GenØk - Centre for Biosafety

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Direktoratet for naturforvaltning
Tungasletta 2
7485 Trondheim
Dato: 11.03.2013

Vedlagt er inspill fra GenØk – Senter for Biosikkerhet om høringer **EFSA/GMO/BE/2012/110** for MON 87427 mais fra Monsanto Company.

Vennligst ta kontakt hvis du har noe spørsmål.

Med vennlig hilsen,

Lise Nordgård
Forsker
GenØk – Senter for Biosikkerhet
lise.nordgard@uit.no

Bidragsytere:

Idun Merete Grønsberg
Forsker
GenØk – Senter for Biosikkerhet

Conny Tümmler
Ingeniør
GenØk – Senter for Biosikkerhet

Vinicius Vilperte
Master student
GenØk – Senter for Biosikkerhet

GenØk - Centre for Biosafety

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Assessment of the technical dossier submitted under EFSA/GMO/BE/2012/110 for approval of MON 87427 Maize from Monsanto Company

Submitted to

Direktoratet for Naturforvaltning

by

Lise Nordgård, Idun Merete Grønsberg, Conny Tummler, Vinicius Vilperte
Centre for Biosafety – GenØk
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KONKLUSJON PÅ NORSK

Vi trekker frem mangler i dossieret som ikke gir grunnlag for en konklusjon om sikker bruk, samfunnsnytten og bidrag til bærekraftighet av MON 87427. Søker har ikke inkludert noe av den informasjonen omkring samfunnsnytten og bærekraftighet til MON 87427 som kreves i den norske genteknologiloven (Appendix 4) for godkjenning i Norge.

Hovedkonklusjon og anbefalinger

Genøk – Senter for Biosikkerhet viser til brev fra Direktoratet for naturforvaltning (DN) angående høring som omfatter MON 87427 for bruksområdene import, prosessering, mat og fòr.

Søker har ikke utført analyser av viktige kjemiske prosesser som erfaringsmessig vites å være aktuelle problemstillinger for denne type genmodifiserte planter (herbicid toleranse medfører akkumulering av pågjeldende stoffer). CP4 EPSPS-proteinet gjør maisplantene tolerante overfor ugrasmidler med virkestoffet glyfosat. I den senere tid har laboratorie forsøk vist at glyfosat kan føre til celleskader, blant annet i humane embryoceller. Undersøkelser har også vist en skadelig effekt på vassdrag og vannorganismer. I tillegg forstyrrer glyfosat næringsstoffomsetninga i jord.

Søker bør på bakgrunn av den nylig publiserte artikkelen av Podevin og du Jardin med tittelen; “Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants”, utvide den molekylære karakteriseringen av denne eventen og se på muligheten for ulike RNA varianter, fusjonsproteiner og del utrykk av P6. Artikkelen har ført til en diskusjon om tidligere godkjenninger av GM-planter har oversett kritiske sikkerhetsspørsmål knyttet til bruken av Cauliflower mosaic virus 35S promotor (P35S) i GM-planter.

Søker har ikke utført foringsforsøk med plantematerialet på forsøksdyr.

Søker gir ikke opplysninger som adresserer vurderingskriteriene bærekraft, samfunnsnytte og etiske aspekter som forutsettes anvendt i den norske genteknologiloven. I denne sammenheng er det viktig å få dokumentert erfaringer med hensyn på effekter på miljø, helse og samfunnsaspekter. Denne type dokumentasjon er ikke vedlagt søknaden om omsetting av mat produsert fra MON 87427 eller inneholdende ingredienser produsert fra MON 87427.

Vår konklusjon er at norske myndigheter ikke godkjenner bruk av MON 87427 i de bruksområder det søkes om.

SUMMARY OF THE ASSESSMENT OF THE TECHNICAL DOSSIER RELATED TO EFSA/GMO/BE/2012/110

As a designated National Competence Center for Biosafety, our mission at GenØk in advice giving is to provide independent, holistic and useful analysis of technical and scientific information/reasoning in order to assist authorities in the safety evaluation of biotechnologies proposed for use in the public sphere.

The following information is respectfully submitted for consideration in the evaluation of product safety and corresponding impact assessment of MON 87427, setting out the risk of adverse effects on the environment and health, including other consequences of proposed release under the pertinent Norwegian regulations.

