Inventory and guidelines for studies on the interactions of the soil microbiota with genetically modified (GM) plants

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Inventory and guidelines for studies on the interactions of the soil microbiota with genetically modified (GM) plants

(Richtlijn bestudering bodeminteracties)

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Preface

One of the issues that COGEM and the Dutch GMO office address in their risk assessments for genetically modified crops are the possible effects on the soil ecosystem. The reason for addressing this issue is obvious: soils represent a valuable life-supporting system providing many ecological services to mankind, including fertility for crop growth, nutrient recycling, retention and filtration of water, and solidity as a substrate for infrastructure. Many of these functions are supported by complex communities of microorganisms and invertebrates. Changes in the composition of these communities or their ecological functions may potentially lead to a diminishing service and this is a concern in risk assessment.

Microbial ecology is a fast growing field that over the last few years has adopted a variety of new, molecular methods to explore the richness of soil communities. It turns out that species diversity and diversity of functions are much greater than thought before. In addition, the results from the NWO-ERGO (Ecology Regarding Genetically Modified Organisms) research program became available over the last few years, in which soil interactions was one of the themes. The question for COGEM and the GMO office was whether these novel methods and insights have provided new ways of thinking regarding the assessment of applications for genetically modified plants.

In this report dr. Semenov and prof. van Elsas present an inventory of existing methods to examine microbial communities, with special attention to the novel methods deriving from e.g. next-generation sequencing and metagenomics. They then discuss how the present status of soil microbial analyses may inform the risk assessment of genetically modified plants. An essential feature of their approach is that they propose a step-wise assessment, where hazard identification is followed by three tiers of analysis, in which successively more comprehensive questions are addressed with every subsequent tier. This approach is illustrated by discussing a number of case studies.

The report provides a valuable addition to the science-based instrumentation that COGEM and the GMO office deploy in the environmental risk assessment of genetically modified crops. In an accompanying letter, COGEM will present its view on the proposed approach for assessment of soil interactions between GM plants and microbiota.

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Executive summary

Microbial communities (bacteria and fungi) are crucial for specific life support functions (LSF) of soil, in particular the biogeochemical cycling of carbon and nitrogen compounds and the protection of plants from pathogens. Protection of the soil LSF, notably the biogeochemical cycling processes, is a key issue in the context of the risk assessment of genetically-modified (GM) plants. However, assessment of the soil microbiota that underlies the soil LSF has long been hindered by a lack of suitable methodology and only recently (advanced) methods that allow studying soil microbial communities in sufficient detail have become available.

This report reviews the current status of the advanced (and more traditional) methodologies used for the in-depth analysis of the soil microbiota, and then proposes guidance for the use of such methods in risk assessment. With respect to the methods, their sensitivities, specificities and usefulness for the assessment of the impact of GM plants are examined. This has yielded a list of methods applicable for risk assessment, the so-called toolbox for the assessment of the risk of GM plants on the soil microbiota (**toolbox for assessing soil interactions**). Use of such methods, in principle, enables a general understanding of the structure and functioning of the microbiota in soil. However, the high complexity of the soil microbiota still impedes a thorough depiction of the nature and function of each of its members, other than in an overall format.

In a generic sense, previous traditional approaches to the analysis of soil LSF provide overall data which are of use in cases where GM plant impacts on soil functioning are suspected. More advanced methods (target gene based quantitative PCR, molecular fingerprintings like DGGE, PLFA, T-RFLP) that focus on underlying functions and organisms are also useful in cases of suspected impact. Analyses of 16S rRNA gene based clone libraries and pyrosequencing are other advanced options able to yield substantive data about the soil microbial community make-up.

The manner in which the toolbox for assessing soil interactions should be employed in the risk assessment of GM crops is then examined. Use of four steps out of a six-step protocol, as advocated by EFSA, is recommended. Thus problem analysis is followed by a characterization of hazard, exposure and, consequently, risk. Then, appropriate selection of methods to be applied should be based on a case-by-case approach, in which the type and depth of the analyses are guided by the presumed impacts of the specific GM plant defined in steps 1-4 of the prior analysis. The risk assessment should thus follow a tiered (step-by-step) approach, entailing theoretical and experimental phases in which the conclusions as to the putative risk of a prior *tier* will set the stage for either further analyses in a next *tier* or stopping of the risk assessment.

A set of examples of GM plants with different presumed hazard is presented to illustrate the approach to risk assessment in theory as well as experimental testing in small systems or different sizes of field experiments. The basis of the proposed scheme – resulting in guidance in GM plant risk assessment - is the flexible selection of tools from the **toolbox for assessment of soil interactions**, in close connection to the requirements of the assessment that relate to the identified risk (problem formulation). The risk assessment of GM plants with respect to the impact on the soil LSF should thus be guided by science-based assessment of effects based on the specific crop/trait combination (problem analysis). If hazard identification indicates a possible effect of GM plants on soil microbial communities, a tiered approach is suggested, in which the outcome of the analyses in each tier will dictate the need for further, more specific, assessments in a next tier. In every next tier, methods for analysis of microbial interactions in soil need to be carefully selected in accordance with the requirements of the tier and the type of hazard identified.

Nederlandse samenvatting

Microbiële gemeenschappen (bacteriën en schimmels) zijn cruciaal voor specifieke "life support" functies (LSF) van de bodem, met name de biogeochemische cycli van koolstof en stikstof componenten en de bescherming van planten tegen pathogenen. Bescherming van de bodem LSF, in het bijzonder de biogeochemische cycli, is een sleutel bij de risicoanalyse van genetisch gemodificeerde (gg-) planten. Een gedegen analyse van de bodemmicrobiële gemeenschappen die ten grondslag liggen aan de bodem LSF is evenwel lange tijd belemmerd geweest door een gebrek aan goede methoden om deze in detail te bestuderen. Dergelijke methoden zijn pas sinds kort voorhanden gekomen.

Dit rapport inventariseert de huidige staat van de methoden die gebruikt worden voor een diepteanalyse van de bodemmicrobiota en geeft aanbevelingen voor hun gebruik in de risicoanalyse. Met name worden de gevoeligheid, specificiteit en het nut voor vaststelling van de effecten (impact) van gg-planten van de methoden onderzocht. Dit heeft geresulteerd in een lijst van methoden die geschikt geacht worden voor risicoanalyses, de zogenaamde "**toolbox**" voor toetsing van effecten van gg-planten op de bodemmicrobiota. In principe levert het gebruik van deze methoden een globaal inzicht in de structuur en functie van de bodemmicrobiota. De hoge complexiteit van de bodemmicrobiota verhindert echter nog een diepe beschrijving van de aard en functie van elk lid van de microbiële gemeenschap.

De voorgaande traditionele aanpak van analyse van de bodem LSF geven algemene gegevens die nuttig zijn voor die gevallen waarin vermoed wordt dat er effecten van gg-planten op het bodemfunctioneren zijn. Echter, geavanceerdere methoden (zoals

quantitatieve PCR of moleculaire fingerprintings zoals DGGE, PLFA en T-RFLP) die zich op onderliggende functies en organismen richten zijn ook nuttig in het geval effecten vermoed worden. Analyses van op het 16S ribosomale RNA gen gebaseerde klooncollecties en 'high-troughput' sequentie-analyses zijn andere geavanceerde opties die substantiële gegevens over de samenstelling van de bodemmicroflora kunnen geven.

De manier waarop de **toolbox** kan worden aangewend wordt vervolgens geanalyseerd. Gebruik van vier stappen uit een 6-staps analyseprotocol afkomstig van EFSA wordt aanbevolen. Zo wordt probleemanalyse gevolgd door karakterisering van gevaar, blootstelling en, concluderend, risico. Daarna moet de juiste selectie van methoden gebaseerd worden op een "case-by-case" aanpak, waarin type en diepte van de analyses bepaald worden door de aangenomen effecten van de specifieke gg-plant, zoals vastgesteld in stappen 1-4 van de analyse. De risicoanalyse zal dus een gelaagde ("tiered") aanpak moeten volgen, waarin de conclusies van een eerdere laag (tier) aan moeten geven wat in een volgende laag nodig is aan testen, of dat het testen wellicht gestopt moet worden.

Een set voorbeelden van gg-planten met verschillend aangenomen gevaar wordt vervolgens gepresenteerd om de risicoanalyseaanpak te illustreren, in theorie en in experimentele tests in kleine systemen, evenals in veldexperimenten van verschillende grootte. De basis voor het voorgestelde schema – resulterend in begeleiding in de risicoanalyse van gg-planten – is de flexibele selectie van methoden uit de "**toolbox**" voor analyse van bodeminteracties, in nauwe connectie met de eisen van de analyse die gerelateerd zijn aan het geïdentificeerde risico (probleemformulering). De risicoanalyse van gg-planten met betrekking tot de effecten op de bodem LSF moet dus geleid worden door een wetenschappelijke analyse van de effecten op basis van specifieke gewas-eigenschap combinaties (probleemanalyse). Als de analyse een mogelijk effect van gg-planten op de bodemmicrobiële gemeenschappen aangeeft, dan wordt een gelaagde aanpak aanbevolen, waarin de uitkomst van elke laag de noodzaak voor volgende, specifiekere, analyses in een volgende laag aangeeft. In elke volgende laag worden methoden voor analyse van de microbiële interacties in de bodem zorgvuldig geselecteerd conform de criteria van de tier en het type gevaar dat geïdentificeerd is.

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Objectives:

- To make an inventory of existing scientific approaches related to the examination of possible effects of (GM) plants on soil microbial communities

- To discuss and review the aforementioned approaches (tools) with respect to their usefulness for GM plant risk analysis

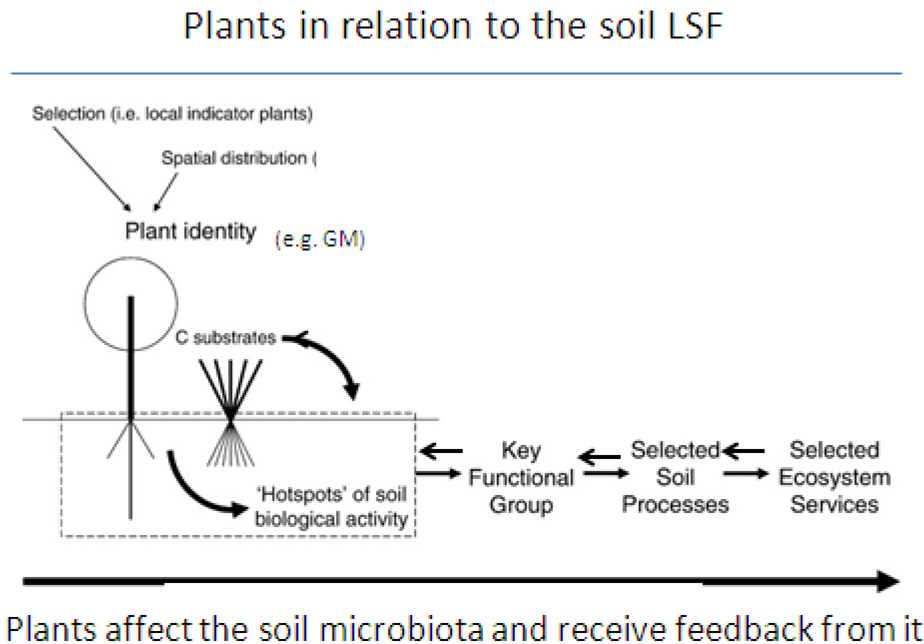
- To develop appropriate guidelines for soil studies in order to monitor and distinguish possible changes in the structure and functioning of soil microbial communities under (GM) plants

1. Introduction

Soil constitutes a living entity

Soil is a living entity, in which the microbial communities (bacteria and fungi) lie at the basis of key processes that serve life on the planet (life support functions (LSF), reviewed in van Elsas *et al*. (2007). See Figure 1 below. For sustainable agriculture, it is important to safeguard these communities (called the soil microbiota) in the soil, because of their role in the soil LSF. The latter include, in particular, the biogeochemical cycling of carbon, nitrogen and other elements, the protection of plants against phytopathogens and the general maintenance of soil ecosystem stability and resilience. The importance of the soil microbiota has also been recognized by the EU, as microbial parameters of soil are at the core of current EU recommendations for the monitoring of soil organic matter (crucial for the soil microbiota) and diversity (Van Camp *et al*, 2004). Moreover, the risk assessment of GM plants in the EU includes an assessment of possible effects on biogeochemical cycles and soil microbial interactions.

