degradation in air (for fumigants and other volatile active substances).
8	**Ecological studies on the active substance**
8.1	*Effects on birds*
8.1.1	Acute oral toxicity.
8.1.2	Short-term toxicity – eight-day dietary study in at least one species (other than chicken).
8.1.3	Effects on reproduction.
8.2	*Effects on aquatic organisms*
8.2.1	Acute toxicity to fish.
8.2.2	Chronic toxicity to fish.
8.2.3	Effects on fish reproduction and growth rate.
8.2.4	Bioaccumulation in fish.
8.2.5	Acute toxicity for *Daphnia magna*.
8.2.6	*Daphnia magna* reproduction and growth rate.
8.2.7	Effects on algal growth.
8.3	*Effects on other non-target organisms*
8.3.1	Acute toxicity to honeybees and other beneficial arthropods (e.g., predators).
8.3.2	Toxicity to earthworms and to other soil non-target macro-organisms.
8.3.3	Effects on soil non-target micro organisms.
8.3.4	Effects on other non-target organisms (flora and fauna) believed to be at risk.
8.3.5	*Effects on biological methods for sewage treatment*

1.3 Necessary tests

The directive 91/414/EC only gives the data requirements but does not describe the tests that would fulfil them. The applicant needs to decide which tests are most appropriate. According to Mensink, Smit and Montforts (2008), "Nowadays, tests submitted for regulatory purposes will most often be performed according to the OECD Guidelines for the Testing of Chemicals, which include most relevant internationally agreed test methods used." These OECD guidelines are summarised in Appendix 3.

Tests will have to be performed with artificially produced proteins, as the proteins can never be obtained in significant quantities from the plant expressing them. Chitinase can be produced by solid-state or liquid substrate fermentation using *Bacillus subtilus*. Proteins such as GNA lectin and EPSP synthase, normally expressed in plants, can be produced in vitro by placing the genes encoding for the protein in micro-organisms. These micro-organisms can be grown in a liquid substrate. The protein then needs to be extracted from this medium. These in vitro produced proteins can be used for testing, on the condition that they are the same as those produced by the plant. This should be tested. The in vitro produced protein will be addressed as the 'active substance'. The active substance has to be brought into a formulated product, which contains the active substance but also co-formulants or additives, components with specialised characteristics that are needed to make and keep the pesticide efficient. The type of application may require a different formulation. For instance, when sprayed, the active substance needs to be protected against UV light, thereby increasing its persistence on the leaf. Additives may also be needed to obtain good spreading of the formulation on the leaves.

2 Approach

In this report a comparison will be made between a protein (the crop protection agent of natural origin) assumed to be sprayed with a conventional sprayer and the protein expressed in the GM plant. In this report the protein is either chitinase, GNA lectin or EPSP synthase.

1. Chitinase is produced by bacteria in order to break down the chitin in competitive fungi, thereby having a fungicidal action. Chitinase can also have a insecticidal action by degradation of the chitin of insects. The mode of action depends on the type of chitinase.

2. GNA lectine is a protein that is produced by the snowdrop *Galanthus nivalis* and has an insecticidal effect.

3. EPSP synthase is a mutant protein formed by the plant which gives the plant resistance against herbicides. Actually, this protein does not meet the definition of a crop protection agent. A herbicide is considered a crop protection agent, however the EPSP is not herbicidal but it protects the crop from the herbicidal action of a crop protection agent. This casus has been included for comparative reasons.

The identities of the proteins (chitinase, GNA lectin and EPSP synthase) are of extreme importance in identifying the necessary data requirements and need to be well described. The description of the characteristics includes:

- Biological properties (i.e., origin, environmental requirements (pH, temperature, humidity))
- Biochemical properties
- Mode(s) of action and function
- Stability

In section 3, templates will be used to identify tests that can be used for the GM plants.

As a start, it should be realised that each of the three GM plants: potato, sugar beet and oilseed rape, differ in their phenology. For instance, sugar beet forms thick roots and does not flower in its first year, while oilseed rape does not form thick roots and is usually cultivated flowering in the first year. These crop-specific plant stages are important in the identification of the data requirements. A flowering crop might excrete proteins in the nectar and pollen of the flower, necessitating data requirements for bees. In section 2.1, all plant stages of sugar beet, potato and oilseed rape are assembled into an imaginary plant. These plant stages will be further used in the template for comparisons of sprayed proteins and proteins expressed by GM plants (section 2.2).

2.1 Relevant plant stages of the GM plant

In Table 2, the relevant plant stages are presented. As all these stages will not be present in one single plant species, the combination of all these stages is for an imaginary plant. The information on the plant stages of sugar beet and potato was derived from Van den Brink et al. (2008). The information on the plant stages of oilseed rape was from Harper (1973).

Table 2. All possible plant stages of sugar beet, potato and oilseed rape and the imaginary plant

	Imaginary plant	**Sugar beet**	**Potato**	**Oilseed rape**
	plant stages selected for further comparisons			
		Cultivation		
1	Seed	Seed	Seed	Seed (4-6 d)
2	Tuber		Tubers	
3	Seedling	Seedling		Seedling (4-6 d)
4	Young plant	1st year young plant forming a thick root	Young plants forming horizontal stolons[7] which produce a potato at the end of the stolon	Rosette (18-25 d)
5	Mature plant until flowering[1]			Stem elongation (4-7 d)
6	Flowering plant	2nd year plant forming flowers[2,3,4], nectar and pollen. Pollen can be spread by wind and insects 8 to 9 km Bolters (flowering 1st year plants)[5]	Flowering[7,8] (only pollen, no nectar) and berry/seed forming plants	Flowering plant (nectar and pollen) (7-14 d)
7a	Seed forming plants		Seed forming plants[9]	Seed forming plants
7b	Tuber/thick root forming plants		Mature plants with a diversity of bigger and smaller potatoes	
7c	Fruit producing plants/trees			
7d	Nut producing trees			
		Harvest		
		Harvest of roots	Harvest of tubers[10]	Harvest of seeds
		After harvesting		
8	Left over material on top and in the soil	Small left over beets, leaves and beet heads are left in the field	Foliage, berries and roots and small left over potatoes	Foliage left as ground cover during the winter and ploughed back into the soil
		Bolters (regrowth from beet heads and small beets)[6]	Volunteers (regrowth of potatoes in other crops)	Spill of seeds after harvest

[1] Many crops like endive, leek, kale and sprouts are harvested before flowering. Stages 4 and 5 are not relevant.

[2] Second year plants are not grown in the Netherlands.

[3] At occasions of low temperatures in the 4-5 leaf stage, the length of this period and the day length, a first year plant may form flowers. Flowers may also be formed in weed beets that grow from seeds that were already present in the soil or from weed beets seeds present in the sowing material.

[4] Seed is produced in France and Italy.

[5] Cultivated sugar beets normally behave as biennials. In their first year of growth they form a rosette of leaves and form a substantial succulent root. Only in their second year do they bolt (= form a flowering stalk). Occasionally, bolters are first year plants. Bolters are also second year beets that are left in the field by accident. These are also called weed beets: first year bolting is a trait found in wild beets.

[6] Usually, the left over material is ploughed into the soil. Beets are harvested after the first year of growth. According to GAP, bolters are being eliminated effectively and do not need to be considered in the registration procedure.

[7] There is an enormous variation among potato strains/races concerning flowering, seed- and berry production. Seeds can remain fertile in the soil for over 10 years.

[8] Bumblebees and carabids are important pollinators.

[9] Some varieties produce a lot of seed, some do not flower at all or produce little seed. This is variety dependent.

[10] Foliage is sprayed to death or sheared mechanically before the harvest.

2.2 General template for comparison protein and GM plant

In section 3 a general template will be created. In the general template, information on the identity of the 'model' protein is unknown. Biological, physical, chemical and technical properties are however essential, as they are the starting point for the risk assessment and specifically the evaluation of the possible exposure of the non-target organisms to the protein.
Because the information on identity is not available for the model protein, all data requirements need to be worked out.

In Figure 1, this general template is positioned at the left-hand side of the figure (STEP 1). In first instance, the data requirements for the imaginary protein used as a crop protection product will be given. Next, these data requirements and tests will be transposed to the imaginary GM plant. For this imaginary GM plant, all possible plant stages of the GM plant as defined in Table 2 will be taken into consideration. Thus, the data requirements given in the general template can be considered to be complete and can be used as a starting point when working with a specific protein, other than the three cases elaborated in this report.

In the following step (STEP 2) the general template is transposed to each of the three cases in chapter 4 on the right-hand side of Figure 1.

Note that the templates for the GM plant are shaded in grey. This shading has also been used in chapters 3 and 4.

Proteins expressed by GM plants are to be evaluated according to Directive 91/414. Tentatively, some proteins may be evaluated according to the fourth stage of the re-evaluation programme. Chitinase and GNA lectin show similarities with substances on the list of the fourth stage. This fourth stage specifically offers possibilities for waivers.
The Directive 2001/36 EC cannot be used because proteins are not living micro-organisms.

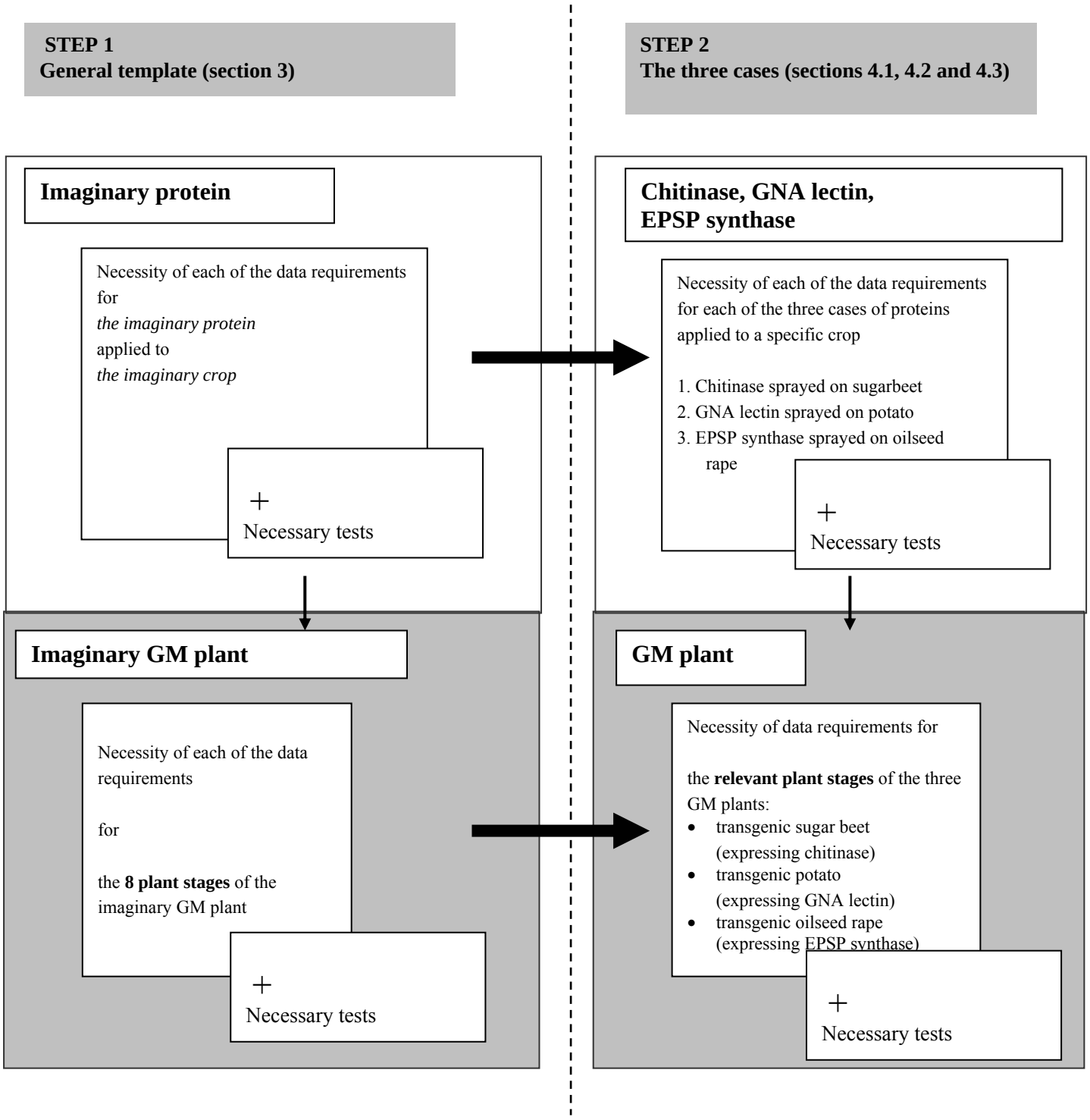

Figure 1. Scheme showing a two-step plan to achieve a description of the data requirements and tests for each of the three cases.

3 The general template

In this chapter, a general template will be made for each of the data requirements (Tables 3 to 10). The upper, white part of each template is specifically written for the unknown protein, being a crop protection agent of natural origin. In the grey shaded part of each template below, all possible plant stages of an imaginary GM plant are being considered. In chapter 4 the general templates will be further elaborated for each of the three cases.
General templates will be made for two types of data requirements in the dossier:

Fate and behaviour (section 7 in the dossier, see Table 1)
Effect on and exposure of non-target organisms (section 8 in the dossier, see Table 1).

3.1 Data requirements for fate and behaviour

Table 3. General template: data requirement 7.1. Fate and behaviour in the environment.

	Protein used as a sprayed substance	**Tests specifically for the protein**
	In general, the properties of the protein are the basis for the evaluation of fate and behaviour in the environment (soil, water and air).	
.	Degradation in soil: Yes, information on degradation in soil should be presented for the protein being the active ingredient of the formulation unless it can be proven that the soil will not be exposed after spraying.	Questions to be answered: • Is the protein naturally present in the soil, • If yes, what are the natural background levels? • Is it necessary that the test can differentiate between different forms of the protein (for example six distinctive molecules of chitinase with different activities are present) *Fourth stage re-evaluation:* Concentrations of the protein applied as a crop protection product are probably high relative to the natural background level. A waiver can be presented if relevant information is available in the literature or from practical experience showing that the concentration of the protein is declining to natural background levels. Otherwise, tests have to be presented on persistence of the protein. *91/414/EC:* Test on degradation in the soil. Possible test are: ➢ OECD 307: aerobic and anaerobic transformation in soil ➢ C. 23: aerobic and anaerobic transformation in soil If persistence of the protein is > 100[1] days), field data will be requested.

