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EXECUTIVE SUMMARY

The influence of biotechnology in agriculture is growing rapidly. The engineering of genetically modified plants (GMPs) has increased in both the variety and number of GMPs produced in recent years. This has great potential for future agriculture, but asks for well-defined risk assessment as well. To date environmental risk assessments regarding the cultivation of GMPs have mainly addressed aboveground effects. However, plants are the primary source of energy to life, both above and below ground, and soils are key to essential functions of terrestrial ecosystems, such as support of plant growth, and nutrient cycling. This study has sought to identify soil functions or groups of micro-organisms that are most relevant for determining and monitoring effects of GMPs on terrestrial ecosystems.

The **diversity** of species and processes in soil ecosystems is huge. It is impossible to include all of this enormous variety in tests regarding the effects of GMPs on soil ecosystems. Therefore, we have tried to identify species and groups of species as well as microbe-mediated processes that might be used as indicators for effects of GMPs on the soil ecosystem. Chapter 2 provides a description of these species and processes in soil, and the next chapter describes available techniques to measure these. The report presents an overview of research addressing effects of GMPs on soil ecosystems in chapter 4, and ends with suggestions for indicators and future research. In addition, this study also included a workshop, which was held in January of 2002, dedicated to discussion of GMP effects on soil ecosystems, and the results are integrated into the text of this report and given as appendices.

This study has mainly focused on the effects on **bacteria, fungi and microbe-mediated soil processes**, because these are the most important parameters for the functioning of the soil ecosystem. Soil micro-organisms are exposed to GMPs and GMP products in several ways. They can be directly exposed to the GMP roots, or indirectly to GMP exudates or dead plant material. These different exposure routes and exposure times are important in determining how soil microbial communities and processes will be affected.

In order for **indicator species and processes** to be useful in determining the effects of GMP introduction, they must be comprised of vulnerable microbial groups or processes that have a low degree of functional redundancy in the system and are important to the proper functioning of the ecosystem. Potential microbial indicator groups identified in this study include antagonistic bacteria, mycorrhizal fungi, ammonia-oxidising bacteria, and nitrogen-fixing bacteria, and indicator processes include decomposition of recalcitrant organic compounds and soil suppressiveness. Choice of relevant indicators depends on the system, in particular soil texture and structure and system parameters such as moisture retention, pH, and plant species. For example, mycorrhizal fungi might make highly relevant indicators in low-input systems, whereas wood-degrading basidiomycetes might be more appropriate in forest systems. We suggest to use a **two-pronged approach** for testing the effects of GMPs on soil ecosystems, using **specific tests** directed at appropriate indicators, should they be identifiable, as well as more **general tests** targeting broader aspects of the total microbial community to assess general or unpredicted effects.

Many, but not all, of the **research** that has been conducted on the effects of GMPs on soil micro-organisms and processes have found some changes in soil microbial communities or processes. However, most studies have failed to link the effects directly to the influence of GMPs. It is also not clear how relevant the microbial parameters studied thus far are for the functioning of the examined soil ecosystems. Thus, it may be that certain GMPs have undesired effects on soil-borne micro-organisms, but this must be demonstrated on a per case basis using clearly comparable and relevant indicators of disturbance to the soil ecosystem.

Soil ecosystems are highly dynamic, being exposed to numerous natural and anthropogenic fluctuations, including seasons, weather patterns and varying agricultural practices. Such variation results in large and frequent changes in the conditions to which soil organisms are exposed, resulting in considerable fluctuations in the size, composition and activity of the microbial community in soil. Determining the size and scope of such ‘normal’ variation is agricultural systems is critical for establishing a proper **baseline,** to which observed effects can be related.

A wide variety of **techniques** has been applied in GMP research, making comparison between studies difficult. Many studies have used plate count techniques, either solely or in combination with other techniques. Plate count techniques have been shown to be a useful tool to detect changes in specific microbial groups, but only target a small fraction of the total microbial community. Several molecular techniques such as T-RFLP, DGGE and SSCP have also been applied and show promise for informative and routine use. Some of these molecular techniques can be applied to detect general shifts in microbial community structure as well as changes in specific dominant microbial populations.

Development of new techniques for soil research will continue to improve our understanding of soil ecosystems, enabling a better assessment of baseline and effects in the future. The **conclusions and suggestions** mentioned in this report are therefore not absolute. Many new techniques are being developed and improved and may be (more) suitable for routine testing in the (near) future. This report has sought to make an inventory of the knowledge to date and to identify the most suitable testing procedures that are presently available. These procedures should be evaluated regularly in the future to keep them up to date. Given the necessity to standardise protocols and tests, it is recommended that clear guidelines be established for all institutions charged with conducting tests on the effects of GMPs.

SAMENVATTING

Het invloed van biotechnologie in de landbouw neemt sterk toe. De laatste jaren is het aantal en de variatie van genetisch gemodificeerde planten (GMP's in dit rapport) sterk toegenomen. Dit biedt vele mogelijkheden voor de landbouw in de toekomst, maar vraagt tegelijkertijd ook om goede risicoanalyses. Tot nu toe worden in risicoanalyses van de toepassing van GMP voornamelijk bovengrondse effecten gemeten. Planten zijn niet uitsluitend de belangrijkste energiebron voor bovengronds leven, maar ook voor ondergronds leven. De bodem herbergt vele organismen, die een essentiele rol vervullen voor vitale functies zoals plantengroei, nutriënten kringlopen en de zuivering van water. Dit rapport beoogt de essentiële bodemfuncties en/of -groepen te identificeren die van belang zijn voor het bestuderen en monitoren van effecten van genetisch gemodificeerde planten op bodemecosystemen.

Er is een zeer **grote diversiteit** aan soorten en processen in bodemecosystemen. Het is onmogelijk om deze enorme variatie in zijn geheel te bepalen. We hebben daarom geprobeerd soorten, groepen van soorten, en microbiële processen te identificeren, die gebruikt zouden kunnen worden als indicatoren voor de effecten van GMP's op bodemecosystemen. Hoofdstuk 2 beschrijft deze soorten en processen en hoofdstuk 3 behandelt de technieken waarmee ze gemeten kunnen worden. In hoofdstuk 4 wordt een overzicht gegeven van studies op dit onderzoeksgebied en tenslotte eindigt het rapport met suggesties voor indicatoren en toekomstig onderzoek. Daarnaast is er in januari 2002 een workshop georganiseerd over de effecten van GMP's op bodemecosystemen. Hiervan zijn de resultaten te vinden in de bijlagen.

In dit rapport ligt de nadruk op de effecten op **bacteriën, schimmels en bodemprocessen**, omdat dit de belangrijkste parameters zijn voor het functioneren van het bodemecosysteem. Bodemmicro-organismen kunnen op verschillende manieren worden blootgesteld aan GMP's of GMP-producten. Ze kunnen direct worden blootgesteld aan de plantenwortels of indirect aan de GMP-exudaten of dood plantenmateriaal. Deze verschillende routes beïnvloeden de micro-organismen en de bodemprocessen op verschillende manieren. Per route verschilt de blootstellingtijd en ook het deel van de microbiële gemeenschap dat wordt beïnvloed.

Indicatorsoorten en -processen worden in dit rapport gedefinieerd als soorten en processen die een belangrijke rol spelen in de bodem systemen waarin zij voorkomen en die mogelijkerwijze gevoelig zijn voor effecten van GMPs. Microbiële indicatorgroepen, die in dit rapport behandeld worden zijn antagonistische bacteriën, mycorrhiza schimmels, ammoniumoxiderende bacteriën en N_2-bindende bacteriën. Indicator processen zijn de decompositie van moeilijk afbreekbare natuurlijke organische verbindingen zoals houtvezels en onderdrukking van bodemziekten. Indicatoren zijn afhankelijk van het systeem, vooral van de bodemtextuur en -structuur en systeemparameters zoals vochtigheid, pH en plantensoort. Bijvoorbeeld mycorrhizal schimmels zijn mogelijke indicatoren in low-input systemen, en houtafbrekende basidiomyceten in bossystemen. Wij stellen voor om een **tweeledige aanpak** te gebruiken voor het testen van GMP's met testen voor **specifieke indicatoren** en **algemene testen** voor de effecten op de gehele microbiële gemeenschap, en niet voorziene effecten.

Er zijn in de meeste, maar niet alle, **studies naar de effecten van GMP's** op bodemmicro-organismen en -processen, veranderingen in bodemgemeenschappen of -processen gevonden. Maar de meeste studies zijn er niet in geslaagd deze veranderingen direct in verband te brengen met de invloed van de GMP's.

Een belangrijk aspect hierbij is de vaststelling van een '**baseline**'. De omstandigheden in een bodem veranderen vaak en sterk - denk daarbij aan een droge grond waarop een flinke regenbui valt-, waardoor ook de omvang, samenstelling en activiteit van de microbiele gemeenschap in de bodem sterk verandert. Om effecten en de gevolgen van de groei van GMPs op het bodemeco-systeem goed te kunnen vaststellen is het noodzakelijk dat er een gedegen inzicht is in de natuurlijke variatie in de microbiele gemeenschap in de bodem als gevolg van abiotische factoren en de groei van (niet gemodificeerde) planten.

Er zijn veel **verschillende technieken** toegepast in het onderzoek naar de invloed van GMPs op de bodem tot nu toe, wat het vergelijken van de onderzoeksresultaten het trekken van algemene conclusies lastig maakt. In veel studies is gebruik gemaakt van plaattellingen, zowel als enige techniek als in combinatie met andere technieken. Plaattellingen zijn geschikt voor het waarnemen van veranderingen in de microbiële gemeenschap, maar kunnen slechts een beeld geven van een klein (het cultiveerbare) deel van de totale gemeenschap. Moleculaire technieken zoals SSCP, DGGE en T-RFLP zijn ook toegepast. Deze kunnen informatief zijn en geschikt voor routinegebruik, voor zowel algemene veranderingen in de microbiële gemeenschap, als veranderingen in specifieke dominante populaties.

De ontwikkeling van nieuwe methoden en technieken zal in de toekomst het inzicht in het functioneren van bodem organismen en van bodemecosystemen in het algemeen ongetwijfeld verbeteren. De **conclusies en suggesties** die genoemd zijn in dit rapport zijn daarom dan ook niet definitief. In dit rapport is een inventarisatie gemaakt van de huidige kennis en op basis daarvan is geprobeerd om de beste testprocedures te selecteren. Het doel van het rapport is te dienen als adviesrapport voor verder onderzoek en de ontwikkeling van regels en testprocedures voor de effecten van GMP's op bodemecosystemen. Gelet op de snelle ontwikkeling van nieuwe methodologieen in de biologie en de bodemmicrobiologie in het bijzonder dienen de hier voorgestelde testprocedures regelmatig geëvalueerd te worden en dient gestreefd te worden naar internationale standaardisatie.

CHAPTER 1. INTRODUCTION

1.1 Aim

To date environmental risk assessments regarding the cultivation of genetically modified plants have mainly focused on aboveground effects, such as the effects on wild relatives and non-target herbivores. However, plants are the primary source of energy to life both above and below ground. Although soils are key to essential functions of terrestrial ecosystems, such as support of plant growth, nutrient cycling and drinking water purification, not much is known about the effects of genetically modified plants (GMPs) on soil ecosystems. This report aims to identify soil functions and/or groups of micro-organisms that are most relevant for studying and monitoring effects of GMPs on terrestrial ecosystems, as well as to assess whether adequate methodologies are available to determine the possible effects due to the use of GM crops under field conditions. This may contribute to risk assessment procedures for the introduction of GMPs into the environment with focus on potential effects on soil (micro-) organisms and processes. The identification of key processes will be based on the relevance to vital soil ecosystem functions as well as methodological accessibility.

1.2 History of genetically modified plants

Stanley Cohen and Herbert Boyer opened the age of genetic modification in the 1970's, with the first engineered transfer of genetic material from one organism to another. The discovery of the possibility to change genetic characteristics of an organism led to high expectations. However, as early as 1974 scientists drew attention to possible risks involving genetic modification in a letter to the US National Academy of Sciences (Berg et al., 1974), which asked the scientific community for a voluntary deferment of cloning experiments with certain animal viruses, toxins and oncogenes. This letter also called for an international conference to discuss these issues. The conference was held in 1975 and resulted in a recommendation both to continue the research and to develop a safety code concerning the research on genetic modification.

The first genetic modification of plants occurred over 15 years ago, and since then a number of techniques have been developed for the introduction of a wide variety of desired traits into numerous plant species (Tsaftaris et al., 2000). Most current research in this field is directed toward the improvement of food crops such as potatoes, maize and sugar beets; the remaining work concerns non-food crops such as cotton, tobacco, ornamental plants and those used to produce pharmaceuticals.

Plants are typically modified for:
▪ resistance traits, for instance against herbicides, pathogens and diseases
▪ increased tolerance to (a-)biotic stress, like drought, cold or heat
▪ enhanced quality or quantity of the product.

Biotechnology has great potential to advance agricultural practices. However, the creation and use of genetically modified crops is a controversial issue. Supporters argue that genetic modification is indispensable to meet the food demands of the world's increasing human population. Genetically modified crops often allow farmers to achieve higher yields on the same field. Also, the introduction of genetic modifications increasing plant tolerance to (a-) biotic stress or other plant properties may allow for the use of soils previously unworthy of agriculture. This could greatly increase the area of productive land, especially in the Third World. Furthermore, the introduction of plant modifications to increase resistance against insect herbivores and fungal pathogens could significantly decrease our reliance on toxic chemicals.

Many people have been opposed to the use of genetically modified plants on moral grounds (Gaskell et al., 1999). Although the moral debate concerning the use of gene technologies is certainly far from settled, these issues fall outside the scope of this report, which will concentrate on potential environmental risks. Opponents of genetic modification often argue that transgenic organisms or products can be dangerous. However, the possible effects of genetically modified organisms on the environment are still largely unknown. It is impossible to predict all (long-term) effects. Potential unwanted side effects involved with GMP release include effects on non-target organisms, and development of resistance in target organisms. Other possible hazards could be a selective advantage for a wild relative arising from the transfer of genes of a GMP to sexually compatible plants, pollen mediated allergenicity and toxicity, and increased survival, establishment and dissemination of GMPs. Moreover, the direct or indirect effects of GMP consumption by humans are unknown. It has been argued widely that all genetically modified organisms should be more thoroughly tested before they are used for field experiments or commercial use. Another concern is that Third World countries may become overly dependent on and exploited by western companies, due to the monopoly of companies on these technologies. An additional concern is specific for modifications that increase tolerance to herbicides, as use of such crops may actually increase the use of chemical herbicides.

Another major public concern is the possibility for horizontal gene transfer (HGT), whereby the genetic material that is introduced into the GMP is non-sexually transferred to other organisms (Kidwell, 1993). For instance, genes introduced into the plant may be incorporated into the genetic material of micro-organisms in the soil. Although inter-domain HGT has been shown to occur in laboratory studies (Dröge et al., 1998), the transfer of recombinant genes from genetically modified plants to plant-associated or soil-borne bacteria probably occurs at extremely low frequencies in the field (Dröge et al., 1998; Hoffmann et al., 1994). In fact, several studies have failed to detect HGT between plants and bacteria (Paget and Simonet, 1994; Smalla et al., 1994; Schlüter et al., 1995; Broer et al., 1996). However, the expression of the acquired genes and the subsequent selection pressure on the host species might be of more importance in determining the consequences of HGT between GMPs and soil organisms (Nielsen et al., 1998, 2001). In cases where the genetic information is already commonly present in the soil, the potential transfer from transgenic plants to the soil bacterial community might be less serious, as little is added to the naturally occurring gene pool subject to potential transfer events (Dröge et al., 1998). In contrast it will be more serious in case of new antibiotic resistance genes. Risk assessment should therefore not focus on the process of gene transfer, but rather on the origin and function of the genetic material and potential consequences of HGT. HGT therefore affects the subject of this report only as far as its products elicit measurable changes in microbial indicator processes and organisms.

Through 2001, 213 permits for field experiments in the Netherlands were granted, approximately 1300 permits for contained use of genetically modified organisms, and 33 applications for market introductions have been granted or are being processed (information provided by the Netherlands Ministry of Housing, Spatial Planning and the Environment). The main crops for which genetic modifications have been introduced are potatoes, turnips, cauliflower, maize, sunflowers, sugar beets, and carrots.

1.3 Genetic modification

A genetically modified organism is defined as “an organism, with the exception of human beings, of which the genetic material has been altered by means that are not possible by nature through reproduction and/or natural recombination" (directive 2001/18/EC). In traditional plant breeding, two parental plants are crossed. The offspring will therefore contain a combination of genetic information from both parents, but the breeder cannot control the contributions of the two parents with regard to specific traits. Consequently a cross to introduce a desirable trait is likely to produce numerous undesirable traits as well. With genetic modification it is possible to speed up the process of plant breeding and improve the precision. Furthermore, it is possible to cross species barriers. A desired trait can be transferred from one species into another, even if they are not closely enough related to allow a sexual cross.

Genetic information is typically introduced into plants via one of two transformation strategies: the use of *Agrobacterium* as a biological vector and direct gene transfer techniques.

Agrobacterium tumefaciens is a soil bacterium that causes crown gall in plants. The bacterium has a natural ability to alter the genetic material of plant cells so that outgrowths (or galls) are formed on the plant. The mechanism has been adapted so that desirable genetic information is transferred into the plants rather than that which promotes the formation of galls. The *Agrobacterium* may also transfer some of its own DNA (inheritance information) into the plant DNA. In order to transform a plant with a desired piece of DNA, this DNA must first be introduced into the *Agrobacterium* vector strain. The indirect gene transfer technique using *Agrobacterium* is today the most widely used. This is because of its simplicity and efficiency. However, there are limitations. *Agrobacterium* has a limited host range (herbaceous dicotyledonous species) and is less effective on monocotyledonous and woody species. However, *Agrobacterium* has also been used recently to transform important non-host species, demonstrating that host range limitations are not absolute (Tsaftaris et al., 2000).

Direct gene transfer techniques introduce foreign DNA into cells by chemical, physical or electrical means. The most commonly used methods to date have been electroporation, particle bombardment, ultrasound, microinjection and lasers. Electroporation uses short pulses of a strong electric field to cause small, transient holes in the plant cells, allowing the DNA to enter the cells through cell pores. This method works best in plant tissues without a cell wall. In particle bombardment, the DNA-fragment is shot into the plant cells with gold or tungsten particles as carriers from a DNA cannon. Ultrasound-induced transformation uses ultrasound waves to induce DNA uptake into protoplasts, suspension cells, and tissues. Microinjection is the direct delivery of DNA into plant cells using a microsyringe. Laser-mediated transformation uses laser beams to create openings in cell compartments and organelles thereby allowing DNA entry (Tsaftaris et al., 2000).

Only a small proportion of the cells is successfully genetically transformed by any of these approaches. Marker genes, which comprise DNA fragments coding for easily selectable traits, are therefore often linked to the DNA coding for the desired genetic modifications to allow for the rapid identification of successful transformants. Markers are typically based on resistance against antibiotics or herbicides, thereby allowing for a simple identification of transformants based upon plant growth in the presence of the appropriate antibiotic or herbicide.

1.4 Risk analysis and legislation

The aim of risk assessment is to provide a basis for rational decision making, allowing the community to maximally benefit from biotechnology, without jeopardising safety. Basic principles for legislation are identification of hazards and their evaluation. Risk is a product of two quantities: the likelihood of the realisation of the identified hazards and the severity of the possible consequences (De Maagd, 2001). Adequate implementation of safety principles and mechanisms requires a distinction to be made between what one needs to know and what one would like to know. Also, risk management provisions should be in proportion to the identified risk. In the case of genetically modified plants, a case-by-case approach is necessary to determine the specific risks involved, since each GMP introduction contains a unique set of circumstances.

It is impossible to prove complete safety under all circumstances (zero-risk). To keep risks as small as possible a maximum transparency of research and policy-making is needed, as well as continuous knowledge development, responsible decision making about concrete applications, and accurate monitoring (Tiedje et al 1989).

Two European Community regulations were delivered in 1990, the Council Directive 90/219/EEC on the contained use of genetically modified micro-organisms and the Council Directive 90/220/EEC on their deliberate release into the environment. These directives include regulations for the permission for research on GMOs (in laboratories as well as in the field). The new directive 2001/18/EC has recently replaced directive 90/220/EEC. The directive requires each proposed field trial to be evaluated individually and all field releases require a permit by the national authorities. The testing of new crops should follow a step-by-step approach. The scale of use should only be increased gradually when it becomes apparent that it is safe to do so after monitoring possible environmental effects related to specific aspects of the GM crop. The field trials have to be monitored, and a report has to be sent to the appropriate authorities in the country issuing the permit. Reports are required to register all observed abnormalities and should include information on properties such as plant growth, flowering and fitness.

To date, risk assessment has been hampered by a lack of knowledge on the functioning of soil ecosystems. As outlined in the beginning of this chapter, the effect of GMPs on soil ecosystems is an important and until recently ignored issue, especially relevant for plants with modifications targeting soil-borne organisms, such as those coding for fungal-resistance and antibacterial compounds. In the next chapter, the importance of the soil environment for the functioning of terrestrial ecosystems will be discussed in more detail and several key species and processes will be discussed. Chapter 3 describes different methods to measure possible indicators of the effects of GMPs on soil ecosystems. Chapter 4 presents several examples of studies addressing the effects of genetically modified plants on different soil parameters and an overview of studies in this field of research. Finally, chapter 5 will draw upon the previous chapters to propose suitable ways to determine such effects and identify knowledge gaps.

CHAPTER 2. KEY ISSUES IN PLANT-SOIL ECOSYSTEMS

2.1 Introduction

This chapter will focus on plant-soil interactions in terrestrial ecosystems, as a basis for assessment of the effects of GMPs on soil ecosystems. Plants are the primary source of life in soil. They provide necessary nutrients, protection, and determine the microclimate for soil organisms in the uppermost soil layers. Plants therefore have a big influence on the size and composition of the microbial community and the distribution of micro-organisms. Micro-organisms are the dominant soil organisms both in terms of biomass and activity (respiration, nutrient cycles), accounting for over 80% of the total biomass (excluding roots) of most soil ecosystems. They largely determine the functioning of terrestrial ecosystems, and they have direct interactions with plants, thereby creating a strong feedback between plants and micro-organisms. Therefore, this chapter, and in general this report, will focus on the microbial community and microbial processes in soil. In this chapter a description of relevant plant-soil ecosystem interactions and soil characteristics and processes will be given. This will provide a basis for understanding the consequences of potential perturbation of the soil ecosystem due to the growth of genetically modified plants. In combination with information on available methodology, it will be used to design appropriate protocols to assess the risks of genetically modified plant cultivation on soil ecosystems. Firstly, general information will be provided on relevant characteristics of the soil-plant ecosystem. Secondly, this chapter will deal with specific groups of micro-organisms and microbial processes that might be used as indicators of the effects of GMPs.