Figure 1. Plants, in relation to their selection and spatial distribution, affect soil processes, which are largely (but not excusively) carried by soil microbiota. They also receive feedback from the latter.



Developments in the methodology used to describe the soil microbiota

The last ten years have witnessed an explosive development of ever more sophisticated tools and their application to study the living soil (van Elsas & Boersma, 2011). In this report, we address both the relevant traditional and advanced methodological tools. In the traditional soil microbiological approaches, data on the culturable part of the soil microbiota have been derived from growth of cells in liquid cultures or of colonies on Petri plates. However, these methods have strong limitations. Most importantly, only a small fraction of the microbial community in soil can be accessed on the basis of cultivation. A sensible way to understand the complexity of soil microbial communities was therefore the development of direct molecular assessments, for which pre-extraction of cellular macromolecules such as DNA, RNA and/or fatty acids is a prerequisite (van Elsas & Boersma, 2011). Such cultivation-independent molecular-based methods can reveal the structure of complex microbial communities in soil, as well as specific microorganisms and genes therein (e.g. Inceoglu *et al*., 2010). Current molecular-based methods (if properly used) decrease knowledge limitations and highlight possible differences and changes in soil microbial communities under various conditions, e.g. under the impact of GM plants.

The potential impact of genetically modified (GM) plants on the soil microbiota

Genetic modification of plants can facilitate modern crop production, resulting in enhanced crop yield, resistance to pests and diseases, and tolerance to herbicides (Liu *et al*., 2005). However, there are concerns about the potential adverse effects of GM plants, in particular with respect to their effects on the soil microbiota. General knowledge of the “normal” functions within a soil may provide risk assessors with the necessary background information for risk assessment of GM plants. In the light of their key importance for soil quality and functioning, this report focuses on soil microbial communities and how these may be impacted by GM plants.

The interaction between GM plants (related to the GM traits expressed by these) and soil microorganisms, resulting in a possible impact on the processes carried by the soil microbial community, represents a key issue to be considered in risk assessment

(Icoz & Stotzky, 2008; Mocali, 2010). Clearly, the significance of the impact of a GM plant will depend on which soil functions are affected. Moreover, it is important to which extent the impact (in comparison to that of a non-GM plant) will persist after removal of the plant, i.e. at harvest (Bruinsma *et al*., 2003). Some GM plants produce compounds that are foreign to the soil environment and in such cases it is important to monitor the effects of the release of such compounds on the soil microbiota (Shen *et al*., 2006). Such effects will likely depend on the activity of the compounds (e.g. proteins; Oger *et al*., 1997) and the quantities that are released and persist in the soil. Also, when GM plants require changes in agricultural management (e.g. different usage of pesticides or fertilizers), the soil microbiota may be affected by the altered management practices (Enwall *et al*., 2007). On the other hand, various agricultural management strategies (e.g., plowing, crop rotation) are already known to strongly affect the soil microbiota. Moreover, different forms of non-GM plants can also cause significant changes in the structure and diversity of soil microbial communities. Hence, any GM plant impact needs to be weighed against a proper reference situation, also called a ***baseline***. The issue of the baseline is elaborated below.

As we will see in this report, various effect levels of GM plants on microbial communities in soil have been reported, ranging from no effects to different statistically significant effects (Brusetti *et al*., 2004; Turrini *et al*., 2004; Castaldini *et al*., 2005; Shen *et al*., 2006; Icoz and Stotzky, 2008; Hannula *et al*., 2012; Chun *et al*., 2012; Fliessbach *et al*., 2012; Prischl *et al*., 2012). However, the occurrence of significant differences between effects of GM and isogenic reference plants, which may be discerned at a particular moment in time (often during GM crop development), does not necessarily pose a risk to the environment. Therefore, the weighing of the true ecological meaning of a particular measured impact needs careful scrutiny.

<u>*Assessment of GM plant impact*</u>

Depending on its modification, a specific GM plant may affect soil microbial communities or their ecological functions in a particular way, related to the modification. Therefore, it is important to consider how each GM plant type may affect soil microbial communities or their ecological functions, in relation to the modification. As each GM

plant may represent a unique case, a case-by-case approach is to be followed in the risk assessment of GM plants. In this approach, each GM plant is assessed on the basis of the specific modification (specific crop-trait combination), the intended use and the environment into which it will be introduced. Several scenarios are possible. First, GM plants may express novel genes leading to new proteins or metabolites. Such gene products may possess, to a greater or lesser extent, antimicrobial properties or may affect the soil microbiota in other (indirect) ways. Second, GM plants may have altered biochemical composition (such as changed metabolic pathways), which may result in effects on the soil microbiota. Third, GM plants may have undergone modifications that are, by themselves, not expected to affect microbial communities (e.g. changes in flower colour, herbicide tolerance). To analyze the impact of GM plants, specific hypotheses need to be formulated addressing the question how the specific plant-trait combination may affect the soil microbiota. For each event, such a hypothesis then should be tested in a tiered (step-by-step) manner, in which the outcome of each tier is leading the plans for the next tier.

What are currently accepted practices in GM plant impact testing? First, given a suspected impact as a result of the modification, tests are done in laboratory set-ups. Then, given a perceived necessity to pursue testing in an ecologically relevant setting, tests are laid out in the field, according to an established classification scheme: type I (maximally 1 ha), II (intermediate size, larger than 1 ha) and III (large-scale) field trials. There are different requirements in terms of potential for spread of the transgene and mitigation measures between these different types of field trials. Our opinion here is that testing of the impact on the soil LSF is appropriate in all three types of field trials, but might be concentrated in type I trials, as these provide a first assessment of potential impact in a field situation.

The baseline of soil functioning, leading to the concept of normal operating range (NOR)

In the context of the potential effects exerted by GM plants on the soil microbiota, the ***baseline*** of soil functioning should be defined. This baseline needs to depict the variation within a soil system, resulting from natural or agronomical (cropping, cultivar variations) factors. Given, the high functional redundancy across the soil microbiota and function,

the use of selected key low-redundancy nitrogen cycling groups such as ammonium oxidizers (Kowalchuk and Stephen, 2001) and (symbiotic) nitrogen fixers as indicators alerting to a disturbance, has been advocated. Next to these, groups of higher redundancy such as decomposers and phosphate solubilizers may be recommended in the light of their importance for soil carbon and phosphorus cycling. By and large, such indicators may feed into the so-called baseline, and, if sufficient information becomes available, the baseline information may be a good basis to formulate a **normal operating range** (**NOR**), which encompasses thorough information of the variation in multiple parameters that is normally encountered in the soil / cropping system (Pereira e Silva *et al*., 2013). In cases in which such a thorough dataset is not available, a baseline can be based on data from common practice with the currently-used near-isogenic plant line or a suite of lines grown under similar cropping conditions (such lines are thus used as references).

Ideally, in future databases, the variation in soils captured in the NOR is depicted as a sequential occurrence of maxima and minima in relevant variables (***indicators***, as argued in the foregoing) that define the rates of LSF processes. Such ups and downs thus determine the 'natural' limits of variation in soil functioning (Arshad and Martin, 2002). Given the presence of multiple functions in soil ecosystems, assessments of "normality" of the soil (e.g. in multivariate analyses) may include a range of soil attributes which, when considered together, provide a descriptor of the overall biological function of soil (Karlen *et al*., 1994; Villamil *et al*., 2007; Romaniuk *et al*., 2011). The NOR concept can be applied in GM plant risk assessment to determine whether or not a specific aspect of the soil is within the natural or agronomical variability of the system or must be seen as an effect outside the normal variability. In the latter case, it should be further determined if this effect is biologically significant or not.

Aim of this study

The aim of the current study, commissioned by COGEM, was to obtain an overview of the currently available tools (the toolbox) that allow to measure the effects exerted by (GM) plants on the living soil. We therefore set out to:

1) make an inventory of existing scientific approaches related to the examination of possible effects of (GM) plants on soil microbial communities
2) discuss and review the aforementioned approaches (tools) with respect to their usefulness for risk analysis their strengths and weaknesses
3) develop appropriate guidelines for soil studies in order to monitor and distinguish possible changes in the structure and functioning of soil microbial communities under (GM) plants in field trials

2. Inventory of the current toolbox to assess the living soil and effects of GM plants - developments and applications

The successful observation of effects of GM plants on the soil microbial community depends strongly on:

(1) the methods used and

(2) the experimental set-up, i.e. the plants/soils used and the conditions under which the experiments are performed (Brusetti *et al*. 2004).

To meet the first point, it is of key relevance to avail of a set of robust and reliable methods that are fine-tuned to evaluate GM plant effects. An overview of the methods developed to evaluate complex soil microbial communities is given in a recent review by Van Elsas and Boersma (2011). The toolbox for assessing the soil microbiota described in this overview includes both traditional and advanced molecular methods. To meet the second point, the experimental design of each experiment needs to be careful and should take into account a best professional judgement of the predicted impact of the GM plant (problem formulation).

Considering the traditional approaches, the soil microbiota can be measured by cultivation-based techniques such as dilution plate counting, most probable number (MPN) determination and, at a community level, in an approach called CLPP (community-level physiological profiling). The latter method utilizes the BIOLOG system (Garland, 1997). These methods, e.g. plate countings, select for organisms that are

readily culturable on the media used. They allow to determine the physiological characteristics of dominant organisms in either pure or mixed populations. However, the determination of microbial diversity from analysis of isolates has become impopular, because the procedures are laborious and only limited numbers of isolates can realistically be studied. On the other hand, the community-level physiological activities can be assessed in high throughput using - for instance – physiological microplate assays offered by BIOLOG (Garland, 1997).

In addition, overall measurements of metabolic rates of the soil microbiota can be taken, such as by soil respiration measurements (e.g. in SIR – substrate-induced respiration). SIR provides a glimpse of potential *in situ* microbial activity (Icoz *et al*., 2007). Another possible approach is to monitor specific microbial processes, e.g. in biogeochemical cycling, via measurements of activities of enzymes associated with the process studied (Icoz and Stotzky, 2008). Finally, the size of the microbial biomass can be determined using direct microscopic counts following a staining procedure, or by Jenkinson's classical chloroform fumigation/extraction method.