		1: The criterion of 100 days is derived from the Uniform Principles of Chemicals (European Commission. 1997). In this document the criteria for the risk assessment of chemical substances are described.
	Sorption and mobility: The possible spread of the protein in relevant environmental compartments has to be evaluated, unless it can be justified that exposure of the particular environmental compartments to the protein is unlikely to occur. Tests should be performed in at least three soil types.	*Fourth stage re-evaluation:* A waiver can be submitted if relevant information is available in the literature or from practical experience showing that the protein is not mobile in soil or if it can be argued that the protein is not mobile. 91/414/EC: Mobility of chemicals in the soil can be assessed by deriving the Kom from ➢ Adsorption study using a batch equilibrium method (OECD 106). immobile Kom > 100 slightly mobile Kom 20 - 100 moderately mobile Kom 5 - 20 mobile Kom 1 - 5 highly mobile Kom 1 In case the protein is very mobile, a second tier column leaching study can be used and in the third tier, a field lysimeter study. ➢ OECD 312: leaching in soil columns. ➢ Soil columns (BBA-part IV, 4-2): leaching experiments (BBA, 1986). ➢ Thin or thick layer chromatography (TLC) experiments. ➢ OECD Guidelines. no. 22: OECD Guidance Document for the Performance of Outdoor Monolith Lysimeter Studies. The necessity for these tests is for proteins however, very unlikely.
	Degradation in water: The protein can reach the water via drift (via the carrier seed/pollen or through the air), runoff and drainage. Yes, information on degradation in water should be presented for the protein being the active ingredient of the formulation. Studies on biodegradation, hydrolysis and photolysis are requested Information is not required when exposure of the surface water is not expected.	*Fourth stage re-evaluation:* Information on degradation in water should be given for the protein (as an active ingredient of the formulation). The applicant can provide a waiver underpinning the rapid degradation of the protein in water with relevant information from the literature. *91/414/EC:* An aquatic photolysis and hydrolysis study are commonly used for chemicals ➢ OECD 316: phototransformation of chemicals in water – direct photolysis. ➢ OECD 111: photolysis as a function of pH. ➢ OECD 308: water-sediment study.
	Degradation in air: The protein can reach the air by drift after	*Fourth stage re-evaluation:* In the imaginary case the protein is volatile (which

	spraying. Yes, information on degradation in air should be presented for the protein being the active ingredient of the formulation. This is however only necessary for fumigants and other volatile active substances in aerosols. If the vapour pressure is very low, volatilisation is not expected and a test need not be performed.	is not expected for a relatively large molecule), the applicant can provide a waiver underpinning the rapid degradation of the protein in air with relevant information from the literature. In the likely case that the protein is not volatile, tests are not necessary. *91/414/EC:* Test guidelines are not available.
plant stage	**GM plant expressing the protein**	**Tests specifically for GM plants**
	Degradation in the plant: Persistence of the protein in the plant is unique for GM plants. The protein is continuously formed in GM plants during growth. In the plant tissue the degradation rate is unknown and subjected to the matrix of the tissue into which it is embedded. Once the protein is excreted or leaks out of the plant, the protein will be subjected to the outer environment, similar to the degradation of the protein applied as a crop protection agent of natural origin.	*In general* following questions could be posed. The answers are helpful in determining the possible exposure to each of the defined plant stages: 1. Are proteins expressed in each plant stage (for example, only expression in the leaves and not in the seed)? 2. Is degradation of the protein in the plant matrix different from degradation of the protein in the soil? 3. Can the concentration of the protein build up in the plant tissue in case of a low degradation rate of the proteins and continuous production of proteins? 4. Can metabolites be formed as a result of interaction with plant tissue?
2-8	Degradation in soil: Yes, data on degradation are relevant, assuming that the protein is actively excreted by the roots or assuming that the protein passively leaks from the roots. Not only the protein can be released, but also metabolites/intermediates as a result of interaction with plant tissue. The possibility of continuous excretion and/or leakage to the soil is different from the application of the protein as a crop protection agent of natural origin, which will be applied once or multiple times at regular intervals. It is assumed that the protein is excreted/leached by all plant stages. The 'seed at planting' is not the plant stage that is expected to release the highest concentrations of proteins. This plant stage does not need to be taken into consideration.	*Fourth stage re-evaluation:* If it is expected that protein activity is reduced quickly, the applicant can provide a waiver underpinning this with relevant information from the literature. This data requirement is then considered to be satisfactorily fulfilled. *91/414/EC:* The GM plant is in the field for more than 100 days. This means that there is possibly a continuous excretion to the soil. This means that concentrations of the protein in the soil do not necessarily follow normal degradation according to first order degradation but increases to an accumulated plateau concentrations might also occur. Typical for the GM plant: • Continuous excretion of protein into the soil is possible. • Increase of concentrations of the protein/kg soil due to increase of below soil plant mass is possible. • It is not possible to express the excretion of protein in mg/kg soil. For the control agent applied at the plants it can be expressed in kg product/ha. The knowledge of a concentration expressed in kg/ha or mg/kg^2 soil is essential for choosing a concentration

		range in ecotoxicity tests. • The protein might be present as a natural background concentration (e.g., similar proteins excreted by bacteria). TESTS <u>Step 1</u>. Test to determine whether the protein is really excreted or leaks into the soil. This should be done for several plant stages. If excretion/leakage does not occur, further tests are not necessary. If excretion/leakage occurs, then proceed with step 2. <u>Step 2</u>. Determine DT50 of the protein in three soils according to OECD 307: aerobic and anaerobic transformation in soil. The following questions should also be answered: • Data on natural background levels of the protein. It should be realised that the proteins not necessarily occur in the soil naturally. • Does the concentration of the protein/metabolites decline to natural background levels and in what stage of the crop? Or are accumulated plateau concentrations reached? If still present after harvest at concentrations above the natural background level, the soil has to be sampled at regular intervals. <u>Step 3</u>. Determine the concentration of the protein in the rhizosphere soil at regular intervals during the growth of the GM crop. A normal crop should serve as a control. This leads to a concentration of the protein/kg soil for each GM plant stage. This information is necessary for ecotoxicity testing of soil (micro)organisms. [2]: The concentration of an applied product expressed in mg/kg is calculated by assuming 1500 kg soil/m^3 and distribution of the applied product within the upper 5 cm of the soil.
all plant stages stage 1 less relevant	<u>Sorption and mobility:</u> The possible spread of the protein in relevant environmental compartments has to be evaluated, unless it can be justified that exposure of the particular environmental compartments to the protein is unlikely to occur. Tests should be performed in at least three soil types.	No test is necessary.

	One possible situation should be evaluated: Mobility of the protein after excretion/leakage from leaves/roots/ left over material. As EPSP synthase does not have a target in the soil, this data requirement is not considered to be necessary.	
all plant stages stage 1 less relevant	Degradation in water: Yes, information on degradation in water should be presented for the protein being the active ingredient of the formulation. The protein can reach the water through drift **(carrier seed/pollen or through the air)** or via run-off and drainage. . Information is not required when exposure of the surface water is not expected.	*Fourth stage re-evaluation:* What are the expected quantities of the protein in water after drainage, run-off or a burst of pollen/seeds? These estimated concentrations have to be used for aquatic tests. In a first simple laboratory test in which seed/pollen is brought into water, the presence of the protein in water can be determined. In case the protein cannot be measured or the DT50 of the protein is very short, aquatic tests need not be performed. *91/414/EC:* An aquatic photolysis and hydrolysis study are commonly used for chemicals: ➢ OECD 316: phototransformation of chemicals in water – direct photolysis. ➢ OECD 111: photolysis as a function of pH. ➢ OECD 308: water-sediment study.
3, 4, 5, 6, 7ac, 8 not 1 and 2	Degradation in air: The protein can only reach the air though volatilisation from leaves, seeds, fruits and left over material. Volatilisation is however not possible for a relatively large molecule. This route is however not to be evaluated under this data requirement. No data requirement is necessary for degradation in air.	*Fourth stage re-evaluation:* Tests are not necessary. *91/414/EC:* Tests are not necessary.

1 seed at planting
2 tuber
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees
8 left over material

3.2 Data requirements for ecotoxicology

Table 4. General template: data requirement 8.1.
Effects on birds and mammals (8.1.1, 8.1.2 and 8.1.3)

	Protein	**Tests specifically for the protein**
	Yes, data requirement for the protein being the active ingredient of the formulation unless it can be proven that birds and mammals are not exposed to the protein.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to birds and mammals. *91/414/EC:* • An acute oral toxicity test deriving an LD50 is minimally required for one bird species. Studies testing chemicals are usually performed with *Colinus virginianus, Anas platyrhynchos* or *Coturnix coturnix japonica.* • A subacute oral toxicity test deriving an LC50 is also required for one bird species (in the near future this test will no longer be necessary). • A (semi)chronic oral toxicity test deriving a NOEC needs to be performed when exposure takes place during the breeding season or when the exposure is expected to be repetitive. For mammals, the data from the toxicological dossier can be used. TESTS: ➢ OPPTS 850.200 Avian Acute Oral Toxicity Test (OPPTS, 1996). ➢ Avian dietary toxicity (5-day) test in a quail species or in mallard duck: OECD 205 Avian Dietary Toxicity Test. ➢ OECD 206 Avian Reproduction Test.
plant stage	**GM plant expressing the protein**	**Tests specifically for the protein**
1	Yes, data requirement for the protein expressed by this GM plant stage unless it can be proven that birds and mammals do not feed from planted seeds and are thus not exposed.	Before testing birds it is necessary to know whether the protein is expressed in seeds. Only if this is the case are studies are necessary. *Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that **the protein expressed by the seeds** is not toxic to birds and mammals. *91/414/EC:* • An acute test should be performed with *Colinus virginianus, Anas platyrhynchos* or *Coturnix coturnix japonica.* • Chronic tests are required when exposure is chronic or repetitive. However, since 'seeds at sowing' are only available to birds and mammals during a very short period, chronic exposure is not actual.

		For the tests the protein is incorporated into the feed. It should be taken into consideration that the assimilation efficiency from plants could be different from that from feed. In other words: less or more protein could become available to the bird/mammal when incorporated in feed than in plants.
2	Yes, data requirement for the protein expressed by this GM plant stage unless it can be proven that birds and mammals do not feed from tubers and are thus not exposed.	Before testing birds and mammals it is necessary to know whether the protein is expressed by the tubers. Only if this is the case are studies are necessary.
3-4	Yes, data requirement for the protein expressed by these GM plant stages unless it can be proven that birds and mammals do not feed from seedlings and young plants and thus are not exposed.	Before testing birds it is necessary to know whether the protein is expressed by seedlings and young plants. Only if this is the case are studies necessary. Further similar to stage 1 (seeds at sowing).
5	Yes, data requirement for the protein expressed by the mature GM plant stage unless it can be proven that birds and mammals do not feed from the mature plant stage and thus are not exposed.	Before testing birds it is necessary to know whether the protein is expressed by mature plants. Only if this is the case are studies necessary. *91/414/EC:* • An acute test should be performed with *Colinus virginianus, Anas platyrhynchos* or *Coturnix coturnix japonica.* • Chronic tests are required when exposure is chronic or repetitive. This may well be the case for mature plants. For the tests the protein is incorporated into the feed. It should be taken into consideration that the assimilation efficiency from plants could be different from that from feed. In other words: less or more protein could become available to the bird/mammal when incorporated in feed than in plants.
6	Similar to plant stage 5.	Similar to plant stage 5.
7	Yes, data requirement for the protein expressed by these GM plant stages unless it can be proven that birds and mammals do not feed from seeds, nuts and fruits and are thus not exposed.	Before testing birds it is necessary to know whether the protein is expressed by seeds, nuts and fruits. Only if this is the case studies are necessary. Further similar to stage 1 (seeds at sowing).
8	Yes, data requirement for the protein expressed by these GM plant stages unless it can be proven that birds and mammals do not feed from left-over material and are thus not exposed.	Before testing birds it is necessary to know whether the protein is expressed by left-over material. Only if this is the case are studies necessary. Further similar to stage 1 (seeds at sowing).

1 seed at sowing

2 tuber at planting

3 seedling

4 young plant

5 mature plant until flowering

6 flowering plant

7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees

8 left over material

Table 5. General template: data requirement 8.2.

Effects on fish (8.2.1, 8.2.2, 8.2.3, 8.2.4)

Effects on Daphnia magna (8.2.5, 8.2.6)

Effects on algal growth (8.2.7)

	Protein	**Tests specifically for the protein**
	Yes, data requirement for the protein being the active ingredient of the formulation unless it can be proven that aquatic organisms are not exposed to the protein. Exposure may occur through drift, runoff and drainage, resulting in concentrations of the protein in the surface water.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to aquatic organisms. *91/414/EC:* An assessment of toxicity is necessary, unless it can be justified that fish will not be exposed. TESTS Acute test are minimally required. Chronic test are necessary when exposure is chronic or repetitive. This also depends on the identity of the protein and its fate and behaviour in the exposed compartment. The solubility of the protein in water has to indicate whether aquatic tests are feasible. **Acute toxicity to fish:** ➢ OECD 203 Fish, Acute Toxicity Test. ➢ C. 1 acute toxicity for fish. **Chronic toxicity to fish:** ➢ OECD 204 Fish, Prolonged Toxicity Test: 14-Day Study. **Fish reproduction and growth rate:** ➢ OECD 229 Fish Short-Term Reproduction Assay. ➢ C. 14 Fish juvenile growth test. **Bioaccumulation** A test for bioaccumulation is only necessary when the log Kow >3. Proteins can be expected to be biodegradable and bioaccumulation testing seems inapplicable to proteins. Moreover, proteins are expected to be too large to pass the cell membrane. **Acute toxicity to invertebrate aquatic organisms:** acute toxicity (24 and 48-hour) for *Daphnia* preferably (*Daphnia magna*): ➢ OECD 202 Daphnia sp. Acute Immobilisation Test and Reproduction Test. ➢ C.2 Acute toxicity for *Daphnia.* ➢ **Chronic toxicity to invertebrate aquatic organisms:** ➢ OECD 211 *Daphnia magna* Reproduction Test. ➢ C. 20 *Daphnia* reproduction test. **Effects on algal growth:** ➢ OECD 201 Alga, Growth Inhibition Test. ➢ C.3 Algal inhibition test. **Effects on aquatic plants:** Tests only have to be performed when the protein has an herbicidal

		action. ➢ OECD 221 *Lemna* sp. Growth Inhibition Test. Aquatic OECD tests were designed to be performed with relatively stable substances.
plant stage	**GM plant expressing the protein**	**Tests specifically for the GM plant**
1 and 2	No data requirement, as exposure to aquatic organisms is considered minimal relative to other plant stages since the volume of seeds and tubers is less than the volume of a (mature) plant. Further, proteins expressed by these plant stages are unlikely to reach the surface water.	**Tests are not necessary.**
6, 7a	Yes, data requirement. Certain seeds and pollen can reach water carried by the wind. Once in the surface water, the protein can be excreted or leaks from these plant stages. No data requirement when it can be proven that seeds and pollen do not reach the water.	*Fourth stage re-evaluation:* Exposure of aquatic organisms is not possible when seeds are too heavy to be transported through the air but remain on the soil and when pollen is not spread by the wind (no wind pollinators). A waiver may be presented. *91/414/EC:* For seeds and pollen that reach the water (depending on the crop species) the theoretical quantity should be calculated. The next step should be that possible protein release into the water should be estimated. If this is not possible, tests with fish, *Daphnia magna* and algae should be performed. Test with aquatic plants should be performed when the protein has an herbicidal action. It should be questioned whether chronic tests are necessary. Are seeds released in one burst or in a longer period? If released in one burst, it can be questioned whether chronic testing is necessary. The exposure is possibly only acute. This question should be answered in relation with the data requirement on persistence in water. Tests are similar to those described above.
3-4 7bcd 8	The protein can be excreted or leaks from these plant stages. The protein may reach the water through runoff and drainage. Dispersal through the air is not applicable to any of these stages of the GM plant.	*Fourth stage re-evaluation:* Degradation and mobility of the protein in soil should be taken into consideration (see data requirements 7). If these are negligible, a test does not need to be performed. In case the protein does reach the surface water, a waiver can be submitted when it can be proven with data from the literature that the protein is not toxic to aquatic organisms. To underbuild the waiver, the theoretical quantity of the released protein in the surface water can be estimated. *91/414/EC:* Degradation and mobility of the protein in soil should be taken into consideration (see data requirements 7). If degradation is short and mobility is weak, a test does not need to be performed. Otherwise acute and chronic tests with fish species should be

		performed. It should be questioned whether chronic tests are necessary. Are seeds released in one burst or in a longer period? If released in one burst it can be questioned whether a chronic test is necessary, as the exposure is possibly only acute. This question should be answered in relation with the data requirement on degradation in water.
5	Data requirement, as proteins leak from the dying roots and foliage.	Similar to stage 3-4, 7bcd, 8.