2.2 General a-biotic and biotic characteristics of soil-plant systems

This section will provide information on the general biotic and a-biotic characteristics of the soil ecosystem to help provide a comprehensive context within which to view specific soil properties. A choice is made for those items that have relevance for the present subject (*i.e.* they are influenced by or do influence the growth of crops at the conditions prevailing in the Netherlands and Western Europe). This includes items such as soil structure, disturbances by agricultural practices, the nitrogen cycle etc., whereas items such as the sulphur cycle and agriculture in submerged soils (as used for rice cultivation) are not included.
While important to the general functioning of the soil system, the general properties described below are probably too broad to be used as indicators for effects of GMPs.

2.2.1 Soil structure

Soils contain varying proportions of clay, silt and sand particles (Brady, 1984), the proportions of these different size particles constitute the texture of the soil. Soil texture is a very important property for the ecology of micro-organisms because it describes the surface area that is available as a habitat for the growth of micro-organisms. Soils with greater clay compositions have much more surface area available for the growth of micro-organisms than soils with high sand concentrations, because clays are much smaller particles than sands (Atlas and Bartha, 1998). The combination of solid matter, liquid and gas distribution in soil determines its structure. Soil components are aggregated into aggregates of dfferent sizes and chemical composition. The soil structure provides shelter, surfaces for adhesion and different microclimates. Capillary formation in soil is complex, and affects the flow of water and air through soil. Some are so small that even bacteria cannot enter. Others provide shelter for micro-organisms against predators, like protozoa and nematodes because they are not able to enter and provide protection against chemical influences as well.

Differences in physical conditions influence the distribution of (micro-)organisms. Drought-resistant organisms, like many Gram-positive bacteria, often reside on the exterior of the aggregates. Inside the aggregates are Gram-negative bacteria and anaerobic organisms that are less resistant against drought. Over 80% of the micro-organisms is adsorbed to organic matter and clay minerals. Adsorption of micro-organisms is a complex phenomenon. It depends on the type of clay material, cations, pH and the characteristics of the micro-organisms. Inside the aggregates the colonies are dependent on the diffusion of nutrients. Therefore the colonies usually stay small.

2.2.2 Chemical properties

Soils contain vastly differing concentrations and chemical forms of organic carbon, inorganic and organic nitrogen and available inorganic phosphorus (Brady, 1984), and these biochemical properties influence soil micro-organisms (Alexander, 1977). Soil organic matter is derived from the remains of plants, animals and microbes (Bear, 1964). Humic substances, which are those portions of the soil organic matter that have undergone sufficient transformation to render the parent material unrecognisable, are of particular importance. Although humic materials typically constitute less than 10% by weight of mineral soils, they can represent the majority of organic matter in organic soil layers.

The composition of the soil atmosphere (atmosphere-lithosphere interface) also varies greatly between soils. The soil atmosphere exists in the porous spaces between soil particles. At times the soil pores are filled with water, displacing the soil atmosphere. Some soils or soil layers are oxic, that is, the soil atmosphere contains oxygen, whereas others are anoxic, that is, there is no free oxygen in the soil atmosphere. Even in oxic soil layers, there are anoxic regions devoid of free oxygen (Sexstone et al., 1985). The oxygen content of the soil atmosphere determines to a large extend the types of metabolism that can occur and the chemical transformations that the indigenous micro-organisms can carry out. Concentrations of CO_2 in the soil atmosphere commonly are one to two orders of magnitude higher than in the aboveground atmosphere. Gas diffusion and microbial respiration affect the concentrations of both CO_2 and O_2 in the soil atmosphere. In oxygen-deficient soils, other gases, including CH_4 from methanogenesis and H_2S from anaerobic sulphate reduction, occur in high concentrations in the soil atmosphere (Atlas and Bartha, 1998). The biochemical properties of the soil environment are highly heterogeneous, containing numerous biochemical gradients and contrasting conditions within very small spatial and temporal scales. This heterogeneity allows for a wide variety of biochemical processes to be carried out, but makes it difficult to predict and model the exact impact of disturbance on the total soil system.

2.2.3 Physical disturbance

Disturbances in the soil can have considerable consequences for the life and activity of soil-borne organisms. Due to physical disturbances, such as ploughing, unstable aggregates may fall apart and new ones may be formed. The niches of many (micro-)organisms may be lost, leading to cell death, which in turn releases organic matter for use by other organisms. The mixing of soils may also increase the accessibility of organic matter or other nutrients for soil organisms, thereby stimulating the activity of micro-organisms. For example, the number micro-organisms can become twenty to thirty times higher within a few days of ploughing. Also the ratio between bacteria and fungi (the most dominant microbial life forms in soil) can change as the result of ploughing and soil management. The distribution of water in soil can change dramatically as well. Physical disturbances are also caused by more natural causes like the digging activities of molls, earthworms and growing plant roots. Environmental fluctuations such as freezing and thawing or drying and wetting can also greatly influence soil conditions. Wetting of a dried soil leads to a temporary increase in activity, as seen from the increased output of CO_2 or enhanced N- mineralisation. Similar results have been observed after the thawing of frozen soil. Several mechanisms have been put forward to explain these observations. It is generally accepted that soil aggregates are disturbed due to the changes in gas pressure after re-wetting or thawing. This disruption renders soil organic matter more available for utilisation by microbes. These microbes are in turn more accessible to predators, leading to enhanced turnover of carbon and nutrients.

2.2.4 Soil microbial communities

Many bacterial genera occur in soil (Gray and Parkinson, 1968). A higher proportion of Gram-positive bacteria is found in soil than in marine of freshwater habitats, but in absolute numbers Gram negatives pre-dominate also in soil. Common bacterial genera found in soil include *Acinetobacter, Agrobacterium, Alcaligens, Arthrobacter, Bacillus, Brevibacterium, Caulobacter, Cellulomonas, Clostridium, Corynebacterium, Flavobacterium, Micrococcus, Mycobacterium, Pseudomonas, Staphylococcus, Streptococcus* and *Xanthomonas.* However, there are wide differences in the relative proportions of individual bacterial genera found in different soil types with different plant species (Alexander, 1977; Westover et al., 1997; Grayston et al., 1998; Dunfield and Germida, 2001), and most results to date have relied on culture techniques, which only reflect a small fraction of the total bacteria in soil (see below). Only a small fraction of the bacteria in the soil may be active at a given time, as starvation and dormancy may be the rule as opposed to the exception. Nutrients present in the rhizosphere (see below) and residuesphere (the soil influenced by decaying organic matter), can act to alleviate these starvation conditions for those bacteria that can take advantage of these localised conditions.

Actinomycetes (hyphal-forming bacteria) can compose 10-33% of the bacteria in the soil (Alexander, 1977), with *Streptomyces* and *Nocardia* being the most abundant genera. *Micromonospora, Actinomyces* and other actinomycetes are indigenous to soils but generally are present in low numbers. Actinomycetes are relatively resistant to desiccation and can survive under conditions of drought in desert soils. They favour alkaline or neutral pH and are sensitive to acidity. Myxobacteria are found in soils and on forest litter. Representative genera of myxobacteria typically found in soils include *Myxococcus, Chondrococcus, Archangium* and *Polyangium.*

Important photoautotrophic bacterial populations in soil include the cyanobacterial genera *Anabaena, Calothris, Chroococcus, Cylindrospermum, Lyngbya, Microcoleus, Nodularia, Nostoc, Oscillatoria, Phormidium, Plectonema, Schizothrix, Scytonema* and *Tolypothrix.* Some of these genera, such as *Nostoc,* contribute fixed forms of both nitrogen and organic carbon in some soil habitats. Although the majority of energy in soil systems is derived from plants, energy from photoautotrophic bacteria can supplement energy input significantly in some systems. In addition, a number of autotrophic organisms in soil can use chemical reactions for energy. These chemolithotrophs, such as ammonia-oxidising, nitrite-oxidising and sulphate-reducing bacteria, are critical in nutrient cycling but total energy inputs into the system are quite small.

Availability of fixed forms of nitrogen is an important limiting factor in soil for microbial activities and growth of higher plants. Micro-organisms are responsible for the fixation of atmospheric nitrogen for input into organic systems. However, nitrogen fixation is an energetically costly exercise, and must be coupled to energy-deriving reactions. Some cyanobacteria can use energy from photosynthesis to fuel nitrogen fixation on soil surfaces without plant growth, thereby contributing to soil nitrogen and stability. Some heterotrophic free-living soil bacteria, most importantly members of the genus *Azotobacter*, can use energy from organic substrates to drive the fixation of atmospheric nitrogen to fixed forms of nitrogen. Some anaerobic bacteria, such as *Clostridium* species, fix nitrogen using energy derived from fermentation reactions. However, the most important sources of fixed nitrogen in many soils come from plant-microbe interactions, where *Rhizobium* or *Bradyrhizobium* species fix atmospheric nitrogen within the nodules of certain plant roots.

Fungi constitute a high proportion of the microbial biomass in many soils (Domsch et al., 1980). A very broad spectrum of fungi can be found in soil, mostly as indigenous and sometimes as allochthonous organisms. Soil fungi may occur as free-living organisms or in mycorrhizal association with plant roots. Fungi are found primarily in the top 10 cm of the soil and are rarely found below 30 cm. They are most abundant in well-aerated and acidic soils. The most frequently isolated fungi from soils are members of fungi imperfecti, such as species of *Aspergillus, Geotrichum, Penicillium* and *Trichoderma,* but numerous other ascomycetes and basidiomycetes also occur in high numbers. Some soil fungi, especially those found in association with plant roots, are difficult if not impossible to isolate and identify.

Most fungi in soil are opportunistic. They grow and carry out active metabolism when conditions are favourable, which implies adequate moisture, adequate aeration and relatively high concentrations of utilisable substrates. Many soil fungi metabolise carbohydrates, including polysaccharides and even allochthonous fungi that enter the soil can often grow on the major components of plant residues; relatively few fungal species, though, are able to degrade lignin (Garrett, 1981). A number of fungi are facultative or obligate anaerobes, but there is very little known about the ecology of these organisms.

As with bacteria, dormancy is a typical condition of soil fungi. Some fungi have been shown to remain dormant but viable for decades. In the absence of available substrates, they are inactive. Numerous fungi and oomyctes occur in soil as specialised dormant structures, which include sporangiospores, conidia, oospores, ascospores, basidiospores, chlamydospores and scleriotia. Fungal mycelia may also be metabolically inactive in soil.

The majority of soils inhibits fungal growth and the germination of fungal spores to some extent, a phenomenon known as soil fungistasis (Lockwood, 1977; Lockwood and Filonow, 1981). Fungistasis occurs in most soils, except some deep subsurface soils, highly acidic soils and cold-dominated soils. The addition of readily decomposable organic materials reduces the level of fungistasis, allowing fungal spores and other propagules to germinate and fungal mycelia to resume active growth. Soil fungistasis appears to be biological origin and is thought to depend on the activity and composition of the microbial community as well as the nutrient status of the soil (Atlas and Bartha, 1998; De Boer et al., 2002).

The majority or our knowledge of microbial ecology in soil ecosystems to date stems from studies which rely on the culturing of micro-organisms. However, it is estimated that less that 5% of the total bacteria in soil are culturable using currently available methods (Amann et al 1995). The recent introduction of culture-independent molecular techniques in microbial ecology has however given us a new perspective in the study of soil-borne micro-organisms (see review in chapter 3). A number of molecular surveys have been conducted on soil-borne microbial communities (Borneman et al., 1996, 1997; McCaig et al., 1999). Two major conclusions that can be drawn from such molecular strategies are 1) soil contains a tremendous amount of microbial diversity, including numerous phyla that are not, or poorly, represented in culture collections, and 2) culture-based techniques may be biased in favour of those taxa that can grow well under laboratory growth conditions (WardRianey et al., 1995; Rheims et al., 1996; Hugenholtz et al., 1998; Felske et al., 1999). Thus, the generalisations given above are mostly based upon only a small and perhaps unrepresentative fraction of the total community. Despite these shortcomings of culture-based techniques, most of the general principles and processes known for microbial ecology in soils remain valid. Rather, the influx of molecular techniques into microbial ecology has rather served to complement existing methods by providing an additional perspective, and the most complete understanding of soil ecosystems is achieved by a combination of both culture-dependent and culture-independent techniques.

It should also be noted that the influx of molecular data in microbial systematics has redefined the classification of many micro-organisms. Thus, while the broad trends discussed above refer to some common microbial designations (e.g. pseudomonads), it should be realised that molecular data have often resolved such groups into several, sometimes distantly related taxa. Such redefining does not influence studies directed at classical groups made on the basis of functional similarity, but will seriously affect molecular approaches, which often rely on phylogenetic relatedness to target specific microbial groups.

2.2.5 The rhizosphere

The rhizosphere is the zone of soil influenced by plant roots. The structure of the plant root system largely influences the size and composition of the rhizosphere microbial community (Nye and Tinker, 1977; Russell, 1977; Bowen, 1980; Lynch, 1982). This influence has typically been referred to as the rhizosphere effect. The interactions of plant roots and rhizosphere micro-organisms are dictated to a large extent by the heterogeneous and dynamic soil environment. Key processes influencing the rhizosphere include water uptake by the plant, release of organic chemicals to the soil by the roots, microbial production of plant growth factors and competition for available mineral nutrients. The space within plant roots, often referred to as the endorhizosphere, is also colonised by certain micro-organisms, and such non-pathogenic endophytic organisms can have a positive, neutral or deleterious effect on plant growth. Since the majority of endophytes originate from rhizosphere/rhizoplane microbial communities (Hallman et al., 1997), they will not receive separate attention in this report except in the cases of specific functional groups of plant symbionts.

Bacteria often occur as microcolonies on the root surface and on soil particles, and the regulation of many important bacterial activities, such as the production of antibiotics, induction of pathogenesis, or specific plant-microbe interactions is density dependent. Microbial populations can change their behaviour in response to cell density in a process referred to as quorum sensing. Quorum sensing is mediated by the recognition of specific compounds secreted by a given microbial population, so that expression of the genes involved in the density dependent processes only occurs when a certain critical bacterial density has been achieved. Processes controlled by quorum sensing are therefore often of prime importance in plant-microbe and microbe-microbe interactions.

There is a higher proportion of Gram-negative, rod-shaped bacteria and a lower proportion of Gram-positive rods, cocci and pleomorphic forms in the rhizosphere than in root-free soil (Rovira and Campbell, 1974; Woldendorp, 1978; Campbell, 1985). There is also a relatively higher proportion of motile, rapidly growing bacteria, such as pseudomonads, in the rhizosphere as compared to elsewhere in soil. In many cases, this increase is directly influenced by plant root exudates, which favour micro-organisms with high intrinsic growth rates. Organic materials that are commonly released from roots include amino acids, keto acids, vitamins, sugars, tannins, alkaloids, phosphatides and other substances (Rovira 1969). Roots surrounded by micro-organisms excrete many times as much organic material as sterile roots. Although a few of these materials inhibit micro-organisms, most stimulate microbial growth. The influence of materials released by the plant into the soil is evidenced by the observation that bacterial populations within the rhizosphere have markedly different nutritional properties than bacteria in root-free soil. Many rhizosphere bacteria require amino acids for maximal growth and it is likely that root exudates supply these compounds.

Successional changes in the rhizosphere microbial community occur as the plant grows from seed germination to maturity. During plant development, a distinct rhizosphere succession results in rapidly growing, growth-factor-requiring, opportunistic microbial populations. These successional changes correspond to changes in the materials released by the plant roots to the rhizosphere during plant maturation. Initially, carbohydrate exudates and mucilaginous material support the growth of large populations of micro-organisms within the grooves of the epidermal plant cells, on the root surface and within mucilaginous layers surrounding the roots. As the plant matures, autolysis of some of the root material takes place as part of normal root development and simple sugars and amino acids are released into the soil, stimulating the growth of pseudomonads and other bacteria with high intrinsic growth rates.

Just as plant roots have a direct effect on the surrounding microbial populations, micro-organisms in the rhizosphere have a marked influence on the growth of plants. In the absence of appropriate microbial populations in the rhizosphere, plant growth may be impaired (Lynch, 1976; Dommergues and Krupa, 1978; Campbell, 1985). Microbial populations in the rhizosphere may stimulate plant growth in a variety of ways, including increased recycling and availability of mineral nutrients, synthesis of vitamins, amino acids, auxins, cytokinins and gibberellins and by suppression of potential plant pathogens through competition and the development of amensal relationships based on production of antibiotics (Nieto and Frankenberger, 1989; Alvarez et al., 1995).

It is important to realise that the same mechanisms by which beneficial micro-organisms are attracted to the roots, *i.e.* the availability of root-released organic compounds as substrate of biosynthesis and energy, also operate for harmful plant pathogenic organisms. Most plant pathogenic bacteria or fungi are also attracted by root released products, thereby facilitating processes that lead to infection of the roots. Competition for these root released substrates between non-pathogenic and pathogenic microbes, is therefore a critical factor in disease development and plant health.

Micro-organisms in the rhizosphere influence the availability of mineral nutrients to the plants, sometimes by limiting concentrations of inorganic nutrients before they can reach plant roots and in other cases by increasing the availability of inorganic nutrients to the plant (Barber, 1978). For instance, plants have been shown to exhibit higher rates of phosphate uptake when associated with rhizosphere micro-organisms than in sterile soils (Campbell, 1985). The principal mechanism of increasing phosphate availability is the microbial production of acids that dissolve apatite (a common mineral group including calcium fluophosphate), releasing soluble forms of phosphorus (Atlas and Bartha, 1998). With respect to the delivery of nutrients to the plants, plant-microbe symbiosis is of particular importance. The two types of symbiotic interactions that are probably most important for plant-soil systems are the symbioses that plants have with N_2-fixing bacteria such as *Rhizobium*, *Bradyrhizobium* and *Frankia*, and with mycorrhizal fungi (see 2.3.1 and 2.3.2 for more information).

2.2.6 Decomposition

Through the process of decomposition of organic matter, energy is provided to organisms and nutrients are released for uptake by both micro-organisms and plants. Bacteria and fungi are the primary decomposers of organic matter in soil. The retention of organic matter in soil is controlled mainly by environmental variables such as moisture regime and temperature, the chemical constitution of the organic matter, and by the spatial distribution of and physico-chemical interactions with other soil constituents (Oades, 1988). The decomposition of organic matter in soil is the net result of a number of turnover processes in which the microbial biomass acts as a key intermediate station. Materials are taken up, converted into new products and subsequently released either actively or passively. Numerous transformation processes occur simultaneously and are interdependent. Soil structure and texture are dominant controls over decomposition of organic matter by microbes. This is reflected in the differences in decomposition rates between different types of soil. Finer, clay-rich soils show on average, slower decomposition rates and higher retention of organic matter than coarse, sandy soils.

2.2.7 Nitrogen cycle

An overview of the N-cycle is given in Figure 2.1. Nitrogen is a constituent of amino acids, nucleic acids, amino sugars and their polymers, and therefore essential to all life on earth. However, it occurs in the biosphere mainly in inert forms such as N_2. In order to make it available for living organisms it needs to be transformed by either industrial processes or by natural *biological fixation*. Industrial fertiliser use has increased the amount of fixed nitrogen entering many terrestrial ecosystems, bringing potentially negative effects of eutrophication of natural habitats, contamination of drinking water, and undesired effects on vegetation. The biological fixation of molecular nitrogen is carried out by several free-living bacterial genera, some of which may be rhizosphere-associated, and by several bacterial genera that form mutualistic associations with plants (Evans et al., 1991; Postgate, 1992; Benson and Silvester, 1993). In terrestrial habitats, the symbiotic fixation of nitrogen by rhizobia accounts for the largest contribution of combined nitrogen. The rates of nitrogen fixation by symbiotic rhizobia are often two to three orders of magnitude higher than rates exhibited by free-living nitrogen-fixing bacteria in soil. For example, rhizobia associated with an alfalfa field may fix up to 300 kg N per hectare per year, compared to a rate of 0.5-2.5 kg N per hectare per year for free-living *Azotobacter* species (Dalton, 1974; Burns and Hardy, 1975).

Many plants, animals, and micro-organisms are capable of *mineralisation (or ammonification)*, a process in which organic nitrogen is converted to ammonia. Nitrogen in living and dead organic matter occurs predominantly in the reduced amino form, and the mineralisation of organic nitrogen is critical to continued ecosystem productivity (Blackburn 1983). *Immobilisation (or assimilation)*, which is the reverse of mineralisation, also occurs as the result of microbial activity in soil. Both NH_4^+ and NO_3^- can be taken up by microbes to meet their demand for nitrogen and be assimilated into organic compounds. The processes of mineralisation and immobilisation occur simultaneously in soil as the result of the intracellular activities of different microbial populations,
The process of *nitrification* involves the oxidation ammonia to nitrite, which is in turn oxidised further to nitrate:

$$NH_4^+ + 1½\, O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

$$NO_2^- + ½O_2 \rightarrow NO_3^-$$

Nitrification, specifically ammonia oxidation, can be a rate-limiting step in the nitrogen cycle and has important consequences for soil ecosystems (Prosser, 1989). Nitrification yields protons thereby reducing soil pH. The positively charged substrate of nitrification tends to bind negatively charged clay particles in soil, yet the negatively charged products can freely migrate in the soil water. Thus, nitrification can lead to leaching of nitrogen from soil ecosystems. Also, plants differ in their ability to take up different nitrogen sources, with most plants preferring nitrate to ammonia. Thus, nitrification will influence nitrogen availability to plants, and plants in turn compete with nitrifying bacteria for available ammonia. Nitrification is also the first step in the loss of fixed nitrogen from soil ecosystems via denitrification (see below). The dynamics of nitrification are therefore critical to the nitrogen balance in soil, which is especially critical in areas receiving an increasing input of nitrogen-rich fertilisers.