This submission is structured to address specific provisions for an impact assessment required under the Norwegian Gene Technology Act of April 1993, focusing on the requirements in Appendix 2 - Principles for environmental risk assessment pursuant to sections 13-16 of the regulations, and Appendix 4 - Evaluation of ethical considerations, sustainability and benefit to society, cf section 17 of the "Regulations relating to impact assessment pursuant to the Gene Technology Act" of December 2005, pursuant to section 11 cf section 8. The information presented here may be applicable to more than one provision in different appendices.

We have targeted our critique to address the information needs under the relevant provisions that relate to our particular area of competence in biotechnology assessment as comprehensively as possible. Lack of commentary on our part towards any information under consideration should not be interpreted as specific endorsement of that information.

Specific recommendations

Based on our findings, we propose a few specific recommendations, summarized here and detailed in the critique below.

- The Applicant should explain the mechanism that triggers the male sterility.
- Considering recent scientific findings the Applicant should extend the molecular characterization of the event by examining the possibility for different RNA variants, fusion proteins and partial expression of P6.
- The Applicant should provide additional data using a comprehensive set of smaller probes in order to evaluate the genetic stability of the event; southern blot studies for generational stability should follow the same methodology as the others southern blot analysis (i.e. using the same probes); longer exposure times for Southern Blots. are recommended if indicated sample or control bands are not clearly distinguishable;
- The Applicant should include molecular profiling techniques to allow a more thorough study of the insert genetic stability over multiple generations
- The Applicant should provide all the primer sequences that were used for on the sequencing studies; the electropherograms should be provided as well in order to check the quality of the sequences; generational sequencing studies should have been conducted.
- The Applicant should use the plant produced CP4 EPSPS and not the *E.coli* produced CP4 EPSPS to assess the safety of the protein
- The Applicant should present results of a long term exposure-/feeding study using MON 87427 before the product is released on the marked for food/feed consumption.

Overall recommendation

From our analysis, we find that the deficiencies in the dossier do not support claims of safe use, social utility and contribution to sustainable development of **MON 87427**. **Critically, the Applicant has not included any of the required information to assess social utility and sustainability as required in Appendix 4 of the Norwegian Gene Technology Act, which would be necessary for consideration of approval in Norway.** Hence at minimum, the dossier is deficient in information required under Norwegian law. A new application or reapplication should only be reconsidered with the delivery of the information requests recommended here, including any additional information deemed significant by the Norwegian authorities.

Therefore, in our assessment of MON 87427, we conclude that based on the available data supplied by the Applicant, the Applicant has not substantiated claims of environmental safety satisfactorily or provide the required information under Norwegian law to warrant approval in Norway at this time.

ASSESSMENT OF THE TECHNICAL DOSSIER RELATED TO EFSA/GMO/BE/2012/110

About the event

The genetically modified maize line MON 87427 was developed through *Agrobacterium*-mediated transformation to provide tissue-selective glyphosate tolerance to facilitate the production of viable hybrid maize seed.

The intended technical effect of the modification in MON 87427 corn is to confer tolerance to the herbicide glyphosate in a tissue-selective manner. To accomplish this objective, Monsanto introduced a gene, *cp4 epsps*, which encodes 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) protein into a conventional corn cultivar (LH198 × HiII). The CP4 EPSPS protein confers tolerance to the herbicide glyphosate.

A specific promoter and intron combination (*e35S-hsp70*) is used in the expression cassette for the *cp4 epsps* gene that results in CP4 EPSPS protein production in vegetative and female reproductive tissue like leaves, stalk and root tissues and tissues that develop into grain and silk, providing tolerance to glyphosate within these tissues.

Reduced production of CP4 EPSPS in specific male tissues results in these tissues being sensitive to the herbicide glyphosate and thus eliminates the need for detasseling during the production of hybrid seed corn

Assessment findings

Herbicides

Glyphosate tolerance

Event MON 87427 expresses a *CP4EPSPS* gene from *Agrobacterium sp. line CP4* that confers tolerance to herbicides products containing glyphosate.

In recent years glyphosate has received more risk-related attention due to negative effects on both aquatic and terrestrial ecosystems (Blackburn and Boutin 2003, Ono et al 2002, Solomon and Thompson 2003), and also because of constantly increasing number of glyphosate herbicide applications since the introduction of this chemicals in 1971 (Dill et al 2010, Cuhra et al 2012).