Considering the limitations of traditional approaches, cultivation-independent molecular approaches have often become the prime methods of choice to assess soil microbial communities (Johnsen *et al*., 2001; Van Elsas and Boersma, 2011). Most often, these methods allow a depiction of the microbial communities all-at-once, meaning that overall descriptions of the collective soil microbiota are obtained. As argued, the development of these approaches constitutes a great leap forward in helping to understand the living soil as well as the impact of GM plants on it (Bruinsma *et al*. 2003; Sanvido *et al*. 2007). However, such methods depend on the prior representative extraction of cellular macromolecules (e.g. nucleic acids or phospholipid fatty acids) from soil. It is known that particular biases can occur in the extraction, such as incomplete or biased extraction (Simonet, personal communication). The ensuing analysis tools, i.e. polymerase chain reaction (PCR)-based techniques such as denaturing gradient or temperature gradient gel electrophoresis (DGGE/TGGE), terminal restriction fragment length polymorphism (T-RFLP) analyses, analysis of 16S rRNA gene clone

libraries, quantitative PCR (qPCR), microarray hybridization and direct pyrosequencing, are very powerful, yet will only show what has been extracted successfully. In other words, potential biases of the extraction phase are visualized in the analytical phase. A different angle at the soil microbiota is offered by fluorescent in situ hybridization (FISH), in which the nature and localization of target cells can be determined using specific probes, often based on the 16S rRNA gene. However, each of the direct molecular analysis tools comes with its own specific limitations as to sensitivity, specificity and potential bias, as outlined (Lee *et al*., 2011; Van Elsas and Boersma, 2011). Hence, there are clear issues of bias, sensitivity and specificity that need to be addressed when applying particular (sets of) methods to monitor the soil microbiota.

In addition to the nucleic acid based methods, there are other approaches that analyze the soil microbiota on the basis of the collective cellular macromolecules. These constitute phospholipid fatty acid analysis (PLFA) and ester-linked fatty acid analysis (ELFA). For both PLFA and ELFA, total lipids are extracted from soil suspensions (using different buffers), after which the extracts are analyzed by gas chromatography. These two methods are comparable in their discriminative power, however ELFA is faster and easier than PLFA (Hinojosa *et al*., 2005). A major issue here is the fact that the fatty acids that underlie the community patterns often lack discriminative power, meaning that only a crude split-up of the community into different types can be obtained.

An overview of the methods suitable for analyzing the soil microbiota in the context of risk assessment of GM plants is shown in Table 1. In the next sections, we examine each of the techniques as to their applicability in a risk assessment scheme for GM plants. For each method we summarize its suitability in terms of pros and cons with respect to GM plant risk assessment.

Table 1. Review of methods to study the microbiota of soil (optimized from van Elsas & Boersma, 2011)

Method	Specific for (species/ community/ system)	Major pitfall (interpretational)	Interpretation of results	Advantages (technical)	Disadvantages (technical)
Dilution plating	Species/ community	Only culturable microorganisms visible (only 1% of community).	Limited, no information on soil health and functioning. Estimate of the nature/diversity of a limited number of strains in the community	Easy, cheap, ability to further analyze colonies including metabolic characteristics or whole genome sequence	Low resolution. Not representative. Morphological differences hard to distinguish, laborious analysis necessary.
Chloroform fumigation / extraction	Community	No information on community changes.	Estimate of microbial biomass. No information on microbial community, functional or phylogenetic changes.	Cheap and fast.	Low specificity.
SIR (substrate induced respiration)	Community	Relies on activity of microorganisms, which can be low	Information on active, dominant community responding to substrate	Easy and cheap	Low sensitivity.
FISH (fluorescence in situ hybridization)	Species	Opaque nature of soil and relative low cell densities hamper in situ visualization	Information on active, dominant community members	*in situ* technique: interactions and location visible	Background fluor. in soil. Low resolution and low-throughput.
PCR/qPCR	Species/ Community	Reliant on existing primers and typically restricted to specific phylogenetic groups of interest	Proxies of organisms or genes amplified and/or quantified	Routine techniques of high sensitivity; allow detection and/or quantification	Several PCR biases and artifacts, i.e. inhibition, incomplete coverage
DGGE/ TGGE*	Phylogenetic (species/ community)	Only species >1% abundance are visible.	Estimate of the structure of limited (dominant) microbiota in the community	Well optimized and easy, bands excised for identity check.	Inter-gel comparison difficult. Artifacts.

	Functional (species/ community)	Limited information on functional genes hampers primer design	Information on functional genes, but not on activity of those genes. Relationship between functional gene shift and functioning often unknown	Same as above; Higher resolution than phylogenetic DGGE/TGGE	Same as above
SSCP*	Species/ Community	Similar to DGGE/TGGE	Similar to DGGE/TGGE	No need for gradient or GC-clamp	Laborious preparation of sample
T-RFLP*	Species/ Community	Terminal restriction fragment not species-specific.	Similar to DGGE/TGGE	Easy comparisons between samples, possibility of obtaining different fingerprints from same sample	Identification difficult
Clone libraries*	Species/ Community	Cloning bias.	Often based on 16S rRNA gene. Information on diversity, but not on activity.	Currently high throughput, direct information on sequences. Sensitive.	Manual checking of sequences laborious.
Phylochip/Geochip microarrays*	Species/ Community	Cross-hybridisations, difficult data analysis	Large amounts of information, but no information on function/activity.	All-in-once analysis in high-throughput. High potential for comparative studies	Costly, not possible to detect novel sequences
High throughput sequencing*	Species/ Community	Interpretations affected by artifacts/sequencing errors	Large amounts of information on members of the community at sequence level	All-in-once analysis in high-throughput. High potential for comparative studies	Method is error-prone.

* PCR is the basis of these molecular techniques and its pitfalls and shortcomings are also relevant for these techniques.

2.1. Traditional (cultivation based and microscopic) methods and application in GM plant impact assessment

2.1.1. Plating on selective media

Cultivation-based approaches such as traditional plate counting on general or selective media (next to most probable number (MPN) counting) select for specific organisms that are readily culturable on the medium used. The methods suffer from problems due to the (slightly or strongly) selective nature of any medium and incubation conditions used, competition with other microorganisms during incubation and growth conditions that do not mimic those in natural environments (Luo *et al*., 2004).

Examples of use in GM studies: Cultivation-based methods did not show any consistent statistically significant differences between the microbiota in soils planted with GM maize expressing Cry1Ab or Cry3Bb1 proteins and the near-isogenic (non-*Bt)* maize, with respect to the numbers of culturable aerobic bacteria (including actinomycetes), Gram-negative bacteria, cellulose- and chitin-utilizing microorganisms, nitrifying and denitrifying bacteria and fungi. Similarly, enzyme activities during four consecutive years of maize cultivation were also not different (Icoz *et al*., 2007). Also, no consistently significant differences in bacterial counts were detected in a greenhouse study comparing *Bt* and non-*Bt* maize (Brusetti *et al*. 2004). On the other hand, Xue *et al*. (2005) showed that the ratio of Gram-positive to Gram-negative bacteria was lower in soil with *Bt* corn than in soil with near-isogenic non-*Bt* corn. Numbers of colony-forming units (CFUs) in soils with conventional maize were about one-third of those treated with transgenic maize straw (Cry1Ab protein) (Mulder *et al*., 2006). Turrini *et al*. (2004) found that root exudates of *Bt* corn reduced the hyphal growth of arbuscular mycorrhizal fungi as compared with root exudates of non-*Bt* corn. Moreover, a better colonization by fungi (spores and hyphae) was observed in residues of *Bt* cotton than in residues of non-*Bt* cotton.

Pros: Easy and cheap

Cons: Only culturable microorganisms are detectable, which are often a minority in soil. Limited information on soil functioning. Low resolution.

2.1.2. Chloroform fumigation / extraction

During chloroform fumigation, soil samples are fumigated to lyse most of the microorganisms. Chloroform kills microbes, but does not solubilize organic matter in soil. It is assumed that 24 h of chloroform fumigation is enough to lyse most of the sensitive microorganisms. The released cellular components are compared to those of a similar unfumigated soil sample, which is the control. Subtracting the values of the unfumigated control sample from those of the fumigated sample provides a value which is used as a proxy for the carbon in the microbial biomass. We are unaware of any application in GM plant impact assessment. The method yields information on the total size of the microbial biomass, and has a relatively low resolution.

Pros: Cheap and fast.

Cons: No information on microbial community changes. No information on functional or phylogenetic changes.

2.1.3. SIR (substrate-induced respiration) and BIOLOG physiological profiling

SIR is the measurement of (increased) soil respiration following addition to soil of oxidizable substrates like glucose, glutamic acid or amino acids. The method measures the soil's physiological respiration such as CO_2 production or O_2 consumption, to quantify potential microbial activities. It provides a quick estimate of the size of the living soil microbial biomass. The levels of production and consumption can be measured immediately following the addition of the substrate, and thus the method is quick (Sparling, 1995). SIR has been used in combination with chemicals designed to act as selective inhibitors, such as cycloheximide (suppressing soil fungi), to suppress specific parts of the microbial communities (Williams and Rice, 2007). The method has an

analysis time of approximately 1-3 hours, depending on the apparatus used to measure soil respiration.

BIOLOG plates, used in CLPP, allow to monitor the activity of the soil microbiota on a range of substrates in a microtitre plate set-up. Several plate formats are available, either in 95- or 31-well format (ecoplates). The method enables the simultaneous measurement of activity on a range of substrates rather than one-at-a-time.

Examples of usage of SIR and/or BIOLOG CLPP for GM studies: Selective inhibition-SIR showed higher ratios of fungi to bacteria in samples from *Bt* cotton residues than in those from near-isogenic controls (Gupta *et al*., 2002; Gupta and Watson, 2004). An increased activity of the soil microbiota after the addition of *Bt*-cotton straw in comparison to conventional straw was shown via BIOLOG ecoplates (Mulder *et al*., 2007).

Pros: The methods can be conducted fairly quickly, with analysis times of approximately 1-3 (SIR) or 48 h (BIOLOG CLPP, including incubation time).

Cons: Limited information of soil functioning. Low sensitivity.

2.2. Molecular methods

The majority of current molecular analyses of the soil microbiota is preceded by direct soil nucleic acid (or fatty acid; see later) extractions (except for FISH - fluorescence in situ hybridization). The extraction methods that allow access to soil nucleic acids commonly yield both DNA and RNA that are released from soil microbial communities. Most analyses have targeted soil DNA instead of RNA, the reason being the greater stability of DNA during extraction procedures. However, RNA-based protocols are in use in ribosomal marker-based studies and in studies on messenger RNA (Urich *et al*., 2008). Several extraction kits optimized for soil DNA guarantee robustness with respect to the representativeness, quantity and quality of the DNA that is obtained (van Elsas & Boersma, 2011). A key issue here is that such kits will deliver microbial community DNA which includes that from dead or moribund cells, not allowing a clear depiction of

communities that are active. Another issue is the impossibility to tease out all community DNA from a soil sample, thus leading to incomplete sampling. Nevertheless, the study of different aspects of the soil microbiota is spurred by PCR-based amplification of selected target genes. Such target genes include taxonomic markers such as the 16S ribosomal RNA (rRNA) gene, or functional gene markers like *nifH* (encoding a subunit of dinitrogenase reductase, a key enzyme for nitrogen fixation) and/or *amoA* (a subunit of ammonia mono oxygenase, a key enzyme for ammonium oxidation, the first step of nitrification).

2.2.1. FISH

FISH uses fluorescent DNA-based probes that bind to the RNA in the ribosomes, with which they show high degrees of sequence complementarity. FISH is widely used in the field of microbial ecology, to assess the localization and number of microorganisms in natural systems. Preparing DNA probes for one species (which can be selected as an indicator organism) and performing FISH with this probe allows to visualize the distribution of this specific species within a soil or rhizosphere sample. Preparing probes for more than one species (e.g. using two different fluorescent probes, giving two colors) can visualize the co-localization of these species in the soil sample.

Pros: Possibility to highlight interactions and locations of specific microbial species in complex (soil) samples.

Cons: Limited information of soil functioning. Only active organisms are visible. Low resolution and low-throughput.

