Methods to study potential effects of genetically modified plants on soil microbial diversity and soil functioning

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Samenvatting

Genetisch gemodificeerde planten (GMP) vormen wereldwijd een steeds groter aandeel van het landbouwareaal. Dat brengt kansen met zich mee voor onder andere het milieu en de voedselvoorziening van de wereld, maar vraagt ook om een solide risicoanalyse. GMP kunnen zowel boven- als ondergronds een invloed hebben op hun directe omgeving, en de gevolgen daarvan moeten goed worden onderzocht.

Dit rapport richt zich op het leven ondergronds. De organismen die de bodem bevolken spelen een essentiële rol in de gezondheid van de bodem en daarmee in het leven op deze aarde. Ze zijn verantwoordelijk voor processen zoals grond- en waterzuivering en recycling en levering van nutriënten aan planten. Ze spelen een belangrijke rol in alle elementencycli alsmede bij het op peil houden van bodemvruchtbaarheid, bodemstructuur en ziektewering. In dit rapport wordt ingezoomd op de methoden die gebruikt worden om de mogelijke effecten van GMP's op de diversiteit en populatiesamenstelling van de bodemmicrobiota, alsmede het bodemfunctioneren te bestuderen.

Het rapport begint, na de 'terms of reference' (sectie I), met een voorbeeld van een onderzoek naar het effect van GMP's op het microbiële leven in de bodem (sectie II). Het beschrijft de resultaten, om vervolgens de vragen en kanttekeningen te beschrijven die een risico-analysator (Engels: "risk assessor") bij het onderzoek kan plaatsen. Dit om de taak van de risk assessor, een van de doelgroepen van dit rapport, te ondersteunen. Daarna worden in sectie III de stappen beschreven die voorafgaan aan het grootste deel van de beschreven analysetechnieken die op directe DNA extractie berusten, t.w. bodembemonstering, DNA extractie en PCR amplificatie. In sectie IV worden vervolgens de meest gebruikte technieken voor de analyse van de bodemmicrobiota en haar functioneren beschreven. In deze sectie wordt ook geanalyseerd wat elk van de technieken laat zien, hoe de verkregen gegevens geïnterpreteerd kunnen worden, hoe specifiek en gevoelig de techniek is en wat de claims zijn van de onderzoekers die de desbetreffende techniek gebruiken.

De technische ontwikkelingen in de analyse van de microbiële gemeenschap van de bodem gaan de laatste jaren razendsnel. Dit rapport begint met de beschrijving van traditionele technieken (chloroformfumigatie, plaattellingen), om via de huidige - in het onderzoek routinematig toegepaste - technieken ('DNA-based fingerprinting, clone libraries') naar zeer geavanceerde nieuwe technieken ('DNA microarrays, pyrosequencing') te gaan, die pas in enkele recente studies gebruikt zijn. De vooruitgang die de nieuwe technieken met zich meebrengen is meerledig. Zo geven deze een steeds vollediger beeld van de diversiteit en populatiesamenstelling van de bodemmicrobiota en zijn er steeds minder vertekeningen ('biases') die de resultaten onbetrouwbaar maken. Tevens worden de technieken steeds specifieker, gevoeliger en sneller.

Ondanks deze grote recente vooruitgang, blijven de effecten van de met voornoemde technieken waargenomen veranderingen in de bodemmicrobiota op de bodemgezondheid en het bodemfunctioneren grotendeels onbekend. Met de technieken kunnen op verschillende niveaus veranderingen in de samenstelling van de microbiota in de bodem worden vastgesteld, variërend van gering tot groot. Maar wat deze veranderingen betekenen voor het bodemfunctioneren (alsmede voor een oordeel aangaande de bodemgezondheid) blijft onduidelijk. Dit vooral omdat (1) veel processen 'redundant' zijn, d.w.z. in meerdere organismen voorkomen, en (2) er nog steeds maar een klein deel van de bodemmicrobiota inzichtelijk is gemaakt en onderzocht. Het is derhalve van veel bodemprocessen nog onvoldoende bekend welke en hoeveel soorten bacteriën ervoor verantwoordelijk zijn. Daarom moet bij de interpretatie van alle onderzoeksresultaten altijd de grootst mogelijke zorgvuldigheid worden betracht. Een "overstatement" van wat de resultaten nu eigenlijk zeggen over het geheel ligt op de loer en de risk assessor moet waken voor geprecipiteerde conclusies.

Op grond van bovenstaande moge duidelijk zijn dat, ondanks de grote vooruitgang die is geboekt in de methoden voor analyse van de samenstelling van bodemmicroflora, het effect van de veranderingen in de microbiële diversiteit op het bodemfunctioneren nog onvoldoende bekend is (N.B.: dit ligt dus **niet** aan de technieken, maar vooral aan de **kennislacunes** t.a.v. de omvang en betekenis van de functionele diversiteit en redundantie in de bodem). Derhalve blijven de implicaties van veranderingen voor bijvoorbeeld de weerstand en de veerkracht van de bodem vooralsnog enigmatisch.

De verwachting is dat, in de komende jaren, niet zozeer optimalisatie en verbetering van de technieken de grootste vooruitgang zullen brengen, als wel een beter begrip van de relatie tussen soortsamenstelling van microorganismen en het functioneren van de bodem.