1 seed at sowing
2 tuber at planting
3 seedling
4 young plant
5 older plant until flowering
6 flowering plant (pollen)
7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees
8 left over material

Table 6. General template: data requirement 8.3.1. Effects on bees

	Protein	**Tests specifically for the protein**
	Yes, data requirement. The toxicity tests are necessary for the protein unless it can be proven that bees are not exposed to the protein in the intended crop.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to bees. *91/414/EC:* Contact and oral tests are necessary. **Acute toxicity to bees:** ➢ OECD 214 Honeybees, Acute Contact Toxicity Test. ➢ OECD 213 Honeybees, Acute Oral Toxicity Test. ➢ EPPO guideline 170 ((EPPO, 2001). ➢ C. 16 Honeybees – acute oral toxicity test. ➢ C. 17 Honeybees – acute contact toxicity test.
plant stage	**GM plant expressing the protein**	**Tests specifically for the GM plant**
1	No data requirement. Bees do not come into contact with seeds.	Tests are not necessary.
2	No data requirement. Bees do not come into contact with tubers.	Tests are not necessary.
3	No data requirement. Bees do not come into contact with seedlings.	Tests are not necessary.
4	No data requirement. Bees do not feed from young plants.	Tests are not necessary.
5	No data requirement. Bees do not feed from larger plants until flowering.	Tests are not necessary.
6	Yes data requirement: Bees consume nectar and pollen from flowering plants.	Before performing tests with bees, it should become clear whether the protein is indeed expressed in pollen and nectar. If not, tests are not deemed necessary. *Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant

		information is available in the literature or from practical experience showing that the protein is not toxic to bees. *91/414/EC:* Contact and oral tests in case the protein is present in pollen and nectar.
7a-d	No data requirement. Bees do not feed from seed, tubers, fruits and nuts.	-
8	No data requirement. Bees do not feed from left over materials on the field.	-

1 seed at sowing
2 tuber at planting
3 seedling
4 young plant
5 older plant until flowering
6 flowering plant
7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees
8 left over material

Table 7. General template: data requirement 8.3.2. Effects on terrestrial arthropods other than bees

	Protein	**Tests specifically for the protein**
	Yes, data requirement. Information on toxicity to arthropods other than bees must be reported for the use of the protein being the active ingredient of the formulation.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to beneficial arthropods. *91/414/EC:* Laboratory tests with *Aphidius rhopalosiphi* and *Typhlodromus pyri* will be requested in the first tier. If there is a risk, extended laboratory tests will be conducted with leaf dwelling species such as *Orius laevigatus, Chrysoperla carnea, Coccinella septempunctata* and *Aleochara bilineata* and soil dwelling species such as *Poecilus cupreus*. If there is a risk in the second tier, field tests are requested. **Acute toxicity for other beneficial arthropods:** ➢ Tests for the first tier for *Typhlodromus* and *Aphidius*. ➢ Test for *Typhlodromus pyri* (Blümel et al., 2000a). ➢ Test for the aphid specific parasitic wasp *Aphidius rhopalosiphi* (Mead-Briggs et al., 2000). ➢ Test for *Typhlodromus* and *Aphidius* (Grimm et al., 2001). **Tests for extended laboratory tests:** ➢ Test for *Aphidius rhopalosphi* (Mead-Briggs et al., 2009). ➢ Test for *Coccinella septempunctata* (Schmuck et al., 2000). ➢ Test for *Chrysoperla carnea* (Vogt et al., 2000). ➢ Test for spiders (*Pardosa* spec.) (Heimbach et al., 2000b). ➢ Test for the parasitic wasp *Trichogramma cacoeciae* (Hassan et al., 2000). ➢ EPPO guideline for *Encarsia formosa* (EPPO, 1989). ➢ EPPO guideline for *Phytoseiulus persimilis* (EPPO, 1992). ➢ EPPO guideline for *Trichogramma cacoeciae* (EPPO, 1993).

		Chronic toxicity tests: ➢ Chronic tests (laboratory and extended) for the rove beetle *Aleochara bilineata* (Grimm et al., 2000). ➢ OECD 226 Predatory mite (*Hypoaspis (Geolaelps) aculeifer*) reproduction in soil. **(Semi)-field tests:** ➢ Test for the parasitic wasp *Aphidius rhopalosiphi* (Moll et al., 2002). ➢ Test for phytoseiid mites (Candolfi et al., 2000). ➢ Field test for predatory mites (Blümel et al., 2000b). ➢ Field test in vineyards for *Typhlodromus pyri* (Boller et al., 1988). ➢ Field test for the beetle *Poecilus cupreus* Heimbach et al., 2000a). **Test guidelines (also describing tests)** Test guidelines of Candolfi et al., 2000 (Candolfi et al., 2000). Test guidelines of Candolfi et al., 2001 (Candolfi et al., 2001). Test guidelines of Barrett et al., 1994 (SETAC/ESCORT) (1994). A test for detrivores is available in the form of a test for Collemba, the mite *Hypoaspis* and Enchtraeids. According to the test sequence with regard to soil organisms for persistent substances a test only needs to be performed when the triggers for the standard arthropods tests are met. ➢ OECD 232 Collembolan Reproduction Test in Soil. ➢ ISO method 11267 (1999) Collembola Test. ➢ OECD 220 Enchytraeid Reproduction Test. ➢ Test with the gamasid mite *Hypoaspis aculeifer* (Bakker et al., 2003).
plant stage	**GM plant expressing the protein**	**Tests specifically for the GM plant**
3, 4, 5, 6, 7	Non-target beneficial arthropods[1] like parasitising wasps, predatory mites and ladybirds may be indirectly exposed to the protein by parasitising/feeding from leaf eating and leaf sucking insects.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to beneficial arthropods. Target insects like aphids are continuously feeding from GM plants, ingesting the protein continuously during their lives. Beneficials that feed from these target insects can therefore be exposed chronically. Therefore, chronic tests need to be performed as well.
1	Indirect exposure via seed eating insects is not a worst case scenario	
8	Yes, data requirement for leaf litter eating arthropods (detrivores) but only when the triggers for the standard arthropod tests are met.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to detrivores. *91/414/EC:* A test for detrivores is available in the form of a test for Collemba, the mite *Hypoaspis* and Enchytraeids. ➢ OECD 232 Collembolan Reproduction Test in Soil. ➢ ISO method 11267 (1999) Collembola Test. ➢ OECD 220 Enchytraeid Reproduction Test. ➢ Test with the gamasid mite *Hypoaspis aculeifer* (Bakker et al., 2003). Apart from acute tests, chronic test should be performed as well as exposure is continuous.

1 seed at sowing
2 tuber at planting
3 seedling
4 young plant
5 older plant until flowering
6 flowering plant
7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees
8 left over material

Table 8. General template: data requirement 8.3.2. Toxicity to earthworms

	Protein	**Tests specifically for the protein**
	Yes, data requirement. Earthworms may directly feed on organic material that is exposed to the protein. Information on toxicity to earthworms must be reported.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to earthworms. *91/414/EC:* Acute tests are requested. If persistence is triggered (DT90 ≤100 d and ≤ 3 applications per season) sublethal tests have to be performed. **Toxicity to earthworms** ➢ OECD 207 Earthworm, Acute Toxicity Tests. ➢ ISO 11268-1, Earthworm acute toxicity test (ISO, 1993). ➢ C.8 Toxicity for earthworm: artificial soil test. ➢ ISO 11268-2, Earthworm chronic toxicity test (ISO, 1998). ➢ OECD 222 Earthworm Reproduction test (*Eisenia fetida/Eisenia andrei*).
plant stage	**GM plant expressing the protein**	**Tests specifically for the GM plant**
1	No data requirement. Not a relevant plant stage. Earthworms do not feed from seeds**.** They do actively drag leaves into their burrow. It is possible that seeds adhere to leaves. However, this will only occur occasionally and it is therefore not necessary to fulfil this data requirement.	Tests are not necessary.
2-7	Yes, data requirement. Earthworms may directly feed from decomposing roots and leaves in which the protein is assumed still to be present or they may feed from material in which excretions of the protein have been diffused. Information on toxicity to earthworms must be reported.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to earthworms. *91/414/EC:* Acute tests are requested. If persistence is triggered (DT90 ≤100 d and ≤ 3 applications per season) sublethal tests have to be performed. Tests are the same as given above. In the case of GM plants, the exposure to earthworms can be considered to be chronic, as the protein is assumed to be produced continuously and excretions occur during the whole growing season. It is not necessary to discern between the different plant stages 2-7, as

		the excretions are continuous.
8	Yes, data requirement. Earthworms may directly feed from left over decomposing roots and leaves in which the protein is assumed still to be present or they may feed from material in which excretions of the protein have been diffused. Information on toxicity to earthworms must be reported.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to earthworms. *91/414/EC:* Acute tests are requested. If persistence is triggered (DT90 ≤100 d and ≤ 3 applications per season) sublethal tests have to be performed. In the case of GM plants, the exposure to earthworms can be considered to be chronic, as the protein is assumed to be present in the left over material for a longer period of time or will leak from the left over material. This last stage is probably the worst case for earthworms, as the whole root system starts to decompose after harvest. Conclusion: only stage 8 needs to be considered for risk assessment.

1 seed at sowing
2 tuber at planting
3 seedling
4 young plant
5 older plant until flowering
6 flowering plant
7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees
8 left over material

Table 9. General template: data requirement 8.3.3. Effects on non-target micro-organisms in the soil

	Protein	**Tests specifically for the protein**
	Yes, data requirement. Non-target micro-organisms may be exposed after applications to the crop and the bare soil. The protein may also be applied as a seed dressing. When it can be proven that there is no exposure, a test is not necessary.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to non-target micro-organisms in the soil. If the protein naturally occurs in the soil and the application rate is well below the approximate number of naturally occurring fungi in the soil, the risk to non-target soil-micro-organisms is low. A waiver can be submitted. *91/414/EC:* TESTS: **Effects on soil non-target micro-organisms** ➢ OECD 216 Soil Micro-organisms, Nitrogen Transformation Test. ➢ OECD 217 Soil Micro-organisms, Carbon Transformation Test. ➢ C.21 Soil micro-organisms: nitrogen transformation test. ➢ C.22 Soil micro-organisms: carbon transformation test. Nitrification and respiration tests such as requested for chemicals are never used for testing proteins in monographs.

		Tests are chosen on a case by case basis. Possible tests are studies to determine enzyme activity. The effect on the growth of non-target fungi and bacteria can also be determined, expressed in CFU/g soil dw. The non-target organism can be the total fungi/bacteria, a specific species such as *Pseudomonas* or a specific mycorrhiza.
plant stage	**GM plant expressing the protein**	**Tests specifically for the GM plant**
1-8	Yes, data requirement. Micro-organisms contain chitin and can be affected by the protein. Proteins can be excreted from seeds and roots of all plant stages. Excretion from the seeds is expected to be much lower than from the roots.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to non-target micro-organisms in the soil. *91/414/EC:* In the first instance, it should become clear whether the protein is excreted by the roots of the different plant stages. If so, the concentrations of the protein will probably exceed the background concentration. Therefore, a test must be submitted. Possible tests are: • enzyme activity tests; • culture plate tests to determine the effect on the growth of non-target fungi and bacteria, expressed in CFU/g soil dw; • nitrification and respiration tests.

1 seed at sowing
2 tuber at planting
3 seedling
4 young plant
5 older plant until flowering
6 flowering plant
7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees
8 left over material

Table 10. General template: data requirement 8.3. Effects on terrestrial plants
8.3.4 Effects on terrestrial plants

	Protein	**Tests specifically for the protein**
	Yes, data requirement, but only if the mode of action of the protein is herbicidal. Information on effects on plants should be presented for the protein being the active ingredient of the formulation as plants (wild or agricultural plants) in neighbouring fields can be exposed through drift of the aerial applications.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to plants. *91/414/EC:* Plant tests are not required in the European Commission. Tests are only needed in case of herbicides. Following 91/414/EC, an emergence and a vegetative vigour test are requested. These tests could be optional for proteins as well. ➢ OECD 208 Terrestrial Plant Test: Seedling

		Emergence and Seedling Growth Test. ➢ OECD 227 Terrestrial Plant Test: Vegetative Vigour Test.
plant stage	**GM plant expressing the protein**	**Tests specifically for the GM plant**
6	Yes, pollen excreting the protein may reach other plants through pollination by bees and other arthropods or through wind pollination.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that the protein is not toxic to plants. *91/414/EC:* OECD 208 is not considered to be feasible, as applications are made to the bare soil. With the attained knowledge on degradation in the soil of the protein, exposure to emerging seedlings is considered to be negligible. OECD 227, the Vegetative Vigour Test is possible with pollen collected from the GM plant. The test should be slightly adapted, as the expected exposure is via the air under dry conditions. Applications should be made with a dry powder formulation containing pollen.
1, 2, 3, 4, 5, 7, 8	All other plant stages have no possibilities to have effects on other plants.	*Fourth stage re-evaluation:* No tests are required, as exposure is not expected. *91/414/EC:* No tests are required, as exposure is not expected.

1 seed at sowing

2 tuber at planting

3 seedling

4 young plant

5 older plant until flowering

6 flowering plant

7a-d seed and tuber/thick root forming plants, fruit and nuts producing plants/trees

8 left over material

4 Elaboration of the three cases

4.1 Chitinase expressed by sugar beet

Physical chemical characteristics
Chitinases are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitin.

Producers of chitinase
Chitinase is produced in nature by chitin-containing organisms like insects, crustaceans and fungi, but also by a variety of bacteria and higher plants (Watanabe et al., 1992).
Several chitinases have been characterised: endochitinases, exochitinases (EC 3.2.1.14), β-N-acetylglucosaminidases and chitobiases (EC 3.2.1.30) (Flach et al., 1992).

Function
Chitinases degrade chitin (a polysaccharide), a structural component in the cell walls of most fungi. Chitin also occurs in various invertebrates (e.g., insects, arachnaea), where it serves a structural function similar to that of cellulose in plants. Some chitinases also display a more or less pronounced lysozyme activity (Flach et al., 1992).
Watanabe (1992) describes several roles of chitinases. The role of chitinases found in fungi, crustaceans and insects is the modification of the organism's chitin. In insects, for example, the old cuticle containing chitin is reabsorbed with the moulting fluid (Flach et al., 1992).
Bacteria produce chitinase to be able to utilise chitin as a source of carbon and energy. Plants are thought to produce chitinase as part of their defence mechanism against fungal pathogens. The chitin of these pathogens is degraded by the plant's chitinases.

The function of chitinase in this report is linked to the bacterium *Bacillus circulans* WL-12. *Bacillus circulans* WL-12, isolated as a yeast cell wall-lytic bacterium, secretes a variety of polysaccharide-degrading enzymes in the culture medium (Watanabe et al., 1990). Six distinct chitinase molecules were detected in the culture supernatant (A1, A2, B1, B2, C, and D). It was concluded that chitinase A1 is the key enzyme in the chitinase system of this bacterium.

Targets
Targets of chitinases produced by *Bacillus circulans* WL-12 are chitin-containing organisms like insects and fungi.