Denitrifying nitrate reducers convert nitrate through nitrite to nitric oxide (NO), and nitrous oxide (N_2O) to molecular nitrogen. The denitrification sequence is as follows:

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

Although a wide range of bacteria is capable of denitrification given the proper conditions, bacteria of the genera *Pseudomonas* and *Alcaligenes* are thought to be the primary denitrifying micro-organisms in soil. Usually, a mixture of nitrous oxides and nitrogen is evolved, depleting the environment of fixed nitrogen. The proportion of the denitrification products is dependent on both the denitrifying micro-organisms and on the environmental conditions. The lower pH of the habitat, the greater the proportions of nitrous oxide formed. An adequate supply or an oversupply of reducing equivalents favours formation of molecular nitrogen.

Denitrification most often occurs under strictly anoxic conditions or under conditions of reduced oxygen tension. Some denitrification may occur in generally oxic environments if these contain anoxic microhabitats (Hutchinson and Mosier, 1979). Nitrification and denitrification in soil often occur in close proximity, allowing a substantial part of the NO_3^- formed by nitrification to diffuse into the anoxic denitrification zone, where it is reduced to NO, N_2O and N_2 (Nielsen et al., 1996; Atlas and Bartha, 1998).

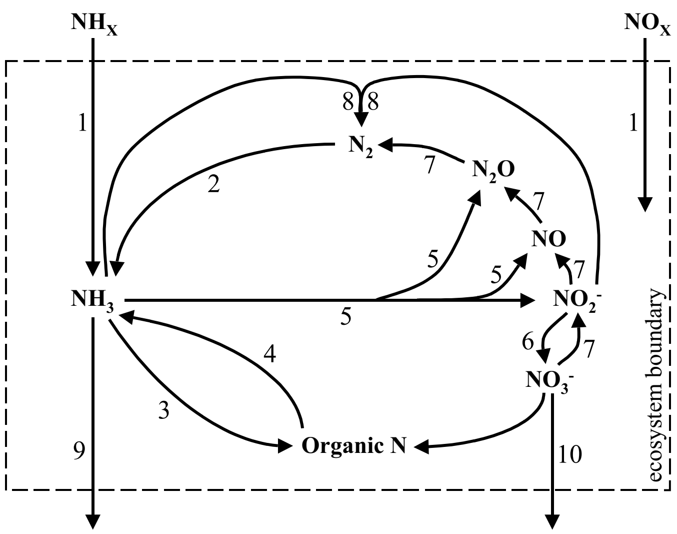

Figure 2.1 Nitrogen cycling and turnover:
1) atmospheric input
2) nitrogen fixation
3) immobilization (assimilation)
4) mineralization (ammonification)
5) aerobic ammonia oxidation
6) nitrite oxidation
7) denitrification
8) anaerobic ammonia oxidation (annamox)
9) volatilization
10) nitrate leaching.

After Tietema et al, 1992.

2.2.8 Phosphorus cycle

Phosphorus is an essential element in all living systems, and can be often be a limiting resource for plant growth. Within biological systems, the most abundant forms of phosphorus are phosphate esters and nucleic acids. Phosphate diester bonds form the links within nucleic acid molecules. Phosphate also forms an essential portion of the ATP molecule, and the hydrolysis of phosphate from ATP to ADP forms the basis for most energy transfer reactions within biological systems. Phospholipids, which contain hydrophilic phosphate groups, are essential components of cell membranes.

The microbial cycling of phosphorus for the most part does not alter the oxidation state of phosphorus (Stewart and McKercher, 1982), but micro-organisms do play a large role in altering the availability of this essential nutrient. Most phosphorus transformations mediated by micro-organisms can be viewed as transfers of inorganic to organic phosphate or as transfers of phosphate from insoluble to mobile compounds, as exemplified by the role of mycorrhizal fungi in assisting plants with phosphorus uptake. Various micro-organisms have evolved transport systems for regulating acquisition of phosphate from the environment. Although phosphate is normally not reduced by micro-organisms, it appears that some soil and sediment micro-organisms may be capable of utilising phosphate as a terminal electron acceptor under appropriate environmental conditions (Atlas and Bartha, 1998).

2.2.9 Plant pathogenesis and pathogen suppression

Soil-borne bacteria and fungi cause many of the most important plant diseases, resulting in decreased crop yield, reduced seed production or plant death. In general, the pathogen first makes contact with the plant. Then the pathogen enters the plant and the infecting micro-organism starts growing. The plant subsequently develops plant disease symptoms, like abnormal growth (stunted, excessive, or tumerous growth), loss of foliage, loss of photosynthetic ability, loss of turgur or even death. Most plant pathogens that are dispersed by air make contact with the stems or leaves of the plant. However, soil-borne plant pathogens rely on either passive or active growth to plant roots, pure chance of contact with an extending plant root, or transport by soil animal vectors, such as nematodes. Upon contact with plant roots, bacterial and fungal pathogens typically penetrate through stomata, wounds or weaker areas of roots (e.g. branch points or root tips).

Most pathogenic bacterial species must maintain intimate contact with their host plant tissues for the greater part of their life history, because they do not form resting stages. Some opportunistic pathogenic bacteria can survive in soil, and cause disease only when sufficient population size has been formed. Many bacteria use seeds for the transient period in the soil.

Fungi cause the most economically important plant diseases. Most fungi have a complex life cycle, part of which is spent in the infected host plant and part outside the host plant. The production of resting spores allows fungi to survive prolonged periods outside the host plant, thereby avoiding unfavourable growth conditions and /or extreme environmental conditions. Fungi produce a great variety of spore types, many of which can be dispersed via the air. Environmental conditions, like temperature, moisture and soil pH, influence the pathogens, the host plants and the plant disease development. Optimal conditions for the pathogens do not necessarily mean optimal disease development, as this also depends on the susceptibility of the plant.

Most soils have natural capacities to suppress germination and growth of plant pathogenic fungi, a phenomenon often referred to as general fungistasis. Fungistasis can be considered as the first level of disease suppression. Not all fungi respond in the same fashion to the fungistasis capacity of a soil. The level of fungistasis is thought to be governed by the nutritional state of the soil (i.e. via competition with the pathogen for nutrients), as well as the production of various biological products (i.e. enzymes and antibiotics) by specific microbial community members (De Boer et al 2002). In addition, some soils are known to exert specific suppression of particular pathogenic fungi, which in some cases is due to the presence of a particular antagonistic population of micro-organisms. Suppressive soils are soils where disease development on or in the susceptible host is suppressed, even though the pathogen is present in the soil or is introduced. Examples of disease suppression involve antagonists against the causative agents of Take All Decline and *Fusarium*-wilt.

2.3 Potential indicators

In the following section several specific microbial groups will be discussed separately in more detail. The diversity of micro-organisms in soil is huge. Rough estimates indicate the presence of 10,000 - 15,000 different species of bacteria per gram of soil. As mentioned before, we know only a small fraction of these organisms. Therefore, it is necessary to focus on representative portions of the microbial community. The choices for these groups are based on their potential use as indicators of perturbation caused by GMP introduction. Factors contributing to the potential suitability of a microbial group as an indicator include its sensitivity to change, importance for key soil functions and processes, and level of functional redundancy (i.e. the level to which the group's function can be taken over by other organisms in the system).

The vulnerability of the groups mentioned below, is mainly based on present knowledge on the specificity of the organisms involved in certain activities and thus of the relatively small redundancy within this process. Before these organisms and their activities will be described we will first discuss, generally, the use of indicators for the assessment of effects of GMPs on terrestrial ecosystems.

To monitor the effects of transgenic plants it will be necessary to make choices, with respect to the organisms and processes to be monitored. Monitoring the effects of transgenic plants can be simplified by choosing one or several indicators, key species or key processes to indicate the effects of the introduction of the GMP. The use of an indicator has the advantage of being easier, cheaper and faster, than trying to monitor the complete soil ecosystem. However, it is important to realise that indicators cannot cover all effects on an ecosystem. The indicator(s) may miss effects on non-target organisms or processes. Another drawback is the difficulty in assessing the cause-reaction relationship. Although correlative data may link GMP introduction with changes in microbial community, it is often difficult to rule out other potential confounding factors. Furthermore, we still know little about the buffering power, functional redundancy and resilience of soil systems, but the degree of functional redundancy can be high (Degens, 1998; Griffiths et al., 2000b). It is therefore often difficult to determine if a detected change is actually detrimental for the ecosystem, and in some cases our choices will necessarily remain fairly arbitrary.

An indicator should meet certain criteria. An indicator needs to be sensitive over a useable dynamic range. Within this broad range, the change observed in the indicator should correspond well with the actual perturbation, a so-called one to one-function (Lenders et al., 1997). If several indicators are selected it is preferable that these indicators have different ranges. For example, if the indicators have different sensitivities, one indicator will show a reaction even after little exposure and others only after prolonged exposure (Lenders et al., 1997). Proper experimental design is necessary in order to isolate the use of the GMP as the sole variable. If the cause and reaction are correlated this does not directly imply that the observed effect is caused by the genetic modified plant, but could be caused by other variables, like climatic changes or soil type. Thus, proper controls are essential to exclude confounding factors.

For the determination of the effects of GMPs on the soil ecosystem a set of parameters involved in vital ecosystem functions has to be identified. The determination of the indicators should consider the following important criteria (Torstensson, 1997; Vos et al., 2000):

- scientific validity
- ecological significance
- reproducibility
- costs
- suitability for (routine) testing
- extrapolation possibilities
- availability of proper controls

There is a trade-off between these different criteria. For example, when an exceptionally informative new technique is developed, it may still not be suitable for routine use due to high costs or highly specialised methodological needs.

Based on these criteria we have chosen several groups that could possibly be suitable indicators. It should be realised that in this report indicators are primarily defined as groups of organisms or processes vital to the functioning of soil ecosystems, which are putatively vulnerable for the impact of GMPs. Their vulnerability is thought to be due to the relatively low redundancy in the species involved, so that the loss of species or the inhibition of their activity due to effects of GMPs might result in the loss of important functions. These groups are mycorrhizae, symbiotic N_2-fixing bacteria, antagonists, wood-decaying fungi and nitrifying bacteria. These groups are described in more detail below. Other groups or processes were not chosen because there is a too high redundancy of the organisms involved (e.g. denitrification or ammonification) or their importance is less for the situation under consideration, *i.e*. Dutch and European agriculture (e.g. methane-oxidising bacteria or sulphate reduction)

2.3.1 Mycorrhizal fungi

The importance of mycorrhizae was perhaps best stated by Harley who stated, that 'the study of plant nutrition in the absence of mycorrhizae is the study of artefact' (Harley, 1971). Mycorrhizae are mutualistic plant root-fungus associations, in which the fungi actually become integrated into the physical structure of the roots (Harley, 1965; Cooke, 1977; Dommergues and Krupa, 1978; Powell, 1982; Campbell 1985; Allen 1991). The fungus derives nutritional benefits from the plant roots, contributes to plant nutrition and does not cause plant disease. Mycorrhizal associations differ from other rhizosphere associations between plants and micro-organisms by the greater intimacy and organisation of the plant-fungus relationship. The mycorrhizal association involves the integration of plant roots and fungal mycelia, forming integrated morphological units. Most of the vascular plants form mycorrhizae and only a minority of plants are nonmycorrhizal. The widespread existence of mycorrhizal associations between fungi and plant roots attests to the importance of this interaction.

The two most important types of mycorrhizal associations are ectomycorrhizae (Marks and Kozlowski, 1973; Marx and Krupa, 1978) and endomycorrhizae (Sanders et al., 1975; Hayman, 1978). In ectomycorrhizae, the fungus (an ascomycete or basidiomycete) forms an external pseudoparenchymatous sheath more than 40 µm thick and constituting up to 40% of the dry weight of the combined root-fungus structure (Harley, 1965). The fungal hyphae penetrate the intercellular spaces of the epidermis and of the cortical region of the root but do not invade the living cells. The morphology of the root is altered, forming shorter, dichotomously branching clusters with reduced meristematic regions. In contrast to the predominantly exogenous ectomycorrhizae, endomycorrhizae invade the living cells of the root, which become filled with mycelial clusters (Harley, 1965). Of the various micro-organisms colonising the rhizosphere, mycorrhizal fungi occupy the unique ecological position of being partly inside and partly outside the host. The part of the fungus within the root does not encounter competition with other soil micro-organisms. Some endomycorrhizae form arbuscules, which are structures involved in substrate exchange between fungus and host. These arbuscular mycorrhizal fungi (AMF) form associations with the majority of terrestrial plants. They comprise a discrete group recently designated the Phylum *Glomeromycota* (Schüssler et al., 2001) and can have a profound impact on vegetation dynamics and diversity (Gianinazzi and Schepp 1994; Smith and Read, 1997; Van der Heijden et al., 1998).

Mycorrhizal associations can be highly sensitive to various perturbations including physical disturbance, changes in soil nutrition due to fertiliser use, and changes in plant species (Pankhurst et al., 1997). Especially AMF may be a highly suitable indicator group because of their widespread importance, susceptibility to disturbance, and functional and phylogenetic cohesion. One should however keep in mind that some crop plants become less dependent on mycorrhizal associations with increased intensity of agricultural practices (i.e. fertiliser use and tillage).

2.3.2 Symbiotic N_2-fixing bacteria

One of the most important mutualistic relationships between micro-organisms and plants involves the invasion of the roots of suitable host plants by nitrogen-fixing bacteria, resulting in formation of a tumour-like growth called a nodule. Within the nodule, the nitrogen-fixing bacteria are able to convert atmospheric nitrogen to ammonia, which supplies the nitrogen required for bacterial and plant growth (Postgate, 1992; Dillworth and Glenn, 1991). The fixation of nitrogen in plant nodules is of extreme importance for the maintenance of soil fertility.

The fixation of atmospheric nitrogen depends on the nitrogenase enzyme system (Dillworth and Glenn, 1991; Stacey et al., 1992; Palacios et al., 1993; Peters et al., 1995). Nitrogenase is very sensitive to oxygen and is irreversibly inactivated on exposure to even low concentrations of O_2. Nitrogen fixation, therefore, is often restricted to habitats in which nitrogenase is protected from exposure to molecular oxygen. In addition to nitrogen reduction, the nitrogenase complex forms one H_2 for every N_2 reduced and can also reduce other substrates such as acetylene (Atlas and Bartha, 1998).

Although rhizobia occur as free-living heterotrophs in soil, they are not typically dominant members of soil microbial communities and do not fix atmospheric nitrogen in this state. Under appropriate conditions, however, rhizobia can invade root hairs, initiate the formation of a nodule and develop nitrogen-fixing activity. The association between rhizobia and plant roots is specific, with mutual recognition between the two compatible partners based on chemotactic response and specific binding to the root hair prior to invasion and establishment of the root nodule. The legume plant root recognises the right population of rhizobia, which in turn recognises the right kind of leguminous root. Within the rhizosphere, plant roots supply the rhizobia with compounds that are transformed by them to substances involved in the initiation of the infection process and subsequent nodule development (Fahrareus and Ljunggren, 1968). Establishment of an adequate rhizosphere population of rhizobia is an absolute prerequisite for infection.

The process of nodule formation is the result of a complex sequence of interactions between rhizobia and plant roots (Solheim, 1984; Brewin, 1991). Flavonoids or isoflavonoids secreted by the host plants induce the expression of a number of nodulation *(nod)* genes in the cognate rhizobial bacteria. The products of *nod* genes are enzymes involved in the biosynthesis of species-specific, substituted lipooligosaccharides, called Nod factors. These signal compounds, which are released by induced rhizobial cells, elicit the curling of plant root hairs and division of meristematic cells eventually leading to the formation of root nodules. Rhizobia respond by positive chemotaxis to plant root exudates and move towards localised sites on the legume roots. (Atlas and Bartha, 1998)

Rhizobia are sensitive to perturbation (Pankhurst et al., 1997), they are important for nitrogen fixation and methods for measuring rhizobia are available. Therefore, we consider rhizobia to be suitable indicators.

2.3.3 Antagonists

Antagonists are organisms that exert a damaging effect on another organism (Campbell, 1989), for instance by the production of antibiotics or lytic enzymes or competition. This can be important for the protection of a plant against diseases. There are a large number of antagonists in most soils. Yet, the relative abundance of antagonistic micro-organisms depends on the agricultural practice applied. We will briefly describe a few species of antagonists, which are frequently observed in soils.

Fungi of the genus *Trichoderma* are particularly well known for their antagonistic potential. *Trichoderma* spp. can be act as both antagonistic and decomposing fungi. Several characteristics explain the frequent use of *Trichoderma* species as biocontrol agents. One of the most obvious reasons is the worldwide distribution of the genus. Furthermore, *Trichoderma* are easy to isolate, exhibit fast growth rates, and are versatile in their nutrient, pH and temperature requirements (Gebhardson and Larsson, 1991). Although *Trichoderma* species are often used in studies of antagonism and biological control, they have not typically been included risk assessment studies. However, Glandorf et al (1997) suggested *Trichoderma* as a possible indicator for risk assessment studies on the effects of transgenic plants producing antifungal proteins.

The two bacterial genera that have been intensively studied for their antagonistic potential are *Bacillus* and *Pseudomonas*. *Bacillus subtilis* is a Gram-positive bacterium that has been used to control several types of fungal diseases associated with different crops (Priest, 1993). Ahrenholtz et al (2000) measured increased killing of *B. subtilis* on the hair roots of T4-lysozyme producing potatoes. Members of the gram-negative bacterium *Pseudomonas* are a predominant group in the rhizosphere of many crops. Some species cause plant disease; many are closely associated with plants and have antagonistic properties. Many species produce antibiotics and diffusible fluorescent pigments, siderophores, with a great affinity for iron. In alkaline soils of low iron availability, the pseudomonad may deprive the pathogen of iron. For this reason, siderophore-producing pseudomonads can be used in the biological control of soil-borne plant pathogens. Actinomycetes, especially of the genus *Streptomyces,* have also frequently been studied for their antagonistic, and are known for their production of antibiotics and lytic enzymes.

Antagonistic organisms are present in a wide range of genera, and the variety in species within the genera mentioned above is large. Thus, phylogenetic markers provide little information regarding this property. Nevertheless, shifts within the populations of species with antagonistic properties have been observed due to the cropping system applied, which may have important implications with respect to antagonistic potential (Garbeva et al., unpublished results). Based on these observations and the great importance of natural soil suppression for terrestrial ecosystems, these groups of organisms could act as important indicators in response to GMP introduction if analyses are properly targeted.

2.3.4 Wood-decaying fungi

The turnover rate of aromatics in nature is a major factor in determining soil organic matter (SOM) dynamics. Aromatics are the chemical basis of lignin, which is the stabilising compound in plants. The structure of lignin is based on the phenyl propanoid unit, which consists of an aromatic ring and three-carbon side chain. Formed by polycondensation, lignin, is not formed by a specific enzyme but in a chemical reaction involving phenols and free radicals.

The decomposition of lignin is primarily attributed to fungi. The colour of the decayed substrate is indicative of the mode of attack. White-rot fungi are the most active lignin-degrading micro-organisms, resulting in the degradation of all wood components to CO_2 and H_2O. Brown-rot fungi degrade the polysaccharides associated with lignin and remove the CH_3 subgroups and R-O-CH_3 side chains; this leaves phenols behind, which on oxidation turn brown. Representative organisms include *Poria* and *Gloeophyllum*. Another group of fungi, the soft-rot fungi, like *Chaetomium* and *Preussia*, are important in wet situations and appear to degrade hardwood lignins more effectively than softwoods (Paul and Clark, 1989).

The low redundancy in this group of organisms, their relatively great sensitivity to disturbance and the importance of the process of lignin degradation for the functioning of soil ecosystems, make this group of organisms to be highly suitable as indicator

2.3.5 Nitrifying bacteria

The process of nitrification appears to be limited for the most part to a restricted number or activity of autotrophic bacteria (Focht and Verstraete, 1977; Hooper, 1990). Different microbial populations carry out the two steps of nitrification - that is, the formation of nitrite and the formation of nitrate. Normally, however, the two processes are closely coupled and an accumulation of nitrite does not occur. The oxidation of ammonia to nitrite and the oxidation of nitrite to nitrate are both energy-yielding processes. Nitrifying bacteria are chemolithotrophs and utilise the energy derived from nitrification to assimilate CO_2.

To date, all ammonia-oxidising bacteria (AOB) detected in terrestrial environments, by both conventional as well as molecular strategies, belong to a narrow clade within the ß-subclass of the *Proteobacteria,* consisting of two genera, *Nitrosospira* (containing former genus designations *Nitrosolobus* and *Nitrosovibrio*) and *Nitrosomonas*. Although *Nitrosomonas europaea* is the best characterised species of AOB, probably due to the relative ease with which it can recovered in pure culture, numerous studies have suggested that *Nitrosospira* species are dominant in terrestrial ecosystems (Kowalchuk and Stephen, 2001). Recent molecular studies have revealed a large diversity among AOB in terrestrial ecosystems and it appears that certain groups of AOB may be indicative for specific environmental conditions (Kowalchuk and Stephen, 2001).

The property of nitrite oxidation is spread across several bacterial groups, making comprehensive molecular studies of this group more difficult. Culture-based studies point to *Nitrobacter* as the most important nitrite-oxidising genus in soil systems (Bock et al., 1990), although role of the genera *Nitrospira, Nitrospina*, and *Nitrococcus* is still not well studied. Nitrite oxidation may be more susceptible to stress conditions, as nitrite accumulation may occur under various stress conditions (Bollag and Kurek, 1980), but ammonia oxidation is thought to be the rate limiting step in nitrogen turnover in most terrestrial ecosystems (Prosser, 1989).

Some other micro-organisms, including heterotrophic bacteria and fungi, are capable of a limited oxidation of nitrogen compounds, but heterotrophic nitrification does not appear to make a major contribution to the conversion of ammonia to nitrite and nitrate ions in most agricultural systems. Relatively few microbial genera are involved in the process of nitrification, and environmental stress can severely affect this process (Atlas and Bartha, 1998). Due to the relatively low redundancy and their influence on an important nutrient cycle, nitrifying bacteria are suitable indicators.

CHAPTER 3. METHODOLOGY

The microbial community represents the largest source of biological diversity on the planet. With an estimated ten thousand microbial species and over 10^9 microbial cells per gram of soil, monitoring all aspects of the soil-borne microbial communities represents a nearly impossible task. Also, micro-organisms are inherently difficult to study due to their small size, lack of distinguishing morphological characters, and the fact that the vast majority (>95%) cannot yet be cultivated (as mentioned in chapter 2). Fortunately, certain microbial processes or microbial groups can provide important insight into the system as whole, thus shining some light into the black box of soil-borne microbial communities.