2.2.2. PCR and qPCR

Classical PCR can only be used in a qualitative or semi-quantitative way. When used in a quantitative manner, we speak of quantitative PCR (qPCR; Holland *et al*., 1991). The principle of this method lies in the generation of a signal that accumulates with the amount of amplicon produced in each cycle of the PCR and can be monitored in the

course of the reaction. Sensitive instruments have been developed that allow the real-time detection of the signal. The number of cycles needed to attain a certain threshold level is used as a measure of the amount of pre-existing template, which can be calibrated on the basis of a pre-established calibration line with standard target DNA. Currently, qPCR is widely used on DNA extracted from soil, allowing the quantification of either total or specific soil bacteria via 16S rRNA or functional genes. The method is subjected to the same biases as PCR based on soil-extracted DNA (Van Elsas and Boersma 2011). It provides a picture of target gene abundance, which however should be regarded as implicitly biased or distorted; therefore, to assess GM plant impact, qPCR is to be used essentially in a comparative fashion. Moreover, PCR or qPCR will not detect any gene of the same function with aberrant sequence.

Examples of usage for GM plant studies: Dias *et al*. (2012) recently applied qPCR-based *amoA* gene quantification to a field cropped to potato, including a GM variant (altered starch composition). They found a clear effect, which did not relate to the plant genotype (including the GM plant) *per se*, but rather to the type of cultivar planted.

Pros: Routine technique of high sensitivity which allows detection and quantification.

Cons: Sensitivity depends on primer choice. There are several PCR biases and artifacts, including inhibition.

2.2.3. Microbial community fingerprintings

The PCR products generated by 16S rRNA or functional gene amplification can be analyzed by the aforementioned fingerprinting methods, i.e. DGGE (Muyzer *et al*., 1998), TGGE (Muyzer *et al*., 1998), T-RFLP (Kuske *et al*., 2002), single-strand conformational polymorphism (SSCP) (Schwieger & Tebbe, 1998) and ribosomal internal spacer analysis (RISA) (Ranjard *et al*., 2000). These methods enable the direct fingerprinting of soil microbial communities at different levels of resolution. DGGE analysis of PCR amplicons (PCR-DGGE) has been most widely accepted, whereas SSCP and RISA (or automated RISA) are used to a smaller extent. In PCR-DGGE, similar-

sized amplicons generated by PCR are separated on the basis of differences in their nucleotide sequences, especially their G+C content. This is done on polyacrylamide gels with denaturing gradients. PCR-DGGE has thus been optimized for soil DNA, constituting a reliable method to characterize soil microbial communities. Depending on the primers used, it can highlight the microbial communities (mainly dominant species) at the level of either the 16S rRNA gene or a functional gene (such as *nifH*). The ability to reamplify and sequence particular bands in the patterns allows for the identification of the microbial types or genes that underlie these bands. However, different sequences may display similar migratory behavior in the gel, thus giving rise to coinciding bands. It is also possible that the presence of multiple melting domains within the same molecule may cause bands to appear fuzzy on gel. Finally, some organisms contain several ribosomal operons, between which micro-heterogeneity may exist. Since functional redundancy among bacteria in soil is high, community shifts observed via PCR-DGGE gels do not *a priori* provide information on changes in soil functioning or quality. Therefore, functions of which the genes are harbored by only one or few bacterial species offer better perspectives to find impacts, as disturbances influencing such low-redundancy groups may have a greater influence on soil functioning than those affecting highly-redundant groups.

In analyses of the soil microbiota, T-RFLP has come up as another robust method. It is based on the detection of (labeled) single terminal restriction fragments per each sequence amplified directly from an environmental sample DNA (Kuske *et al*., 2002). The ensuing fingerprints, characterized by peak-valley patterns, depict the microbial communities assessed. T-RFLP is technically robust, however lacks the discriminative power of DGGE, given the fact that terminal restriction sites often coincide between species.

All fingerprinting methods have the intrinsic problem that they show only the most dominant representatives of the microbial populations assessed. Whereas the methods thus allow visualizing changes in the major populations in soil, we realize more

and more that also members of the rare biosphere may have strong effects on the functioning of ecosystems.

Examples of usage for GM plant studies: Dias *et al* (2012) recently investigated the bacterial communities under potato, including an amylose-free GM potato. Their PCR-DGGE based analyses did not reveal any effect of the GM plant variety on these communities (as compared to the comparator plants), whereas they did find a major effect of time (plant developmental stage). Chun *et al*. (2012) conducted a 2-year field study and an analysis via PCR followed by T-RFLP to assess the impacts of GM rice (resistance to protoporphyrin oxidase inhibiting herbicides) versus the near-isogenic counterpart on soil bacterial and fungal communities. No differences were observed in the diversities and compositions of the microbial communities between GM rice and its non-transgenic counterpart. Instead, community variation was dependent on growth stage and year. In a three-year field study (Hannula *et al*., 2012), the impact of different potato cultivars - including a GM potato (amylose-free) - on rhizosphere fungal communities was investigated using T-RFLP on the basis of PCR with fungal-specific primers. Additionally, fungal biomass and extracellular fungal enzymes (laccases, Mn-peroxidases and cellulases) were quantified. Plant growth stage and year had the strongest effect on both diversity and function of the fungal communities while GM was the least explanatory factor.

The *nifH* gene has been used to study the impact of GM-Bt white spruce on soil nitrogen-fixing communities via PCR-DGGE (Lamarche *et al*., 2007). The authors did not find a significant effect of the GM plant on nitrogen-fixing communities. Finally, consistent significant differences were detected between Bt and non-Bt maize in 16S rRNA assessments of culturable rhizosphere bacteria by PCR-DGGE (Tan *et al*., 2010).

Pros: Easy, robust and optimized methods. In PCR-DGGE, highly discriminative bands can be excised for sequencing to identify the species.

Cons: Only over 0.1-1% abundance organisms are visible. No information on soil functioning. Possibility of occurrence of artefacts.

2.2.4. Clone libraries

In clone library analyses, PCR-generated amplicons are ligated into a suitable vector plasmid. Then, the resulting constructs are introduced into an *Escherichia coli* host by transformation. After growth of single colonies that received vectors with insert, cloned amplicons can be isolated by plasmid extraction, sequenced and the sequences analyzed by comparison to databases. The sensitivity of clone library analyses, with respect to understanding the community make-up, is somewhat higher than that of the fingerprinting techniques. This is mainly due to the sequences being analyzed separately, and hence single sequences from abundant or less abundant species are well detectable. Clone libraries have relatively high resolution but do not allow a quick overview of the difference in community make-up between samples, as is the case with fingerprinting techniques. Therefore, often there is a need to combine methods. It is important to recognize the cloning bias that is inherent to the technique, i.e. DNA fragments are ligated into a vector plasmid with possibly differential efficiencies.

Examples of usage for GM plant studies: Dias *et al* (2012) applied clone library analysis to a field containing GM and non-GM potato lines and found that differences between amylose-free GM line and the common variants were ephemeral. In another study, analyses of 16S rRNA gene clone libraries indicated that bacterial community structures differed between soils from a non-GM plot and a GM plot. The bacterial community of the plot with the GM rice (modified to express ABC-TPSP, a fusion of trehalose-6-phosphate synthase and phosphatase) was less diverse than that of the non-GM plot (Lee *et al*., 2011).

Pros: High throughput, sensitive, providing direct information on community make-up from sequence analyses.

Cons: Large cloning bias. A lot of information on microbial community structure but no information on activity or on functioning.

2.2.5. DNA microarrays

In DNA microarray analysis, soil DNA (often after a PCR amplification step) is fluorescently labeled and brought into contact with a microarray of probes. Two developments have led to the current routine use of two types of microarrays (chips), i.e. (1) the Phylochip (phylogenetically based), and (2) the Geochip (functionally based). See van Elsas and Boersma (2011). Sequences in the soil DNA that are homologous to the probes present on the microarray will bind via hybridization and at the positions of their homologous counterparts. After hybridization, the signals on the chip are digitally analyzed. Microarray hybridization has great potential, including the possibility to generate a so-called universal microarray which would describe soil quality or health. However, it is important to ascertain which probes are selected to be part of the chip. Moreover, positive detection will depend on the probes that are present on the chip and unknown organisms or genes will therefore not be detected by using chips that are based on database sequences (Yergeau *et al*., 2012). We are unaware of any application in GM plant impact assessment.

Pros: High throughput. High potential for comparative studies

Cons: Large amount of information, however no information on soil functioning. Not possible to detect novel sequences.

2.2.6. High throughput sequencing

Several powerful novel sequencing techniques have been developed over the last decade (Rothberg *et al*., 2011), such as Roche 454-based/pyrosequencing, Illumina/Solexa genome analyzer and Ion Torrent sequencing. These high-throughput technologies seem very suitable for massive parallel sequencing of soil metagenomes and metatranscriptomes (Wall *et al*., 2009). Thus, Roche 454-based pyrosequencing consists of multiparellel sequencing by synthesis, in which pyrophosphate that is released is detected in an enzymatic cascade ending in luciferase and detection of the emitted light. Pyrosequencing bypasses three bottlenecks of classical sequencing, namely library

preparation, template preparation and the actual capillary sequencing. The Illumina/Solexa and Ion Torrent platforms work by different principles. The sensitivities of the three high-throughput sequencing platforms, collectively called next-generation sequencing (NGS), are mainly determined by the efficiency and unbiased nature of the sequencing, which directly uses soil DNA. Therefore, the soil DNA extraction method strongly determines the representation and eventual bias of the data. A major advantage of the three sequencing methods is that many new sequences will be discovered, thereby giving novel insight into the soil microbiota and its functioning (Elshahed *et al*., 2008). Currently, a shift can be perceived in the literature from the community fingerprinting based techniques to methods based on NGS. However, there is evidence that fingerprinting methods yield information similar to the high-throughput sequencing information.

Examples of usage for GM studies: In a study by Inceoglu *et al* (2010), the rhizospheres of six potato cultivars, denoted Aveka, Aventra, Karnico, Modena (GM variant, amylose-free tuber), Premiere and Desiree, at three growth stages (young, flowering and senescence) were examined by bacterial amplicon sequencing, in addition to corresponding bulk soils. Around 350,000 sequences were obtained (5,700 to 38,000 per sample). Principal components analyses revealed that rhizosphere bacterial communities were significantly different from those from corresponding bulk soil in each growth stage. Furthermore, plant cultivar effects were found in the young plant stages, whereas these became insignificant in the flowering and senescence stages. Besides, an effect of time of season was observed for both rhizosphere and bulk soils. However, the putative effect of the GM plant (Modena) was insignificant.

Pros: High throughput. Large amounts of information. High potential for comparative studies.

Cons: Laborious methods. Sequencing errors, in particular in pyrosequencing and Ion Torrent. Large amount of information on the community at sequence level, limiting replication and statistics. Expensive.

A summary of the here described methods is provided in Table 2 (below). In this Table, we also highlight the strengths and weaknesses (pros versus cons) of each method.