This study
In this study, we focus on chitinase as produced by *Bacillus subtilus* WL-12. For chitinase, the data requirements for an application of the non-living chitinase in sugar beet will be addressed. Necessary tests will be evaluated for the transgenic sugar beet. For sugar beet, not all plant stages that were defined for the imaginary plant in Table 3 are relevant. For instance, there is no flowering stage in one-year old sugar beet. The following stages are considered:

1 seed at sowing
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

4.1.1 Data requirements for fate and behaviour

Table 11. Chitinase: data requirement 7.1. Fate and behaviour in the environment

	Chitinase	**Tests specifically for chitinase**
	In general, the properties of the protein are the basis for the evaluation of fate and behaviour in the environment (soil, water and air).	A waiver can be presented if relevant information is available in the literature or from practical experience. Otherwise, tests have to be presented on fate and behaviour.
.	Degradation in soil: Conform general template.	Questions to be answered: • Is chitinase naturally present in the soil? (Yes, other chitinases are being produced by soil bacteria). • If yes, what are the natural background levels of the chitinase? • Is it necessary that the test can differentiate between different types of chitinase? *Fourth stage re-evaluation:* Conform general template. *91/414/EC:* Conform general template.
	Sorption and mobility: Conform general template.	*Fourth stage re-evaluation:* Conform general template. *91/414/EC:* Conform general template.
	Degradation in water: Conform general template.	*Fourth stage re-evaluation:* Conform general template. *91/414/EC:* Conform general template.
	Degradation in air: The molecule chitinase is too large to possibly volatilise into the air.	*Fourth stage re-evaluation:* Tests are not necessary. *91/414/EC:* Tests are not necessary.
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for the GM sugar beet**
	Degradation in the plant: Conform general template.	Conform general template.
3-8	Degradation in soil: Conform general template.	Conform general template.

stage 1 less relevant		
3, 4, 5, 7b, 8 stage 1 less relevant	Sorption and mobility: Conform general template.	Conform general template.
all plant stages stage 1 less relevant	Degradation in water: Chitinase can reach the water through drift (carrier seed/pollen or through the air) or via run-off and drainage. Conform general template.	Conform general template.
3, 4, 5, 7b, 8 stage 1 less relevant	Degradation in air: The molecule chitinase is too large to possibly volatilise into the air.	Conform general template.

1: The criterion of 100 days is derived from the uniform principles of chemicals.

1 seed at sowing
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

4.1.2 Data requirements for ecotoxicology

Table 12. Chitinase: data requirement 8.1 (8.1.1, 8.1.2 and 8.1.3). Effects on birds and mammals

	Chitinase	**Tests specifically for chitinase**
	Yes, data requirement although the mode of action of chitinase is fungicidal.	**Tests are necessary.** **Conform general template.**
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for the GM sugar beet**
1-8	Yes, data requirement although the mode of action of chitinase is insecticidal.	Tests are necessary. Conform general template.

1 seed at sowing
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

Table 13. Chitinase: data requirement 8.2.
Effects on fish (8.2.1, 8.2.2, 8.2.3, 8.2.4)
Effects on *Daphnia magna* (8.2.5, 8.2.6)
Effects on algal growth (8.2.7)

	Chitinase	**Tests specifically for chitinase**
	Yes, data requirement although the mode of action of chitinase is fungicidal. Conform general template.	Tests are necessary. Conform general template.
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for GM sugar beet**
All stages	Yes, data requirement although the mode of action of chitinase is fungicidal. From none of the plant stages is spread of chitinase to the water surface expected. The most likely way of transport is by air through wind pollination, but sugar beet does not have a flowering stage. It should however be confirmed by fate tests that chitinase is not mobile and that the route via drainage is therefore not expected.	*Fourth stage re-evaluation:* No tests are required. A waiver can be submitted showing that the action of chitinase is solely fungicidal. *91/414/EC:* Tests are not necessary, since there is no exposure is expected.

1 seed at sowing
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

Table 14. Chitinase: data requirement 8.3.1. Effects on bees

	Chitinase	**Tests specifically for chitinase**
	Conform general template. In addition: The exoskeleton of insects is made of chitin. Contact or ingestion of chitinase may conflict with chitin metabolism.	Conform general template. In addition: Normally, oral tests are performed by adding the active substance to sugar water. It should be tested whether it is possible to prepare a solution with the protein. In the case that the properties of the protein change in the sugar solution or when the protein sticks to the wall of the feeding tube, another way of oral feeding should be sought.
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for the GM sugar beet**
1, 3, 4, 5, 8	Conform general template.	Conform general template.
7a-d	Conform general template.	Conform general template.

1 seed at sowing
3 seedling
4 young plant

5 mature plant
7b thick root forming plants
8 left over material

Table 15. Chitinase: data requirement 8.3.1. Effects on terrestrial arthropods other than bees

	Chitinase	**Tests specifically for chitinase**
	Conform general template. In addition: The exoskeleton of insects is made of chitin. Contact or ingestion of chitinase may conflict with chitin metabolism. Beneficial non-target arthropods do not directly feed on the sugar beet but may indirectly suffer from adverse effects through tri-trophic interactions: beneficial non-target arthropods feeding on or parasitising insects that feed from chitinase treated sugar beets. Information on toxicity of chitinase to non-target arthropods other than bees must be reported. the key question is whether chitinase when consumed by the prey/host, can effect the beneficial non-target insects.	Conform general template.
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for the GM sugar beet**
1	Conform general template.	Conform general template.
1, 3, 4, 5, 7b	Conform general template. In addition: The plant stages of most concern are the young plant and the thick root forming plants, as prey populations as well as the populations of the non-targets only start to build up from the seedling stage. The key question is whether chitinase when consumed by the prey/host, can effect the beneficial non-target insects. [1]These species are tested for the risk assessment of chemical pesticides.	Conform general template. In addition: Insects like aphids are continuously feeding from GM sugar beets, ingesting chitinase during their lives. Beneficials that feed from these insects can therefore be exposed chronically. Therefore, chronic tests need to be performed as well.
8	Same as in template.	Same as in template.

1 seed at sowing
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

Table 16. Chitinase: template: data requirement 8.3.2. Toxicity to earthworms

	Chitinase	**Tests specifically for chitinase**
	Yes, data requirement. Earthworms may directly feed on organic material that is exposed to chitinase, although the mode of action of chitinase is fungicidal	*Fourth stage:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that chitinase is not toxic to earthworms, as the mode of action is fungicidal. *91/414/EC:* Conform general template.
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for the GM sugar beet**
1, 3, 4, 5, 7b, 8	Yes, data requirement. Earthworms may directly feed on organic material that is exposed to chitinase, although the mode of action of chitinase is fungicidal.	*Fourth stage:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that chitinase is not toxic to earthworms, as the mode of action is fungicidal. *91/414/EC:* Studies conform template.

1 seed
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

Table 17. Chitinase: data requirement 8.3.3. Effects on non-target micro-organisms in the soil

	Chitinase	**Tests specifically for chitinase**
	Yes, data requirement. Non-target micro-organisms may be exposed after applications to the crop and the bare soil. Chitinase may also be applied as a seed dressing. When it can be proven that there is no exposure, a test is not necessary. In addition: **Micro-organisms contain chitin and can be affected by chitinase.**	Conform general template.
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for the GM sugar beet**
1	No data requirement. The possible excretion of chitinase is much smaller than in the older plant stages.	At first, it should be clear whether chitinase is excreted by the seeds. If not, micro-organisms will not be exposed to chitinase and a waiver can be submitted. Otherwise, tests are necessary like those proposed for the protein.
3, 4,	Yes, data requirement. Micro-organisms	*Fourth stage re-evaluation:*

7b, 8	contain chitin and can be affected by chitinase released (actively or passively) by roots or passively by left over material.	In the first instance, it should become clear whether chitinase is excreted by the different plant stages. If not, micro-organisms will not be exposed to chitinase and a waiver can be submitted. Otherwise, tests are necessary like those proposed for the protein in the general template. *91/414/EC:* Conform general template.

1 seed at sowing
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

Table 18. Chitinase: data requirement 8.3.4. Effects on terrestrial plants

	Chitinase	**Tests specifically for chitinase**
	No data requirement for chitinase, as the mode of action is not herbicidal (plants do not contain chitin and cannot be affected by chitinase).	Tests are not necessary.
plant stage	**GM sugar beet expressing chitinase**	**Tests specifically for the GM sugar beet**
1-8	No data requirement.	Tests are not necessary.

1 seed at sowing
3 seedling
4 young plant
5 mature plant
7b thick root forming plants
8 left over material

4.2 GNA lectin expressed by potato

Physical chemical characteristics
Lectins are a very heterogeneous class of carbohydrate binding (glycol)proteins, which are capable of reversible binding to at least one specific class of mono- or oligosaccharides (Hogervorst, 2006).

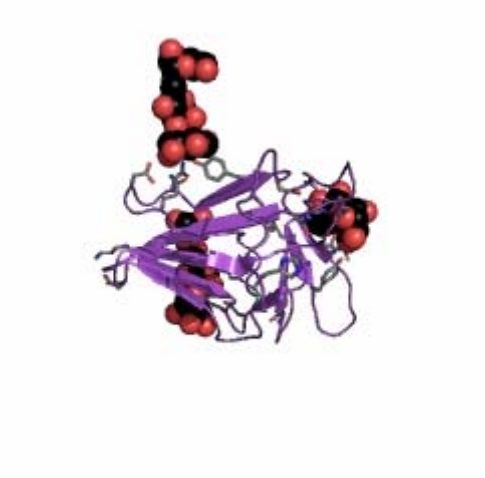

Figure 2. Molecular structure of mannose-specific agglutinin (lectin) from snowdrop (*Galanthus nivalis*) bulbs.

Producers of lectin
Lectins are ubiquitous in plants, animals and micro-organisms. In general, lectins bind to glycoconjugates but there are also chitin-binding lectins (Peumans and Van Damme, 1995).

Function
Plant lectins play a role in the plant itself but also interact with glycoconjugates of other organisms. Among others, lectins are involved with plant defence. The exact mechanism of the action of plant lectins is not known.

Targets
Targets of lectins are all organisms that contain exposed glycoconjugates. Plant lectins play a role in defence against different kinds of plant-eating organisms and are inhibitory to certain fungi (Chrispeels and Raikhel, 1991).

This study
For this study we focus on GNA, snowdrop lectin. The snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) is probably the most widely studied lectin. GM potato plants in which the gene encoding GNA has been engineered show partial resistance against phloem-sucking insects such as aphids (Hemiptera, Sternorrhyncha) (Down et al., 1996; Gatehouse et al., 1996), but also other Hemiptera such as planthoppers and leafhoppers. This indicates that these toxins are translocated within the phloem sap (Kehr, 2006). GNA has a broad range of activity, as it also effects lepidopteran pests, a coleopteran herbivore and root-knot nematodes (see references in Hogervorst, 2006).

Data requirements
For GNA lectin the data requirements for an application of GNA lectin in potato will be addressed. Necessary tests will be evaluated for the transgenic potato.
For potato, not all plant stages defined for the imaginary plant are relevant. The following stages are relevant:

2 tuber
4 young plant
5 older plant until flowering
6 flowering plant
7 ab seed and tuber forming plants
8 left over material

4.2.1 Data requirements for fate and behaviour

Table 19. Template: data requirement 7.1. Fate and behaviour in the environment

	GNA lectin	tests specifically for GNA lectin
	In general, the properties of GNA lectin are the basis for the evaluation of fate and behaviour in the environment (soil, water and air).	
.	Degradation in soil: Conform general template.	Questions to be answered: • Is GNA lectin naturally present in the soil? • If yes, what are the natural background levels? • Is it necessary that the test can differentiate between different forms of the protein (for example, six distinctive molecules of chitinase with different activities are present) *Fourth stage re-evaluation:* A waiver can be presented if relevant information is available in the literature or from practical experience showing that the concentration of the protein is declining to zero. A natural background level is not present in agricultural fields (no snowdrops). It is expected that GNA lectin activity is reduced quickly and degradation into another active metabolite does not occur. If this cannot be proven, tests have to be presented on the degradation of GNA lectin. *91/414/EC:* Conform general template.
	Sorption and mobility: The possible spread of GNA lectin in relevant environmental compartments has to be evaluated, unless it can be justified that exposure of the particular environmental compartments to the	Conform general template.

	protein is unlikely to occur. Tests should be performed in at least three soil types.	
	Degradation in water: Conform general template.	Conform general template.
	Degradation in air: The molecule GNA lectin is too large to possibly volatilise into the air.	*Fourth stage re-evaluation:* Tests are not necessary. *91/414/EC:* Tests are not necessary.
plant stage	**GM potato plant expressing GNA lectin**	**Tests specifically for the GM potato plant**
	Degradation in the plant: Conform general template.	*In general*, the following questions could be posed. The answers are helpful in determining the risks of each of the defined plant stages: 1. Is GNA lectin expressed in each plant stage (for example, only expression in the leaves and not in the seed)? 2. Is degradation of GNA lectin in the plant matrix different from degradation of the protein in the soil? 3. Can the concentration of GNA lectin build up in the plant tissue in case of a continuous production rate but a low degradation rate? 4. Can metabolites be formed as a result of interaction with plant tissue?
2-8	Degradation in soil: Conform general template.	*Fourth stage re-evaluation:* Conform general template. *91/414/EC:* Conform general template. Typical for the GM potato plant: • Possibly continuous excretion of GNA lectin into the soil. • Possibly increasing concentrations of GNA lectin/kg soil as below soil plant mass increases. • It is not possible to determine the excretion of GNA lectin expressed by the GM potato plant in mg/kg soil. For the control agent applied at the plants, it can be expressed in kg product/ha. The knowledge of a concentration expressed in kg/ha or mg/kg soil is essential for choosing a concentration range in ecotoxicity tests. • GNA lectin is not present as a natural background concentration in rural fields (only text in bold differs from general template) TESTS Step 1. Test to determine the presence of GNA lectin excreted/released into soil by several plant stages. If excretion/leakage does not occur, tests

		are not necessary. Step 2. Determine DT50 of the protein in three soils according to OECD 307: aerobic and anaerobic transformation in soil. The following questions should also be answered: • Are accumulated plateau concentrations reached? • Does the concentration of GNA lectin decline to zero and if so, when? • If still present after harvest, soil has to be sampled at regular intervals. Step 3. Determine the concentrations of GNA lectin in rhizosphere soil at regular intervals during growth of the GM potato crop. This gives information on the concentration of GNA lectin/kg soil for each GM potato plant stage. This information is necessary for ecotoxicity testing of soil (micro) organisms.
2-8	Sorption and mobility: Conform general template. In addition, two possible situations should be evaluated: 1. Mobility of GNA lectin after excretion/leakage from leaves/roots/ left over material. 2. Mobility of GNA lectin through a carrier (seed, pollen). If the crop does not produce pollen and seeds, this data requirement does not need to be fulfilled.	Sub 1: in case of excretion to the soil, use adsorption study to derive a K_{om}. In case of excretion to above ground plant parts, GNA lectin will splash to the soil after a shower. This option is already dealt with by performing the adsorption study. If GNA lectin is not mobile on the leaf surface, it will degrade by photolysis. Sub 2: there is no test available to measure the mobility of the protein through carriers. If plant material can drift to neighbouring fields or water surfaces, it is of importance to determine the distance to which the protein can be carried.
2-8	Degradation in water: Conform general template.	Conform general template.
	Degradation in air: Conform general template.	Conform general template.

1: The criterion of 100 days is derived from the uniform principles of chemicals.

2 tuber

4 young plant

5 mature plant until flowering

6 flowering plant

7ab seed and tuber forming plants

8 left over material

4.2.2 Data requirements for ecotoxicology

Table 20. GNA lectin: data requirement 8.1 (8.1.1, 8.1.2 and 8.1.3). Effects on birds

	GNA lectin	**Tests specifically for GNA lectin**
	Yes, data requirement although the mode of action of GNA lectin is insecticidal	*Fourth stage re-evaluation:* As GNA lectin is specific against insects, birds are not at risk and studies are not regarded as necessary. A waiver can be submitted showing that the action of GNA lectin is solely insecticidal. *91/414/EC:* conform template
plant stage	**GM potato plant expressing GNA lectin**	**Tests specifically for the GM potato plant**
3, 4, 5, 7ab, 8	Yes, data requirement although the mode of action of GNA lectin is insecticidal.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that GNA lectin is not toxic to birds because the mode action of GNA lectin is solely insecticidal. *91/414/EC:* Tests conform the template unless it can be proven that birds do not eat any of the plant stages of the potato plant.
2, 6	Tubers are not relevant to birds. Flowering plants are not a worst case plant stage.	