Due to the immense complexity of soil systems, choices have to be made regarding the indicator processes and organisms that readily provide the most useful information about the health and state of perturbation of soil ecosystems. The choice of these indicators should be based upon relevance and importance to the system being studied (see chapter 2), and on methodological accessibility. This chapter will briefly review some of the available methods for studying the possible effects of genetically modified plants on microbial species and processes in soil, including the advantages and disadvantages the various approaches.

3.1 Microbial community dynamics

One approach to measure effects of transgenic plants on micro-organisms is looking at the dynamics of the microbial community. In contrast to measurements of microbial biomass, such approaches often address relative shifts in the community composition and diversity, as opposed to only total population sizes. A variety of approaches have been developed to examine changes in microbial community composition. Some of these address the system as a whole, and give indications of the general similarity of two systems being compared. Others either look at a subset of the community, or can be modified to focus in on a subset of the total community. In general, dominant species are often detected easily, whereas species with a small population size can be overlooked depending on the method used. Thus, it is critical to determine the level at which samples are to be compared and to determine which, if any, specific populations deserve special attention.

While cultivation-based methods have provided a great deal of knowledge concerning the distribution and physiology of micro-organisms, their general usefulness may be limited due to bias, incompleteness and time constraints. This limited window of observation has been extended in recent decades, in particular due to the influx of nucleic acid techniques into microbial ecology. All techniques have their own advantages as well as limitations, and the use of multiple approaches will almost always give a more complete picture of microbial community dynamics than any single technique alone. Nonetheless, given that feasibility constrains the use of all available techniques, choices will have to be made as to which techniques are most appropriate to the samples to be examined and the microbial properties considered most important.

3.1.1 Cultivation-based methods

▪ *Plate count techniques*

This method is based on the cultivation of micro-organisms on different solid media. Media consist of nutrients and a solidifying agent. Often the solidifying agent is agar, because most bacteria lack the enzymes necessary for depolymerising agar. Serially diluted sample suspensions can be spread on top of the medium or mixed with the medium before the plates are poured. On the plate the micro-organisms will grow and can be enumerated after the incubation period. For enumeration it is assumed that each colony is formed from a single cell. Especially for hyphae-forming bacteria, actinomycetes, and fungi this assumption is not justified.

All culture conditions are selective for those micro-organisms that are able to grow under the specific artificial environment provided. Thus, one can increase the coverage of plate count techniques by using several media, each of which selects for a separate fraction of the community. However, even with the use of multiple media, the plate count technique will still fail to detect the majority of viable cells, as many micro-organisms require growth conditions than cannot be mimicked in the laboratory or require other organisms for growth. It is estimated that only 1% to 10% of the total microbial community (Sørensen, 1997) is culturable with the use of currently available culture methods. The non-culturable cells may however still be alive and active in soil and may be critical to the functioning of terrestrial ecosystems.

The plate count technique depends on the cultivation of the micro-organisms on the media used and selectivity therefore depends on the composition of the medium. Less selective media can be used for 'total viable and culturable' counts, for example Luria-Bertani (LB) medium (Vaudequin-Dransart et al., 1995). Miller et al (1989) showed that highest number and largest diversity of soil bacteria was found when using a relatively nutrient poor medium, such as 10% TSA as compared to rich media such as 100% TSA. This might be a reflection of the oligotrophic conditions that micro-organisms typically encounter in soil.

Adding a bacterial inhibitor, can enhance the selection and growth of fungi. In contrast, fungal inhibitors can also be added as a supplement when enumerating bacteria. However the addition of such inhibitors can cause non-target effects, e.g. some fungal inhibitors may also hamper some fungi to a small extent. Other supplements can also been applied to provide added selection. For instance special carbon sources or toxins can be added to give a selective advantage to the micro-organisms which are able to utilise or are resistant to the added substance. Another possibility is to vary the incubation conditions, such as incubation temperature or time to select for specific groups. For instance the incubation of samples at 80ºC is applied to specifically select for spore-forming bacteria, such as Bacilli, which are among the most widespread bacteria in soils. The plate count technique is easy, quick and cheap, but very limited in the range of micro-organisms because of the limitation to culturable micro-organisms.

Once pure cultures have been obtained, a number of methods are available to identify the species represented. As morphological characters are often insufficient for this purpose, other metabolic and molecular techniques have been employed including, BIOLOG, restriction digestion or sequencing of 16S rRNA genes, Enterobacterial Repetitive Intergenic Consensus Sequence-PCR (ERIC-PCR), Random Amplified Polymorphic DNA (RAPD), and Fatty Acid Methyl Esters (FAME) analysis. With the FAME method, Germida et al (1998) could identify 60% of the isolates (based on a similarity index of >0.3). Ritchie et al (2000) also used this method to detect differences between the microbial communities of 3 different soil types, but were not able to distinguish between tillage practices within one soil type. Siciliano and Germida (1999) demonstrated differences between different transgenic and non-transgenic cultivars of canola with FAME analysis of isolated bacteria. The differences detected between the root-associated microbial communities of the transgenic and non-transgenic *Brassica napus* varieties were actually demonstrated to be greater than found for two different non-transgenic *Brassica* species examined, *Brassica napus* and *Brassica rapa* (Siciliano and Germida, 1999).

▪ *Most Probable Number (MPN)*

Again different media can be used to set either several culturable cell counts or counts related to more specific microbial groups. MPN relies on the principle that only a single cell is necessary to create a population of cells, which are in turn capable of changing medium properties to an extent that this growth is detectable. The technique uses serial dilutions. After the incubation period, the growth in the culture is scored either positive or negative; positive indicating the presence of at least one organism able to grow in the medium. The results of the growth in the different dilutions can be used to calculate the most probable number of organisms present in the original sample (Cochran, 1950; Gerhardt et al., 1981; Alexander, 1982). The calculated most probable number corresponds to the number of cells able to grow and produce a detectable result in the culture conditions provided. This carries its advantages and disadvantages. On the positive side, it is possible to design MPN experiments that target a specific property of interest, such as degradation of a certain compound or a detectable biochemical conversion. In this way, bacterial numbers for individual functional groups can be estimated. On the negative side, a positive result in an MPN dilution requires growth. Thus, since the vast majority of cells in a sample may not be culturable, MPN methods will no doubt underestimate cell numbers. Also, MPN techniques assume that a single cell is enough to propagate growth, and that all cells are inoculated into the experiment as single cells. It is known that many organisms are only active above a threshold density or require aggregates for growth, so that MPNs would grossly underestimate the actual number of cells. On the other hand, if cell aggregates are not fully disturbed during the cell suspension step of the experiment, each individual positive dilution in the MPN series may have originated from cell aggregates composed of many cells, again underestimating actual cell numbers. A further disadvantage is that some assays may be very time consuming, especially for slow-growing cells such as autotrophs.

3.1.2 Direct count techniques
Direct count methods do not depend on culturability of micro-organisms. They can be used to look at the arrangement and form of micro-organisms (Paul and Clark, 1989). Most of the techniques use a staining method including a fluorescent dye. Dyes have been applied that specifically link to proteins (e.g. acridine orange, fluorescein isothiocyanate (FITC) and rhodamine), fungal cell wall components (Calcofluor) or nucleic acids (e.g. europium chelate, 4'-6'-diamidino-2-phenylindole (DAPI) and bisbenzimide). The link to the specific cell components leads to a fluorescing reaction. Cells can be seen under a fluorescent microscope, as they represent a unit at which the linkage component is more concentrated than in the surrounding soil. Ahrenholtz et al (2000) used fluorescence microscopy to monitor the killing of *Bacillus subtilis* by transgenic T4 lysozyme producing potato plants. They used a staining technique (staining kits are commercially available) to discriminate between bacteria with intact (green fluorescence) and damaged (red fluorescence) cell membranes. Subsequently, they determined the proportion of red cells with a fluorescence microscope.

Normal light microscopy is not discriminating enough to permit detailed identification of soil microflora (Paul and Clark, 1989). Furthermore most direct count techniques are not very exact, because of the large variation within a sample and the large extrapolation from ng under observation to g or kg of sample and the imprecise staining methods. The greatest disadvantage of microscopic techniques is that lack of identifying characters for most micro-organisms. Although there may be millions of extant bacterial species, only 11 general cell shapes have been identified for bacteria. Fungia also often lack identifying characters, especially in vegetative growth stages that may be prevalent in soils and plants. Thus, microscopic methods can be used to count microbial cells, but not to identify them. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) can overcome some of these limitations by providing a much higher degree of resolution and in the latter case three-dimensional information. However, both TEM and SEM are prohibitively expensive for routinue use in cell counting procedures.

▪ *Fluorescence in situ hybridisation (FISH)*
The ability to identify microbial cells under the microscope has been realised in recent years with the advent of fluorescence in situ hybridisation (FISH; Amann et al., 1995). In FISH fluorescence dyes are attached to specific oligonucleotide probes, whose sequence is designed to target a specific taxonomic grouping of interest. Such probes usually bind 16S rRNA, and the specificity of the fluorescent probe depend on the sequence. Thus, FISH can be directed at a high taxonomic level (such as all bacteria) by targeting conserved regions of the 16S rRNA or at a fine taxonomic level (such as species) by targeting highly variable regions of the 16S rRNA. Those cells in a sample given a positive units fluorescent signal are thus identified to the taxonomic level defined by the probes, and multiple probes (1-4) can be used in one experiment, to detect different microbial groups simultaneously. FISH can be combined with the use of confocal laser scanning microscopy to allow a 3-D reconstruction of a sample, thereby allowing the observer to count cells and gain insight into their *in situ* location in a sample. While this technique offers the best opportunity to count cells of specified microbial groups and visualising microbial interactions, it is often difficult to perform in complex soil environments. Furthermore, it requires the use of highly sophisticated techniques and expensive equipment, making it far from routine.

3.1.3 Nucleic acid-based techniques
Recent decades have witnessed a vast increase in the use of molecular techniques in describing microbial communities. An advantage of molecular techniques is that they do not

depend on cultivation, yet rely on nucleic acids (DNA and/or RNA) extracted directly from the environment. To date, most have involved in molecular analysis is 16S rDNA (Stephen and Kowalchuk, 2002) although the use of protein-encoding genes is also possible and may be used with increased frequency in the future. Depending on the technique and primers used, molecular methods can determine general community structures or specific groups.

▪ *Reverse Array hybridisation*
As the name suggests, reverse sample array hybridisation switches the typical placement of sample and probe with respect to typical hybridisation experiments. In this case, the environmental nucleic acid sample is typically labelled in some way, and allowed to pass of over a surface to which specific defined probes have been fixed. Labelled DNA (or cDNA, if the starting material is rRNA) fragments that hybridise with the attached probe sequences remain in association with the surface, while non-matching sequences are washed away. The depth and breadth of the analysis depends on the number and the taxonomic level of probes spotted onto the hybridisation surface. The number of specific taxon-specific nucleic acids can vary from only a few to potentially hundreds or even thousands, and arrays often use a hierarchical approach. Reverse array hybridisation analysis has been applied to detecting shifts in Domain-level groups (Zheng et al., 1996), within specific bacterial groups (Mobarry et al., 1996), and in the distribution of specific microbial populations within the community (Layton et al., 2000). The data generated by reverse hybridisation can be highly quantitative and does not rely upon the culturing of bacteria or Polymerase Chain Reaction (PCR), which are both prone to biases. As with any probing technique, the analysis is only as good as the quality of the probes in recognising their targets. In practice, a sizeable portion of the total hybridisation signal may remain undefined. In the near future, it is likely that reverse probing of oligo- and polynucleotides bound to microchips will become the dominant form of nucleic acid community monitoring. However, such technologies are still in a stage of development and will probably remain cost prohibitive for the foreseeable future.

▪ *Clone library screening*
The use of PCR and cloning technique has been the principle culture-independent method used in the past decades to uncover novel microbial diversity. PCR primers typically targeting the 16S rRNA gene are used to amplify DNA fragments from environmental nucleic acid extractions. These fragments are subsequently cloned in *E. coli* and the cloned material subjected to sequence and phylogenetic analysis. By comparing the recovered sequences with the database of known sequences, tentative identifications can be made. By comparing the distribution of recovered sequences over the major microbial phyla, one can get a glimpse into the structure and diversity of the microbial community. While this method is ideal for the recovery of novel sequence information, it is not suitable for the comparison of multiple samples because a very large number of clones need to be analysed per sample before a reasonable picture of the microbial community can be achieved.

PCR-based community profiling techniques
In contrast to cloning approaches, community profiling methods seek to produce information concerning the structure of the entire community or a specifically targeted portion thereof, and typically detect only the most dominant microbial populations (>1% of total). Some profiling methods focus on specific molecular targets, which can provide some information on the identity of detected organisms, whereas others are more random and only provide information on the relative difference or similarity between samples. Also key to the usefulness of community profiling methods are the methods of analysing and comparing banding patterns, and a number of strategies have recently been applied to the quantitative comparison of community profiles. Although such efforts have increased the usefulness of such methods, researchers have yet to arrive at commonly accepted methods for interpreting the similarity or difference between community profiles derived from different environmental samples. Also, community profiling methods typically rely upon PCR, thus, while they may be highly sensitive, the potential biases of amplification procedures also need to kept in mind.

▪ *Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE)*
Denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis use the differences in melting properties of DNA fragments and not size to separate the fragments

obtained after multiplication through PCR of indicator DNA. Therefore polymorphisms can be detected in DNA fragments of equal size differing in sequence. Originally used as a method for the detection of mutations, this technique has become a powerful and popular method in the analysis of microbial communities in recent years (Muyzer and Smalla, 1998). A mixture of PCR-amplified DNA fragments is run through a gel with a linear gradient of denaturing chemicals (DGGE) or temperature (TGGE). To stabilise the melting behaviour of the DNA fragments, and to ensure that they do not denature completely during the experiment, a GC-clamp, consisting of a long stretch of mostly G/C base pairs is incorporated into one of the primers and subsequently into the PCR products. As the double-stranded DNA fragments reach a higher level of denaturing chemicals or temperature, they begin to melt at a point in the gel that is determined by the sequence of the fragment, which is of course different for different species. As each DNA fragment denatures it's mobility is dramatically hampered in the gel, whereas fragments that remain double-stranded continue through the gel until they in turn reach their critical denaturing conditions. Thus, a PCR mixture containing several species will in produce a profile in which each band will consist of a population of identical DNA fragments, each presumably derived from one particular species. Although several issues can complicate the interpretation of DGGE/TGGE patterns, these techniques allow for the rapid comparison of the dominant microbial populations between multiple samples. One of the most attractive features of these methods is that individual bands can be excised for sequence determination. Although the fragment size can limit the amount of information that can be recovered, this procedure often provides a good means of band identification.

The scope of the analysis depends on the specificity of the primers used in the PCR. Well-defined PCR-DGGE systems have now been developed for bacteria and to a lesser extent for fungi (Kowalchuk, 1999). In addition, a number of phylogenetic and functional groups of micro-organisms are now accessible via these methods, including ammonia-oxidising bacteria, methane-oxidising bacterial, fluorescent pseudomonads, actinomycetes, cyanobacteria (probably not important in soils), and arbuscular mycorrhizal fungi (AMF), and the development of methods to target other groups is in progress. Although the vast majority of DGGE/TGGE studies have targeted 16S rDNA (or rRNA), it is also possible to target specific protein-encoding genes or gene families (Wawer and Muyzer, 1995). Such methods should become of greater importance in the coming years.

▪ *Single-Strand Conformation Polymorphism (SSCP)*

Like DGGE, Single Strand Conformation Polymorphism (SSCP) uses sequence dependent mobility differences to separate mixed PCR products into constituent DNA populations, and was originally developed for detection of mutations (Hayashi, 1992; Orita et al., 1989). Double- stranded DNA fragments are first denatured into single strands which are then exposed to non-denaturing conditions which allow them to fold in a sequence specific manner. The shape and size of each molecule determine the electrophoretic mobility of these folded single strands in a non-denaturing gel. Ideally, one double-stranded molecule, e.g., PCR product, has three bands, one for each strand and, at the bottom of the gel (with the largest migration distance) a double-stranded product, which is formed by re-annealing of the complementary single strands. However, a single strand can also fold into more than one conformation, which may result in additional bands. The technique makes use of standard electrophoretic equipment and does not require a GC-clamp, like DGGE or TGGE (Tebbe et al., 2001). Initial limitations of this technique for community analysis, which were caused by re-annealing and heteroduplex formations of near complementary molecules, can be eliminated by removing the reverse DNA strand of each PCR product with lambda exonuclease (Schwieger and Tebbe, 1998). This is done with the use of a 5' phosphorylated reverse primer in the PCR. With these modifications, SSCP can be regarded as a highly viable alternative to the more prevalent DGGE or TGGE, since, like for those techniques, group specific community profiles can be generated and single bands of profiles can be identified by DNA sequencing (Peters et al., 2000). Recently, SSCP has been successfully used to compare rhizosphere communities of transgenic and non-transgenic maize (Schmalenberger and Tebbe, 2002).

▪ *Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Terminal Restriction Fragment Length Polymorphism (T-RFLP)*

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is based on cleavage of PCR amplified rDNA products with restriction endonuclease enzymes (Giraffa and Neviani, 2001; Tiedje, et al., 1999). The cleavage products are separated by size using gel electrophoresis. As a fingerprinting method, the results provide only general data of how similar or different multiple communities may be, without prior knowledge of the organisms involved. While this method is simple and inexpensive, it does not give any information as to the nature of any differences detected between samples.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is very similar to ARDRA except that usually one of the primers in the amplification reaction is fluorescently labelled for the subsequent detection of only the terminal restriction fragment. Resulting gel fragments are typically run on an automated sequencer along with size standards. By using the restriction information of existing database sequences and by using a number of restriction enzymes, it is sometimes possible to come to reasonable inferences with respect to the taxonomic placement of detected fragments, and this ability is increasing as available databases become more sophisticated (Liu et al., 1997; Marsh et al., 2000). Although it has the potential to rapidly compare diversity and composition of multiple samples, it usually relies upon the use of rather sophisticated equipment. Also, ideally several restriction enzymes would be used in a single, compound analysis, decreasing the speed with which samples can be processed. Typically, each distinct band in a T-RFLP pattern is treated as an operation taxonomic unit, however it is often impossible to control what the breadth of this unit. The use of fluorescent sequencing technology can however provide a simple and accurate means of determining the intensity of band signals.

▪ *Random Amplified Polymorphic DNA (RAPD)*
Random Amplified Polymorphic DNA (RAPD) is a DNA polymorphism assay based on PCR amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams et al 1990). Primers are usually short (decamers) and have an arbitrary sequence. They detect DNA polymorphisms in the absence of specific nucleotide sequence information, i.e. unknown fragments of DNA are used. RAPD relies on the statistical chance that the complementary primer sites occur somewhere in the genome as inverted repeats enclosing a relatively short stretch of DNA (up to a few thousand base pairs). Typically, a single microbial isolate will produce a complex banding pattern that is unique to a relatively narrow phylogenetic grouping (usually below species). Thus, although RAPD is a good technique for comparing isolated strains, it typically produces an unintelligibly complex mixture of bands when applied to mixed microbial communities.

▪ *Repetitive Sequence-PCR (rep-PCR)*
ERIC-PCR, REP-PCR and BOX-PCR (Martin et al 1992; Louws et al., 1994; Versalovic et al., 1994) are types of repetitive sequence-based PCR techniques (rep-PCR, Versalovic, 1994) which target different genetic elements: the 124- to 127-basepairs (bp) enterobacterial repetitive intergenic consensus (ERIC) sequence, the 35- to 40-bp repetitive extragenic palindromic (REP) sequence and the 154-bp BOX element, respectively. These techniques rely on the analysis of amplified genomic sequences located between interspersed repetitive elements. They involve the amplification of genomic DNA located between the given repetitive sequences. The elements are distributed throughout extragenic regions of the genomes of many bacteria, including many plant-associated bacteria (Versalovic et al., 1991). The unique locations of these elements in bacterial genomes allows discrimination at the genus, species and even strain level based on the electrophoretic pattern of amplification products. The techniques were developed for the analysis of axenic bacterial cultures, but has also been applied to mixed bacterial populations. Similar to ARDRA and RAPD, this can lead to highly complex banding patterns when applied to communities, and although one can make a general determination as to similarity or difference between profiles, any populations-specific information is lost in the complexity.

▪ *rDNA Intergenic Spacer Analysis (RISA)*
rDNA Intergenic Spacer Analysis is a method based on length polymorphism of the spacer region located between the 16S and the 23S rRNA genes amplified with eubacterial primers directly on the DNA extracted from the community (Ranjard et al., 2000). This region is extremely variable in size and sequence even within closely related taxonomic groups. Its

amplification by PCR has been suggested as an excellent tool for strain characterisation, typing, and to a lesser extent for community fingerprinting (Nagpal et al., 1998; Garcia-Martinez et al., 1999; Giraffa and Neviani, 2001).

▪ *Length heterogeneity PCR (LH-PCR)*
This method is very similar to T-RFLP. The methods differ in the way they distinguish between organisms. T-RFLP is based on the variability in the restriction sites, whereas LH-CR is based on the natural length variability of the 16S rDNA sequences. These DNA fragments may be the same size for different taxonomic or functional groups. LH-PCR is not a very specific method, like T-RFLP or ARISA, however it can be used for complex microbial communities, where T-RFLP or ARISA would produce too complex banding patterns to analyse. The method was first used for soil microbial communities by Ritchie et al (2000). They used this method successfully to distinguish 3 soil types and different tillage practices within one soil type. It can be a useful method for rapid analysis of microbial communities.

▪ *Guanine plus Cytosine (G + C) composition and DNA re-association kinetics*
A broad-scale approach for the analysis of the microbial community is the determination of the guanine plus cytosine (G + C) composition, using DNA extracted from the total community in the soil. This can be measured using thermal denaturation procedures (Torsvik et al., 1990; Clegg et al., 1998) or density gradient centrifugation (Holben and Harris, 1995; Tiedje et al., 1999). This method is based on the fact that each species has its own G + C composition, therefore a change in G + C composition reflects a shift in microbial community composition. On the other hand, lack of shift does not give strong evidence of community similarity, as results combine all organisms. The inherent complexity of a sample can also be judged by determining the re-association kinetics of the total DNA extracted from a sample. Less complex samples will re-anneal more quickly than more complex ones, and the total DNA complexity can be estimated with reference to standards of known diversity. Although this technique can be a powerful means to address changes in microbial diversity, it cannot provide any details regarding the nature of the differences between samples. Such methods can also be applied in the direct comparison of total DNA extracted from different samples. The kinetics of cross hybridisation between different DNA samples can be compared to those within a single DNA extraction to give a relative measure of similarity (Clegg et al., 2000; Kozdroj and Van Elsas, 2001). Again, although this can provide a value for the general level of similarity between samples, it provides no information as to the nature of the differences between samples

3.1.4 Biomarker approaches
Structural molecules, other than DNA, can often provide information concerning the identity and physiology of microbial cells. Such molecules, which can be specific for certain micro-organisms or microbial groups are typically referred to as biomarkers. The two groups of molecules most typically used in microbial studies are phospholipid fatty acids (PFLA) and fatty acid methyl esters (FAME).