Studies in animals and cell cultures indicate possible health effects in rodents, fish and humans. Glyphosate given in the feed to pregnant female rats resulted in higher embryonic mortality and aberrations in the skeleton (Dallegrave et al. 2003). Nile-tilapia (Oreochromis niloticus) fed sublethal concentration of Roundup (active ingredient: glyphosate) resulted in a

number of different histopathological changes in organs (Jiraungkoorskul et al. 2003). Experiments with sea urchins exposed to Roundup influenced early cell divisions (Marc et al 2002), effects that have relevance to potential health effects in many eukaryotic organisms, including domestic animals and humans. Exposure to Roundup affected the CDK1/CyclinB regulator which is nearly identical in sea urchins and humans.

Glyphosate has also been shown to negatively affect the differentiation of nerve cells (Axelrad et al 2003). In human placenta cells, Roundup is more toxic than the active ingredient glyphosate (Richard et al 2005). The authors concluded that additional components of Roundup increase the biological availability and accumulation in organisms.

In a recently published study by Seralini et al (Seralini et al 2012) the authors concludes that long term exposure of lower levels of complete agricultural glyphosate herbicide formulations, at concentrations well below officially set safety limits, induce severe hormone-dependent mammary, hepatic and kidney disturbances in rats.

Recommendation:

- The Applicant should include long term exposure-/feeding studies should in a risk assessment before a GM plant product is released on the marked for food/feed consumption.

Molecular characterization:

2.1. General description of the trait(s) and characteristics which have been introduced or modified

The Applicant states that *"MON 87427 utilizes a specific promoter and intron combination (e35S-hsp70)… This specific promoter and intron combination also results in limited or no production of CP4 EPSPS protein in two key male reproductive tissues: pollen microspores, which develop into pollen grains, and tapetum cells that supply nutrients to the pollen. …MON 87427 will produce a male sterile phenotype through tissue-selective glyphosate tolerance"* (p.32). The MON87427 maize appears to contain the same genetic elements (P-35S, I-*hsp*70, TS-*CPT2*, CP4 EPSPS and T-*nos*) present in the second EPSPS gene cassette in the NK603 maize event. Why does the specific promoter-intron combination, results in male sterility in the case of MON87427, but not in the NK603? Furthermore the applicant does not supply any information on the molecular mechanism causing the limited or no expression in the two male reproductive tissues but not in the other parts of the plant.

Recommendation:

- The Applicant should explain the mechanism that triggers the male sterility.

2.2.2. Information on the sequences actually inserted/deleted or altered

A paper by Podevin and du Jardin (2012) with the title; "Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants", was recently published in GM Crops and Food 3: 1-5.

This paper has created a discussion related to if past approvals of GM events have overlooked key safety questions related to the use of the Cauliflower mosaic virus 35S promoter (P35S) in GM plants. In the article, Podevin and du Jardin state that some P35S variants contain open reading frames that when expressed could lead to "unintended phenotypic changes. Gene VI encodes the multifunctional P6 protein that can be divided into four domains (Li and Leiser, 2002). Functions of P6 include nuclear targeting (Haas et al 2008), viral particle binding and assembly (Himmelbach et al. 1996), si- and ds-RNA interference and interference suppression (Shivaprasad et al 2008) and transcriptional transactivation (Koybashi et al 2004; Palanichelvam 2002).

The P35S version inserted into MON87427 maize contain the duplicated enhancer region (Kay et al 1987). Podevin and du Jardin point out that even shorter P35S variants like the one used in MON87427 containing one or more duplications of the 35S enhancer overlap with domain 4 of the P6 protein. Even though the used shorter P35S version "does not appear to result in the presence of an ORF with functional domains" according to Podevin and du Jardin the applicant should be required to study the presence of partial P6 protein and the possibility of chimeric proteins containing P6 fragments.

A study by Rang et al. (2005) revealed the possibility for read-through of the NOS terminator in GTS 40-3-2 soybean resulting in four different RNA variants with the potential to express unknown EPSPS fusion proteins. With respect to the fact that five NOS terminator sequences are present in SYHT0H2 soybean the possibility for read- through resulting in different RNA variants and potential fusion proteins should be studied carefully.