2 tuber
4 young plant
5 mature plant until flowering
6 flowering plant
7ab seed and tuber forming plants
8 left over material

Table 21. GNA lectin: data requirement 8.2.
Effects on fish (8.2.1, 8.2.2, 8.2.3, 8.2.4)
Effects on *Daphnia magna* (8.2.5, 8.2.6)
Effects on algal growth (8.2.7)

	GNA lectin	**Tests specifically for GNA lectin**
	Yes, data requirement for GNA lectin being the active ingredient of the formulation unless it can be proven that aquatic organisms are not exposed to GNA lectin. Exposure may occur through drift, runoff and drainage, resulting in concentrations of chitinase in the surface water.	*Fourth stage re-evaluation:* As GNA lectin is specific against insects; fish are not at risk and studies are not regarded as necessary. A waiver can be submitted showing that the action of GNA lectin is solely insecticidal. *91/414/EC:* Aquatic testing conform general template. Solubility of GNA lectin should be high enough for aquatic testing.
plant stage	**GM potato plant expressing GNA lectin**	**Tests specifically for the GM potato plant**

all relevant stages	Yes, data requirement for GNA lectin being the active ingredient of the formulation unless it can be proven that aquatic organisms are not exposed to GNA lectin. From none of the plant stages is spreading of GNA lectin to the water surface expected. The most likely way of transport is by air through wind pollination, but since the pollen of the potato is mostly spread by insects (Van de Wiel and Lotz, 2004) this is not an option. It should however be confirmed by fate tests that GNA lectin is not mobile and that a route via drainage is therefore not expected.	*Fourth stage re-evaluation:* No tests are required. A waiver can be submitted showing that the action of GNA lectin is solely insecticidal. *91/414/EC:* Tests are not necessary, since there is no exposure is expected.

2 tuber
4 young plant
5 mature plant until flowering
6 flowering plant
7ab seed and tuber forming plants
8 left over material

Table 22. GNA lectin: data requirement 8.3.1. Effects on bees

	GNA lectin	**Tests specifically for GNA lectin**
	Yes data requirement. The toxicity tests are necessary for GNA lectin unless it can be proven that bees are not exposed to GNA lectin in the intended crop. The toxicity of GNA lectin to bees should be tested, as bees may come into contact with flowering potato sprayed with GNA lectin.	*Fourth stage re-evaluation:* Conform general template. *91/414/EC:* Conform general template. Normally, oral tests are performed by adding the active substance to sugar water. It should be tested whether it is possible to prepare a solution with GNA lectin. In the case that the properties of GNA lectin change in the sugar solution or when GNA lectin sticks to the wall of the feeding tube, another way of oral feeding should be sought.
plant stage	**GM potato plant expressing GNA lectin**	**Tests specifically for the GM potato plant**
2	No data requirement. Bees do not come into contact with tubers.	-
3	No data requirement. Bees do not come into contact with seedlings.	-
4	No data requirement. Bees do not feed from young plants.	-
5	No data requirement. Bees do not feed from larger plants until flowering.	-
6	Yes data requirement. Bees feed from flowering plants (pollen and nectar). Toxicity to bees should be tested if GNA lectin is excreted in pollen and nectar.	Conform general template. *91/414/EC:* Contact and oral tests in case GNA lectin is present in pollen and nectar.

		Normally, oral tests are performed by adding the product to sugar water. It should be tested whether it is possible to prepare a solution with GNA lectin. In the case that the properties of GNA lectin change in the sugar solution or when GNA lectin sticks to the wall of the feeding tube, another way of oral feeding should be sought.
7ab	No data requirement. Bees do not feed from seed, tubers, fruits and nuts.	-
8	No data requirement. Bees do not feed from left over materials on the field.	-

2 tuber
4 young plant
5 mature plant until flowering
6 flowering plant
7ab seed and tuber forming plants
8 left over material

Table 23. GNA lectin: data requirement 8.3.2. Effects on terrestrial arthropods other than bees

	GNA lectin	**Tests specifically for GNA lectin**
	Yes, data requirement. Information on toxicity to arthropods other than bees must be reported for the use of GNA lectin, as non-target arthropods are directly exposed to GNA applications or are indirectly exposed by parasitising on hosts or feeding on prey.	Conform general template.
plant stage	**GM potato plant expressing GNA lectin**	**Tests specifically for the GM potato plant**
2	No data requirement. Beneficial non-target insects may find hosts/prey in tubers but it seems to be less worst case in comparison with hosts and prey that can be found on the green parts of the potato plant.	No test required.
4, 5, 6, 7ab	Yes, data requirement as non-target insects can be exposed by direct contact or indirectly by feeding from prey/or parasitisation of hosts.	Leaf: conform the data requirements of 91/414 laboratory tests with *Aphidius rhopalosiphi* and *Typhlodromus pyri* will be requested in the first tier. As non-target insects can be exposed for a long period, extended laboratory tests have to be conducted with leaf dwelling species such as *Orius laevigatus, Chrysoperla carnea, Coccinella septempunctata* and *Aleochara bilineata* and soil dwelling species such as *Poecilus cupreus*. Indeed, sublethal effects were shown in several publications: research by Birch et al. (1999) on the tri-trophic interaction between pest aphids, predatory ladybird and transgenic potatoes showed that no acute toxicity due to GNA lectin plants was observed, although female ladybird longevity was reduced by up to 51%. Direct and indirect sublethal effects were shown for the parasitoid *Aphelinus abdominalis* in the

		tritrophic interaction potato, aphid and parasitoid (Couty et al., 2001)
8	Yes data requirement for leaf litter eating arthropods (detrivores). Only when others are triggered.	A test for detrivores is available in the form of a test for Collemba. See general template.

2 tuber
4 young plant
5 mature plant until flowering
6 flowering plant
7ab seed and tuber forming plants
8 left over material

Table 24. GNA lectin: data requirement 8.3. Effects on other non-target organisms
8.3.2 Toxicity to earthworms
8.3.3 Effects on non-target micro-organisms in the soil

	GNA lectin	**Tests specifically for GNA lectin**
	Yes, data requirement although the mode of action of GNA lectin is insecticidal.	*Fourth stage:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that GNA lectin is not toxic to earthworms, as the mode of action is insecticidal. *91/414/EC:* Studies conform template.
plant stage	**GM potato plant expressing GNA lectin**	**Tests specifically for the GM potato plant**
all relevant stages	**Yes, data requirement although the mode of action of GNA lectin is insecticidal.**	*Fourth stage:* No tests are required. A waiver can be submitted, as the mode of action is insecticidal. *91/414/EC:* Tests as in template.

2 tuber
4 young plant
5 mature plant until flowering
6 flowering plant
7ab seed and tuber forming plants
8 left over material

4.3 EPSP synthase expressed by oilseed rape

Physical chemical characteristics
5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) is one of the enzymes involved in the shikimate pathway. EPSPS catalyses the reaction of shikimate-2-phopshate (S3P) and phopshoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPSPS) and phosphate. The shikimate pathway is involved in the aromatic amino acids biosynthesis of many organisms.

Figure 3. Molecular structure of EPSP synthase.

Producers of EPSP synthase
EPSPS are present in plants, bacteria and fungi but not in animals (Padgette et al., 1995).

Function
EPSPS synthase is part of the shikimate pathway. This pathway is involved in the aromatic amino acids biosynthesis of many organisms. EPSPS in plants is localised in the chloroplasts or plastids.

Targets
EPSPS synthase has no targets.

This study
In this study a mutated form of the EPSPS was used to modify plants, in order to make plants tolerant to the herbicide glyphosate. Glyphosate (N-phosphonomethylglycine), the active ingredient in the herbicides Roundup™ and Touchdown™, is a competitive inhibitor to EPSPS synthase (Figure 2), effectively shutting down aromatic amino acid biosynthesis and also synthesis of other aromatic compounds derived from these amino acids. Glyphosate tolerant crops may therefore be treated with glyphosate to eliminate weeds and other problem plants, while leaving the genetically modified plants unharmed.
Genes expressing mutant EPSP synthase originate from plants that showed resistance against glyphosate. Insensitivity can be caused by an overproduction of the mutant EPSP synthase. This causes a higher competition ability with glyphosate with the PEP binding site. Another possibility is that the mutant EPSP synthase has a higher affinity for the PEP binding site than the natural EPSP synthase. This affinity is also greater than the affinity of glyphosate for the PEP binding site.

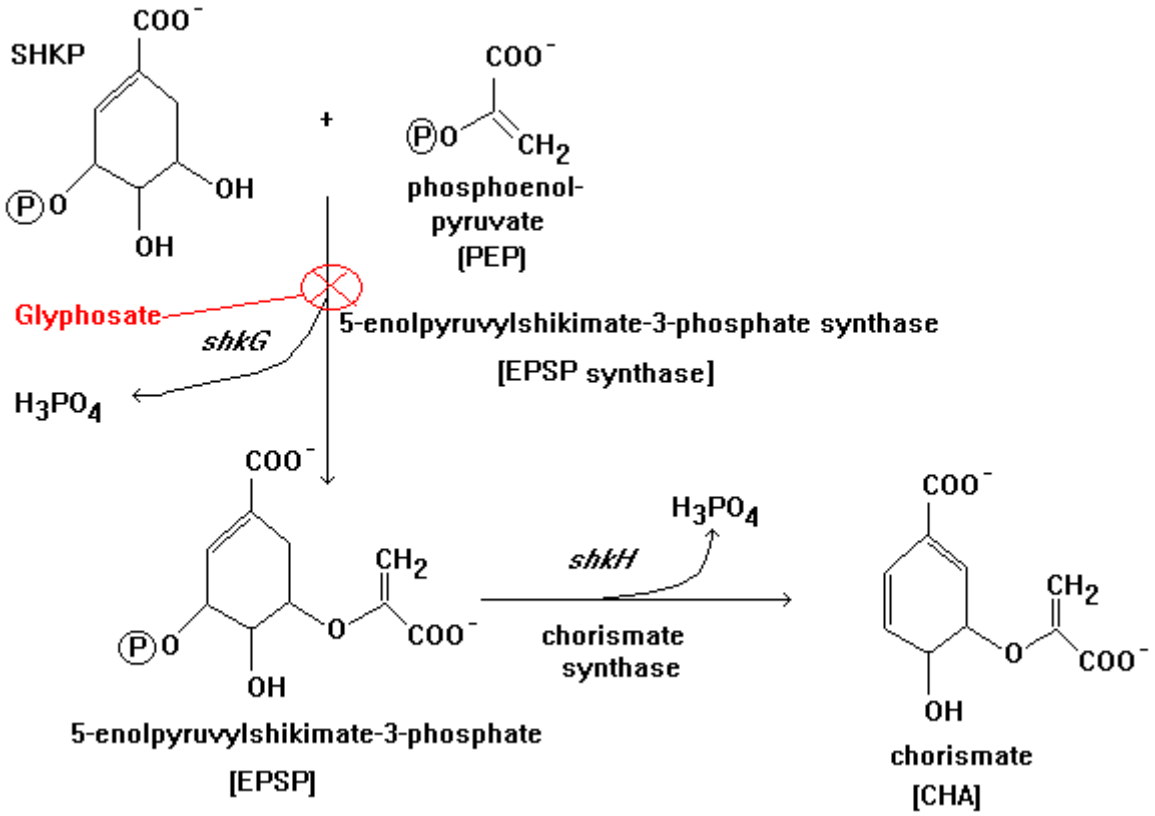

Figure 4. Aromatic amino acid biosynthesis. The shikimate pathway – synthesis of chorismate.

Data requirements

For EPSP synthase the data requirements for an application of mutant EPSP synthase and for mutant EPSP synthase expressed in oilseed rape will be addressed.

A product with mutant EPSP synthase to be sprayed on plants is highly hypothetical. In practice, it is impossible to selectively spray the crop without also exposing the weeds that need to be killed by glyphosate. Moreover, EPSP synthase needs to enter the cells in high enough quantities. This is unlikely to happen when the protein is sprayed on the plant. For sake of the comparison in this report, it is however assumed that mutant EPSP synthase can be sprayed on plants and that the enzyme will enter the chloroplasts of the plant cells. There, in large enough concentrations, it should successfully compete with glyphosate for the binding sites, prohibiting the deregulation of the shikimate pathway by glyphosate.

For oilseed rape, not all plant stages defined for the imaginary plant are relevant. the following stages are relevant:

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

4.3.1 Data requirements for fate and behaviour

Table 25. EPSP synthase: data requirement 7.1. Fate and behaviour in the environment

	EPSP synthase	**Tests specifically for EPSP synthase**
	In general, the properties of ESP synthase are the basis for the evaluation of fate and behaviour in the environment (soil, water and air). EPSP synthase is not a realistic protein to be used in crop protection.	Tests are not necessary.
plant stage	**GM oilseed rape expressing EPSP synthase**	**Tests specifically for the GM oilseed rape**
	Degradation in the plant: Persistence of the protein in the plant is unique for GM plants. In the GM oilseed rape plant, EPSP synthase is continuously formed in the plant during its growth. In the plant tissue, the degradation rate is unknown and subjected to the matrix of the tissue into which it is embedded. Once the protein is excreted or leaks out of the plant, EPSP synthase will be subjected to the outer environment.	Conform general template.
3-8	Degradation in soil: No data requirement since EPSP synthase is locate in the chloroplast and it is assumed that it will not leak from the plant tissue or be excreted actively. Moreover, the soil would be similarly exposed to EPSP synthase if the crop was not genetically transformed. Test requirements need not be fulfilled.	No tests are required.
3-8	Sorption and mobility: Conform general template. The mobility of EPSP synthase should be investigated after excretion/leakage from left over material (after disintegration of chloroplasts therein). Other ways of excretion do not exist, as the EPSP synthase is only present in the chloroplast/plastid. Excretion from leaves is therefore considered non-existent.	Conform general template.
3-8	Degradation in water:	Conform general template.

stage 1 less relevant	The protein can reach the water through run-off and drainage. Yes, information on degradation in water should be presented for EPSP synthase. Studies on biodegradation, hydrolysis and photolysis are requested. Information is not required when exposure of the surface water is not expected.	It seems to be highly unlikely that EPSP synthase reaches the surface water through run-off and drainage. Tests do not need to be performed if it can be argued that this route is unlikely to exist.
	Degradation in air: EPSP synthase can only reach the air though volatilisation from leaves and left over material. Since it not a fumigant and assumedly not a very volatile substance, test requirements need not be fulfilled.	Tests are not necessary.

1: The criterion of 100 days is derived from the uniform principles of chemicals.

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

4.3.2 Data requirements for ecotoxicology

Table 26. EPSP synthase: data requirement 8.1.
Effects on birds (8.1.1, 8.1.2 and 8.1.3)

	EPSP synthase	**Tests specifically for EPSP synthase**
	No data requirement since EPSP synthase cannot effect any pathway in birds. Moreover, applications of EPSP synthase are not realistic.	Tests are not necessary.
plant stage	**GM plant expressing EPSP synthase**	**Tests specifically for the GM oilseed rape**
all relevant stages	No data requirement since EPSP synthase cannot effect any pathway in birds. Moreover, birds are exposed to EPSP synthase by other crops in the same way.	Tests are not necessary.