▪ *Phospholipid fatty acid (PLFA) analysis*
Phospholipid fatty acid (PLFA) analysis can be used to determine the structure and composition of the microbial community, based on the analysis of phospholipid fatty acids. Phospholipid fatty acids are major constituents of membranes of living cells (except for Archaebacteria). They therefore can be used as indicators of viable cellular biomass. PLFA was introduced to soil ecology by Tunlid et al (1989). The lipids are extracted from the soil samples and fractionated into glycolipids, neutral lipids and polar lipids. The polar lipid fraction is transesterified with mild alkali to recover the PLFAs. With gas chromatography the PLFAs are separated, and the peaks are analysed for the quantification and identification (Ibekwe et al 2001).

Specific groups of micro-organisms, taxonomically or functionally related, are characterised by different PLFAs compositions (Laczko et al., 1997). Certain PLFAs can therefore be used as biomarkers for these specific groups and a change in the PLFA composition can indicate a shift in the composition of the microbial community. An advantage of this method is not necessary to cultivate the micro-organisms and therefore provides an estimate of all living micro-organisms present in the soil. It provides quantitative data that can be analysed by a variety of powerful statistical means. However, PFLAs can only detect broad scale changes, as these biomarkers can typically only be used to distinguish at higher taxonomic levels.

In a comparative study of DGGE, CLPP and PFLA, Ibekwe et al (2001) found PFLA to be the most effective method in discriminating between different microbial communities. Lehman et al (1995) however found similar results for their CLPP and PLFA analysis, and Ekblad et al (1998) found a correlation between PFLA results and with the ATP concentration of the soil samples they analysed.

▪ *Fatty acid methyl ester (FAME)*
Fatty acid methyl esters (FAMEs) can be highly specific for microbial species, and their analysis has become a powerful means of identifying cultured micro-organisms. FAMEs are extracted, separated and quantified by gas chromatography and the peaks can be named using commercially available software, like the MIDI Microbial Identification Software. While this method is well suited for the study of bacterial isolates, results become difficult to interpret when applied to complex communities. Since a single species can produce a complex banding pattern, combining the results of a whole community become unintelligible. Similar to some general community profiling methods, community data can only provide a general approximation of similarity of difference between samples, without regard for the nature of the changes.

3.2 Microbial activity

Microbes fulfil several key roles that are vital for the functioning of terrestrial ecosystems. Most important is their role in nutrient cycling, plant growth, purification of drinking water and formation of soil structure. Therefore, risk assessment procedures directed towards soil microbes should also include microbial activity as a key target.

▪ *Substrate usage, CLPP*
As plants release substantial amounts of organic matter from their roots in soil during growth and this release differs between plants and plant growth conditions, it is very likely that transgenic plants are likely to differ in their exudates from their parental plants. Since plant exudates are an important nutrient source for micro-organisms, substrate utilisation might be a good indicator for microbial activity. The difference in plant exudates is likely to cause changes in the community level physiological profiles (CLPP) (Grayston et al 1998), sometimes also referred to as community level substrate utilisation (CLSU) (Widmer et al., 2001). The technique is used for the analysis of microbial communities with Biolog™ microtitre plates (Garland and Mills, 1991).

The method is based on the utilisation of sole carbon sources. Each plate contains 95 wells with different sole carbon sources and 1 well without a carbon source as a control. The reduction of tetrazolium violet produces colour, which is used as an indication of respiration of sole carbon sources. The intensity of the colour can be quantified from digitised images of the microplates (Garland and Mills, 1991). The method provides a rapid fingerprint of the metabolic activity of the microbial community, but it has the disadvantage of being a culture-based method.

There are several examples of studies in which differences in substrate utilisation were detected. Griffiths et al (1999) did indeed observe changes in CLPP of the microbial communities in the soil of transgenic potato producing the lectin GNA and Con A. Donegan et al (1995) detected a shift in microbial usage of substrates as well, while studying the effects of transgenic cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin (Btk toxin). Another study, in which differences in substrate utilisation were observed, is a study on the effects of transgenic alfalfa by Di Giovanni et al (1999). Besides CLPP is applied in many other ecotoxicological studies (e.g. on the impact of soil pollutants) involving the soil microbial community.

▪ *Substrate induced respiration assay (SIR)*
Beare et al (1990) adapted the SIR procedure to measure fungal, bacterial and total microbial mineralisation potentials from plant residues of differing compositions.
The method is based upon the assumption that the utilisation of a generally acceptable substrate by microbial communities depends on the actual physiological condition and the size of the community. Glucose is the substrate used most often, but applied substrates can vary. The substrate is added to soil samples at a defined moisture level and the utilisation is determined by the measurement of CO2 produced in closed jars after a period of several (2-6) hours. The microbial respiration rate is calculated as: (μg CO_2-C g^{-1} dry wt soil from glucose replicate sample) – (μg CO_2-C g^{-1} dry wt soil from control replicate sample)(Beare et al 1990).

Donegan et al (1999) used this method and did not find any differences in SIR values between the two different transgenic plants and the non-transgenic alfalfa plants inoculated with recombinant or wild-type strain *Sinorhizobium meliloti*. Donegan et al (1997) also found no significant differences in SIR values between parental and transgenic tobacco litter or between parental, transgenic and control soil samples, despite finding differences between treatments using other methods. This suggests that SIR is not a very sensitive method, and it may not be able to detect short-term changes in microbial communities. However, the method has been and still is applied in other ecotoxicological studies.

▪ *Enzyme activity*
Soil enzymes originate mostly from micro-organisms (Ladd, 1978), but also from plant roots and animals (Tabatabai, 1994). They catabolise many biochemical reactions in soil and are important for nutrient cycling. There are many different soil enzymes. They differ in chemical composition, function, and sensitivity to changes. Soil enzymes can be suitable indicators because of their sensitivity for changes in soil, simplicity of methods and relationship to soil biology (Dick, 1994; Dick et al., 1996; Bandick and Dick, 1999). Most enzyme activities can be measured by chemical protocols; a few examples are listed in table 3.1.

Table 3.1 Soil enzymes, their main functions and references to methods.

Enzyme	Function	Reference
amidase	Release of inorganic N in the N cycle	Tabatabai, 1994
arylsulphatase	Releases plant available SO_4; and indirect indicator of fungi	Tabatabai, 1994
cellulase	Hydrolyses cellulose	Schinner and von Mersi, 1990; modified by Gander et al, 1994
deaminase	Release of inorganic N in the N cycle	Killham and Rashid, 1986
dehydrogenase (DHA)	Reflects activity of physiologically active micro-organisms	Casida, 1977; modified by Tabatabai, 1994 and Subler et al, 1997
fluorescein diacetate hydrolysis	Broad-spectrum indicator of biological activity (lipases, proteases, esterases)	Zelles et al, 1991
α-galactosidase	Critical role in releasing low Molecular weight sugars	Tabatabai, 1994
β-galactosidase		Tabatabai, 1994
α-glucosidase		Tabatabai, 1994
β-glucosidase		Tabatabai, 1994
invertase	Critical role in releasing low molecular weight sugars	Schinner and von Mersi, 1990; modified by Gander et al, 1994
(acid/alkaline) phosphatase	Hydrolyses phosphate, P cycle	Tabatabai and Bremner, 1969; Tabatabai, 1994
urease	Release of inorganic N in the N cycle	Tabatabai and Bremner, 1972; Tabatabai, 1994

Dehydrogenase activity (DHA) is often used in ecotoxicology as an indicator for the activity of physiologically active micro-organisms. It is, unlike most other soil enzymes, an intracellular enzyme and degrades quickly after cell death. Several enzymes are implicated in DHA of soil microflora (Rossel et al., 1997). From a comparative study including several soil enzymes, Bandick and Dick (1999) concluded that deaminase is a less suitable indicator, because of lack of treatment sensitivity. β-glucosidase was the most consistent indicator of the C cycle enzymes tested by Bandick and Dick (1999). Amidase, α-glucosidase, β-glucosidase, arylsulphatase, FDA and urease all showed consistent results for both air-dried or field-moist soil samples, and therefore suitable for routine testing programs (Bandick and Dick, 1999).

Donegan et al (1999) used acid phosphatase, alkaline phosphatase and dehydrogenase activities to study the effects of transgenic lignin peroxidase-producing alfalfa, transgenic α-amylase-producing alfalfa and parental alfalfa plants and found no differences. Enzyme activities are in some cases sensitive to change and have proven useful tool in ecotoxicological studies. They have also been used as indicators of perturbations caused by inoculation with GM micro-organisms and show potential for monitoring of some aspects of GMP-induced effects (Naseby and Lynch, 1997; Naseby et al., 2000).

3.3 System parameters

▪ *Microbial biomass*

Microbial biomass can be estimated by several techniques, such as respiration, metabolic heat production, adenylate energy content, adenosine triphosphate (ATP) content, enzymatic activities or soil fumigation techniques (Hartmann et al., 1997). Total microbial biomass is frequently measured in effect studies (Donegan et al 1995, 1996). It is a parameter that is relatively easy to measure and easy to detect differences. However, there are some disadvantages of microbial biomass as an indicator. The differences are difficult to interpret. For example the influence of a transgenic plant can cause one species to become very dominant, while the total microbial biomass remains the same, and will therefore wrongly assume there is no or little effect of the transgenic plant. Another approach that might solve this problem is microbial biomass in combination with a second parameter, for instance respiration, measured as the respiration per unit microbial biomass (*e.g.* Hu et al., 2001). This provides information about the microbial biomass and about a function.

Chloroform fumigation extraction is a well established method to measure soil biomass C. With this technique, soil samples are fumigated with alcohol-free chloroform. Chloroform destroys the cell membranes and this allows cell constituents to leak into the soil. After removal of the chloroform the soil samples are extracted with K_2SO_4. The soil suspensions are filtered and the organic C in the soil extracts is measured. The soil biomass C is calculated as the K_2SO_4 extracted C in the fumigated soil minus the K_2SO_4 extracted C in the unfumigated soil (= the flush of extractable C) times a correction factor (Jenkinson and Powlson, 1976; Vance et al., 1987; Wu et al., 1990).

▪ *Soil organic matter (SOM)*

A definition of soil organic matter (SOM) by Stevenson (1994) is that SOM represents the whole of the organic material in soils, including litter, light fraction, microbial biomass, water-soluble organics, and stabilised organic matter (humus). SOM can be measured very precisely applying a thoroughly tested methodology. SOM is an indicator of biological, physical and chemical qualities of the soil. However, it has a very small spatial and short-term temporal variability. The impact of management practices on SOM is usually detectable after several years, only it may take decades (Smith et al., 2000) and it might therefore be less suitable to measure for the effects of transgenic plants.

▪ *Pollution-induced community tolerance (PICT)*

In case of long term pollution of an ecosystem, the ecosystem can adapt to the pollution. For example there might be a shift in the microbial community towards a community that can cope with the pollution. PICT uses tolerance as a measure of pollutant impact. PICT can be used to quantify the toxic effects between the pollution and the ecosystem changes. It is likely for polluted ecosystems that they have a lower diversity, which could also have consequences for the ability of the ecosystem to cope with other environmental stresses. PICT is a method that is used in ecotoxicology studies, but may be adjusted to measure effects of GMPs as well. In ecotoxicology, PICT is often applied to determine the tolerance of the communities against the pollution, in dose-response reactions under laboratory conditions (Rutgers et al., 2001).

In case of GMPs one might think about the application of PICT to determine the impact of different concentrations of the foreign compound produced by the transgenic plant, like T4 lysozyme, and measuring the effect on the soil microbial community. Subsequently, PICT can be used to calculate the potentially affected fraction (PAF) of the microbial community at the different concentrations of the transgenic compounds using statistical extrapolation methods. This PAF curve indicates the fraction of species from the original community that may become affected by the transgenic compound (Van Beelen et al., 2001).

3.4 Summary

In general, all techniques for studying soil ecosystems have both particular advantages, as well as distinct limitations. Combining techniques, especially different types of methods, will in almost all cases lead to a more accurate and well-balanced view of the soil system under study. The methods reviewed in this chapter are summarised in table 3.2.

Table 3.2 Overview of techniques used for the analysis of soil microbial communities and processes.

Technique (acronym)	Specific for			Examples in GMP research	Type of method
	Species / group	Com-munity	System		
Plate count technique	x			Donegan et al, 1996, 1999; Oger et al, 1997, 2000; Lottmann et al, 1999, 2000	culture based
Most Probable Number (MPN)	x				culture based
Fluorescence microscopy		x		Ahrenholtz et al, 2000	Molecular, direct count
Fluorescence In Situ Hybridisation (FISH)	x				direct count
PCR-cloning	x	x			Molecular sequencing
Reverse Array hybridisation		x			Molecular, probing
Denaturing / Temperature Gel Electrophoresis (D/TGGE)	x	x		Lottmann et al, 2000; Heuer et al, 2002	molecular, fingerprinting
Single Strand Conformation Polymorphism (SSCP)	x	x			molecular, fingerprinting
Amplified Ribosomal DNA Restriction Analysis (ARDRA)		x			molecular, fingerprinting
Terminal - Restriction Fragment Length Polymorphism (T-RFLP)		x		Lukow et al, 2000	molecular, fingerprinting
rDNA Intergenic Spacer Analysis (RISA)	x				molecular
Length Heterogeneity – PCR (LH-PCR)		x			molecular
Entero Repetitive Intergenic Consensus sequence – PCR (ERIC-PCR)	x			Di Giovanni et al, 1999	molecular
G + C composition		x			molecular
DNA re-association kinetics		x			molecular
Phospholipid Fatty Acid Analysis (PLFA)		x			fatty acid

Table 3.2 (cont.)

Fatty Acid Methyl Ester (FAME)		x		Siciliano et al, 1998; Siciliano and Germida, 1999; Lottmann and Berg, 2001	fatty acid
Substrate usage (CLPP)		x		Donegan et al, 1995; Di Giovanni et al, 1999; Griffiths et al, 2000a	culture based
Substrate Induced Respiration assay (SIR)		x		Donegan et al, 1997, 1999	respiration
Enzyme activity			x	Donegan et al, 1999; Griffiths et al, 2000a	enzymes
Soil Organic Matter (SOM)			x		soil structure
Pollution Induced Community Tolerance (PICT)			x		soil resilience
Chloroform fumigation extraction		x	x		biomass

CHAPTER 4. EXAMPLES OF STUDIES ON EFFECTS OF GENETICALLY MODIFIED PLANTS ON SOIL ECOSYSTEMS

This chapter provides a review of previous (field) experiments with GMPs, in which specific attention has been paid to the effects of the transgenic plants on soil organisms and processes. It describes the measured parameters (e.g. organisms, community structure, physical or chemical parameters), the techniques used to determine the microbial communities and the results. Since each study has examined different parameters, it is difficult to compare the different studies, but they provide some examples of possible effects of GMPs on soil ecosystems. The last paragraph of this chapter contains a short summary and an overview of studies addressing the effects of GMPs on soil systems.

Table 4.1 Summary of the examples of experiments with GMPs given in this chapter.

First author	Year	Modified plant	Modified trait	Result
Donegan	1997	Tobacco	Proteinase inhibitor I	Effects on populations of protozoa, nematodes and microarthropods
Oger	2000	Birdsfoot trefoil	Opine production	Selective advantage for opine-utilising bacteria
Lottmann	1999	Potato	T4-lysozyme production	No effect of T4-lysozyme production on plant-associated bacteria
Di Giovanni	1999	Alfalfa	Alpha-amylase and lignin peroxidase	Consistent differences in types of bacteria and substrate utilisation
Lukow	2000	Potato	*Barnase/ Barnstar* and *gus* genes	Spatial and temporal effect as well as space × time interaction effects on structural of bacterial communities
Dunfield	2001	Canola	Glufosinate ammonium- and glyphosate-tolerance	GM canola supported different bacterial communities
Vierheilig	1993	*Nicotiana sylvestris*	3 different forms of tobacco chitinase	Reduced susceptibility to colonisation by root pathogen *Rhizoctonia solani*; normal colonisation by root symbiont *Glomus mosseae*

4.1 Ecological impact of transgenic tobacco plants

Donegan et al (1997) studied the possible ecological impact of a field release of genetically modified tobacco plants. They measured several ecological parameters, like the carbon content, persistence of the product of the genetically modified plant and the effects on several groups of soil organisms.

The tobacco plants tested (*Nicotiana tabacum* cultivar Xanthi) contained a genetic modification to constitutively produce proteinase inhibitor I, which is a protein with insecticidal activity. To study the ecological impact, they measured the persistence of the proteinase inhibitor I in the soil and the effect on populations of protozoa, nematodes and microarthropods. Litterbags with leaves of the transformed plants that express the proteinase inhibitor I and litterbags containing leaves of the parental plants, were buried in a field plot. In 2- to 4-week intervals the litterbags, the soil surrounding the litterbags and soil from plots without litterbags (control plots) were sampled for 147 days. In the samples they measured proteinase inhibitor I concentration, litter decomposition rates, carbon and nitrogen content, microbial respiration rates, as well as population levels of protozoa, nematodes, and microarthropods.

After 57 days, the proteinase inhibitor I was no longer detectable with enzyme-linked immunosorbent assays (ELISA) in the samples. By sample day 147, complete decomposition had occurred. In none of the samples were differences in litter decomposition rate detected between transgenic and parental plant litter. The carbon content of transgenic plant litter did differ from the parental plant litter; the carbon content of the transgenic plant litter was significantly lower. The numbers of nematodes in the soil surrounding transgenic plant litterbags were different as well, with higher numbers than in the soil surrounding the parental plant litterbags, and significantly higher numbers than in soil from control plots without plants. Nematode populations also showed a shift towards fungal feeding nematodes in the transgenic soil samples. In the population levels of the microarthropods, the only significant response was found in Collembola population levels. The levels of Collembola in soil surrounding transgenic plant litterbags was significantly lower than in the soil surrounding parental plant litterbags and the control plots. The protozoa assays showed no significant differences. The microbial substrate-induced respiration rates were not significantly different between samples either, but they were significantly different between sample days. For the numbers of insects no significant differences were detected, but this may be due to low sample sizes.

The results revealed that under field conditions differences occurred between decomposing parental and transgenic plant litter as well in the quality of the residual litter. The carbon content is of ecological relevance to microbial decomposers. The low carbon content in the transgenic plant litter may impede decomposition and suppress microbial populations and result in low levels of soil organisms. As for the population levels of the microarthropods only a decrease in the level of Collembola was observed, but this decrease might be of ecological concern. The increase in nematode levels can reflect a shift in trophic group composition. The difference in feeding behaviour of bacterivorous and fungal nematodes on microflora influences the soil ecosystem. Functions like decomposition and nutrient release can be affected by these changes. However, in this study the biodegradation did not seem to be adversely affected, since the decomposition rates of the different plots were comparable. The proteinase inhibitor I persisted for 57 days, but in case of more long-term experiments or commercial use this period could become longer and cause more pronounced effects on the environment.

4.2 The selective advantage introduced by transgenic plants to specific bacterial groups

Oger et al (2000) examined the effects of a genetically modified plant on soil microflora. This study provides an example of effects that can be expected when plants are transformed to produce bacterial growth substrates. They modified *Lotus corniculatus* plants to produce opines and analysed the effect of this opine production on several bacterial groups in the soil.

Lotus corniculatus cv. Rodéo plants were transformed using *Agrobacterium rhizogenes*. The genetically modified plants produced mannityl opines (mannopine, mannopinic acid and agropinic acid) and nopaline. Oger et al used crop rotation to study the influence of the catabolic bias caused by bacterial growth substrates. First wild-type and modified plants were grown in a greenhouse. After 20 weeks the plants were removed from the soil. After these 20 weeks, they counted different types of bacterial groups using seven semi-selective media. The bacterial groups did not differ significantly between the soil used for wild-type and modified plants, except for the mannopine and nopaline-utilisers. The opine-utilisers were present in higher densities in the soil around the root system of the modified plants. This indicated that the growth of *Lotus* plants influenced the bacterial populations present in the original soil mixture.

After removing the plants, the soil was left unplanted or replanted either with wild type Lotus, modified Lotus or wheat. After and during these treatments the bacterial groups were measured again. In the bare soil (both from the modified and the wild type plants) the densities of the bacterial populations were very similar. But the mannopine and nopaline utilisers did differ. Opine-utilising bacteria were present in 5 to10-fold higher densities in transformed plant soil than in wild-type plant soil. In the bacterial populations in the wheat rhizosphere no significant differences were observed between the soil from the modified and the non-modified plants.

In the rhizosphere of the genetically modified plants that were grown in the soil of the modified plants (positive control), the densities of the bacterial groups were comparable to those of the rhizosphere of the non-modified plants in non-modified soil (negative control). The densities of opine-utilisers were higher in the positive control than in the negative control as expected because of the selective advantage induced by the opine-producing plants. In the rhizosphere of the non-modified plants that were grown in modified soil (removing the selective pressure), the densities of the other bacterial groups examined, were similar to the control treatments. Just after the replacement of modified plants with wild-type plants, the mannopine and nopaline utilisers were as numerous as in the rhizosphere of the positive control (GMP). However, the concentration of mannopine utilisers slowly decreased over the 22 weeks reaching an intermediate value between the positive and negative controls. On the contrary, the concentration of nopaline utilisers rapidly decreased to reach a value close to the negative control.

The absence of plant cover did not lead to disappearance of catabolic bias introduced by the transformed plants. The growth of wheat erased the catabolic bias but it did not lead to a decrease in the density op opine utilisers. The replacement of transformed plants with wild-type plants affected only the community of opine utilisers. The decrease in the density of nopaline utilisers clearly showed that the catabolic bias is reversible. However, the results of mannopine utilisers suggest that the bias might be more persistent. This study indicates that the opine-producing plants give the opine-utilisers a selective advantage and this allows them to reach higher numbers in the root zone.