Table 2. Review of characteristics of selected methods to study the microbiota of soil*

Technique		Type	Sensitivity	Technical requirements	Costs	Level of throughput	Automation	Processing time	Reproducibility	Specificity	Quantifiability
Dilution plating		Culture dependent	Medium	Low	Low	Low	Possible	Medium	Low	Low	High
Chloroform fumigation/extraction		Chemical	Low	Low	Low	Low	Not possible	Low	High	Low	Medium-High
SIR (substrate induced respiration)		Chemical	Low	Low-Medium	Low	Medium	Partially possible	Low	High	Low	High
FISH		In situ	Medium	Medium-High	Low-Medium	Low	Possible	Low	n/a	High	High
qPCR*		Molecular	High	High	Medium	High	Possible	High	High	High	High
DGGE/ TGGE*	Phylogenetic	Fingerprint	Medium	Medium	Low	Low	Not possible	Medium	High	High	Low
	Functional	Fingerprint	Medium	Medium	Low	Medium	Not possible	Medium	High	High	Low
SSCP*		Fingerprint	Medium	Low	Low	Low	Possible	High	Medium	Medium	Low
T-RFLP*		Fingerprint	Medium	Medium	Low-Medium	Medium	Possible	Medium	High	Medium	Low
Clone libraries*		Molecular	High	High	High	Low-Medium	Partially possible	High	Medium	High	Low-Medium
Phylochip/Geochip microarrays*		Molecular	High	High	High	High	Partially possible	High	Medium	High	Medium
High throughput sequencing*		Molecular	High	High	High	High	possible	High	High	High	Medium

* PCR is the basis of these techniques and its pitfalls and shortcomings are also relevant for these techniques. Scores are arbitrary (low/medium/high).

3. Environmental risk assessment (ERA) of GM plants - potential effects on the soil LSF

3.1. Risk assessment strategy

GM plants introduced into living (agricultural) soils may affect the functioning (LSF) of soil (in particular, biogeochemical cycling and disease suppression), which are both carried by the soil microbiota. A focus on these two important parameters of the living soil under GM plants is thus warranted.

Generally speaking, risk encompasses two facets, hazard and exposure, and as such is often defined as ***hazard x exposure***. Thus, as a first step of any risk assessment of GM plants, questions about hazard and exposure need to be answered. If there is a hazard and an exposure, the ensuing risk assessment should be based on an analysis of scientific data, resulting in a description of the form, magnitude and characteristics of risk. This is often done in a tiered approach.

Here, as we focus on the risk posed by GM plants to the LSF carried by environment / soil, and in conformity with EFSA (2010), the assessment will include several steps:

1) problem formulation (including hazard identification);

2) hazard characterization;

3) exposure characterization;

4) risk characterization;

5) possible risk management strategies and

6) overall risk evaluation.

Of these, this report will address points 1 through 4 in more detail, with an emphasis on 4, whereas risk management as well as overall risk evaluation clearly fall outside of the remit of this study.

Problem formulation includes the identification of characteristics of the GM plant that may cause adverse effects to the environment / soil (hazards), of the nature of these effects and of pathways of exposure through which the GM plant exhibits its effect on the

soil. It also includes defining assessment endpoints and setting of specific hypotheses to guide the generation and evaluation of data in the next steps (hazard and exposure characterization). In this process, both existing scientific knowledge and knowledge gaps (such as scientific uncertainties) are considered (EFSA, 2010).

Problem formulation is followed by ***hazard characterization***, which pinpoints possible adverse effects due to the exposure of the soil to the GM plant. A key issue here is the type of hazard that may be expected to occur due to the modification. This prediction is based on the nature of the trait and what types of microorganisms might be affected. Then such hazard may be experimentally assessed by testing a (limited) array of soil organisms or processes that are potentially vulnerable to the trait. In particular in cases of GM plants which express new proteins or metabolites, rather specific hypotheses may be formulated and assessed. For instance, plant material and exudates from GM plant can be deleterious for some groups of soil microbes that are vulnerable to these metabolites.

As an example, consider the case of T4-lysozyme producing potato. T4 lysozyme is known to lyse some Gram-positive and Gram-negative bacteria (including plant pathogens such as *Erwinia carotovora)*. Therefore, the testable hypothesis is if other (focal) species might be negatively affected by T4 lysozyme. Testing may proceed by comparing the effects of the GM to those of the non-GM potato (see for more detail Chapter 4.1). Alternatively, literature data or data from other experiments can be used to assess the potential effect on the focal species.

One should then determine to what degree the GM plant interacts with the focal species (***exposure characterization***), allowing to conclude on the likelihood of this potential negative effect. Taking the same example of the T4 lysozyme potato, effects on the soil microbiota are most likely to occur for those plant parts to which the soil/environment is mostly exposed. For that matter, the roots (in which the T4 lysozyme is meant to be expressed) are the major plant parts. However, in general, plant material that remains in the soil following harvest is also important. A key issue is therefore how such plant parts affect the soil microbiota differentially from the near-isogenic

counterpart plant. This testing is normally based on comparative analyses with the near-isogenic parent in the same soil, and impacts on the soil microbiota are to be taken into account if they are significantly different with the GM plant (related to the presumed direct effect).

Based on the potential negative effects (hazard) and the likelihood that the effect can occur (exposure), the risk of the GM plant for the studied focal species in the soil is estimated (***risk characterization***). Risk characterization is defined as the weighing of the factors hazard (type, severity) and exposure (duration, degree of contact with the exposed organisms) in a mathematical or descriptive manner, in order to make a professional judgment of acceptability. For soil interactions, it refers to the estimation of the degree and duration of the potential disturbance of the soil LSF, as well as the potential of the system to regain the LSF (resilience).

3.2. The use of a tiered approach for risk assessment of GM plants

In the aforementioned four steps of GM plant risk assessment, use of a ***tiered approach*** is advocated. Such a tiered approach is most easily sketched as having a layered structure (tiers or levels zero through 2 or 3). At level zero (tier *zero*), a specific hazard is to be identified and a potential problem formulated. Then, at level (tier) 1, testing of the putative impact is to be performed, in which a series of test methods (Tables 1 and 2) is selected that clearly identify, as a first approach, which targets may be affected (so-called ***indicator organisms*** or functions) and to what extent. Data on the actual effects are thus expected to be produced under tier *1*. If the outcome of such tests does not show 'unacceptable' effects, the GM plant impact may be judged to be within acceptable limits, exempting one from doing further testing. Here, acceptability needs to be defined, which is best done as: "not causing unacceptable harm to the system". This is at the judgment of competent authorities and based on the best scientific judgment of the case. If (after the first tier) there is still reason for concern or uncertainty (e.g. the impact falls outside acceptable limits), one can go to tier *2*, which will use a suite of other methods to

place an in-depth focus on, e.g., the specificity and severity of the effect, its breadth in terms of organisms / processes affected and its overall effect on the soil LSF (e.g., possible effect of the GM plant on a larger range of microbial groups/functions). This tier will thus yield "deeper" data that allow to assess the intricacies of the effect and whether this is at an acceptable level. If the latter is the case, one may assume that, in spite of the existence of a discernible GM plant effect, this effect is within acceptability. If the effects are outside the acceptable level and are considered to be biologically relevant, a clear risk is identified and the investigator must face the possibility that the risk is too high. Alternatively, additional (deeper) testing may be required, leading to tier 3. In this tier, for instance, the impact at the level of the whole complexity of the system may be analysed. It is up to the responsible people (risk assessor / applicant) to decide whether a third tier (tier *3*) of analysis is/are necessary.

In addition, risks may be managed or mitigated in ***risk management*** strategies which can reduce the risk to a level which is considered acceptable. Whether an effect of a GM plant as compared to the non-GM plant (for example on a focal species) is to be considered as a risk, the magnitude of the effects and the likelihood of their occurrence have to be taken into account and these effects should be compared to those of various agricultural practices (e.g. use of pesticides; crop rotation; crop varieties). Finally, conclusions have to be drawn on the risk of intended and unintended effects on overall functionality of soil microbial community and its diversity as well as whether or not the levels of risk exceed acceptable threshold levels.

3.3. Method selection and the comparator

3.3.1 Choice of methods – case-by-case approach

If a risk of a GM plant on the soil ecosystem is identified, methods for testing should be supplied. Depending on the specific trait of the GM plant, the expected effect on the soil microflora should provide guidance to make use of those tools from the suite of methods that are available (Tables 1 and 2). Combinations of methods should be selected that are

best suited to answer the specific questions that may have arisen in prior considerations about potential impact. The testing needs to be done in a comparative fashion, in other words, potential impacts of GM plants need to be compared to impacts of comparator plants. In the following, we discuss the guidance in the use of the available toolset to assess impacts of GM plants on the soil LSF and define the principles that will lead us towards the relevant testing protocols.

3.3.2. The comparator – baseline of the cropping system

In general, the potential impacts of GM plants on the soil microbiota are to be compared against those of comparator (non-GM) plants. For this, near-isogenic plant lines are often employed, but using a suite of lines of the same crop species is also a good option. The natural variation under either one or the suite of plant lines may provide the so-called *baseline* of the respective cropping system. Any effect of the GM plant variety to be tested is thus to be weighed against such a baseline.

Baseline development leading to the concept of a soil NOR - Recently, the concept of a soil's normal operating range (NOR) has been developed in terms of a model that provides a proposal for quantification of a range of soil parameters (Pereira e Silva *et al*., 2013). The usefulness of such a NOR in a monitoring framework relies on its ability to define what is deemed normal in a soil. Thus, only (changed) soil parameters that fall outside the boundaries of the NOR are considered to be of potential concern. The NOR is to be considered an extension of the concept of baseline; it includes a larger number of variables. Given the fact that most soils can be characterized as being multifunctional, this implies a multi-focused view on the system. In a sensible use of the soil NOR in GM plant risk assessment, it is important to select those variables which are directly related to potential adverse effects of specific GM plants (Schloter 2003; Bruinsma *et al*., 2003). Briefly, the process parameters that are selected as most relevant should

(1) be relevant to the ecosystem under study and

(2) reveal a fair response to factors that would put the system outside of the NOR.

Box 1 – Definition of a soil normal operating range (NOR)

To define the NOR, an integrated procedure based on multivariate statistical techniques such as principal component analysis or regression can be used. The approach can be adopted from the assessment of the effect of pesticides on aquatic systems. Such an approach has been launched by Kersting (1984) for analyzing several variables simultaneously. A similar approach has been suggested for the detection of ecotoxicological effects of soil pollutants (van Straalen, 2002). Mathematically, the trait-based approach can include numerous parameters. It can thus be depicted as a multidimensional space of n dimensions, where n is the number of parameters measured in the system, its borders representing the NOR (Fig. 2). Moreover, the strength of the "stress", or how much a soil is outside the NOR, can be determined by the distance between the "stressed" soil situation and the border of the multidimensional space of NOR. If the soil is in an undisturbed state, all combinations of the parameters fall within the NOR. Despite the utility of the proposed model, it should be clear that it is informative rather than predictive. Thus the NOR approach could be used as a tool that allows users to detect changes in the soil, which might indicate impacts that go beyond normal soil functioning.