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

Table 27. EPSP synthase: data requirement 8.2.
Effects on fish (8.2.1, 8.2.2, 8.2.3, 8.2.4)
Effects on Daphnia magna (8.2.5, 8.2.6)
Effects on algal growth (8.2.7)

	EPSP synthase	**Tests specifically for EPSP synthase**
	Mutant EPSP synthase cannot effect any pathway in fish, freshwater invertebrates.	Tests are not necessary.
plant stage	**GM oilseed rape expressing EPSP synthase**	**Tests specifically for the GM oilseed rape**
all relevant stages	No data requirement since EPSP synthase cannot effect any pathway in fish or freshwater invertebrates. Moreover, fish and Daphnia are exposed to EPSP synthase by other crops in the same way.	Tests are not necessary.

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

Table 28. EPSP synthase: data requirement 8.2.7. Effects on algal growth

	EPSP synthase	**Tests specifically for EPSP synthase**
	Yes, data requirement for the EPSP synthase being the active ingredient of the formulation, unless it can be proven that algae and water plants will not be exposed. In the imaginary case that EPSP synthase will be sprayed, it will be in combination with glyphosate. When algae and water plants are exposed to both glyphosate and EPSP synthase, only glyphosate will be toxic. EPSP synthase will (assuming that it enters the chloroplast) only help to prevent the deregulation of the Shikimate pathway.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented if relevant information is available in the literature or from practical experience showing that EPSP synthase is not toxic to aquatic organisms. *91/414/EC:* Tests are not necessary, as EPSP synthase cannot be toxic to algae and water plants.
plant stage	**GM plant expressing EPSP synthase**	**Tests specifically for the GM oilseed rape**
all relevant stages	No data requirement since mutant EPSP synthase cannot negatively effect the Shikimate pathway in algae and water plants. See above.	Tests are not necessary.

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

Table 29. EPSP synthase: data requirement 8.3.

8.3.1 Effects on bees and on terrestrial arthropods other than bees
8.3.2 Toxicity to earthworms

	EPSP synthase	Tests specifically for EPSP synthase
	No data requirement as the Shikimate pathway does not exist in bees, non-target terrestrial arthropods and earthworms.	Tests are not necessary.
plant stage	**GM oilseed rape expressing EPSP synthase**	**Tests specifically for the GM oilseed rape**
all relevant stages	No data requirement since EPSP synthase cannot effect any pathway in bees, non-target terrestrial arthropods and earthworms. Moreover, these organisms are exposed to EPSP synthase by other crops in the same way.	Tests are not necessary.

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

Table 30. EPSP synthase: data requirement 8.3.3. Effects on non-target micro-organisms in the soil

	EPSP synthase	Tests specifically for EPSP synthase
	Yes, data requirement as the Shikimate pathway exists in fungi. Not a data requirement for bacteria. However, exposure to EPSP synthase does not deregulate the Shikimate pathway.	No tests are necessary.
plant stage	**GM oilseed rape expressing EPSP synthase**	**Tests specifically for the GM oilseed rape**
1-8	Yes, data requirement as the Shikimate pathway exists in micro-organisms. However, fungi are exposed to EPSP synthase by other crops in the same way.	Tests are not necessary.

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

Table 31. EPSP synthase: data requirement 8.3.4. Effects on terrestrial plants

	EPSP synthase	**Tests specifically for EPSP synthase**
	Yes, data requirement for the EPSP synthase being the active ingredient of the formulation, unless it can be proven that plants will not be exposed. Plants will be exposed after imaginary application of mutant EPSP synthase. Exposure to EPSP synthase will be in combination with glyphosate. However, only glyphosate will be toxic. EPSP synthase will (assuming that it enters the chloroplast) only help to prevent the deregulation of the Shikimate pathway.	*Fourth stage re-evaluation:* If there is exposure, a waiver can be presented explaining that EPSP synthase cannot be toxic to algae and water plants. *91/414/EC:* Tests are not necessary, as EPSP does not have a herbicidal mode of action. It only prevents the toxic action of glyphosate.
plant stage	**GM oilseed rape expressing EPSP synthase**	**Tests specifically for the GM oilseed rape**
All plant stages	None of the plant stages are relevant. Neighbouring crops will not be exposed as EPSP synthase is located within the chloroplasts of the mutant crop. Moreover, EPSP synthase does not have an herbicidal mode of action.	Tests are not necessary.

1 seed at sowing
3 seedling
4 young plant
5 mature plant until flowering
6 flowering plant
7a seed forming plants
8 left over material

5 Comparison among crops and cases and usefulness of tests

The tables in chapter 4 are too extended to easily compare the three cases in relation with the GM crops. As a tool to compare the three cases, Tables 32 and 33 have been prepared. These tables indicate whether tests are needed for the evaluation of the protein expressed by the GM plant. The test requirement has been specified for the plant stage and for the aspects of fate and behaviour (numbers 1-4) and non-target groups (numbers 1-8).

Table 32. Tool for the comparison of fate and behaviour tests requirements for GM plant/protein combination

Y	N	**Chitinase/ Sugar beet**				**GNA lectine/ Potato**				**EPSP synthase/ Oilseed rape**			
	Data requirement	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
plant stage													
1	Seed at planting	N	N	N	N					N	N	N	N
2	Tubers					**Y**	**Y**	**Y**	N				
3	Seedling	**Y**	**Y**	**Y**	N					N	N	N	N
4	Young plant	**Y**	**Y**	**Y**	N	**Y**	**Y**	**Y**	N	N	N	N	N
5	Mature plant	**Y**	**Y**	**Y**	N	**Y**	**Y**	**Y**	N	N	N	N	N
6	Flowering plants					**Y**	**Y**	**Y**	N	N	N	N	N
7a	Seed forming plants									N	N	N	N
7b	Tuber/ thick root forming plants	**Y**	**Y**	**Y**	N	**Y**	**Y**	**Y**	N				
8	Left over material	**Y**	**Y**	**Y**	N	**Y**	**Y**	**Y**	N	N	**Y**	N	N

Aspect
1 = degradation in soil
2 = sorption and mobility
3 = degradation in water
4 = degradation in air

■ = this plant stage is not present in the GM plant
Y = yes, a test is necessary
N = no, a test is not necessary

Conclusions that can be drawn from Table 32:

1. Plant stage 1, the seed at planting, is not a stage that is of concern in any of the three cases.

2. For EPSP synthase, no tests are required. EPSP synthase is located in the chloroplast/plastide of plants. Secretion from these organelles is not likely.

3. For chitinase and GNA lectin there are data requirements for degradation in soil for all relevant plant stages, apart from the seed at planting. If these proteins would be evaluated as a chemical crop protection product, degradation tests in three soil types would have to be performed. The evaluation of the degradation of GM plant produced proteins needs another approach, as two processes must be taken into account. The first process is the degradation of the protein in the

soil (comparable with chemical plant protection products). A second process is ongoing during the life span of the GM plant: proteins may be excreted into the soil at all plant stages. The actual concentration of the protein in the soil is the equilibrium between the rate of degradation of the protein in the soil and the rate of excretion of the protein into the soil. In theory, plateau concentrations may occur or the protein may even accumulate. A DT50 should be determined. The derived DT50 will give an answer to the question of whether degradation is faster than the rate of excretion to the soil. A practical way of addressing this data requirement is to:

- take samples from rhizosphere soil from one or more plant stages and determine whether the protein is excreted into the soil. If the protein is not excreted, no further tests are necessary;
- measure concentrations of the protein in the soil at regular intervals during the life span of the crop, in case of proven excretion of protein into the soil. A differentiation can be made between rhizosphere soil and bulk soil. In case degradation is faster than the rate of excretion into the soil, the highest concentrations of the protein are to be expected in the rhizosphere soil. Additionally, degradation in these samples can be followed in time, as with these sampled data the DT50 can be calculated in the absence of excretions of protein to the soil;
- question whether studies should be performed in as many as three soil types as required for chemical control agents. This should be evaluated on a case-by-case situation.

4. For chitinase and GNA lectin there are data requirements for sorption and mobility for all relevant plant stages apart from the seed at planting. It should be questioned whether studies are really necessary, as the degradation studies may already indicate whether mobility in soil is present. As proteins are likely to be highly degradable, mobility is probably not an important issue.

5. For chitinase and GNA lectin there are data requirements for degradation in water for all relevant plant stages apart from the seed at planting. Proteins can only reach the water through drift of seed and pollen or via run-off and drainage. In principle, photolysis experiments can be used to determine degradation in water. However, it should first be estimated if drift, run-off and drainage lead to quantifiable concentrations of the protein in water before requiring tests for the degradation of the protein in water. Preparing a waiver would be most realistic to answer this data requirement.

6. For degradation in air, neither tests nor waivers have to be presented as it can be assumed that the proteins do not volatise into the air as their molecular weight is too great.

Table 33. Tool for the comparison of ecotoxological tests requirements for GM plant/protein combination

		Chitinase/Sugar beet						**GNA lectine/Potato**						**EPSP synthase/Oilseed rape**						
	Group of organisms	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Plant stage																				
1	Seed at planting	**Y**	N	N	N	**Y**	**Y**							N	N	N	N	N	N	N
2	Tubers							N	N	N	N	**Y**	**Y**							
3	Seedling	**Y**	N	N	**Y**	**Y**	**Y**							N	N	N	N	N	N	N
4	Young plant	**Y**	N	N	**Y**	**Y**	**Y**	**Y**	N	N	**Y**	**Y**	**Y**	N	N	N	N	N	N	N
5	Mature plant	**Y**	N	N	**Y**	**Y**	**Y**	**Y**	N	N	**Y**	**Y**	**Y**	N	N	N	N	N	N	N
6	Flowering plants							N	N	**Y**	**Y**	**Y**	**Y**	N	N	N	N	N	N	N
7a	Seed forming plants													N	N	N	N	N	N	N
7b	Tuber/ thick root forming plants	**Y**	N	N	**Y**	**Y**	**Y**	**Y**	N	N	**Y**	**Y**	**Y**							
8	Left over material	**Y**	N	N	If	**Y**	**Y**	**Y**	N	N	If	**Y**	**Y**	N	N	N	N	N	N	N

Group of organisms:
1 = birds
2 = aquatic organisms
3 = bees
4 = other non-target arthropods
5 = earthworms
6 = soil micro-organisms
7 = terrestrial plants

■ = this plant stage is not present in the GM plant
Y = yes, a test is necessary
N = no, a test is not necessary
If = test only requested if trigger for non-target arthropods is met.

Conclusions that can be drawn from Table 33:

1. Tests for EPSP synthase are not necessary for any of the non-target organisms. The difference between EPSP synthase produced by a GM plant or a normal plant is only the higher affinity for the binding site. This difference is not expected to have consequences for non-target organisms. Moreover, EPSP synthase does not deregulate the Shikimate pathway and will thus not negatively effect the non-target organisms plants, algae and fungi. Another argument is that EPSP synthase is located in the chloroplast and excretion by roots and leaves seems unlikely.

2. Aquatic organisms. Tests are not necessary for aquatic organisms for any of the proteins.

3. Bees. Tests for bees are only required in a flowering crop of GM potato. Sugar beets do not have a flowering stage in their first year until harvest. Normally, oral tests (OECD 213, EPPO guideline 170 and C.16 (EC)) are performed by adding the active substance to sugar water. Acute contact tests are performed according to OECD 213 or C.16 (EC). In the acute contact toxicity test, a solvent is generally used to administer the test substance. Maximum dosage volume should not exceed 5 mL per bee, to allow for adequate volatilisation of the solvent.

   These contact and oral tests could be used for testing GNA lectin expressed by the GM potato plant. Only in the case that the properties of the protein change in the sugar solution or when the protein sticks to the wall of the feeding tube, should another way of oral feeding be sought.

   An important problem encountered when testing the effect of GNA lectin is that it is unknown at what concentration the bees have to be tested. According to OECD 214, five doses in a geometric series, with a factor not exceeding 2.2 and covering the range for LD50, are required for the test. As the concentration of the protein in the nectar, pollen and leaves is unknown, a single dose test at a high concentration may be performed as an alternative. When no effects are observed at this high concentration, the concentrations of GNA lectin in pollen and nectar will not effect bees either. Another approach is to first determine the concentration of the protein in pollen, nectar and leaves (relevant for contact toxicity). In case the protein is not present, bee tests do not have to be performed at all. This approach needs validated extraction and analytical methods. When these are not available, the approach via bee testing is the only option.

4. Non-target arthropods. Chitinase and GNA lectin expressed by the GM sugar beet and the GM potato plant, respectively, may have contact toxicity effects on non-target arthropods through direct contact with protein excretions on the leaves. Perhaps more importantly, indirect exposure occurs by host parasitisation, feeding on prey or feeding on aphid honeydew containing GNA lectin).

   In the tests sequence with regard to soil organisms for persistent substances (see Appendix 4) tests are commenced with first tier acute tests with two standard species, the parasitic wasp *Aphidius rhopalosiphi* and the mite *Typhlodromus pyri*. These species are predators of leaf-dwelling target insects. These standard tests are on glass plates on which the test substance has been sprayed. The experiments last for two days for *A. rhopalosiphi* and for seven days for *T. pyri*. After 7 days the female wasps are individually transferred to aphid infested plants where they are given the opportunity to parasitise the aphids during 24 hours. Reproduction *T. pyri* is assessed over a further seven-day period on the same Petri dish.

   An acute test is in principle interesting for the testing of proteins of GMOs in order to know if the proteins are acutely toxic to the two standard species. However, the problem arises that the test concentration is unknown.
   The following questions arise.
   - Are proteins excreted onto the leaves?
   - If so, what can be the possible concentration of the protein on the leaves?
   - Is the protein excreted by all plant stages (seedling to mature plant) in similar concentrations?

   These questions need to be answered by measuring the concentration via validated extraction and analytical methods.
   To overcome these problems, the option can be considered to use an extended laboratory test for *A. rhopalosiphi* in which toxicity and reproduction is tested on plants (Mead-Briggs et al., 2002; Mead-Briggs et al., 2009). In this test the parasitic wasp is exposed to residues on the plant for 2 days. Thereafter, females are again transferred to aphid infested plants. These aphids are not exposed to the test substance. The disadvantage of this test for the testing of proteins

expressed by GM plants is that toxicity through direct contact is tested. Perhaps more importantly, the indirect effects through parasitisation and predation need to be tested.
This extended laboratory test for *A. rhopalosiphi* can be adopted for testing the proteins expressed by GM plants: The test plant should be the GM plant infested with suitable hosts. These hosts, contrary to the original test, must have been exposed to the GM plant for a certain period of time. Here, the problem arises that a suitable combination between plant-host *A. rhopalosphi* must be found. This might not be possible for each GM plant.

For *T. pyri* an extended laboratory test is also available in which the contact test is performed on excised leaves that are put on a Petri dish. The problem encountered here is that the leaf may need turgor in order to excrete the proteins. This problem can be solved by using whole plants. The major problem that will be encountered is that it will be very difficult to retrieve the mites and their progeny, making a whole plant test less suitable.

In conclusion, only one test is available when following the decision scheme of chemical crop protection agents, which is a modified extended laboratory test the for the parasitic wasp *A. rhopalosiphi*. Some extra tests need to be available for GM plant expressed proteins. Common second tier tests are extended laboratory tests for *Coccinella septempunctata* (Schmuck et al., 2000) and *Chrysoperla carnea* (Vogt et al., 2000). These tests will also need adaptions as well for testing proteins expressed by GM plants.

Only if triggers are met in the studies with the standard species, do studies with the 'real' soil organisms such as Collembola need to be performed. These detrivores that feed from organic left over material may be affected directly by chitinase and GNA lectin.