4.3 Influence of transgenic potato plants on plant-associated bacteria

The first study on the effect of plant modification directed towards the enhancement of resistance against soil-borne bacteria was performed by Lottmann et al (1999). They transformed potato plants to produce T4-lysozyme (a bacteriolytic enzyme) to enhance their resistance to *Erwinia carotovora* subsp. *atroseptica*. The effect of these transformed plants on potentially beneficial bacteria in the rhizo- and geocaulosphere was analysed in field experiments. The aerobic culturable bacteria were determined with aerobic plate counts. They screened for bacteria antagonistic against *E. carotovora* and *Verticillium dahliae*, and for indole-3-acetic acid (IAA)-producing bacteria. The IAA-production and antagonistic activity were chosen to monitor the main functions of potentially beneficial bacteria. Further root biomass was also determined. They used three sampling times a year and did the experiments in two succeeding years.

Although they did find differences in the number of bacteria between the different sampling times, which indicated that the statistical method was suitable, they did not detect any effect of the T4-lysozyme production. In none of the experiments did they find any changes in potentially beneficial plant-associated bacterial populations. However, plate count techniques only detect viable and culturable organisms, which are only a small percentage of the total bacterial population. In later experiments, they extended the procedure with a molecular analysis (Lottmann et al., 2000). They used DGGE to study the effects of plant-produced T4-lysozyme on the establishment of two potentially beneficial bacteria in the rhizo- and geocaulosphere of transgenic and non-transgenic potatoes and the impact of the introduced bacteria on the indigenous rhizosphere and geocaulosphere bacteria, but did not find a negative effect.

4.4 The use of Biolog GN metabolic fingerprinting and ERIC-PCR to assess the impact of transgenic plants on rhizosphere bacterial communities

Di Giovanni et al (1999) examined the effects of one parental and two genetically modified alfalfa plants (*Medicago sativa* L.) on the soil microflora, one modified for the expression of alpha-amylase and one for lignin peroxidase genes. The study compared parental and transgenic alfalfa rhizosphere bacterial communities. In this study, they analysed the rhizosphere bacterial communities of the plants by substrate utilisation and DNA fingerprinting. They used Biolog GN metabolic fingerprinting and enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR) for DNA-fingerprints of the substrate-specific populations and of bacterial communities of a substrate utilised similarly by all of the alfalfa rhizosphere communities.

The bacterial densities varied very little between experiments. Cluster analysis and Principle Component Analysis revealed plant genotype-specific differences in the rhizosphere bacterial community metabolic fingerprints. The parental plants and the alpha-amylase plants showed some overlap in the fingerprints, the lignin peroxidase plants formed a distinct cluster in several experiments. These differences were consistent. When the Biolog GN microplate substrate bacterial communities were compared differences in substrate utilisation of α–cyclodextrin, D-alanine, and L-ornithine were found and no difference in utilisation was observed for dextrin. Cluster analyses of ERIC-PCR showed consistent differences in types of bacteria enriched from the rhizospheres of the different plants. Comparison of ERIC-PCR fingerprints revealed the limitation of the Biolog plate technique. The fingerprints obtained from the substrate wells showed that a limited number of bacterial populations was responsible for the substrate oxidation in the wells. This study shows that transgenic plant genotype may affect the rhizosphere bacterial communities, but the ecological consequences of the effects are still unknown.

4.5 The use of T-RFLP to assess spatial and temporal changes in the bacterial community structure

The study performed by Lukow et al (2000) aimed to examine whether T-RFLP is an appropriate technique to monitor highly diverse soil bacterial communities. To do so they used modified potato plants with Barnase genes, which should cause cells infected with pathogens to commit suicide, and Barnstar genes, to minimise the detrimental effects on non-infected tissue, and other plants with *gus* genes coding for β-glucuronidase. With T-RFLP they compared soil samples with samples from pooled cells of Biolog microtitre plates, and analysed variation in time and space.

The results showed highly significant differences in T-RFLP patterns in space and time. Especially in the *gus* plants, which resulted in high temporal, but little spatial variation. The T-RFLP patterns from pooled cell fractions of Biolog microtitre plate showed a maximum of 3 different T-RFs, in contrast to the community fingerprint patterns from soil which each showed between 44 and 53 T-RFs. The T-RFLP analyses resulted in highly complex, but also highly reproducible community fingerprint patterns. In conclusion was suggested that T-RFLP could be a suitable method for monitoring impact of transgenic plants on highly diverse bacterial communities. The results indicate that T-RFLP is favourable to cultivation-based methods like Biolog, because of the focuses only on a minor (culturable) fraction of the total community.

4.6 Bacterial communities in eight different varieties of field-grown *Brassica* sp.

Dunfield and Germida (2001) studied the diversity of bacterial communities in eight different commercially available varieties of canola (oilseed rape, *Brassica* sp.) during two years on four different field locations. For this study they used four genetically modified *Brassica napus* varieties modified for broad spectrum herbicide-tolerance (glufosinate ammonium-tolerance and glyphosate-tolerance) and four conventional varieties (one of which was imidazolonone-tolerant and one *B. rapa* variety). The root material and soil was collected on various dates when the plants were at the flowering stage of growth. They studied the diversity of bacterial communities in the rhizosphere and root interior with CFU counts, fatty acid methyl ester analysis and community level physiological profiles with Biolog Gram-negative microplates.

Neither canola variety nor soil type affected total CFU counts. In the FAME analysis and CLPP differences were observed. Principal component analysis indicated that the root interior and the rhizosphere bacterial community associated with the genetically modified variety Quest was different from the conventional varieties Excel and Fairview. This difference was noted at different field sites and during two growing seasons. The other genetically modified canola varieties did not confirm this finding. The differences observed for the other genetically modified and conventional varieties appeared to be influenced by soil type.

4.7 Effect of transgenic plants expressing chitinase on a pathogenic and on a symbiotic fungus

Vierheilig et al (1993) studied the colonisation of transgenic *Nicotiana sylvestris* plants by a root pathogen and a mycorrhizal symbiont. They used plants modified for expression of different forms of *Nicotiana tabacum* chitinase. As a control they used *N. sylvestris* plants transformed with a vector containing no insert. The plants were inoculated with either *G. mosseae* or *R. solani*, or neither, these controls received the same stocks of soil or agar, used for the inoculation, without the fungi. The infection was estimated by visually scanning the root samples in the stereo microscope for fungal structures according to the gridline intersection method. Root fresh weight of inoculated plants was measured as well.

Genetically modified plants constitutively expressing vacuolar tobacco chitinase, independent of the presence or absence of the hevein domain, showed reduced susceptibility to colonisation by root pathogen *Rhizoctonia solani*. However they were still normally colonised by the root symbiont *Glomus mosseae*. Thus, in this case it appears possible to make use of the antifungal properties of chitinase without reducing the beneficial symbiotic interactions with mycorrhizal fungi.

4.8 Summary

Most of the studies which were conducted in order to determine the effects of GMPs on soil micro-organisms and processes revealed some effects. GMPs have been found to effect bacteria (Ahrentholtz et al., 2000; Di Giovanni et al., 1999; Donegan et al., 1995 and 1999; Dunfield et al., 2001; Lukow et al., 2000; Oger et al., 1997 and 2000; Siciliano et al., 1999), non-target fungi (Donegan et al., 1995), target fungi (Clausen et al., 2000; Maddaloni et al., 1997; Vierheilig et al., 1995), enzyme activity (DHA)(Griffiths et al., 1999), substrate utilisation (Di Giovanni et al., 1999; Donegan et al., 1999; Griffiths et al., 1999), and decomposition (Hopkins et al., 2001).

However, the case-specific nature of results is highlighted by the fact that a number of studies on the effects of GMPs on soil micro-organisms and processes have failed to find significant effects. No GMP-induced effects were found upon examination of mycorrhizal fungus populations of the species *Glomus mosseae* associated with tobacco (Vierheilig et al., 1993 and 1995). Other studies could not detect any effects on bacterial community structure (Donegan et al., 1999; Lottmann et al., 1999 and 2000; Oger et al., 2000; Saxena et al., 2001; Heuer et al., 2002), respiration (Donegan et al 1997 and 1999), or the fungal community (Saxena et al., 2001).

A wide variety of techniques has been applied in GMP research, and an overview of these studies is presented in table 4.2. Many studies have used plate count techniques, either solely or in combination with other techniques. Plate count techniques have been shown to be a useful tool to detect changes in specific microbial groups (Oger et al., 1997 and 2000). Several molecular techniques, such as ERIC-PCR, T-RFLP, SSCP and DGGE have also been applied and show promise for informative and routine use (Di Giovanni et al., 1999; Lukow et al., 2000; Heuer et al., 1999). Some of these molecular techniques can be applied to detect general shifts in microbial community structure as well as changes in specific dominant microbial populations. CLPP has been shown to be a useful tool for assessing changes in microbial community structure (Donegan, 1999; Dunfield and Germida, 2001). However, this is limited to the changes in culturable micro-organisms. Di Giovanni et al (1999) also showed ERIC-PCR fingerprinting to be more sensitive than CLPP in distinguishing between microbial communities associated with different plant genotypes. Although this methodological variation makes comparison between studies difficult, some general trends can be recognised, and list of review articles on the subject is provided in table 4.3.

Table 4.2 Overview of research conducted on the effects of transgenic plants on soil parameters.

First author	Year	Modified plant	Modified trait	Main results
Ahrenholtz, I.	2000	Potato	T4 lysozyme-production	Increased killing of *Bacillus subtilis* on the hair roots of transgenic potato plants
Boisson-Dernier, A.	2001	*Medicago truncatula*	*Agrobacterium rhizogenes* transformed GusA and nptII	Transformed roots of Medicago truncatula can be nodulated successfully by *Sinorhizobium meliloti* and can be colonised by endomycorrhizal fungi, such as *Glomus intraradices*
Broglie, K.	1991	Tobacco and canola	Bean chitinase	Increased ability of tobacco to survive in soil infested with the fungal pathogen *Rhizoctonia solani* and delayed development of disease symptoms. Canola was also found to be more resistant to root rot disease of *R. solani*
Clausen, M.	2000	Wheat	Antifungal protein KP4	Antifungal activity against *Ustilago maydis*
Di Giovanni, G.D.	1999	Alfalfa	Alpha-amylase and lignin peroxidase	Consistent differences in types of bacteria and substrate utilisation
Donegan, K.K.	1995	Cotton	*Bacillus thuringiensis* var *kurstaki* endotoxin production	Increase in total bacterial and fungal population; suggested not to be caused by the Btk toxin production but another change in plant characteristics
Donegan, K.K.	1996	Potato	*Bacillus thuringiensis* var *tenebrionis* endotoxin	minimal differences between the soil microflora of transgenic plants and the microflora of chemically and microbially treated commercial potato plants
Donegan, K.K.	1997	Tobacco	proteinase inhibitor I	Effects on populations of nematodes and microarthropods, and carbon content; no difference in soil microbial respiration
Donegan, K.K.	1999	Alfalfa	alpha-amylase or lignin peroxidase production	Distinct metabolic fingerprints by soil bacterial communities, higher population levels of culturable bacteria, higher soil pH levels (for GM lignin peroxidase). No effect on protozoa, nematodes and microarthropods and DNA fingerprints of indigenous soil bacteria and rates of substrate induced respiration
Dunfield, K.E.	2001	Canola	glufosinate ammonium-tolerance and glyphosate tolerance	GM canola supported different microbial community than non-GM line
Escher, N.	2000	Corn	*Bacillus thuringiensis* var *kurstaki* endotoxin (Cry1Ab)	Lignin was decomposed more quickly in transgenic corn. Bacterial growth on faeces of *Porcellio scaber* fed on transgenic corn was up to 60% lower than on faeces of *P. scaber* fed on non-transgenic corn
Gebhard, F.	1999	Sugar beet	Kanamycin and glufosinate ammonium resistance	DNA of transgenic sugar beet was detectable for several months in soil under field conditions and in soil microcosms with introduced transgenic plant DNA

Griffiths, B.S.	1999, 2000a	Alfalfa	lectins Con A and GNA production	No effect on protozoa, nematodes; only consistent effects increase of dehydrogenase activity, and difference in CLPP of microbial community at harvest (but did not persist from one season to the next)
Heuer, H.	2002	Potato	T4-lysozyme	Environmental factors related to season, field site, or year, but not the T4-lysozyme expression influenced rhizosphere communities
Hopkins, D.W.	2001	Tobacco	lignin biosynthesis	Material from all of the modified plants decomposed more rapidly than material from the wild-type plants
Lottmann, J.	1999	Potato	T4 lysozyme production	No effect of T4 lysozyme production on plant-associated bacteria
Lottmann, J.	2000	Potato	T4 lysozyme	No negative effect on the establishment of the antagonistic plant-associated strains *Pseudomonas putida* and *Serratia grimesii*; no significant effect on indigenous bacteria
Lottmann, J.	2001	Potato	T4 lysozyme	Cluster analysis of phenotypic and genotypic features did not reveal correlations between bacterial isolates and transgenic character of plants
Lukow, T.	2000	Potato	*Barnase*/*Barnstar* and *gus* genes	Spatial and temporal effects as well as space x time interaction effects on structural composition of bacterial communities
Lusso, M.	1996	Tobacco	β - 1,3-glucanase	Increased resistance of the foliage of T2 progeny of GMP to the glucan-containing fungi *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotiana*. Resistance was not observed when the T2 progeny were challenged with tobacco mosaic virus, tobacco etch virus or tobacco vein mottling virus
Maddaloni, M.	1997	Tobacco	maize ribosome-inactivating protein b-32	Increased tolerance against infection by the soil-borne fungal pathogen *Rhizoctonia solani*; Fungal growth of *R. solani* inhibited
Masoud, S.A.	1996	alfalfa	acidic glucanase (Aglu1) and a rice basic chitinase (RCH10)	GM alfalfa did not appear to negatively affect the *Rhizobium*/alfalfa interaction or to have increased resistance against *Stemphylium alfalfae* or *Colletotrichum trifolii*. However, it did reduce disease severity caused by the oomycete pathogen *Phytophthora megasperma*
Murray, F.	1999	cotton and tobacco	*Talaromyces flavus* glucose oxidase	Expression reduced fungal infection by *Rhizoctonia solani* and *Verticillium dahliae*, but was phytotoxic as well
Neuhaus, J.M.	1992	*Nicotiana sylvestris*	β - 1,3-glucanase	The plants did not exhibit increased susceptibility to *Cercospora nicotianae* infection
Oger, P.	2000	Birdsfoot trefoil	Opine production	Selective advantage for opine-utilising bacteria, no effect on other bacteria
Oger, P.	1997	Birdsfoot trefoil	Opine production	GM opine producing plants alter the cultivable root-associated bacterial community
Palm, C.J.	1994	Cotton	*Bacillus thuringiensis* var *kurstaki*	Methodology for measuring persistence of Btk toxin in soil

			endotoxin	
Saxena, D.	2001	Corn	*Bacillus thuringiensis* toxin production	No significant differences in percent mortality and weight of earthworms, in numbers of nematodes and protozoa and in CFUs of culturable bacteria and fungi
Siciliano, S.D.	1998	Canola	glyphosate tolerance	FAME and Biolog profiles of endophytic and rhizosphere communities of transgenic canola were different from non-transgenic cultivars in the same field
Siciliano, S.D.	1999	Canola	glyphosate tolerance	Lower diversity in bacterial root-endophytic community. More *Flavobacterium* and *Pseudomonas* isolates and less *Bacillus*, *Micrococcus*, and *Variovorax* isolates in the root interior. And less *Arthrobacter* and *Bacillus* isolates in the rhizosphere of GMPs
Tahiri-Alaoui, A.	1994	Tobacco	chitinase	change in mycorrhizae
Vierheilig, H.	1993	*Nicotiana sylvestris*	3 different forms of tobacco chitinase	Reduced susceptibility to colonisation by root pathogen *Rhizoctonia solani*; normal colonisation by root symbiont *Glomus mosseae*
Vierheilig, H.	1995	Tobacco	antifungal pathogenesis-related protein expression	Expression of pathogenesis-related proteins did not affect the time course or final level of colonisation of the roots by the vescicular-arbuscular mycorrhizal fungus *Glomus mosseae*

Table 4.3 Examples of literature studies and reviews

First author	Year	Topic
Abbott, R.J.	1994	Ecological risks
Angle, J.S.	1994	Biodiversity and population-level considerations
Cannon, R.J.C.	2000	Risks and benefits of Bt transgenic crops
Glandorf, D.C.M.	1997	Influence of antibacterial and antifungal protein production
Morra, M.J.	1994	Recommendations for risk assessment
Nielsen, K.M.	1998	Review on horizontal gene transfer from plants to terrestrial bacteria
O'Callaghan, M.	2001	Impacts of GMPs and GMMs on soil biota
Punja, Z.K.	1993	Effects of plant chitinases on fungal diseases
Salmeron, J.M.	1998	Research results with implications for developing bacterial and fungal disease resistant crops
Thomson, J.A.	2001	HGT from GM crops to bacteria and mammalian cells
Tomlin, A.D.	1994	Recommendations for risk assessment
Trevors, J.T.	1994	Transgenic plants and biogeochemical cycles
Wolfenbarger, L.L.	2000	Ecological risks and benefits

CHAPTER 5. POSSIBLE STRATEGIES AND FUTURE RESEARCH

5.1 Research goal

The aim of this report has been to identify soil functions or groups of micro-organisms that are most relevant for assessing effects of GMPs on terrestrial ecosystems. This may be used to evaluate the potential effects of genetically modified crops on soil ecosystems, with special attention to micro-organisms and microbially driven processes. In this chapter we will propose possible strategies for testing the effects of GM crops on soil microbes. Furthermore, we have also sought to point out gaps in knowledge and available technology thereby identifying areas of future research that would improve our ability to assess GM crop-induced effects.

5.2 Evaluation strategy

When assessing effects of GM crops on soil ecosystems we suggest to address the following points of consideration in order to gather information on the background of the GM crop to be introduced and the potential consequences of the introduction of the GM crop into the ecosystem.

Points of consideration include:

1. Environmental conditions of the system into which the GMP is introduced, like soil type, pH, water retention, vegetation, and the surrounding environment (e.g. forest, lakes, cultivation).

2. The microbial community present in the soil system. What are the dominant groups and what the vulnerable groups?

3. The nature and origin of the gene(s) introduced into the plant. When and in which organ(s) of the plant (e.g. roots, leaves) does this gene come to expression? Does the mode of action of the inserted genetic material act in relation to a very specific organism of the system (e.g. targeting the action of a specific plant pathogen) or does it confer a more general property that may affect a whole range of organisms?

4. The exposure of soil-borne micro-organisms to the GM product (depends on the life-span of the plants and persistence of plant products in the ecosystem and on the expression of the gene in the leaves or roots and whether the expression is constitutive or induced).

In many cases, it is possible to predict microbial groups and / or processes in particular soil systems that might be most susceptible to a given GM crop introduction. However, given our incomplete knowledge of soil-borne microbial communities and how plants induce changes in these communities, it is not possible to fully predict where GMP-induced effects will occur in the microbial community.

The answers to the above questions determine which soil micro-organisms or processes might be expected to be affected by the GM introduction in question (see 5.3). In some cases it may be that no specific micro-organisms or processes can be identified that are likely to be affected. Regardless of the predictions that can be made based upon knowledge of the soil system and the GMP being introduced, our understanding of potential effects is incomplete and unforeseen effects cannot be ruled out. Therefore, a number of parameters could be addressed in all cases (see 5.3), to guard against missing (significant) changes in sol ecosystems that may not be directly predicted from the answers of the above questions.

One possibility would therefore be to use a two-pronged approach to study of potential effects of GM crop introductions:

- Identification and assessment of specific microbial groups and processes that are most likely to be susceptible to a given GM crop introduction, taking into account the origin and function of the inserted gene and knowledge of the soil system where the plants are to be introduced.
- Implementation of general analyses that may detect effects outside the scope of predicted microbial groups and processes. Such analyses should evaluate the total microbial community structure and vulnerable species and processes, preferably by a combination of techniques. If such analyses reveal unexpected effects, these can then be analysed in greater detail.

During a workshop dedicated to discussion of GMP effects on soil ecosystems, which was held in January 2002 in Rhenen, The Netherlands (see appendices) and included scientists who are currently involved in this research area, this approach was accepted as the best procedure for field trials with GMPs given the present state-of-art. There was more discussion during the workshop about the approach after commercialisation of GMPs. It was agreed that long-term surveillance is desirable after GMPs have passed the field trial stage, but such surveillance would be scaled back in comparison with original testing, limited by feasibility issues and applied only to situations where long term effects are expected

It is impossible to test the full extent of effects of GM crop introduction, due to practical considerations of time, space and human resources. For (future) testing protocols it will be necessary to determine sampling times, frequency, and spatial scales. Although this is outside the scope of this report it has been discussed in the aforementioned workshop and some of the conclusions from these discussions can be found in appendix 2 enclosed in this report.

5.3 Suggested tests for GMP research

The choice of target groups and processes for the assessment of expected effects differs for each GMP. When specific organisms or processes are expected to be affected these (groups of) organisms and processes should be measured explicitly. This could be one or several of the indicator groups and functions that are mentioned in chapter 2.3, as these indicators are thought to be most indicative of GMP-induced effects. Workshop participants also agreed upon the indicator criteria and choices, as given in chapter 2.3. These indicators should be both experimentally accessible and represent vulnerable properties of the microbial system being examined. Vulnerability is defined here in terms of limited size, variation, and adaptive potential of the part of the microbial community under consideration. The methodology depends on the groups that are identified (see 5.2) as being most likely to be affected. Some groups can be easily measured with plate counts, whereas others will be better determined by nucleic acid techniques, such as those making use of group-specific PCR primers. Such a pre-selection of potentially affected groups or processes is designed to limit the scope of necessary research and focus efforts on the most relevant topics.

For instance, GMPs modified to express general anti-fungal genes to combat fungal pathogens, might also affect other non-target fungal populations, such as mycorrhizal fungi, wood-decaying basidiomycetes or antagonistic *Trichoderma* species. Likewise, genetic modifications affecting plant nitrogen metabolism might be expected to affect bacteria involved in nitrogen cycling such as ammonia-oxidising bacteria and nitrogen fixers. In contrast, some plant modifications, such as for greater resistance to frost, might not suggest any specific microbial responses, although unforeseen changes, for instance due to changes in plant litter quality might still occur, thus warranting at least a cursory examination of the total microbial community. Since the microbial communities associated with different plant species can be very different, the sensitive groups and processes in a soil ecosystem will depend not only on the origin and function of the inserted gene, but also on the plant species. For instance, methane-oxidisers might be an important and vulnerable group specifically in systems of rice cultivation.