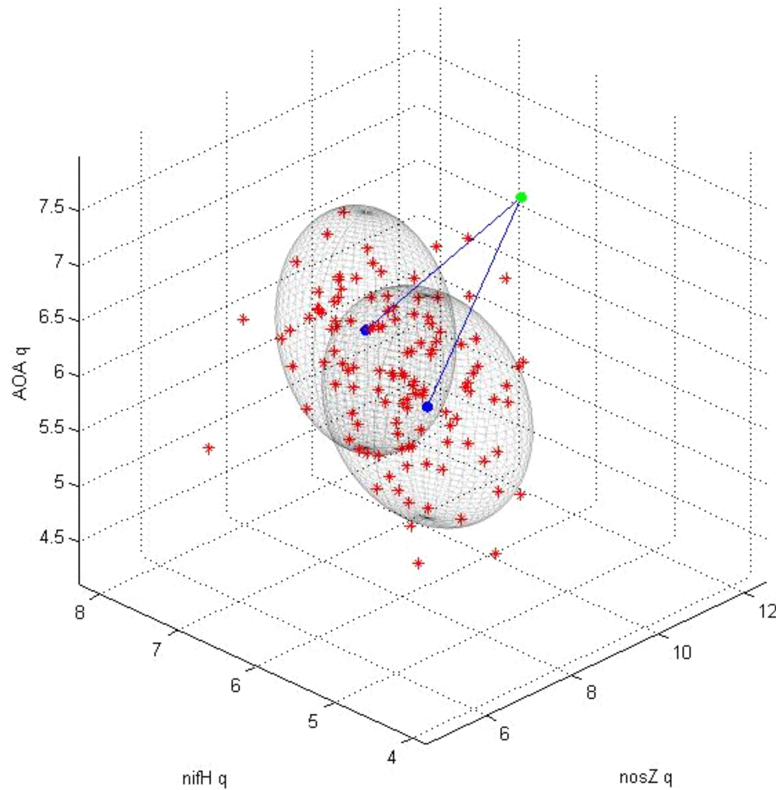


Figure 2: Representative example of a NOR of soils showing three of the 22 dimensions (22 variables). The upper ellipsoid characterizes the NOR for clay soils while sandy soils is represented by the lower ellipsoid. The ellipsoids represent the borders of the NOR for three variables (abundance of AOA, nifH and nosZ). Red crosses are observed values which characterize the NOR. The blue line is the distance between the center of the NOR (blue dot) and the investigated soil (green dot). It is important to mention that the distance that reflects how much the selected soil (green dot) is outside the NOR is the distance between the green dot and the edge of the three-dimensional sphere.

3.3.3 The need to address sensitive processes

The effects of particular impacts that place the soil LSF under pressure outside of the comparator / baseline / NOR might not be detected easily. To enable the earliest possible detection of impact, we argue that the baseline / NOR should be defined primarily by selected and particularly non-redundant or lowly-redundant (and therefore impact-sensitive) key functional groups, e.g. the ammonium oxidizers (Kowalchuk and Stephen,

2001; Mendum, 2002), methane oxidizers (Hanson and Hanson, 1996), nitrogen fixers (Pankhurst *et al*., 1995), sulphur mineralizers (Deng and Tabatabai, 1997; Schmalenberger *et al*., 2008) and/or mycorrhizal fungi, as well as possible plant disease antagonists. Of these, the nitrogen fixers are possibly of highest redundancy and therefore less suitable as a truly sensitive functional group. However, their key role in soil ecosystems provides a plea for their use. The approach to using sensitive groups is to be guided by educated guesses with respect to identifiable effects on such communities (which may - as yet - not always be available). Due to generally low functional redundancies, changes in different components of the relevant microbial communities will be translated more promptly into changes in the related processes. Thus, the potential of such organismal groups and functions to allow detection of subtle changes in the ecosystem make them good candidates for providing an early warning of the degradation of a key process in soil functioning. From such functions, nitrogen fixation (focus on e.g. the rhizobia, thereby lowering functional redundancy) and ammonium oxidation (first step of nitrification) have been advocated as providing quite suitable proxies, in particular in the context of the assessment of the risks of GM plants (Kowalchuk *et al*., 2003; Bruinsma *et al*., 2003).

4. Case studies with respect to the assessment of effects of GM plants on the soil LSF

4.1. General considerations

The specific nature of the transgene in a GM plant should provide guidance in defining the approaches/methods that are most suitable to assess the potential effects on the soil LSF and microbiota. The baseline / NOR for the cropping system should serve as the comparator, but in several cases the near-isogenic parent plant may be suitable. The primary questions here are:

(1) is testing of the specific GM plant necessary, and, if so,

(2) what types of methods apply to determine effects on the soil LSF and microbiota?

Let us work with a hypothetical case, i.e. a GM plant with modification X. The case encompasses all possibilities concerning the risk of GM plants. Thus, the modification, as far as we understand it, may or may not be intended to exert an effect on the soil LSF or on its associated microbiota. In the case of no intended / expected effect on the soil microbiota, and if a worst-case scenario excludes the occurrence of (direct or indirect) unintended adverse effects, no tests would be felt to be necessary as the risk for the soil microbiota would be considered to be negligible. However, in cases where there is potential for an impact on the soil microbiota, testing may become necessary up to the judgment of the investigator. Hence, one must carefully weigh the potential hazard and exposure terms, resulting in a ***risk characterization***, as outlined in the foregoing. Here, the best professional judgment as to what type of impact may be expected is necessary. In case testing is deemed necessary, the scheme as under ***Section 3.1.*** will be followed. In practice, soils from the GM plants with modification X and from the baseline plants (e.g. near-isogenic counterparts) are subjected to selected analyses (Tables 1 and 2), such as of the low-redundant key microbial groups, as described in the foregoing. The premise is that a lack of impact on these sensitive key groups signifies a lack of impact on the whole soil microbial system.

Above, we advocated the use of a tiered approach in the assessments (***Section 3.2***), these being comparative between the GM and comparator lines. In tier zero, a potential hazard may have been identified. Then, as a first step, tier 1 may compare the abundances of specific ***indicators*** of the sensitive processes using selected direct DNA-based molecular tools (e.g. qPCR to test for abundances of the selected bacterial groups next to a selected molecular fingerprinting method). These analyses will yield information about (the absence of) impacts on these selected key indicator groups. If the effect of the GM plant is minor (and thus acceptable) in comparison to the near-isogenic line and (if tested) to other commercial varieties, this may be a reason to conclude that the effects are negligible. Consequently, tier 2 is not necessary. However, in case of an impact that causes concern, the assessment must move to tier 2, in which more focused

and potentially deeper testing (collecting the relevant methods for such testing) will have to take place. And here, the cycle may repeat, as outcomes from tier 2 may provide concerns to be answered under tier 3, in which one should address the full complexity of the system (system-level approach).

Note: to define indicators, a case-by-case subset of microbial species/functional groups - that are relevant to the soil system should be selected for consideration in the risk assessment of the GM plant. The selection should be based on several criteria, including the ecological relevance of the species/functional groups, presumed susceptibility to GM plant stressors and/or testability. For instance, one may select low-redundancy key groups, such as the ammonium oxidizers (basis is the *amoA* gene), the nitrogen fixers (for instance, rhizobia, the basis is the *nifH* gene) and/or the mycorrhizae.

In the sections below, examples are given to show how the case-by-case tiered approach might work. We used the term "scientific trigger" to indicate tier zero, whereafter tiers 1 and on follow as a logical development.

4.2. T4-lysozyme producing potato

In the 90-ies, potato (*Solanum tuberosum*) has been engineered to express the T4-lysozyme gene. This represented a strategy of control of a variety of economically important bacterial crop diseases (Düring, 1996). T4 lysozyme affects Gram-positive and Gram-negative bacteria either by its muramidase activity against the cell wall component murein or by a non-enzymatic mechanism which may involve the disruption of membranes (Düring *et al.*, 1999). It was shown that plant-associated bacteria were indeed affected, in that the susceptibility of the T4-lysozyme potato plants to infection by *Erwinia carotovora* was significantly reduced (Düring *et al.*, 1993). Moreover, a detectable amount of T4-lysozyme was released from the roots, causing bactericidal activity at the root surface (Ahrenholtz *et al.*, 2000). Also, the effects on different species varied significantly under *in vitro* conditions (de Vries *et al.*, 1999).

Scientific trigger (tier zero): Possible adverse effects due to the T4-lysozyme released by the GM plant on Gram-positive and Gram-negative bacteria in the soil microbiota. The presence of this enzyme in the rhizosphere might lead to altered nutrient cycles and/or changes in the structure and activity of soil bacterial communities. In a worst-case scenario, such changes might lead to decreased sustainability of soil functioning. Due to the anticipated influence on bacteria, tests should focus on these and tests for soil fungi are not *a priori* required.

Tier 1: Testing of the abundance and structure of the soil bacterial communities under the influence of T4-lysozyme producing potato and its near-isogenic counterpart (which might include more than one conventional potato varieties) is recommended. Inclusion of more potato lines makes the data more robust. Given the expected effect of T4-lysozyme on many (Gram-positive and Gram-negative) soil bacteria, selection of key sensitive or important functional bacterial groups is adequate. A selection of lysozyme-sensitive bacteria of the aforementioned groups that participate in key soil processes is warranted. Thus, for the soil under study, ammonium oxidation and nitrogen fixation are key processes and hence sensitive bacteria belonging to these functional groups are good candidates to be measured. The techniques to be selected are e.g. fingerprintings such as PCR-DGGE focusing on *amoA* or *nifH* genes, next to gene/organism-specific qPCR assessments to address abundances. These methods may thus highlight possible changes in the abundances and community structures of the ammonium oxidizers and nitrogen fixers. Another focus could be on disease-suppressing bacteria, however our current knowledge on these is limited. Possibly, a functional (pathogen suppressiveness) test would be useful. If no differences are found in this comparative approach, no further testing may be needed, as the identified potential problem appears not to lead to a significant ecological effect. However, if tier 1 identifies a measurable impact, then tier 2 should attempt to better characterize the impact and risk.

Tier 2: The magnitude of the effects found under tier 1 should be investigated at a deeper and broader level under tier 2. Thus, adequate information about the severity of the possible drop-out of key functional or disease-suppressive (or otherwise ecologically

important) species needs to be obtained, using selected tools (Tables 1 and 2). Moreover, a linkage to process parameters should be sought, e.g. testing soil respiration (SIR), ammonium oxidation, nitrogen fixation or disease suppression. In exceptional cases, clone libraries or pyrosequencing can be applied to distinguish the breadth of the bacterial groups affected by the T4-lysozyme producing potato in comparison with near-isogenic counterparts. However, if there is suspicion of imminent serious deleterious effects on the soil LSF, such concerns may lead one to consider negation of the application or application of a third assessment tier.

Tier 3: In a third tier, the duration and gross system-level impact of the GM plant is to be investigated, pulling the appropriate techniques for such an assessment out of the toolbox. Thus, tier 3 will not differ fundamentally from tier 2, with the exception that methods are used that allow a much deeper understanding of the problem (e.g. duration, breadth, ecosystem impact).

4.3. Hordothionin-producing apple tree

Among the diseases that affect apple cultivation in Europe, the most prominent one is apple scab, a fungal disease caused by *Venturia inaequalis*. To diminish the application of fungal treatments, trees can be genetically modified to obtain resistance against apple scab using a gene coding for the antifungal compound hordothionin. Hordothionin is present in the leaves of barley (Florack and Stiekema, 1994) and its coding gene can be introduced into apple by genetic modification. Being small (approx. 5 kDa), the proteins inhibit the *in vitro* growth of a number of fungi (e.g. *Fusarium graminearum*) and bacteria. Only the scion of the GM trees is modified, the root stock is not.

Scientific trigger (*tier* zero): Although hordothionin, which is produced by GM apple tree scions, can have effects on fungi and bacteria, most of the interactions will occur on the surface of apples and leaves. Thus, the possible influence of hordothionin on the soil microbial community is limited to cases where leaves or fruits are in contact with the soil.

Therefore, the exposure of the soil microbiota to hordothionin may be relatively low. Moreover, hordothionin is a natural substrate in barley and hence the soil microbiota under barley is already in contact with hordothionin. However, in a worst-case scenario, biogeochemical cycles in parts of the soil might be affected, and therefore an analysis of the risk of the application is needed. First, the analysis thus addresses the effects from a theoretical perspective: what type of hazard and exposure is there, and how far-reaching and long-lived can hazard and exposure possibly be?