5. Earthworm tests.

The guidance document on terrestrial ecotoxicology (is to be revised in the near future) proposes the following triggers in the decision to use tests for sublethal effects on earthworms:

- The test is not required when both the DT90 is less than 100 days and the number of applications is less than 3.
- The test is always required if the DT90 is above 365 days (regardless of the number of applications).
- The test is always required if the number of applications is greater than six (regardless of persistence)
- If the DT90 is between 100 and 365 days and/or the number of applications is between 3 and 6, a case-by-case decision is made.

Accordingly, the assumption that the concentration of the protein in soil reaches plateau concentrations necessitates sublethal tests for earthworms and tests for soil micro-organisms.

It needs to be considered whether acute tests for earthworms can indeed be skipped. When performing a sublethal test with earthworms, the problem arises that the concentration of the protein in the soil is unknown.
The reproductive test for earthworms is a static test where the test substance is applied to the system only once at the beginning of the test. For testing proteins expressed by GM plants this static system is not adequate, as the concentration in the artificial soil will increasingly differ from field concentrations during the 4-week reproduction test.
A possible solution to the problem, regular renewal of the soil, is not feasible in reproduction testing. The eggs and juveniles will be damaged by the renewal process. As an alternative, it is possible to proceed to a higher tier testing level, where earthworms are tested in a microcosm

experiment. Such a test can be performed in containers where earthworms are exposed to GM plants expressing the protein. Standardisation of such a microcosm experiment is the challenge (plant stage, plant distance, type of soil, homogenous soil samples for protein measurements and regular measurements).

6. Soil micro-organisms test.

Normal tests for soil micro-organisms are the nitrification test and the respiration test.

- OECD 216 Soil Micro-organisms, Nitrogen Transformation Test
- OECD 217 Soil Micro-organisms, Carbon Transformation Test

These test are performed over a 28-day period and the trigger is effect >25%.
In this test, the test substance is applied to the soil once, at the beginning of the experiment. These tests could be used for the protein. However, the exposure would not be chronic and therefore less suitable to detect effects. These tests would require modification to mimic chronic exposure. One option is to investigate the possibility of daily renewal of the protein concentration in the soil. The disadvantage would be the disturbance of the soil, altering the processes.
From evaluation practice it has been observed that effects are only found for fumigants which have also have the function to kill all soil microbial life. It is seriously doubted that the proteins in the three cases of this report would induce any effects on soil micro-organisms that become apparent in nitrogen and carbon transformation tests. Other tests that could be used are tests to determine enzyme activity. There is, however, no standardised test available.

6 Conclusions

Some problems are apparent in the applicability of tests (fate and exotoxicology) for GM plant testing.

Concerning fate tests, the main issue that arises is that the exposure of the environment is unknown. Proteins are assumed to be formed by the plant more or less continuously but it is unknown to what extent these will be emitted to the soil and air.
Risk assessment has to deal with a potential long-term exposure. Concentrations may remain at a certain level during the lifespan of the crop and this plateau concentration is relevant for reproduction in risk assessment. This is both relevant for the soil testing as well as for ecotoxicological testing

Necessities for soil testing:

- determination of the DT50 of protein in soil without excretions to the soil;
- concentrations of the protein expressed by the GM plant in the soil have to be measured during the life span of the crop at regular intervals (resultant of rate of degradation and rate of excretion);
- knowledge on binding to soil particles and its effect on the bioavailability of the protein in soil;
- suitable extraction techniques and validated methods of analysis need to be available;
- exactly the same protein as the one expressed by the GM plant must be available in technical form, in order to be able to conduct standardised ecotoxicological tests (see also 1.1.2).

Applicability of fate tests in soil and water for testing proteins expressed by the GM plant:

- OECD 307: aerobic and anaerobic transformation in soil (see Appendix 3 for links to OECD)
- OECD 106: adsorption study
- OECD 312: leaching in soil columns
- Soil columns (BBA-part IV, 4-2): leaching experiments (BBA, 1986)
- OECD 316: phototransformation of chemicals in water – direct photolysis (US EPA, 1982).
- Photolysis study EPA-540/9-85/014 Guideline
- OECD 111: hydrolysis as a function of pH.
- OECD 308: water-sediment study

The tested concentrations need to be high enough to obtain enough data points during the study. For instance, for transformation tests in soil, at least four data points need to be available in order to calculate the DT50 by first-order degradation kinetics. Problems may arise when the protein is volatile (not expected for a protein), not soluble in water at a concentration that can be measured analytically, or when the protein has a high affinity to the surface of the incubation system. For chitinase and GNA lectin, physical-chemical information was not found and it cannot be evaluated whether problems will arise when performing fate tests.

Concerning ecotoxicity tests: in the risk assessment of chemicals, acute tests are performed in the first tier. In most cases second tier, chronic tests are only performed when triggers are met in the first tier. In case of proteins expressed by GM plants, the question arises whether acute tests are useful as exposure to birds, bees, non-target arthropods, earthworms and soil micro-organisms is probably chronic during the growth of the crop.
Chronic tests need to be adapted from existing tests. Problems that will be faced are:

- determination of the concentration to be tested. This problem could be solved by testing at (unrealistically) high concentrations. If no effects occur it is not necessary to determine the exact concentrations;
- possible fast degradation of the test substance in the test, while exposure should be continuous. Renewal of the test substance in soil may be too difficult to overcome (for instance, tests with earthworms, Collembola and soil dwelling insects).

There are certainly possibilities to use fate and ecological testing for the testing of proteins expressed by GM plants. The ecological tests in particular may need strong adaptations, which may force the testing to a higher tier level right away.

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rivm

8 List of terms and abbreviations

CFU	Colony Forming Units
DAR	Draft Assessment Report
Directives	Can be found on http://ec.europa.eu/food/plant/protection/evaluation/legal_en.htm
DT50	Time needed for 50% degradation of the test substance
DT90	Time needed for 90% degradation of the test substance
Fourth stage of re-evaluation	Regulation (EC) 1112/2002 - Official Journal L 168, 27.06.2002 Commission Regulation of 20 June 2002 laying down the detailed rules for the implementation of the fourth stage of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC. *Re-evaluation of old substances* Before Dir. 91/414 was in force, PPP registration was exclusively under national authority. Under Directive 91/414, a transitional period was included in which all 'old substances' were re-evaluated. This re-evaluation proceeded in four stages. The 4th and last stage of re-evaluation included a more diverse range of products and uses than the mainstream pesticides covered by the earlier stages of the review. The substances on this list are considered to be substances of lesser concern, such as substances used in human foodstuffs/animal feeding, plant extracts, animal-derived products, commodity substances, pheromones and other semi-chemicals and micro-organisms including viruses, rodenticides and mole control agents and pesticides used on stored plant products. The deadline for product notification was October 2003 and the deadline for dossier submission was June/October 2005. The 4th stage was scheduled to be completed in 2008. Commission Regulation (EC) No 1112/2002 laid down the detailed rules for the implementation of the fourth stage of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC. See Appendix 2 for the active substances falling under the 4th stage.
GAP	Good Agricultural Practice
GM plant	Genetically Modified plant
Koc	Soil Organic Carbon-Water Partitioning Coefficient. The soil organic carbon-water partitioning coefficient is the ratio of the mass of a chemical that is adsorbed in the soil per unit mass of organic carbon in the soil per the equilibrium chemical concentration in solution. It is the 'distribution coefficient' (Kd) normalised to total organic carbon content. Koc values are useful in predicting the mobility of organic soil contaminants; higher Koc values correlate to less mobile organic chemicals while lower Koc values correlate to more mobile organic chemicals.
Kom	Kom = Koc /1.72
Kow	The octanol/water partition coefficient (Kow) is defined as the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system. Chemicals with low log Kow values (e.g., less than 3) may be considered relatively

	hydrophilic; they tend to have high water solubility, small soil/sediment adsorption coefficients and small bioconcentration factors for aquatic life. Conversely, chemicals with high log Kow values (e.g., greater than 3) are very hydrophobic. Such substances may cause secondary poisoning in birds, mammals and fish.
LC(D)50	The median lethal concentration/dose (i.e., the concentration/dose of substance that is estimated to be lethal to 50% of the test organisms). The LC50 and its 95% confidence limits are usually derived by statistical analysis of mortalities in several test concentrations, following a fixed period of exposure. The duration of exposure must be specified (e.g., 96-h LC50).
NOEC	No observed effect concentration. The highest concentration of a test substance to which organisms are exposed, which does not cause any observed and statistically significant adverse effects on the organism compared with the controls.
Waiver	Normally, each data requirement needs to be fulfilled with a suitable test. A waiver is a document in which data from the open literature and other possible circumstantial evidence can be used to explain why risks are not expected for the item of this particular data requirement. For instance, when the substance, regarding its mode of action, will not have negative effects on a particular non-target organism.

Appendix 1. Identity of the three cases

Table A.1. Proteins, their active substances and function translated and adapted from Mensink 2006). The cases presented in this table will be elaborated in this report

CASUS	CROP of the application	ACTIVE SUBSTANCE of the protein	function	GM plant equivalent	REMARKS
1A	sugar beet	chitinase	Resistant to a fungal pathogen and has insectidal action	Transgenic sugar beet	Chitinase decomposes chitin membranes and cell walls by hydrolysis of the β-1,4-glycoside bonds of chitin (polymers of N acetyl glucosamide). Chitin can be exo- or endogenous. Chitinase is not known as an active substance of a crop protection agent of natural origin in the Netherlands but it is in some other countries outside the EU. Genes of chitinase can be isolated from viruses, bacteria, fungi, plants and insects. This casus works with a chitinase from bacterial origin (*Bacillus circulans* WL-12). For this casus the formulation is assumed to be sprayed with a conventional beamer.
1B	sugar beet	*Bacillus circulans* WL-12	Resistant to soil fungal pathogens	Transgenic sugar beet	*Bacillus circulans* WL-12 is a Gram-positive bacterium identified as being lytic for yeast and fungal cell walls. The bacterium has been reported to secrete multiple chitinases into culture medium containing chitin as an inducer. Among these chitinases, A1 encoded by the *chiA* gene is thought to be the key enzyme in the chitinase system of this bacterium, because chitinase A1 (ChiA1) is produced most abundantly and exhibits the highest activity as to the hydrolysis of colloidal chitin and a high affinity to insoluble chitin. *Bacillus circulans* WL-12 is assumed to attack chitin via extracellular chitinase. *Bacillus circulans* WL-12 is not known in the Netherlands as an active substance. This fictive formulation with *Bacillus circulans* WL-12 serves as comparison with the formulation containing chitinase only (casus 1A). For this casus the spores are assumed to be sprayed with a conventional beamer.
2	potato	GNA lectin	Resistant to insects	Transgenic potato	Lectins are a heterogeneous group of (glyco)proteins. They agglutinate cells by the formation of glycol conjugates. They may have a plant or mammal origin. The very toxic ricine is a plant lectin. This casus starts with a lectin from plant origin: the GNA lectin (GNA stands for

CASUS	CROP of the application	ACTIVE SUBSTANCE of the protein	function	GM plant equivalent	REMARKS
					Galanthus rivalis agglutimine). This lectin is not known in the Netherlands as a crop protection agent of natural origin. For this casus, the formulation with the lectin is assumed to be sprayed with a conventional beamer.
3	oilseed rape	EPSP synthase[2]	Tolerant of the herbicide glyphosate	Transgenic oilseed rape	This mutant of EPSP synthase shows a higher affinity with a binding site in the Shikimate pathway than glyphosate. It can be isolated from diverse micro-organisms but also from traditionally bred crops with a low affinity for glyphosate. This casus assumes a bacterial origin of the transgene for EPSP synthase (*Agrobacterium tumefaciens* CP4). Transgenic glyphosate-tolerant oilseed rape is used in several countries (a.o. under the name of Roundup Ready Canola in the US and Canada). The formulation with EPSP synthase is assumed to be sprayed with a conventional beamer. More information about the role of EPSP synthase in the Shikimate pathway and the mode of action of glyphosate is found on pages 44-45 of this report.

[2] 5-enolpyruvaatshikimate-3-fosfaat synthase.

Appendix 2. List of the fourth stage

Active substances covered by the basic notification for the fourth stage of the work programme provided for in Article 8(2) of the Directive

All active substances (including any variants thereof such as salts, esters or amines) that were on the market before 25 July 1993 except those which are covered by:

— Regulation (EEC) No 3600/92,

— Regulation (EC) No 451/2000,

— Annex II to this Regulation,

Notwithstanding the above exceptions, substances which were previously considered to be covered by Directive 98/8/EC of the European Parliament and of the Council (1) but which, following clarification of the scope of the Directive, are now considered to fall within the scope of Directive 91/414/EEC and were included in Regulation (EC) No 451/2000, may be notified under Article 4. This applies in particular to substances authorised as disinfectants, i.e., products applied indirectly (for example, for the disinfection or the disinfestation of empty store rooms or other structures and articles like greenhouses, growing houses, containers, boxes, sacks, barrels, etc.) where the purpose of the use is to destroy organisms exclusively and specifically harmful to plants or plant products and after the treatment only plants or plant products will be grown or stored in the treated structures.

All substances belonging to the following categories have to be notified even if they are not mentioned in the table further below:

— active substances of which the use is authorised in human foodstuffs or animal feeding stuffs in accordance with EU legislation,

— active substances which are plant extracts,

— active substances which are animal products or derived thereof by simple processing,

— active substances, which are or will be exclusively used as attractants or repellents (including pheromones). Active substances, which are or will be exclusively used in traps and/or dispensers, in conformity with Council Regulation (EEC) No 2092/91 (2) concerning organic farming.