The sizes of some of the used probes (Probe 1 with 1.2 kb, Probe 3 with 1.5 kb, backbone probe 5-7 with 1.8, 1.5 and 1.7 kb) in the Southern Blot analyses are considered too long and can lead to false negative results. The strength of the interaction between probe and target is based on the number of bonds that form between the single strand of DNA (probe) and the matching recombinant DNA (target). A long probe that binds perfectly to a short insertion will not be strongly bound and may be washed off depending on the stringency of the wash. This might be the reason why the fragment listed above is not detected or not clearly visible in the Southern Blots.

In the Southern Blot analysis, the applicant states that *"Conventional control genomic DNA digested with Nco I (Figure 6, lane 1 and lane 8) and hybridized with Probe 1 and Probe 4 (Figure 3) produced endogenous hybridization bands of ~6.1 kb and ~4.1 kb." (p.42).* The 6,1 kb band, if present, is very weak and hardly distinguishable (Figure 6, p.45). Furthermore a faint band around 1,2 kb is visible that is not listed. Also, lanes 2 and 9 should show a 5,5kb band, which cannot be distinguished from the 6,1 kb band. Longer exposure times and even longer gel runs are recommended to make the bands clearly distinguishable. The applicant also concludes that the bands visible in the conventional controls "most likely resulted from

hybridization with endogenous maize *hsp70* intron sequence". Since probe 1 and 4 were used in the same hybridization solution this cannot be assumed since the binding of either probe could result in those bands. Both probes should therefore be used in separate Southern Blots.

All Southern Blot pictures (Figure 6, p.45; Figure 7, p.46; Figure 8, p.47; Figure 9, p.49) are lacking the molecular weight marker.

The Applicant states, *"Although the other Sph I segment from the plasmid vector (~ 7.1kb) contains a small portion of the Probe 3 sequence, it was not detected under these assay conditions."* (p.44). An adjustment of the Southern Blot conditions or use of a different probe is recommended in order to visualize the presence of the fragment.

The Applicant states in page 42 that "*The positive hybridization control was spiked at 0.1 and 1 genome equivalents to demonstrate sufficient sensitivity of the Southern blot"*. However, in Figure 9, lane 5 (p.49), only one of two bands is clearly distinguishable. Longer exposure times or adjustments to the Southern Blot conditions are recommended in order to achieve the sensitivity claimed by the applicant.
Figure 13 (p.63) shows the southern blot analysis to examine insert stability in multiple generations. Only two probes (probes 1 and 4) and one digestion approach (*Nsi*I) was used for this analysis. Furthermore, the 9,8 kb endogenous band present in the molecular characterization Southern Blot using probe 1 and 4 (Fig. 6, p.45) is not visible. Longer exposure times or adjustments to the Southern Blot conditions are recommended to ensure consistency in the date.

The use of Southern Blot as the only method to examine the genetic stability of MON 87427 is not advisable since they can only give information on the gross structure and copy number of the insert. Small rearrangements and small deletions as well as point mutations that might result in the formation of new ORFs or changes in the expressed protein will not be detected (De Shrijver et al. 2007). The use of molecular profiling techniques (Heinemann et al, 2011) is highly recommended. Furthermore Fig. 5 on page 39/40 indicates that different generations were used for molecular analysis and protein expression studies. The use of the same generation for both analyses is however recommendable since small deletions and rearrangements might occur after multiple generations (De Shrijver et al. 2007).

Recommendation:

- The Applicant should explain the mechanism that triggers the male sterility.
- Considering recent scientific findings the Applicant should extend the molecular characterization of the event by examining the possibility for different RNA variants, fusion proteins and partial expression of P6.
- The Applicant should provide additional data using a comprehensive set of smaller probes in order to evaluate the genetic stability of the event; southern blot studies for generational stability should follow the same methodology as the others southern blot analysis (i.e. using the same probes); longer exposure times for Southern Blots. are recommended if indicated sample or control bands are not clearly distinguishable;
- The Applicant should use molecular profiling techniques to allow a more thorough study of the insert genetic stability over multiple generations.

(ii) Organization and sequence of the inserted genetic material at each insertion site
The Applicant states that *"The PCR products were sequenced using multiple primers, including primers used for PCR amplification. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry."* (Arackal et al. 2010 – p.15). The fragments generated by the PCR amplification are considered to big (2.9, 3.0 and 1.3 kb) to be used for direct sequencing with BigDye terminator. If intern primers were used (as stated by the applicant), their sequences are not available.