Tier 1: The GM plant carries a broad-spectrum antimicrobial protein and therefore key soil microbiota may be affected in scion-soil contact situations. The risk assessor may consider to test whether key sensitive bacterial and fungal groups (similar to the aforegiven example, and occurring in soil under hordothionin-producing apple trees) might be affected. If needed (up to a prior judgment of necessity given time and money constraints), a comparative analysis of GM impact with that of near-isogenic counterparts on soil can highlight possible changes in the key soil microbiota. The testing of soil samples impacted by apples or leaves from GM versus near-isogenic parent trees will proceed along the lines set in case study 1, with the difference that the focus is placed on fungal next to bacterial groups. Thus, in contact situations, fungal communities can be assessed using e.g. inhibition assays or 18S rRNA gene based PCR followed by DGGE or DGGE. The focus may be placed on mycorrhizal fungi. Given the fact that impacts on bacteria (and archaea) might occur, nitrogen fixers and ammonium oxidizers are again the candidates to be measured for potential impact. Thus, qPCR and PCR-DGGE focused on *nifH*, *amoA* genes and mycorrhizae may be performed to highlight possible changes in the selected key soil microbiota. In cases in which no significant differences between the contacted communities under the GM versus near-isogenic parent apple tree are found, no reason for concern will remain. However, if there are major shifts as a result of the modification, extra tests may be required (tier 2).

Tier 2: In case of significant effects in tier 1, the depth, breadth and timing of the impact on the soil LSF (biogeochemical cycling and disease suppression) is to be considered. For this, suitable techniques are to be selected. For instance, SIR, which distinguishes the

respiratory activity of the soil microbiota, is recommendable, as it addresses a major generic soil function important for general activity impacting all soil LSFs. Moreover, in the case of suspected impacts on (potentially disease-suppressive) soil fungi, the use of tests addressing disease suppressiveness of the soil can be cogitated. In cases concerns relate to dropped-out fungal species, fungal clone libraries may analyze the types of fungi that are impacted. The outcome of tier 2 should provide collective data that allow an educated assessment of whether the putative risk / impact can be considered to be acceptable. In cases of concern and a need-to-know more, a third tier of testing can be included (see example 4.2).

4.4. Chitinase-producing plants

Many plant diseases are caused by fungal (as well as insect) pathogens, both of which are sensitive to chitinases. Therefore, chitinase-producing disease-resistant GM plants are currently under development as non-chemical alternatives to pathogen control (Graham & Sticklen, 1994). However, chitin is also a component of beneficial soil fungi or insects.

Scientific trigger (*tier* zero): Chitin is the main structural component of fungal cell walls and therefore chitinase production by GM plants has the potential to change the structure of the fungal communities, in particular in the rhizosphere. Such changes might lead to a different biogeochemical cycling or availability of nutrients in soil due to a rebalancing of nutrient cycles. Moreover, in cases phytopathogen-suppressive fungi are impacted, the disease-suppressive nature of the soil system might change. Also, particular chitin-containing soil insects may be affected. Whereas this scientific trigger is real, it should be weighed against the natural occurrence of chitinase activity in soil communities, which may be roughly estimated at 1-5% of the soil microbiota.

Tier zero: A definition of the potential problem is obtained under tier zero. Chitinases are already being produced by the microbiota present in the soil and so there is a natural background. However, their release from plant roots may change the ecological impact,

affecting sensitive groups of fungi. Of these, saprotrophic as well as mycorrhizal fungi, next to pathogen-suppressive ones, are prime choices of key fungi to be considered for testing. Will these be affected by the chitinase produced by the GM plant? In a first attempt, the theoretical basis of such an effect, which thus outweighs the natural background of chitinases, is to be examined. Second, key fungi that are predicted to occur in the target soil should be considered (and selected for testing) as to their sensitivity to the chitinase. In cases these theoretical analyses yield an outcome of no or negligible risk, there would be no need to move to tier 1.

Tier 1: A recommended further step (if a hazard is identified in tier zero) is to examine the impact on the soil fungi that are potentially impeded by the GM plant. For instance, direct tests may be performed in microcosms, in which qPCR is used (provided suitable fungal primers are available) to assess fungal abundance changes. Phylogenetically-defined fingerprintings such as 18S rRNA gene based PCR-DGGE can also focus on the structure of the soil fungal communities, identifying key functional fungal groups. Sequence analysis of identifiable bands from the DGGE gels can then follow. The comparative analyses will thus highlight changes in fungal abundances and community structures between the GM and near-isogenic plant lines. The relevance of any such impact is to be judged by the risk assessor, who may judge that the ecological impact is either significant or negligible (in which case no extra tests are required). However, testing under tier 2 may be invoked in cases of significant impact; tier 2 should thus attempt to better characterize the impact and risk.

Tier 2: A potential impact on all or particular fungi may be further investigated concerning impact on the soil system, e.g. with respect to functioning in terms of biogeochemical cycling (e.g. carbon compound degradation or mineralization, soil respiration) or suppressiveness towards particular soil pathogens. For these aspects, the appropriate tests are to be selected from the **toolbox**. The outcome of the testing may either lead to a liberation of the application in case the impact on soil function is judged to be negligible (for instance, because the effect is ephemeral or small), or lead to the conclusion that even deeper testing is needed under tier 3.

Tier 3: In cases the impact on soil function measured under tier 2 is judged to be too severe or strong to allow the GM plant application, it needs to be further investigated with respect to duration, spatial effect, ephemerity and degree of impact on global biogeochemical cycles. This will entail testing along the same lines set under tier 2, however on a more thorough basis. The final outcome will either lead to negation of the application (impact judged to be too severe) or to liberation (impact judged to be acceptable).

4.5. Potato changed in starch composition

The starch in conventional potato tubers consists of two components - amylopectin and amylose. In industrial processes, only the thickening properties of amylopectin are required, while amylose is undesirable in many products. Moreover, the chemical modification and separation of these two components is associated with a high consumption of energy and water. Therefore GM potatoes were developed in which the amylose content is lowered due to RNAi-mediated suppression of a tuber-specific *kg*z gene (Inceoglu et a., 2012).

Scientific trigger (tier zero): GM potatoes with changed starch composition due to RNAi-mediated suppression of an endogenous gene are, in theory, not expected to adversely affect the soil microbial community, since only the composition of (tuber-internal) starch is changed in the GM potato. However, there may be unintended effects resulting from the imposed changes of plant physiology that are reflected in altered root exudation or setting patterns. Such alterations might be thought of as being within the range of normal potato cropping practice, in which case the risk assessment would conclude – under tier zero – that no uncommon impacts are to be expected on theoretical grounds. However, in those cases in which the changed exudation would surpass the baseline / NOR of potato cropping, an examination of key soil functions carried by bacterial and fungal communities (thus involved in soil LSF), at the levels of abundance and community structures, may be warranted. Low-redundancy / key relevant groups,

such as ammonium oxidizers and nitrogen fixers, are then to be assessed in order to address such potential impact. Recent work on such potential impact on ammonium oxidizers, however, indicated that plant cultivar type as well as location in the field (plot), rather than GM plant per se, affected the community of ammonium oxidizers. Taking all considerations into account, an educated judgment is needed here (on theoretical or experimental grounds) as to whether tier 1 testing is warranted or not. If so, the focus needs to be on the same key sensitive groups recommended under example 4.2.

Tier 1: Under tier 1, the hypothesis of changed root-released compounds of a GM plant beyond what is normal in the cropping system, leading to an altered microbiota is to be tested. Thus, both biochemical analyses and fingerprinting methods to assess key bacterial and fungal community structures (focusing on key groups involved in soil LSF such as ammonium oxidizers and nitrogen fixers) are required. In addition, the sizes of the bacterial and fungal biomasses may be assessed using the appropriate techniques, e.g. qPCR or direct microscopy. In cases of no differences between the GM and near-isogenic parent or other comparator plants, tier 2 is not needed. However, it needs to be performed in cases of any significant change.

Tier 2: In cases of impacts of the GM potato plant on the sizes of the bacterial or fungal communities or of the functional communities (ammonium oxidizers, nitrogen fixers), analysis of the relevant soil function affected (for total communities: respiration; for functional communities: the specific function) needs to be performed. In cases of impact on community structures or diversity, an in-depth analysis of the eventual lost organisms needs to be done, using identification of DGGE gel band identity. Following identification of the change, an educated estimation of the hazard and risk is to be performed, leading to a conclusion on negligibility or not of the risk.

4.6. Carnation with altered flower color (blue carnation)

Carnation (*Dianthus caryophyllus)* has been modified with a trait, which only affects its flower color (shifted to blue). Carnation cannot naturally produce the blue pigment delphinidin because part of the anthocyanin biosynthetic pathway is absent. Therefore, it is impossible to produce blue carnation by traditional breeding. In this case, the applicant provided information that introduction of the *f3'5'h* gene enables the production of the blue pigment delphinidin. The *f3'5'h* gene encodes the flavonoid 3'5' hydroxylase (F3'5'H) enzyme which converts dihydrokaempferol (DHK) to dihydromyricetin (DHM). Both products can be used as substrates by the dihydroflavonol 4-reductase (DFR) enzyme of carnation. The orange/red pigment pelargonidin is produced if DHK is converted and the blue pigment delphinidin is produced if DHM is converted. The *Cytb5* gene that was introduced in IFD26407-2 encodes cytochrome b5 which increases the activity of F3'5'H.13. An increased activity of F3'5'H results in a higher production of DHM and therefore in a higher amount of delphinidin. The GM plant expresses its altered color only in the flowers. Hence – in a worst-case scenario of potential impact, i.e. flowers entering a soil and being broken down by a soil microflora – there would be an effect on the soil microflora which would only be marginally different from that of a non-blue carnation plant. In this case (hypothetical information) the applicants provided data revealing that growth and root setting characteristics, next to exudation patterns, turned out to be very similar to those of the near-isogenic parent or of a suite of wild lines. Alternatively, they provided evidence for the GM carnation not being outside of the band width of such characteristics, so that no concern would exist that any indirect effect of the modification would be present in the GM plant.

Scientific trigger (tier zero): The hazard and exposure terms of the risk equation are judged to be both zero or near-zero. Even in the case of the soil being exposed to fallen flower leaves, there would be an effect that would not surpass that of a non-blue carnation plant. Hence, there would be no scientific trigger to do any testing of impact on any part of the soil microbiota, given the apparent absence of a hazard component (hazard equals zero). The plant is predicted to impact the soil much like the parent or any

accepted comparator plant. In other words, the putative impact of the modification is expected to not go beyond the baseline defined by the normal cultivation of carnation.

Keynote: plant roots are the major drivers of rhizosphere microbial communities and root exudate amounts and patterns confer the underlying mechanisms, and hence attention for root exudate patterns of GM plants is important, in particular if there is a scientific reason to suspect that major changes, i.e. those beyond the normality of the crop, have occurred. However, given the very drastic changes in root setting, physiology and exudation that are brought about by classical breeding, such effects in GM plants will most often be within the baseline (normality). In such cases, there appears to be no scientific trigger to specifically address the putative GM plant effects on the soil microbiota.

5. Discussion

In this report, we examine the suite of currently available methods that allow the study of the soil LSF and relevant microbiota, and recommend a strategy of making use of these methods in the risk assessment of GM plants. As such we provide ***guidelines for the assessment of the putative effects of GM plants on the soil LSF***, as a tiered approach to the potential problem, coupled to the selection of methods from the toolbox for soil analyses that is presented.

The overriding aim of the GM plant risk assessment is the preservation of the ***life support functions*** of the soil, which are carried largely by the soil microbiota. To assess the impact of a GM plant on the soil LSF and microbiota, it is necessary to select those methods that are best able to detect any adverse effects of the GM plant in comparison to a comparator (non-GM) plant. Then, the selection of the testing protocol is best guided by best professional judgement as to what the impact of the GM plant on the soil LSF might be (***problem formulation***).