The most suitable parameters to measure when specific effects of GMPs on soil microbial populations and functions are expected, based on the literature review (see 2.3) and the results of the workshop, are presented below. The main criteria for the selection of these groups and processes are relevance to the ecosystem, vulnerability, and/or low redundancy. As mentioned before, their use in the evaluation of GMP effects should be dependent on the type of plant, modification and knowledge about the cropping system, which will indicate the groups that are likely to be affected.

Relevant and accessible indicators:

- Nitrifying bacteria, particularly ammonium-oxidisers
- Mycorrhizal fungi
- Symbiotic N_2-fixing bacteria
- Wood-decaying fungi
- Antagonists (e.g. pseudomonads, *Trichoderma*)

For the detection of less predictable effects on microbial groups and processes, more general analyses could be applied. We propose the application of a polyphasic methodology to determine effects on the soil microbial community. The indicators and techniques we identified are described in more detail in chapters 2 and 3. These parameters were also discussed and accepted as relevant during the workshop, although the parameters were thought to differ in their relevance for specific soils and a distinction has to be made between field trials and commercial release monitoring.

- Broad-targeted nucleic acid-based fingerprinting methods for the detection of shifts in the microbial community: e.g. DGGE with primers specific for bacteria and fungi, or broad groups thereof
- Measurements of general enzyme activities: e.g. dehydrogenase and phosphatase
- Community structure with phospholipid fatty acid analysis (PLFA)
- Total plate counts e.g. with 1/10 tryptose soy agar (TSA)
- Measurement of general soil fungistasis
- Decomposition of recalcitrant organic compounds (e.g. litter bag, cotton strip methods)

When changes in these general parameters are detected, we propose that the affected species or groups be identified, so that further research can be conducted on these groups or species if relevant.

Since soils are highly variable and resilient environments, short-term changes and reversible effects are thought to be of less importance. Long-term changes however, are relevant and therefore it is proposed to apply long-term monitoring (at least over two to three growing seasons) for the identified indicators. This was discussed in detail during the workshop and the results of these discussions are summarised in appendix 2.

Other groups of micro-organisms that could be suitable as indicators are pathogens and methane-oxidisers. However, methane-oxidisers are probably relevant for only a narrow range of agricultural situations, for example rice cultivars, and therefore less relevant for the Dutch agricultural soils. Pathogens are important for agricultural soils, and changes in pathogen pressure can influence the growth of the GMPs and the successive plants. However, pathogens as a group are too diverse to be suitable for use as an indicator. Some specific pathogens could be suitable for use as indicators in specific situations, but before being of general use, additional research is necessary to identify and develop pathogens as indicators.

5.4 Knowledge gaps

Despite the recent development of methodologies and research, there are still significant knowledge gaps in our understanding of the soil ecosystems. New techniques have enabled us the look into the soil, and identify unculturable micro-organisms. We are now able to detect changes in community structure or specific species, but we often do not know the consequences of such changes. In order to be able to answer the 'so what?' question (something has changed, so what are the consequences?), we need to increase our understanding of different groups and link these groups to their functions and the functioning of the ecosystem. Most of the knowledge gaps identified here are general knowledge gaps that are not specific for risk assessment of GMPs. However, with more knowledge on these topics, it will be possible to improve risk assessment procedures.

- Diversity and functions of soil micro-organisms

Despite great advances in soil biology, there are still gaps in the knowledge concerning the diversity of soil organisms. At present, there is still a large proportion of unknown soil micro-organisms that are difficult to measure and study, since at present we do not know how to isolate these microbes from the ecosystem in which they live. Estimates are that only 1-10 % of the total number of bacteria present in soil can be isolated in culture using currently available methods. In fact, species estimates vary from millions to billions, but there are only about 6,000 described bacterial species. Future research should be directed towards accessing this unculturable fraction, and determining their function, activity and spatial heterogeneity. Tools for the study of the identity of the majority fraction of the microbial community in soil are partly available, but need to be expanded, in particular to be able to identify specific groups such as wood-decaying fungi, mycorrhizal fungi, and antibiotic-producing organisms. Novel tools for determining the functional characteristics of soil microbes might become available via the input of modern genomics-approaches, e.g. metagenome library analysis and DNA/RNA chips (see below). During the workshop discussions (appendix 2) linking microbial diversity to functions came out as one of the most important knowledge gaps. Since many micro-organisms are difficult to measure or study, it is difficult to assess their function and importance for the soil ecosystem. This is especially interesting for microbial groups that have been found to be very dominant in soils, but whose functions are still unknown. Also, many functional groups of micro-organisms are polyphyletic, complicating the use of PCR-based strategies. Clearly, the importance of strategies targeting key structural genes or key microbial processes will increase our future ability to study microbes on a functional group basis. Also, a number of recently introduced techniques allow one to couple microbial identification and activity in a single assay (Gray and Head, 2001). Such techniques hold great promise if they can be incorporated into the standard toolbox of the microbial ecologist. We lack both empirical and conceptual knowledge with regard to the spatial and temporal distribution of soil organisms in soil environments. Our inability to characterise and model soil environments at a scale relevant to microbial inhabitants has also inhibited progress in this area. Understanding the factors underlying selection and adaptation of species would improve our knowledge and predictive ability of the functioning of the soil ecosystem.

- Other soil organisms

In addition to direct effects on soil organisms, GMPs can also exert indirect effects via the food web. GMPs may indirectly affect species that feed on bacteria or fungi, by changing the quantity and quality of their food sources. In addition to potential effects of GM-modified products entering the food chain, predators may also be presented with a different proportion or number of bacterial and fungal species to consume. To assess the effects on these species, the persistence of the GM products in micro-organisms and the effects on the soil community as a whole could be established. Moreover, expansion of the test procedures to include soil animals is recommended.

- Natural variation and baseline

For the establishment of proper baselines it is necessary to determine the natural variation within a system. Diversity and activity of soil microbial communities will fluctuate during seasons and differ between years and field sites. Fluctuations due to environmental changes, such as climatic factors or agricultural practices, are often strong and several good examples were provided during the workshop. Considerable efforts should be made to enable the discrimination between natural variation and GMP effects. This is an absolute must for the establishment of a proper baseline. Other factors that should be taken into account when determining a baseline will be crop-cultivar and agricultural practice. The present state of our knowledge on these issues is often still too fragmentary to establish a solid baseline for the assessment of GMP-effects on soil ecosystems.

- Methodology
All techniques for measuring soil organisms have their drawbacks and limitations. Some techniques may just measure the culturable part, some may just indicate changes, but no details. Others may only be of use for very specific groups and all can be subject to biases and error. However, combining different techniques can compensate for some of these shortcomings. Development of new techniques and combining detailed measurements into overall pictures might be a solution for the future. For example micro-arrays might be a promising new method, but at present it is rather expensive and not yet suitable for use in routine testing. For some approaches, new possibilities are much easier to achieve, like the development and use of new sets of specific primers for PCR-based analyses. Improvement of primer sets and technology will be important for microbial groups that lack good systems for molecular evaluation (i.e. improved primers for groups such as the fungi, arbuscular mycorrhizal fungi, lignin-decomposing basidiomycetes, actinomycetes, nitrogen-fixing bacteria and various unculturable bacterial lineages). Also, the development of PCR-based systems to study families of enzyme-encoding genes, and not just rRNA genes, will increase our ability to target functional groups, which may be polyphyletic with respect to standard markers. Another important hurdle is the adaptation of research protocols, which are often costly and time-consuming in their present form, into tests that can be used in routine screening and monitoring procedures. The standardisation of test procedures is essential, as is the development of easy-to-use kits and protocols.

5.5 Postscript

The conclusions and suggestions mentioned in this chapter should not be viewed as absolute or definitive. They are based on the knowledge and techniques available at present. Many new techniques are being developed and improved and will be (more) suitable for routine testing in the (near) future. This report has sought to make an inventory of the knowledge to date and to identify the most suitable testing procedures that are available at present. Its aim has been provide an advisory report for regulations and setting up a standard for testing the effects of GMPs on soil ecosystems. This should be evaluated regularly in the future to keep the procedures up to date. Given the necessity to standardise protocols and tests, it is recommended that technique guidelines be established for all institutions charged with conducting tests on the effects of GMPs. To ensure accurate and objective testing, this might best be conducted under the charge of centralised (e.g. RIVM in the Netherlands).

GLOSSARY

Agrobacterium tumefaciens: bacterium, causes diseases in plants, and possesses the ability to build in own heritable information into plant-DNA. This bacterium is used for genetic modification of plants.
Alpha-amylase: bacterial (*Bacillus licheniformis*) gene.
Amino acid: an amphoteric organic acid containing the amino group NH_2
Antagonist: an organism exerting a damaging effect on another organism.
Barnase: a bacterial ribonuclease from *Bacillus amyloliquefaciens* and coupled with the gst1 promoter it leads to suicide of infected cells (with various types of pathogenic or symbiotic organisms) to prevent spreading of the pathogen.
Barstar: gene construct derived from *Bacillus amyloliquefaciens* and inhibits Barnase synthesis.
Biological diversity: the number of species and their relative abundance within a given area, including also the phenotypic and genetic diversity maintained within the population of these species.
Chitinase: degrade chitin, involved in natural defense of plants against fungal infection.
Classical (conventional) plant breeding: new cultivars are developed by crossing different genotypes of the same or closely related plant species.
Con A: (concanavalin A) a lectin with anti-feedant properties.
Conjugation: a form of gene transfer from one bacterial or yeast cell to another. The transfer is accomplished by products of genes located on a small circular DNA molecule called a plasmid. The process of conjugation is found in nature and also used in genetic modification.
Containment: the condition in which an organism or its genetic material is prevented from freely moving beyond a specific location.
Cry-proteins: Cry(stal) Bt toxins naturally produced in *Bacillus thuringiensis* during sporulation, present in the crystalline inclusions.
Deliberate release: any intentional introduction into the environment of a GMO or a combination of GMOs without provisions for containment such as physical barriers together with chemical and/or biological barriers used to limit their contact with the general population and the environment.
Deoxyribonucleic acid (DNA): genetic material that defines all heritable characteristics of an organism. DNA as two helical molecular backbones composed of sugar molecules (deoxyribose) joined by phosphate molecules. Four bases (Cytosine, Adenine, Thymine, Guanine) join the two helical backbones across the middle of the molecule. Some viruses have ribonucleic acid (RNA) as their hereditary material.
Ecosystem function: The biological, chemical or physical processes occurring in an ecosystem.
Enzyme: proteins that initiate chemical processes.
Eukaryote: contains a discrete, membrane bound nucleus within the cell.
Field trial: an experiment or trial that is performed outdoors or in an uncontained environment.
Food web: the total set of interrelated trophic interactions between organisms in an ecosystem.
Fungicide: a chemical which kills or harms a fungus.
Genetically modified organism: an organism, with the exception of human beings, of which the genetic material has been altered in a way that does not occur naturally through reproduction and/or natural recombination.
Geocaulosphere: tuber surface.
Glyphosate: a herbicide.
GNA: (*Galanthus nivalis* agglutinin) a lectin with anti-feedant properties.
Gram positive: holding the purple dye when stained by Gram's stain.
Gram negative: not holding the purple dye when stained by Gram's stain.
Gram stain: a method for the differential staining of bacteria by treatment with a watery solution of iodine and the iodide of potassium after staining with a triphenylmethane dye (as crystal violet).
Gus genes: genes encoding glucuronidase.
Hazard: a potentially adverse outcome of an event or activity.
Herbicide: a chemical, which kills or harms plants.
Host: an organism harbouring another organism.

Host range: all the possible organisms capable of harbouring a specific organism.
Indicator: variable that may be used as a surrogate for either final or intermediate variables.
Indigenous: originating and growing or living in a particular geographic region or locale.
Indirect interactions: effects of one organism on (an)other organism(s) in the accessible ecosystem that occur through mechanisms involving abiotic or biotic factors.
KP4: (killing-protein 4) antifungal protein.
Lignin: three-dimensional heteropolymer, it has essential structural roles in higher plants and makes a significant contribution to the carbon input to soils.
Marker gene: a gene that facilitates the identification of organisms that have taken up recombinant DNA molecules.
Micro-organism: all groups of organisms of a size < 100 μm.
mRNA: messenger RNA.
Nitrogen fixation: the ability of some bacteria to remove elemental nitrogen from the atmosphere or water and convert it into nitrate, the form of nitrogen that is an essential nutrient for most forms of life.
Non-coding DNA sequences: some of these are DNA sequences that serve as spacer regions (introns) between sequences that are parts (exons) of a complete protein coding sequence; they are spliced out of the message (mRNA) that provide s a cell with complete instructions for assembling the protein. Other non-coding sequences come in a variety of longer and shorter repetitive forms; no cellular function is known for any of them.
Non-target organisms: organisms for which a specific control method has not been developed, but that may be influenced by this method directly or indirectly
Opines: bacterial growth substrates.
Organism: any biological entity capable of replication or of transferring genetic material.
Pathogen: a specific causative agent of disease.
PCR: polymerase chain reaction.
Persistence: the ability to continue through time.
Pesticide: a chemical, which kills or harms a pest, also used as a general term to include all biocides used against pathogens and pests. May be divided according to groups of organisms it is toxic to: e.g. insecticide, fungicide, etc.
Phytophagous: feeding on plant material.
Plasmid: a small circular molecule of DNA that may contain a variety of genes. Found in bacteria although many artificial ones have been made. Some are capable of conducting their own transmission from one bacterial cell to another, and may also cause other plasmids that are not self-transmissible to also move between cells.
Prokaryote: characterised by the absence of a nucleus, nuclear membrane an other membrane-bound organelles.
Protein: consists of amino acids and helps fulfil functions in a living cell.
Proteinase inhibitor I: a protein with insecticidal activity.
Reporter gene: a class of marker genes where the product reacts with a chemical to produce a detectable coloured compound, fluoresces, or emits light and enables a tagged trait to be identified.
Resilience: the ability (of an ecosystem) to recover to a previous state or condition after a major change or disturbance.
Resistance: the ability of either organisms or enzymes to counter the effects of toxic materials or disease or harmful environmental agents.
Rhizosphere: the volume of soil around a root, under the influence of that root, in which microbial activity is increased.
Risk: the probability of a specific hazard (or set of hazards) occurring.
Risk assessment: a process of evaluating risks involving hazard identification, estimating likelihood of its occurrence and magnitude of the consequences.
RNA: ribonucleic acid, chain of nucleotides typically produced using DNA as template with ribose as sugar. Four bases Adenine, Cytosine, Guanine and Uracil.
rRNA: ribosomal RNA.
Saprophagous: feeding on dead organic material.
Suppressive soil: a soil in which disease is reduced or absent, even though the pathogen is present or introduced and a susceptible host is grown.
Symbionts: two or more individuals that interact closely, to the benefit of one or more of the participants.
T4-lysozyme: enzyme, enhances bacterial resistance.

Target organisms: organisms for which a specific control method has been developed.
Transduction: a form of gene transfer among bacteria (found in nature and also used in genetic modification). The transfer is accomplished by a bacterial virus called a bacteriophage (or just phage). After the bacteriophage has replicated (copied itself) numerous times within its host bacterial cell, it forms protein wrapped viral particles containing its own DNA and often some parts of the host and donate the chromosomal DNA sequences to the new host, often changing the genetic makeup of the new host.
Transformation: a form of gene transfer among, for example bacteria; the process is found in nature and also used in genetic modification. During transformation, one bacterial cell copies its DNA and releases the copy into the environment. Another cell takes the free DNA and with some frequency exchanges it for the same region of DNA in its own chromosome. If the process brings in different (variant) forms of the genes, the receiving cel ids said to be transformed.
Wild type: the organism as growing in the natural environment, i.e. before laboratory culture, selection, genetic modification, etc.

ACRONYMS:

ARDRA: amplified ribosomal DNA restriction analysis
ATP: adenosine triphosphate
Bt: *Bacillus thuringiensis*
CLPP: community level physiological profiling
DGGE: denaturing gradient gel electrophoresis
DNA: deoxyribonucleic acid
ERIC-PCR: enterobacterial repetitive intergenic consensus sequence PCR
FAME: fatty acid methyl ester
FISH: fluorescence in situ hybridisation
GM: genetically modified
GMO: genetically modified organism
GMP: genetically modified pant
HGT: horizontal gene transfer
LH-PCR: length heterogeneity PCR
MPN: most probable number
PCR: polymerase chain reaction
PICT: pollution-induced community tolerance
PLFA: phospholipid fatty acid
RAPD: random amplified polymorphic DNA
RISA: rDNA intergenic spacer analysis
RNA: ribonucleic acid
SIR: substrate induced respiration assay
SOM: soil organic matter
SSCP: single-strand conformation polymorphism
TGGE: temperature gradient gel electrophoresis
T-RFLP: terminal restriction fragment length polymorphism
VROM: ministry of housing, spatial planning and the environment (NL)

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Legislation

directive 90/219/EEC
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APPENDIX 1. WORKSHOP PROGRAMME AND ABSTRACTS

Workshop title: The effects of genetically modified plants on soil ecosystems

Date: Workshop, 20 – 22 January 2002
Place: Hotel 't Paviljoen, Rhenen, The Netherlands

Organisers: M. Bruinsma, J.A. van Veen, G.A. Kowalchuk

PROGRAMME

The aims of the workshop were to exchange knowledge, the state-of-the-art in this field of research, and to discuss different topics from the report, to help improve the report. The workshop therefore started with presentations on Sunday and Monday morning about the research of the participants. The titles of the talks and several abstracts are listed below. On Monday afternoon the workshop continued with discussion sessions. In 3 groups 4 different topics were discussed, the topics and results of these discussions can be found in appendix 2. The workshop ended on Tuesday with the presentation and discussion of the results of each group in a plenary discussion session.

Presentation titles

J.A. van Veen	Workshop introduction
M. O'Callaghan	Environmental impacts research in New Zealand
P.R. Hirsch	GM plants and soil microbes: detecting changes in microbial diversity
K. Smalla	Microbial communities affected by transgenic crops?
C.C. Tebbe	Effect of transgenic plants on microbial communities in rhizospheres
Y. Dessaux	Genetically modified plants producing opines and their associated bacterial microflora
E.M.H. Wellington	Horizontal gene transfer amongst soil bacteria
M. Viebahn	Field release of genetically modified *Pseudomonas putida* WCS358r to study the effects on the indigenous soil microflora
E. Smit	The impact of genetically modified biocontrol bacteria on important non-target soil micro-organisms
K. Dunfield	Plant genetics and soil type influence the composition of the microbial community associated with the roots of genetically modified canola (oilseed rape; *Brassica* sp.) grown in Saskatchewan, Canada
B.S. Griffiths	Lessons from a study of the effects of lectin-producing GM potatoes on non-target soil organisms and processes
G. Berg	Effects of transgenic produced T4 lysozyme on potato-associated fungi with antagonistic properties
T.M. Timms-Wilson	Fate and impact of GM-BCA *Pseudomonas fluorescens* on microbial communities

ABSTRACTS

Environmental Impacts Research in New Zealand

M. O'Callaghan

Research on environmental impacts of genetically modified plants in New Zealand has been restricted by a voluntary moratorium on field trials while a year-long Royal Commission into Genetic Engineering was held. The Royal Commission has recently reported back to the New Zealand Government, who have subsequently stated that some field trials will be permitted, but under very tight restrictions. The Royal Commission has called for more research into the effects of genetically modified plants on the environment, in particular noting that there was lack of information on impacts on soil ecosystems. It was possible during the moratorium, to conduct research on non-target effects of GMPs in New Zealand under contained conditions. We have been examining the effect of avidin-expressing tobacco plants on organisms important in decomposition processes in soil (micro-organisms, nematodes and earthworms) and developing methods to monitor the persistence of transgenic DNA in soil. Avidin is a biotin-binding protein, which could impact on organisms other than the target insects. Although this work is in the early stages, the results have not indicated many significant changes in soil biota under transgenic as opposed to non-transgenic plants. Transient changes were seen in the composition of the nematode communities in one soil type. In one of the few field trials that will be conducted in New Zealand this season, we are also monitoring the impact of potatoes expressing the toxin magainin, which protect tubers from the causal agent of soft rot, *Erwinia carotovora*. The magainin is excreted from the plant cells, and could therefore impact on plant associated rhizosphere bacteria, as well as other soil organisms, in particular protozoa and nematodes. Bacterial populations will be monitored using a range of techniques (viable counts, BIOLOG, DGGE) and nematode populations will be enumerated and classified to trophic levels.

Effect of transgenic plants on microbial communities in rhizospheres

C.C. Tebbe

In a recently finished study tried to detect if herbicide resistant maize or sugar beets (GMO) harbour different microbial communities in their rhizosphere than their non-engineered counter-parts. For community analysis we used PCR-SSCP targeting the 16S rRNA genes of bacteria. The plants were cultivated on the same field in crop rotation systems as they are used in common agricultural practice. Results of this study will be presented and it should be discussed if and at what level of resolution monitoring of microbial communities is relevant for risk assessments of GMO.

Genetically modified (GM) plants producing opines and their associated bacterial microflora

Y. Dessaux

Transgenic *Lotus* plants producing one or more compounds termed opines have been generated and installed in a clay-rich soil from the Paris region. The evolution of microbial populations colonizing the root system of the plants was monitored. Essentially, total cell densities were identical whatever the analyzed rhizosphere ("untransformed or transformed"), as were the densities of several easily screenable, microbial groups. However, the density of the microbial community degrading the opines was 30 to 1000 higher at the root system of the plants producing opines (opine bias) than it was at the root system of the untransformed plants. Fine analysis of the structure of the populations of the screened groups also indicates that the evaluation of the consequence of the growth of a GM plant highly depends upon the identification of the “target” population(s). Additionally, the consequences of the growth of the GM plants are transgene-specific. Another series of experiments demonstrated that the opine bias is independent on the nature of the opines, the plant species, and the soil type. Last, rotation experiments involving the replacement of transgenic plants producing opines by their untransformed parents revealed that the duration of the opine bias also depends upon the relevant transgene. On a more fundamental point of view, these experiments also demonstrate that the structure of the microbial microflora in the rhizosphere may be determined by factors of plant origin that may not be produced anymore at the time of the analysis.