"Sequencing electropherograms were rejected if they were of unacceptable quality, particularly with respect to peak shape and intensity. None of the rejected data was inconsistent with the conclusions presented in this report." (Arackal et al. 2010 – p.16). The electropherograms are not available, therefore is not possible to evaluate the quality of the sequences.

"As expected, a ~5.0 kb PCR product between Primer A and Primer B in MON 87427 (Figure 11, lane 3 – p.46) was not amplified in this analysis, since the PCR conditions necessary to generate a product of this size were not used." (p.24). Additional data should be supplied showing the presence of the 5.0kb band under optimal conditions.

The study was conducted only with plants from one generation. Since Southern blot analyses for four generation were conduct, and this analysis is not able to detect small rearrangements, sequencing analysis should have been conducted as well.

Recommendation:
- The Applicant should provide all the primer sequences that were used for on the sequencing studies; the electropherograms should be provided as well in order to check the quality of the sequences; generational sequencing studies should have been conducted.

2.2.3 Information on the expression of the inserted/modified sequence.
The CP4 EPSPS levels were measured in tissue selected from MON 87427 using ELISA. Data from grain and forage are summarized in Table 5 as these two tissues are considered to be the most relevant for food and feed. Other parts of the plant are not mentioned.
The relative standard deviations for the measured levels of CP4 EPSPS in treated maize samples are 25% for grain (both dry and fresh weight measures). While for forage, it is 34 % in fresh weight and 40% in dry weight. This means that there is a bigger spread (higher variability) in the measured activity levels for forage, than for grain. This variability is not commented upon.
For the untreated maize samples, the relative standard deviations are 16 % for both fresh and dry weight of grain. While the relative standard deviations are 31 % in fresh weight of forage and 36 % in dry weight.
Thus, there seem to be a lower variability in the measured activities of CP4 EPSPS in the untreated maize tissues of grain, than in the treated ones. There is no big difference in the CP4 EPSPS levels of treated and untreated tissues of forage.

4. Toxicological assessment

The toxicology assessment in the dossier is based on the general "weight of evidence approach" consisting of

- history of safe use
- lack of structural or functional relationship to proteins that adversely affect human or animal health
- low expression level of the protein in the grain
- rapid digestion of the protein in mammalian simulated gastrointestinal fluids
- lack of acute toxic effect to mammals

On the background of this approach, the applicant states that repeated dose toxicity study and a 90-days feeding study is not necessary to confirm safety.

The argument of long history of safe use is based on the "fact" that this bacterially derived protein has posed no risk to human health since its introduction to food and feeds in 1996 (Delaney et al 2008) and that the actual concentration of the CP4 EPSPS protein is very low in food and feed.

The Applicant has not analysed the question of safety further because of the "weight of evidence" provided in the long history of safe use. Still, the protein is expressed in a new context in this event and a more should be analysed more thoroughly.

Recommendation:

- The protein is expressed in a new context in this event and should be analysed more thoroughly as it differs from the previous events where this protein is expressed.

4.2 Assessment of the newly expressed protein

The assessment of the newly expressed protein is done with a protein from recombinant, bacterial systems, in this case *E.coli*. Thus the protein used is bacterial and not the one from the plant in question. The aim must be to use plant derived version of these proteins. One should always go for the version actually expressed in the gene modifies species as many of the PTMs differ/vary between species, tissues, stage of development and according to environmental variables such as temperature and light intensity (Gomord et al. 2005, Küster et al. 2001).

The safety assessment of the *E.coli* produced CP4 EPSPS is based on old data (Harrison et al. 1996). However, the equivalence data (plant versus bacterial protein) are recent.
The glycosylation equivalence data show that both plant and bacterial version of the CP4 EPSPS protein has faint bands indicating potential glycosylation present (Figure 19). But since the *E.coli* protein previously has been shown to not have glycosylation, they say that what is observed is not glycosylation. They do however not say what else it could be. The apparent molecular mass would increase upon glycosylation, which it in this case seem not to

do. However, the additional bands that were observed should have been analyzed further. Also, it would be interesting to see if a higher exposure time of the membranes would have given more apparent bands at other sizes.
In the stability assays, the *E.coli* version of the protein is used and not the plant version (both for temperature and pH stability).
The acute toxicity study with a single dose of the protein was also performed with the bacterial version of the protein. These data are from 1996 and performed on mice (Harrison et al 1996). Newer data should have been added together with a repeated toxicity study that has been found not to be necessary because of the "weight of evidence" approach used.