Moreover, given the complexity of the soil microbiota and its often high functional redundancy, it is recommended to place a focus on those members of the microbiota (indicators) that are:

(1) important for key processes in the soil LSF, and

(2) not functionally highly redundant.

The selection of such indicators is guided by the principle that – given their limited functional redundancy - any GM plant impact on them may (1) cause potential harm to (the stability of) essential functions in the soil system, and (2) be detectable. Given this consideration, a relatively small subset of lowly-redundant microbial groups involved in the soil LSF might serve as indicator/tester groups. In coherence with Kowalchuk *et al.* (2003) and Bruinsma *et al.* (2003), we proposed herein that such groups may include the ammonium oxidizers and rhizobial nitrogen fixers. Next to these, groups of higher redundancy such as decomposers and phosphate solubilizers may be recommended in the light of their importance for soil carbon and phosphorus cycling. Furthermore, plant growth promoters, mycorrhizal fungi and pathogen suppressants are also important for the soil LSF and provide groups of choice in case the underlying processes need to be tested.

Concerning the systematics of the assessment of the risks posed by a GM plant to the soil LSF, it is recommended that a four-step approach is taken. The ERA should thus start with a clear ***problem formulation*** (scientific trigger) to enable the educated selection of microbial LSF/groups to be tested in respect of effects of a specific GM plant (crop-trait combination). In cases in which clear scientific triggers exist indicating that the GM plant might have a negative effect on the soil microbiota, definition of the ***hazard*** and ***exposure*** terms is necessary. Finally, ***risk*** is to be characterized as the combination of hazard and exposure.

GM plant risk assessment in practice – what and how? One of various examples of the selection of a potentially affected key bacterial group is provided here. However, the risk assessor, on the basis of the list of methods and considerations given in this report, will be asked to select the key targets for testing in each individual case, i.e. using a case-by-case approach.

Given its sensitivity and low functional redundancy, the first step of nitrification, ammonium oxidation, may be severely impacted by stress imposed on the soil system, possibly also by GM plants (Kowalchuk and Stephen, 2001). Hence, ammonium oxidizers are ready candidates for use in soil quality assessments (Kowalchuk and Stephen, 2001; Mendum, 2002). They might, for instance, be especially useful indicators of the potential impact of GM plants that produce compounds toxic for bacteria. Whereas this one example seems to make sense, it is not possible to provide consistent guidelines that are equal for all GM plants. The reason for this contention is obvious: it is not the genetic modification *per se* that should be the reason for concern about GM plant impact, but the nature of the modification as related to the crop that is modified, i.e. the **crop-trait combination**. And this, in comparison to the already-existing variance that is normally present in the cropping system. As each new crop-trait combination (also called ***event***) is inherently different, be it radically or subtly so, there is no general rule with respect to the methods that should be used in the assessment of risks. Indeed, selection of the strategy is to be done **case-by-case** and guided by the expected or hypothesized effect. Hence, for each case the selection of the most suitable combination of measurements (criteria) is recommended, and these should be based on a previously distinguished scientific trigger for testing of the specific GM plant.

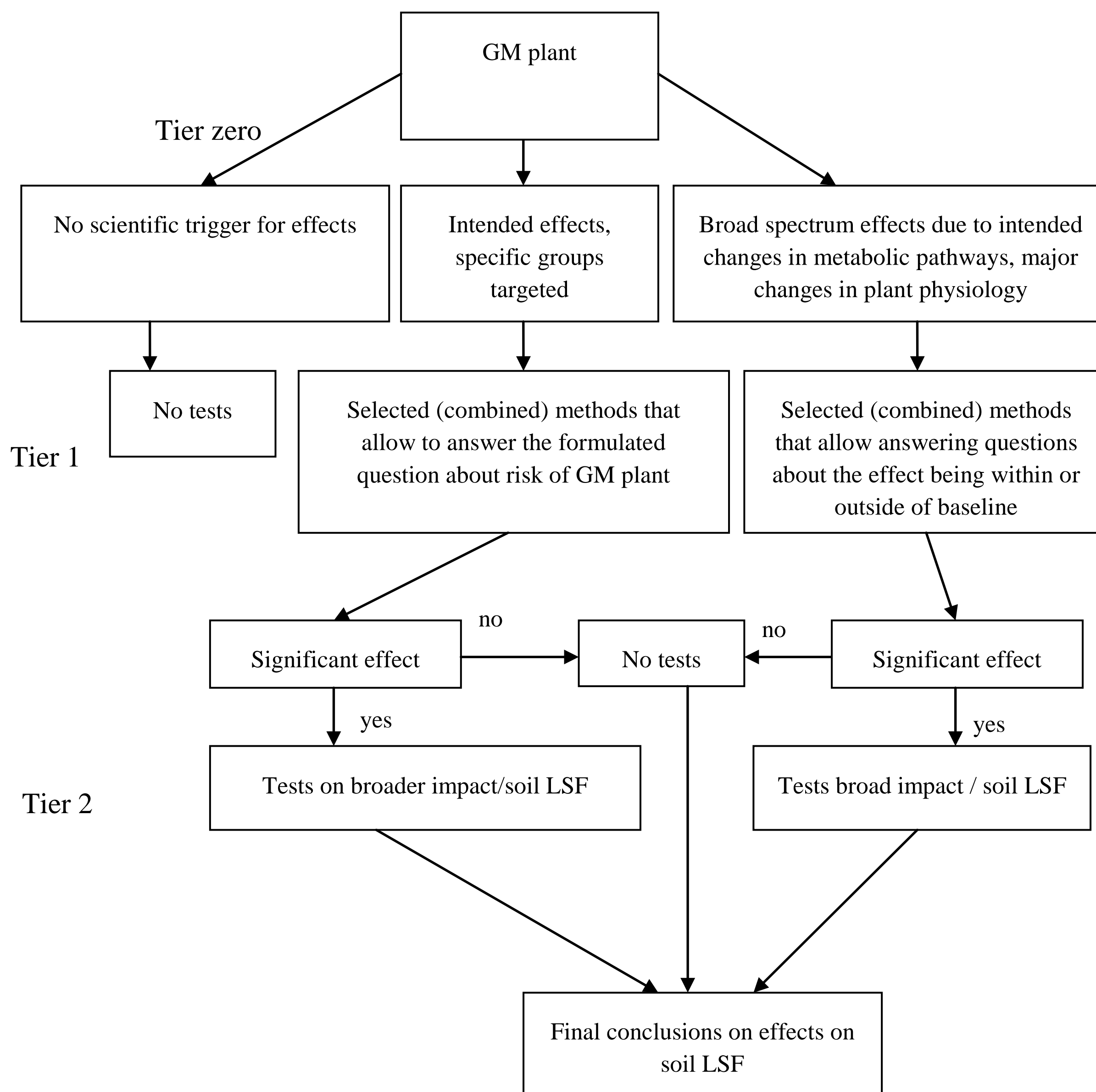


Fig. 2. Some practical suggestions allowing selection of appropriate methods for GM plant risk assessment.

Guidance in the selection of appropriate tools - How can we select the proper tools from the explosively developing toolset to yield a toolset that is appropriate to measure effects on the soil LSF and microbiota? The key question here is whether the tools, or a selected subset, will lead to the objective, i.e. a reliable measurement of the impact of a GM plant

on the soil and a fair assessment of whether such an impact is adverse for soil functioning. Undoubtedly, the best professional judgments have to be made at this point about what the different techniques will be able to offer, the level of certainty reached and therefore also what the toolset is unable to offer. *Section 2*, coupled with Tables 1 and 2, provides overviews of the methods and their potentials and pitfalls. From these Tables and from the foregoing descriptions, we can conclude that an impressive array of traditional and advanced assays exists. From among these, quantitative PCR of specific target groups and molecular fingerprintings (PCR-DGGE, PLFA, T-RFLP) of whole communities - or subsets thereof - offer reliable and feasible techniques to measure effects of GM plants on (selected members of) the soil microbiota. As argued, the choice of methods will have to be guided by an estimation of potential expected effects (***problem formulation***) which will be different per plant/trait combination (see cases). Thus, here the applicant as well as the risk assessor will have important roles in considering what type of testing is crucial as well as feasible, in order to answer the risk question defined in the problem formulation phase.

In the tiered approach that is proposed for the risk assessment, tier zero should thus address, using a direct focused approach, an identification of hazard and exposure, and thus guide the selection of the methods applicable in tier 1, as a first step to estimate the risk. If that risk is present and considered relevant, a second tier may be needed. In case a tier 2 is applied, this will encompass a broader and more extended assessment, for which a suite of techniques is selected that allow to more deeply define the type of impact of the GM plant effect. In other words, we advocate the careful selection of methods that are fine-tuned to address the presumed impact, in a tiered approach, in which tier zero is focused on problem formulation and hazard identification, tier 1 tests specifically address this hazard, and tier *two* (and possibly three) encompass more substantive approaches with the focus on testing in comparison to the baseline set by using comparator plants, such as the near-isogenic parent plant or a suite of plants that determine normality of the cropping system.

6. Recommendations*:

*Note added upfront: the writers of this report understand from the guidelines of this study provided by COGEM, that it is not in the remit of this study to appoint responsible people or institutions for any of the scientific or risk assessment approaches. However, it is also understood that, in common risk assessment practice, there is an interplay between applicants and competent authorities, in order to come a professional judgment of risk.

1. Prior to GM plant risk assessment, the crop-trait combination should be examined on a case-by-case basis as to whether a scientific trigger can be found that leads one to expect an effect on soil LSF and microbial communities (problem formulation). If such a trigger can be defined, the functional groups that are at risk should be identified, in particular if they are sensitive to the presumed effect and are exposed to the GM plant.

2. In cases in which no scientific trigger can be indicated that suggests that the GM plant has an impact on the soil LSF and/or microbial community, no tests of the effect of the GM plant on the soil microbial community are needed.

3. To properly address the questions about the putative effects of GM plants on the soil LSF and microbiota, selection of an adequate toolset from among the methods listed and described in this report (Tables 1 and 2) is required. This toolset should adequately provide answers to the specific question about presumed impact formulated under 1. Obviously, it is understood that this is an inventory at this point in time (05 April of the year 2013) and that this inventory does not include or deliberately wants to exclude future methods that allow an optimal handle at the GM plant risk questions posed.

4. The effect of a GM plant on the soil microbiota is to be weighed against that of a comparator, for instance a near-isogenic plant line, a suite of lines, or a baseline or normal operating range of the respective cropping system. This will enable to assess whether the effect is within or outside of normality for the specific cropping system considered.

5. In case of a formulated problem, a focus on selected key microbial groups (e.g. ammonium oxidizers, nitrogen fixers) is recommended, as these can best provide information about the possible adverse effect of the GM crop on the soil LSF. Use of direct molecular approaches (e.g. fingerprintings, clone libraries, qPCR and pyrosequencing) is then recommended, as these are capable of distinguishing such microbial groups in soil. Detection of significant influences of GM plants on these might take the analyses from tier one to tier two.

6. Very pragmatically, if testing of GM plant impact on the soil is needed, it is important to apply methods that show high sensitivities and specificities. Quantitative PCR and molecular fingerprintings (PCR-DGGE, PLFA, T-RFLP) are often appropriate to assess the sizes of the soil microbiota and the (overall) microbial community structures. Moreover, most classical approaches, which are generally less sensitive, provide general, integrated, results on soil processes important for the soil LSF and, as such, confer additional value to testing.

7. This report has placed a focus on methods to assess the soil LSF carried by the soil microbiota (bacteria and fungi). Thus, the overall soil quality effects of, for instance, earthworms or nematodes has not been considered. Considerations with respect to such parts of the soil biota are possibly needed in other parts of the risk assessment, e.g., addressing non-target organisms.

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