In particular, all substances listed in, or falling within a category listed in the following table, should be notified in accordance with Article 5:

(4E-7Z)-4,7-Tridecadien-1-yl-acetate
(4Z-9Z)-7,9-Dodecadien-1-ol
(7Z-11Z)-7,11-Hexadien-1-yl- acetate
(E)-10-Dodecenyl acetate
(E)-11-Tetradecenyl acetate
(E)7-(Z)9-Dodecadienyl acetate
(E,E)-8,10-Dodecadien-1-ol
(E/Z)-8-Dodecenyl acetate
(Z)-11-Hexadecanole
(Z)-11-Tetradecen-1-yl-acetate
(Z)-13-Octadecanole
(Z)-3-Methyl-6-isopropenyl-3,4- decadien-1yl
(Z)-3-Methyl-6-isopropenyl--9-decen-1-yl acetate
(Z)-5-Dodecen-1-yl acetate
(Z)-7-Tetradecanole
(Z)-7-Tetradecenal
(Z)-8-Dodecenol
(Z)-8-Dodecenyl acetate
(Z)-9-Dodecenyl acetate

(Z)-9-Hexadecenal
(Z)-9-Tetradecenyl acetate
(Z)-9-Tricosene
(Z,E)-11-Tetradecadien-1-yl acetate
(Z,Z) Octadienyl acetate
1,7-Dioxaspiro-5,5-undecan
1-Decanol
2-Phenylphenol (incl. Sodium salt)
2-Propanol
3,7-Dimethyl-2,6-octadien-1-ol
3,7-Dimethyl-2,6-octadienal
4-chloro-3-methylphenol
5-Decen-1-ol
5-Decen-1-yl acetate
6-Benzyladenine
7,8-Epoxi-2-methyl-octadecane
7-Methyl-3-methylene-7-octene-1-yl-propionate
Acetic acid
Acridinic bases
Alkyldimethybenzyl ammonium chloride
Alkyldimethylethylbenzyl ammonium chloride
Aluminium ammonium sulphate
Aluminium sulphate
Amino acids
Ammonium carbonate
Ammonium hydroxide
Ammonium sulphate
Anthraquinone
Azadirachtin
Barium nitrate
(1) OJ L 123, 24.4.1998, p. 1.
(2) OJ L 198, 22.7.1991, p. 1.
27.6.2002 EN Official Journal of the European Communities L 168/19
Biphenyl
Bone oil
Boric acid
Calcium carbide
Calcium carbonate
Calcium chloride
Calcium hydroxide
Calcium oxide
Carbon dioxide
Chlorhydrate of poly(imino imido biguanidine)
Chlorophylline
Choline chloride
cis-7,trans-11-hexadecadienyl acetate
cis-Zeatin
Citronellol
Cystein

Denathonium benzoate
Didecyl-dimethylammonium chloride
Dioctyldimethyl ammonium chloride
Dodecyl alcohol
EDTA and salts thereof
Ethanol
Ethoxyquin
Farnesol
Fatty acids including esters and salts such as (1):
— Decanoic acid
— Ethylhexanoate
— Ethyloleate
— Fatty acid potassium salt
— Pelargonic acid
Fatty alcohols
Folic acid
Formaldehyde
Formic acid
Garlic extract
Gelatine
Gibberellic acid
Gibberellin
Glutaraldehyde
Grease (bands, fruit trees)
Hydrogen peroxide
Hydrolysed proteins
Indolylacetic acid
Indolylbutyric acid
Iron sulphate
Kieselgur (Diatomaceous earth)
Lactic acid
Lauryldimethylbenzylammonium bromide
Lauryldimethylbenzylammonium chloride
Lecithin
Lime phosphate
Lime sulphur
Methyl nonyl ketone
Methyl-trans-6-nonenoate
Naphtalene
1-Naphtylacetamide
1-Naphtylacetic acid
2-Naphtyloxyacetamide
2-Naphtyloxyacetic acid
Naphtylacetic acid ethylester
Nicotine
Nitrogen
Octyldecyldimethyl ammonium chloride
Onion extract
Oxyquinoline
Papaine

Paraffin oil
p-Cresyl acetate
Pepper
Petroleum oils
Pherodim
Phosphoric acid
Phoxim
Plant oils such as (2):
— Coconut oil
— Daphne oil
— Etheric oils
— Eucalyptus oil
— Maize oil
— Olive oil
— Peanut oil
— Pinus oil
— Rape seed oil
— Soya oil
— Sunflower seed oil
Potassium permanganate
Potassium sorbate
Pronumone
Propionic acid
Pyrethrins
Quartz sand
Quassia
Quaternary ammonium compounds
Quinoline derivatives
Repellents (by smell) of animal or plant origin
Resins and polymers
Rock powder
(1) Each fatty acid has to be notified separately but not their variants. (2) Each plant oil has to be notified separately.
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Rotenone
Sea-algae extract
Seaweed
Sebacic acid
Serricornin
Silicates (sodium and potassium)
Silver iodide
Sodium P-toluenesulphon-chloramide
Sodium carbonate
Sodium chloride
Sodium hydrogen carbonate
Sodium hydroxide
Sodium hypochlorite
Sodium lauryl sulphate
Sodium metabisulphite

Sodium o-benzyl-p-chlorphenoxide
Sodium ortho phenyl phenol
Sodium propionate
Sodium p-t-amylphenoxide
Sodium tetraborate
Soybean extract
Soybean oil, epoxylated
Sulphur and Sulphur dioxide
Sulphuric acid
Tar oils
trans-6-Nonen-1-ol
trans-9-Dodecyl acetate
Trimedlure
Urea
Waxes
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ANNEX II
All active substances (including any variants thereof such as salts, esters or amines) covered by the full notification for the fourth stage of the work programme provided for in Article 8(2) of the Directive.
Active substances (including any variants thereof) that were on the market before 25 July 1993 which:
1. are micro-organisms including viruses, including the following:
Aschersonia aleyrodis
Agrotis segetum granulosis virus
Bacillus sphaericus
Bacillus thuringiensis including: (*)
— subspecies *aizawai*
— subspecies *israelensis*
— subspecies *kurstaki*
— subspecies *tenebrionis*
Beauveria bassiana
Beauveria brongniartii (syn. *B. tenella*)
Cydia pomonella granulosis virus
Mamestra brassica nuclear polyhedrosis virus
Metarhizium anisopliae
Neodiprion sertifer nuclear polyhedrosis virus
Phlebiopsis gigantea
Streptomyces griseoviridis
Tomato mosaic virus
Trichoderma harzianum
Trichoderma polysporum
Trichoderma viride
Verticillium dahliae Kleb.
Verticillium lecanii
2. are used as rodenticides (products applied in plant growing areas (agricultural field, greenhouse, forest) to protect
plants or plant products temporarily stored in the plant growing areas in the open without using storage facilities),
including the following:
Brodifacoum
Bromadiolone

Bromethalin
Calciferol
Calcium phosphate
Chloralose
Chlorophacinone
Cholecalciferol
Coumachlor
Coumafuryl
Coumatetralyl
Crimidine
p-Dichlorobenzene
Difenacoum
Difethialone
Diphacinone
Ethanethiol
Flocumafen

(*) Each subspecies has to be notified separately.

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Fluoroacetamide
Isoval
Papaine
Phosphine and phosphine developing compounds such as:
— aluminium phosphide
— calcium phosphide
— magnesium phosphide
— zinc phosphide
Pyranocumarin
Scilliroside
Sodium cyanide
Sodium dimethylarsinate
Strychnine
Sulphaquinoxaline
Thallium sulphate
Thiourea
Tricalcium phosphate

3. are used on stored plants or plant products, including the following:

Cyanides such as:
— calcium cyanide
— hydrogen cyanide
— sodium cyanide
Phosphine and phosphine developing compounds such as:
— aluminium phosphide
— magnesium phosphide

Appendix 3. OECD tests

Tests can be found at the site of the OECD Guidelines for the testing of chemicals:
http://titania.sourceoecd.org/vl=3031321/cl=18/nw=1/rpsv/periodical/p15_about.htm?jnlissn=1607310x

Tests 101 – 123:
http://puck.sourceoecd.org/vl=1575180/cl=27/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n1/contp1-1.htm

Tests 301 – 316:
http://titania.sourceoecd.org/vl=3139694/cl=17/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n3/contp1-1.htm

Table A.2. OECD Tests for fate and behaviour. Tests in bold can be used for testing of proteins

OECD 106	**Adsorption -- Desorption Using a Batch Equilibrium Method**
OECD 111	**Hydrolysis as a Function of pH**
OECD 121	Estimation of the Adsorption Coefficient (Koc) on Soil and on Sewage Sludge using High Performance Liquid Chromatography (HPLC)
OECD 301	**Ready biodegradability in an aerobic aqueous medium**
OECD 302A	Inherent biodegradability: modified SCAS test
OECD 302B	Inherent biodegradability: Zahn-Wellens/EVPA test
OECD 302C	Inherent biodegradability: modified MITI test (II)
OECD 303	Simulation test – Aerobic sewage treatment – A: activated sludge units; B: biofilms
OECD 304A	Inherent biodegradability in soil
OECD 305	Bioconcentration: flow-through fish test
OECD 306	Biodegradability in seawater
OECD 307	**Aerobic and anaerobic transformation in soil**
OECD 308	Aerobic and anaerobic transformation in aquatic sediment systems
OECD 309	Aerobic mineralisation in surface water – simulation biodegradation test
OECD 310	ready biodegradability – CO_2 in sealed vessels (head-space test)
OECD 311	Anaerobic biodegradability of organic compounds in digested sludge: by measurement of gas production
OECD 312	**Leaching in soil columns**
OECD 313	Estimation of emissions from preservative – treated wood to the environment: laboratory method for wooden commodities that are not covered and are in contact with fresh water or seawater
OECD 314	Simulation tests to assess the biodegradability of chemicals discharged in wastewater
OECD 315	Bioaccumulation in sediment-dwelling benthic olgochaetes
OECD 316	Phototransformation of chemicals in water – direct photolysis

Tests can be found at the site of the OECD Guidelines for the testing of chemicals:
http://titania.sourceoecd.org/vl=3031321/cl=18/nw=1/rpsv/periodical/p15_about.htm?jnlissn=1607310x

Tests 202-232:
http://titania.sourceoecd.org/vl=3031321/cl=18/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n2/contp1-1.htm

Table A.3. OECD test for effects on biotic systems. Tests in bold are used in the template

Effects on Biotic Systems OECD	
OECD 201	**Alga, Growth Inhibition Test**
OECD 202	**Daphnia sp. Acute Immobilisation Test and Reproduction Test**
OECD 203	**Fish, Acute Toxicity Test**
OECD 204	Fish, Prolonged Toxicity Test: 14-Day Study
OECD 205	**Avian Dietary Toxicity Test**
OECD 206	**Avian Reproduction Test**
OECD 207	**Earthworm, Acute Toxicity Tests**
OECD 208	Terrestrial Plants, Growth Test
OECD 209	Activated Sludge, Respiration Inhibition Test
OECD 210	**Fish, Early-Life Stage Toxicity Test**
OECD 211	**Daphnia magna Reproduction Test**
OECD 212	Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages
OECD 213	**Honeybees, Acute Oral Toxicity Test**
OECD 214	**Honeybees, Acute Contact Toxicity Test**
OECD 215	**Fish, Juvenile Growth Test**
OECD 216	**Soil Micro-organisms, Nitrogen Transformation Test**
OECD 217	**Soil Micro-organisms, Carbon Transformation Test**
OECD 218	Sediment-Water Chironomid Toxicity Using Spiked Sediment
OECD 219	Sediment-Water Chironomid Toxicity Using Spiked Sediment
OECD 220	Enchytraeid Reproduction Test
OECD 221	Lemna sp. Growth Inhibition Test
OECD 222	**Earthworm Reproduction Test (*Eisenia fetida/Eisenia Andrei*)**
OECD 224	Determination of the Inhibition of the Activity of Anaerobic Bacteria: Reduction of Gas Production from Anaerobically Digesting (Sewage) Sludge
OECD 225	Sediment-Water Lubriculus Toxicity Test Using Spiked Sediment
OECD 226	Predatory Mite (Hypoaspis (Geolaelps) aculeifer) Reproduction in Soil
OECD 227	Terrestrial Plant Test Vegetative Vigour Test
OECD 228	Determination of Developmental Toxicity of a Test Chemical to Dipteran Dung Flies (Scathophage stercoraria L. (Scathophagidae) Musca autumnalis De Geer (Muscidae)
OECD 229	Fish Short-Term Reproduction Assay
OECD 230	21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity and Aromatase Inhibition
OECD 231	Amphibian Metamorphosis Assay
OECD 232	Collembolan Reproduction Test in Soil

Tests can be found at: http://ecb.jrc.ec.europa.eu/testing-methods/annex5/

Table A.4. Test method for ecotoxicity and fate & behaviour according to Annex V to Directive 67/548

	Part C: Methods for the determination of ecotoxicity
C. 1	Acute toxicity for fish
C. 2	Acute toxicity for Daphnia
C. 3	Algal inhibition test
C. 4	Biodegradation: determination of the ready biodegradability
C. 5	Degradation: biochemical oxygen demand
C. 6	Degradation: chemical oxygen demand
C. 7	Degradation: abiotic degradation: hydrolysis as a function of pH
C. 8	Toxicity for earthworms: artificial soil test
C. 9	Biodegradation: Zahn – Wellens test
C. 10	Biodegradation: activated sludge simulation test
C. 11	Biodegradation: activated sludge respiration inhibition test
C. 12	Biodegradation: modified scas test
C. 13	Biodegradation: flow-through test
C. 14	Fish juvenile growth test
C. 15	Fish, short-term toxicity test on embryo and sac-fry stages
C. 16	Honeybees – acute oral toxicity test
C. 17	Honeybees – acute contact toxicity test
C. 18	Adsorption/desorption using a batch equilibrium method
C. 19	Estimation of the adsorption coefficient (Koc on soil and on sewage sludge using high performance liquid chromatography (HPLC))
C. 20	Daphnia reproduction test
C. 21	Soil micro-organisms: nitrogen transformation test
C. 22	Soil micro-organisms: carbon transformation test
C. 23	Aerobic and anaerobic transformation in soil
C. 24	Aerobic and anaerobic transformation in aquatic sediment systems

Appendix 4. Test sequence with regard to soil organisms for persistent substances

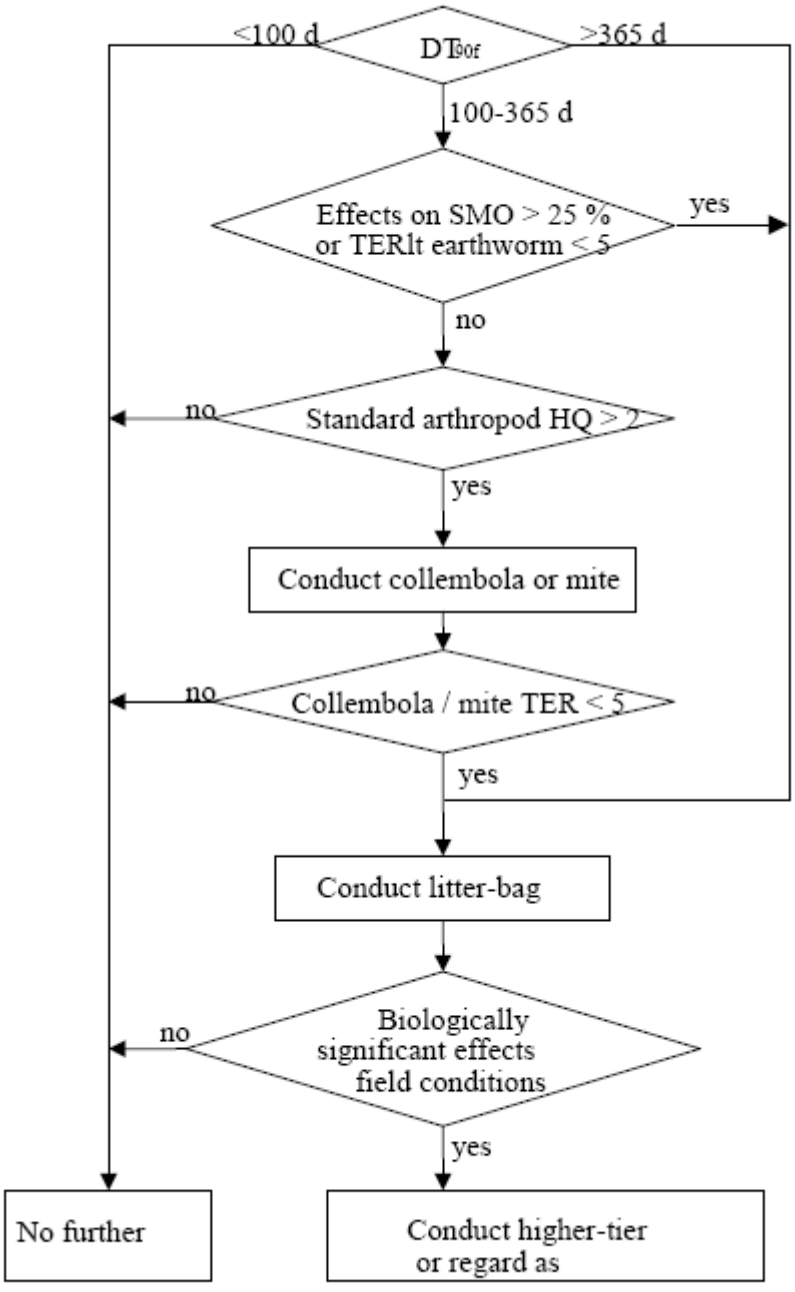

Figure 1. Test sequence with regard to soil organisms for persistent substances (European Commission, 2002).

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