Recommendation:

- The Applicant should use plant version of the protein for the safety assessment studies.
- The Applicant should provide figures that have higher exposure times to show that no additional bands for glycosylation are present.

Social utility and sustainability aspects

In addition to the EU regulatory framework for GMO assessment, an impact assessment in Norway follows the Norwegian Gene Technology Act. In accordance with the aim of the Norwegian Gene Technology Act, production and use of the GMO shall take place in an ethically and socially justifiable way, under the principle of sustainable development. This is further elaborated in section 10 of the Act (approval), where it is stated that

> *"significant emphasis shall also be placed on whether the deliberate release represent a benefit to the community and a contribution to sustainable development"*.

These issues are further detailed in the regulation on consequence assessment section 17 and its annex 4. The Applicant has not provided relevant information that allows an evaluation of the issues laid down in the aim of the Act, regarding ethical values, social justification of the GMO within a sustainable development. Given this lack of necessary information for such an evaluation, the Applicant has not demonstrated a benefit to the community and a contribution to sustainable development from the use of MON 87427. The Applicant should thereby provide the necessary data in order to conduct a thorough assessment on these issues, or the application should be refused.

It is also important to evaluate whether alternative options, (e.g. the parental non-GM version of MON 87427 may achieve the same outcomes in a safer and ethically justified way.

Further, the Norwegian Gene Technology Act, with its clauses on societal utility and sustainable development, comes into play with a view also to health and environmental

effects in other countries, such as where GMOs are grown. For instance, it is difficult to extrapolate on hazards or risks taken from data generated under different ecological, biological, and genetic contexts as regional growing environments, scales of farm fields, crop management practices, genetic background, interactions between cultivated crops, and surrounding biodiversity are all likely to affect the outcomes. Hence it cannot be expected that the same effects will apply between different environments and across continents.

Recommendation:

- The Applicant should submit required information on the social utility of MON 87427 and its contribution to sustainable development, in accordance with the Norwegian Gene Technology Act.

Conclusion

Available information for risk assessment evaluation

This evaluation is based on the Applicant's own submitted information, along with our own expertise in related fields. The relevant scientific literature is very limited in some cases, yet we have tried to extract information from the peer-reviewed literature that may inform the scientific validity of the information under consideration. In situations where lack of knowledge, complexity and uncertainty are high, particularly in relation to unknown adverse effects that may arise as a result of approval for release of a living modified organism into the environment or food supply, the available information may not be sufficient to warrant approval. Further information may address some of these issues, however an accurate description of uncertainties provided by the applicant would provide a more useful basis for assessing the level of risk that may come with regulatory approval of the GMO, taken on a case-by-case basis.

In all cases, product-related safety testing should have an independent and unbiased character. This goes both for the production of data for risk assessment, and for the evaluation of the data.

The lack of compelling or complete scientific information to support the claims of the Applicant documented here highlights the need for independent evaluation of the dossier as performed here, including the raw data produced by the Applicant. We therefore support better transparency and independent review of information to ensure high standards within the regulatory process. This would include any information provided by the Applicant used to justify confidentiality claims on any scientific data. We encourage the authorities to insist on this level of transparency and accessibility to all scientific data (including raw data) to ensure the scientific validity of the information presented.

Overall recommendation

Above we highlight a number of issues in relation to the questionable safe use of MON 87427 that do not justify a conclusion of safe use, social utility and contribution to sustainable development. Critically, the Applicant's environmental monitoring plan lacks sufficient details and descriptions to support the required monitoring activities, and has not included any of the required information to assess social utility and sustainability as required in Appendix 4 of the Norwegian Gene Technology Act, which would be necessary for consideration of approval in Norway. Taken together, these deficiencies fail to address the necessary safety regulations under Norwegian Law, and thus the application is incomplete and should not be approved. A new application or reapplication should only be reconsidered with the delivery of the information requests recommended here, including any additional information deemed significant by the Norwegian authorities.

Therefore, in our assessment of MON 87427 we conclude that based on the available data, the Applicant has not substantiated claims of safety satisfactorily to warrant approval in Norway at this time.

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