Horizontal gene transfer amongst soil bacteria

E.M.H. Wellington

Horizontal gene transfer plays a vital role in the evolution of diverse metabolic pathways for the biosynthesis of secondary metabolites. The functions of such products in soil is unclear but for some, such as siderophores, their role in iron acquisition is clear. Such pathways are capable of biosynthesizing molecules with biological activity capable of intereacting with plants, are involved in cell signalling or its interference and antagonism against a wide range of organisms. Clearly although not essential for growth such metabolites play a critical role in competition and survival in nature. How such pathways have evolved and continue to produce diverse metabolites is of interest because it sheds light on the extent and nature of gene transfer between bacteria in soil both within and between genera.

The impact of genetically biocontrol bacteria on important non-target soil micro-organisms

E. Smit, P. Leeflang, K. Wernars, F. de Souza, R. Landeweert, C. Veenman, M. Viebahn

Bacteria with natural antagonistic properties can be introduced into soil to reduce the occurrence of phytopathogenic fungi. Such bacteria have been genetically modified with genes coding for compounds with antifungal activity to increase their protective effect. However, before these genetically modified micro-organisms (GMM’s) can be used it is imperative to know if they will also affect important non-target micro-organisms in soil. In this study the environmental impact of *P. fluorescens* and *P. putida* strains modified with a gene cluster coding for phenazine and a gene cluster coding for phloroglucinol production was determined. In particular the effects on important fungal groups such as *Fusarium*, the ecto-mycorrhizae and the arbuscular mycorrhizae were assessed.

A molecular screening procedure for 18RDNA sequences from *Fusarium* was applied on wheat rhizosphere samples from a field trial with genetically modified *P. putida*. Results indicated that both the wild type strain and the phenazine producing GMM caused a shift in *Fusarium* species composition. *In vitro* experiments with these *P. putida* strains revealed that the phenazine producing GMM dramatically reduced the growth number of ecto-mycorrhizae on plates. However, quantitative real time PCR data obtained from a microcosm study with the ecto-mycorrhizal species *Paxillus involtus* showed that there was no effect of the *P. putida* or the GMM on its hyphal development in soil and rhizosphere. Microcosm studies were also done with the *P. putida* strains and two arbuscular mycorrhizae, *Glomus mossae* and *Glomus intraradices*. Analysis of the percentage of root infection on maize plants by both species showed that there was no effect of the *P. putida* wild type and the GMM.

Field Release of genetically modified *Pseudomonas putida* WCS358r to study the effects on the indigenous soil microflora

M. Viebahn

The aim of the field study was to determine whether introducing genetically modified microorganisms (GMMs) can cause shifts in the indigenous microflora. *Pseudomonas putida* WCS358r was improved in its biocontrol properties by insertion of the phenazine (phz) or phloroglucinol (phl) biosynthetic gene loci of *P. fluorescens* strain 2-79 and Q2-87, respectively. The GMMs were introduced in the soil as a coating on wheat seed and effects of the GMMs on the natural microflora of wheat roots were compared to effects of the parental strain and an untreated control. During the growing season populations of GMMs in single and combination treatment were lower than those of WCS358r. Apparently, the GMMs had established less well than the parental strain during the first days of the field trial. Phz and phl genes remained stably integrated in the chromosome of the GMMs. Fungal or bacterial populations, determined by plate counts, were not significantly affected. In 1999 the DAPG-producing GMMs caused a transitory shift on fungal and bacterial microflora as determined by ARDRA. This effect was not detected in 2000, probably due to a higher variability in fungal and bacterial populations. In 2000 all bacteria had a positive effect on plant growth, supposedly due to suppression of deleterious microorganisms.

Plant genetics and soil type influence the composition of the microbial community associated with the roots of genetically modified canola (oilseed rape; *Brassica* sp.) grown in Saskatchewan, Canada

K.E. Dunfield

We conducted a three-year field study to assess the effects of herbicide-tolerant genetically modified canola (oilseed rape, *Brassica* sp.) on microbial biodiversity. Four genetically modified and four conventional canola varieties were grown at six locations across Saskatchewan, Canada. The rhizosphere and root-associated microbial communities were characterized throughout the field season via community level physiological profiles (CLPP), fatty acid methyl ester analysis (FAME) and terminal amplified ribosomal DNA restriction analysis (T-ARDRA). Generalizations about the effect of genetically modified plants on the microbial community were not possible since there were significant interactions between plant varieties and field sites. Soil type or crop history at these sites may have affected the microbial community. One variety of genetically modified canola, Quest, consistently influenced the microbial community. However, in most cases Quest seemed to be unique and supported microbial communities quite different from the three other genetically modified and four conventional canola varieties we tested. Our analysis throughout the field season indicated that at certain sites the canola variety influenced the composition of the microbial community, but these alterations were temporary and dependent upon the presence of the plants. Although genetically modified canola plants supported different microbial communities than their conventional counterparts at some field sites, these differences did not persist beyond the field season.

Lessons from a study of the effects of lectin-producing GM potatoes on non-target soil organisms and processes

B.S. Griffiths

Jepson et al (1994, Molecular Ecology 3, 45 - 89) listed 18 processes, 9 microbial species and 42 faunal species used in environmental testing, yet even these were not sufficient for risk assessment and ignored the role of the community. It would be useful to limit tests to organisms likely to contact the transgenic product and to use a tiered approach. Genetic engineers can produce many lines containing the gene of interest and it is relatively easy to test their effectiveness in pest control. However, the logistics, and effort involved, of environmental testing mean it should only be used for lines that are ready for use. Populations and processes should be monitored and decomposition is a key process to study. Other factors that should be considered are temporal samples over a growing season and effects on subsequent crop growth. Control treatments for the plant lines and systems with altered chemical inputs need to be carefully chosen.

Effects of transgenic produced T4 lysozyme on potato-associated fungi with antagonistic properties

G. Berg

Transgenic T4 lysozyme potato plants show enhanced resistance against the plant pathogenic bacterium *Erwinia carotovora* ssp. *atroseptica* [1] but T4 lysozyme was also found to be active against plant pathogenic fungi [2]. During a two-year period (1999/2000) the influence of T4 lysozyme on fungi in the rhizo- and geocaulosphere was investigated under field conditions. Three T4 lysozyme expressing lines (DL10, DL11, DL12), a transgenic control (DC1) and a non-transgenic line (DESI) have been analyzed at different stages of plant development (young plants, flowering plants and senescent plants). To assess potential effects of T4 lysozyme on plant-associated fungi, the abundances of colony forming units, the percentage of antagonistic fungi and their diversity were investigated. A total of 2760 fungal isolates were screened for their potential to antagonize *Verticillium dahliae* in vitro. The percentage of antifungal isolates in the rhizosphere was 24 on average. In the geocaulosphere only 13.6 % of the screened isolates were antagonistic towards *V. dahliae*. Antagonistic isolates were characterized by molecular fingerprinting using BOX-PCR. Computer-based analysis of the fingerprints showed that the majority of groups were heterogenous and contained isolates from all plant lines. Antagonistic isolates were identified by physiological and biochemical methods. Till now 29 species of antagonistic fungi could be identified. The most abundant antifungal genera were *Penicillium* spp., *Trichoderma* spp., *Fusarium* spp. and *Paecilomyces* spp.. The results suggest that transgenic plants producing T4 lysozyme did not affect the abundances, the antagonistic properties and the diversity of root- and tuber-associated fungi.

[1] Düring *et al*. (1993) Plant J. 3, 587-598.
[2] Düring *et al*. (1999) FEBS Lett. 449 (2-3), 93-100.

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APPENDIX 2. DISCUSSION RESULTS OF THE WORKSHOP

During the workshop on the effects of genetically modified plants on soil ecosystems, held in Rhenen, The Netherlands, from January 20th until January 22nd 2002, group discussions were a major part of the programme. Four different topics (indicators, general analyses, testing procedures and future research) were discussed in 3 groups of 7 participants each. This appendix contains the summaries of the results of these discussion sessions of the workshop, including the plenary discussions, in which each group presented results on a per topic basis.

TOPIC 1: INDICATORS

Questions posed to the discussion groups

What makes a good indicator with respect to measure the effects of genetically modified plants on soil ecosystems? In our report we have chosen vulnerability and redundancy as the main criteria. Are these indeed good criteria or are other criteria more important? After setting the criteria, we would like you to discuss which indicators can be identified based on these criteria. Which groups are suitable as indicators and how specific would you define them (e.g. species or genus level) and should the choice of indicators be based on diversity, activity, number or a combination of factors? And how well do we have to understand an indicator to use it?

Discussion results

Criteria for indicators:

- Vulnerability and sensitivity of function or group
- Relevance of species and processes for the ecosystem
- Non-redundant
- Clean and efficient to do
- Should look to the future
- Indicators should be pathogenic as well as beneficial organisms (GMPs should not increase pathogens, neither should they harm beneficial organisms)

Examples of possible indicators:

Rhizobia (symbiotic N_2-fixing bacteria)
- sensitive indicator of e.g. metal pollution
- easily detected (genetically accessible)
- important for certain plants and modifications e.g. legumes

Nitrifying bacteria
- essential for N-cycling in soil
- not plant-associated (bulk soil indicator), autotrophic
- ammonium-oxidisers (genetically accessible)
- Phylogenetically not redundant

AM and other mycorrhizal fungi
- depending on system
- huge impact on plant growth
- genetically accessible
- Negative: lack of knowledge on physiology

Wood-decaying fungi:
- Ratio between microbial carbon and total carbon as indicator of fungal dominance
- Play essential role in decomposition processes (various assays)
- Specific activities (i.e. fungal peroxidases)

Pseudomonads:
- Easy to look at due to culture methods and genetics
- Difficult to attribute a particular function and ecological significance
- Pseudomonads diversity: important for disease suppression

Antagonists:
- Difficult to assess in one assay
- Might be of use when specific antagonist is known

Methane oxidisers:
- Critical in some environments (rice cultivation)
- Ubiquitously present (relevance not known)

General microbial diversity

Plant health – quality, quantity of end product, crop yield

Endophytes (rhizosphere and rhizoplane)

Soil fauna

Soil suppressiveness

Indicators issues:

Indicators should be open for extension of knowledge i.e. functions and taxonomy of indicators

Should indicators be associated with the plants or be present in the bulk soil?
- Bulk soil might not be affected most of the time, however, if it is affected it is a major issue; preferably indicators associated with plants as well as bulk soil indicators

Should indicators be associated with the GMPs or with the successive crop?

The build up of pathogenic potential might be bigger due to GM crop and resistance building. This will influence the successive crop: should this be measured directly or via the successive crop?

Endophytes may reflect plant health of the GM crop, is this relevant?

Plant health as an indicator might not be enough, it does not provide any information about geochemical processes in soil

If an indicator changes, 'so what?' or 'what now?' What should be the consequences of a detected change in an indicator? When is a change a problem? Are the effects reversible?

TOPIC 2: GENERAL ANALYSES

Questions posed to the discussion groups

In our report we have made the distinction between testing for expected effects, based on the origin and function of the inserted gene and the soil system into which the plant is introduced, and the testing for unexpected effects, based on our incomplete knowledge on soil ecosystems (with for example molecular fingerprinting methods or phospholipid fatty acid analyses). We would like you to discuss whether you think that testing for these 'unexpected' effects is necessary or not and which tests would be suitable for this purpose. If, based on the inserted gene and the soil system, no specific effects are to be expected on soil micro-organisms and soil processes would it still be necessary to test either the indicators as identified in discussion 1 or to perform general tests?

Discussion results

Necessity of testing for unexpected effects:

Even if, on the basis of evaluations of putative effects of the insertion, no impact on the soil is expected, we still recommend testing/monitoring of impacts

One should distinguish 2 phases:
- Small-scale testing (indicators topic 1)
- Post-release testing (plant health main criterium)

How to test for these effects:

When general testing, which kind of experiments, which scale?
Case by case or for all kind of releases?

Tests that assess the indicators (as discussed in topic 1) were felt to be suitable in combination with general surveillance

General surveillance:

Community level methods:
- Structural (DGGE, SSCP, T-RFLP, FAME, PLFA)
- Functional: MUF substrate; MPN with high molecular substrates, BIOLOG; qCO_2; respiration of unit of biomass; SIR

One group made a pyramid, illustrating the level of detail and feasibility for different analyses (fig 1).

Other comments:

Commercialisation:
- only for particular cases more than general surveillance is required (only for plants that have traits that are likely to affect the microbial soil community)

Permits granted in the past were mainly based on plate counts, now more comprehensive methodologies have become available, should the permits be re-evaluated? Or is this too late if the crop has already been commercially released and has shown no obvious deleterious effects? Is monitoring with new techniques necessary?

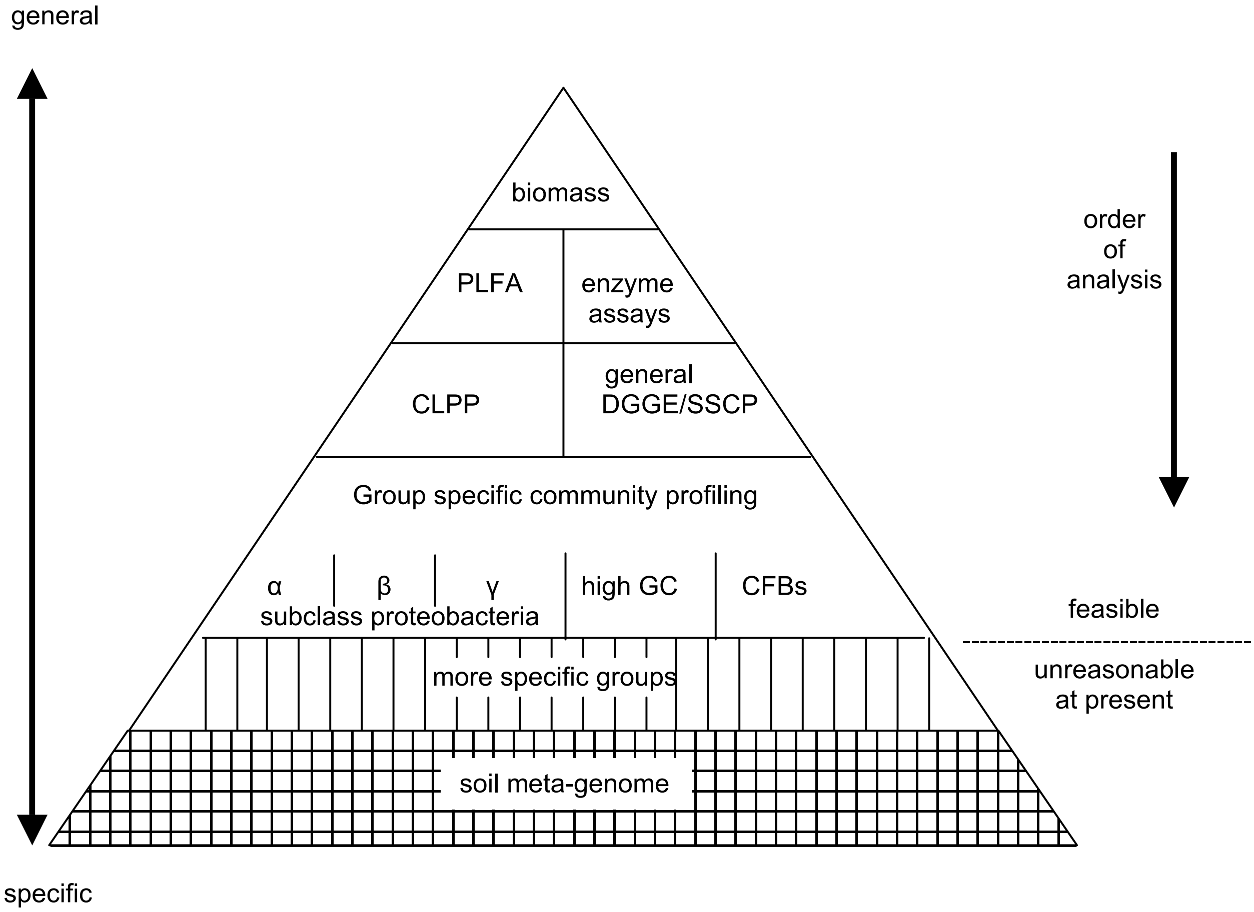

Fig 1. Analysis pyramid illustrating the level of detail and feasibility for different analyses, organised from general to specific tests (Kowalchuk).

TOPIC 3: TESTING

Questions posed to the discussion groups

What are the best testing and monitoring procedures? How do you establish a baseline and incorporate proper controls? On what scale should experiments be conducted both in terms of space (pot, glasshouse, field) and time (days, seasons, years)? And what are the extrapolation possibilities of contained use (pot, glasshouse) to field scale usage? How far do the plants influence the soil, is this limited to the rhizosphere or do they influence the entire field? How should sampling be conducted (number, frequency, place)? At what point should permits be granted? And what should be monitored after granting a permit? How likely are irreversible effects? And are reversible effects acceptable and if so, on what time scale?

Discussion results

Baseline:

Purpose: to detect normal variation in the ecosystem

Suggestions for baseline and controls:

- Parental cultivar of GM crop if available
- Different cultivars to assess natural variation or accepted perturbation
- Many GMPs imply changes in cropping practices/management (e.g. application of herbicides) document both with and without changed management
- Should include the range of soil types that is suitable for the growing crop
- Not only the comparison parental with GM but also different cultivars of the same plant species, and soil types
- Building/introducing a farm (change of land use) will probably have a bigger impact on the soil ecosystem than the differences between different crops or cultivars

2 possible situations:

- New GM variety commonly grown plant
  Baseline: compare substantial equivalents, parental line + management system
- Totally new plant with GM trait (e.g. frost-resistant citrus crops)
  Baseline: some disagreement on this issue: largest acceptable perturbation in the agro-ecosystem e.g. tillage, crop rotation, flooding or look at the most similar cultivation system or the system in native location

Sampling scale:

- First in small scale, but should move faster to field scale - less impedance to field-scale trials
- Emphasis based on the rhizosphere: interface with GMP, but impact on bulk is important as well (this years GM rhizosphere soil will be next years bulk soil and vice versa)
- Pot experiments do have value, but field trials are essential

Extrapolation:

How much can we extrapolate from pot experiments?
- No clear cut answer; pot experiments have their value for testing the methodology; correct answers only from the field

Benefits of a pot experiment
- Needed to assess methodology

- To look at the baseline expression of a trait
- Prerequisite to design a field experiment
- The difference between pot experiments and small-scale field testing is huge, but for a given soil the difference between small-scale field testing and large-scale field testing is not such a big difference

Disadvantage:
- Not predictive for field experiment

Sampling:

- Seasonal comparisons needed - do more than one year
- All groups: testing > 1 year, because of the variation between years (weather influences soil ecosystems), at least 2 or 3 growing seasons/years
- Temporary effects should be considered acceptable - recovery noted, intermediate effect not important, long-term effects matter
- Time course: following plant development, min. 3 sampling times per life cycle
- Need 1 pot experiment (1 life cycle, dependent on situation) and at least 2 complete life cycles in the field
- Number of samples will depend on variation within the system, need enough replicates to see statistical differences
- Number of locations is dependent on purpose of the product
- Effects in successor crops should be assessed
- Focus of risk assessment of potential effects on soil ecosystems should be in the development phase (not the early research phase)
- Select (long-term) monitoring methods based on short-term effects
- Monitoring after market release should be integrated in more general survey/monitoring activities

(Ir)reversible effects:

What do we mean by recovered?
- Is that recovered function, original species, original community and functions? (community/species are more vulnerable than functions)
- The rhizosphere is very dynamic, even dominant groups can appear and disappear

Reversible effects:
- Could become stronger if you plant the same crop again and again (season after season)

Irreversible effects:
- Breeding for resistance in the ecosystem i.e. Bt

Public perception:

Nothing should change, but rhizosphere communities are dynamic: effect of the season, the cropping systems, field site

TOPIC 4: FUTURE RESEARCH

Questions posed to the discussion groups

Where do we go from here? Is it necessary to co-ordinate research on the effects of genetically modified plants (and who would co-ordinate this)? What are the main knowledge gaps at the moment? And what kinds of experiments are necessary to fill the gaps?

Discussion results

Knowledge gaps:

- Link diversity with function (function to diversity)
- Access the 95% non-culturable fraction (activity, function, spatial heterogeneity)
- Determine functional networks/interactions (groups of bacteria)
- Understand factors underlying species distribution and composition (selection in natural bacterial communities)
- Understanding of the role and importance of Archae in terrestrial ecosystems
- Establish baselines (dynamics, compare conventional variety other established agricultural practices), long-term studies to establish extent of natural variation seen under various agricultural practices
- Clarify the effects of introduction of foreign genetic material into the bacterial gene pool adaptive potential and evolutionary trajectories of bacterial population
- How complex are equilibria between communities, what will shape communities?
- Communication within communities, quorum sensing
- What are the important functions for soil?
- What are the criteria defining a healthy soil? (Soil should sustain plant growth, should retain efficiently water and allow growth of various and diverse plant species)
- Effect assessment vs. risk assessment (what is a risk?)
- What is the diversity and impact of endophytic micro-organisms?
- How do you define reversibility?
- Spatial heterogeneity, techniques to differentiate microhabitats

Methods:

- Use of micro-array technology (for rRNA markers as well as other structural genes)
- Linkage between function and taxonomy
- Sets of primers for key functional genes e.g. chi (set (6) of primers will cover chitinase)
- Target diversity in fungi, protozoans, archeae

Research topics:

- Some research is directly necessary/suitable for risk assessment (need-to-know)
- Some research is for deeper insight in soil ecosystems (nice-to-know? Or necessary for understanding of the system and therefore for making decisions about what is nice-to-know and what is need-to-know?)

Objectives:

- Urgent need for fast molecular approach to link taxonomic signature (phylogenetic position) to key function
- Role of groups in rhizosphere
- Extent of functional redundancy
- Do functional groups at community level exist beyond known examples?
- Add to knowledge of impacts of community variation

Co-ordination of research:

- Trans-national research co-ordination does make sense, commercialisation and laws might be EU wide
- There are differences between EU countries, in climate and conditions, but some crops are grown from Mediterranean to the north
- EU co-ordinated research could bring different research fields together (multi-disciplinary research)
- Networks of excellence, working not as competitors, but as complimentary groups
- Globally or locally?