Section I: Terms of reference

The Dutch GM regulatory authority, under the Ministry of Housing, Spatial Planning and the Environment (VROM), by virtue of a call by the Rijksinstituut voor Volksgezondheid en Milieu (RIVM) have commissioned a project briefly entitled "Methods to unravel soil microbial communities" which should investigate which methods are best used when the influence of GM crops on the soil microbial community is to be assessed and to what extent those techniques are able to answer relevant questions concerning soil health and functioning.

The specific questions posed were:

1. **What do current techniques - and data obtained by them - actually tell us about the living soil?**
2. **What is the relevance of such data and how do they relate to the functioning and health of a given soil. In other words, is the microbial population found in the soil capable of performing essential steps in key soil processes (e.g. in element (C,N,P etc.) cycles, in disease suppression and in maintenance of soil structure).**

Approach taken:

Soil microorganisms (bacteria and fungi) are at the basis of most key soil processes that determine (healthy) soil functioning. Assessment of soil microbial communities has traditionally involved cultivation-dependent studies but - during the last two decades - these have been surpassed by a range of cultivation-independent studies, which have diversified into dozens of different applications. All techniques – both cultivation-dependent and cultivation-independent ones – have their strengths and weaknesses. In this report, we sequentially describe what the most commonly used techniques can achieve in analyses of the living soil. We specifically address the questions: what do the techniques tell us, how should the results be interpreted and what are the strengths and weaknesses of the techniques.

Section II: The risk assessor's task, an example.

To set the stage for the strategy used in assembling this report, an example from the literature is first presented. Thus, data obtained in a study on the effects of GM plants on the soil microbiota which were published in the recent literature are dissected in respect of their merits and intricacies (Fig. 1) (Baumgarte and Tebbe, 2005).

One may add that - currently - the most frequently used techniques are molecular ones. This is because more traditional techniques like direct microscopy of microbial cells and cultivation (plate counts) do not - by themselves - provide detailed and overall information on the soil microbiota, let alone about soil health and functioning. Thus, when using microscopy, cell counts or hyphal lengths are determined, but it is impossible to distinguish the different kinds of bacteria or the other organisms (fungi). Moreover, when cultivation is used as the basis, only part of the microbial population will be determined, as only a small percentage of all extant soil microorganisms (mainly bacteria) is commonly culturable. Hence, the example chosen deals with a molecular technique, which is required to obtain a more complete picture of the soil microbiota.

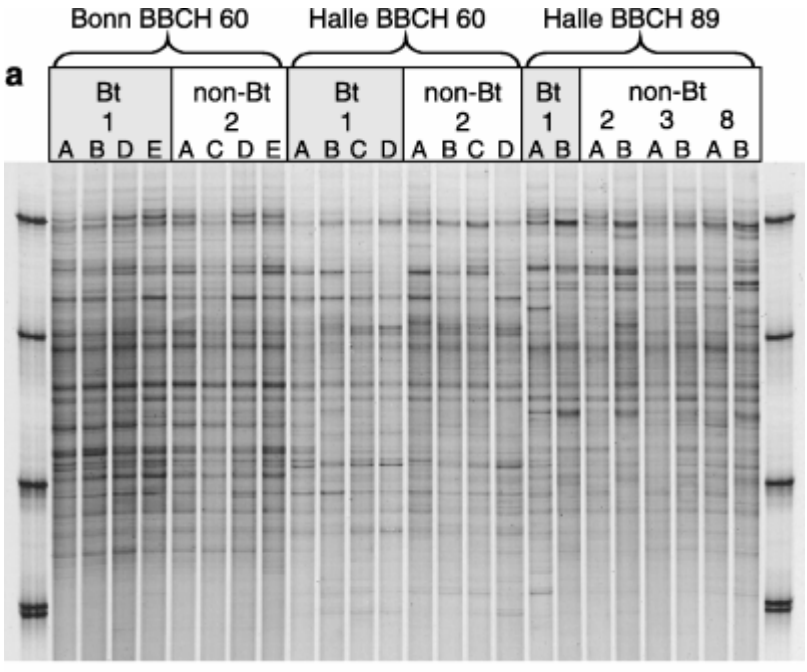


Figure 1: An example of an SSCP fingerprinting analysis.

Figure 1 thus shows a typical result of a soil microbial community analysis in a study on the effects of GMP's on the soil microbiota. A risk assessor confronted with such data has to assess these for their quality and true meaning. In the underlying case, the researchers used a molecular fingerprinting method called 'single strand conformational polymorphism' (SSCP), which is based on soil DNA generated amplicons produced by PCR with (phylogenetically-based) bacterial primers. The aim was to assess whether there were any differences between soil samples from three different fields that had been under genetically modified (GM) crops – in this case Bt maize (maize containing a *Bacillus thuringiensis* insect toxin gene) - versus under the parental counterpart. The main question the researchers addressed was what the data obtained tell us about the health and functioning of the soil. In other words, would there be a difference between soil health and functioning for GM versus non-GM crops? This is an important question when it comes to assessing the risk of introducing GM crops into the environment.

If we look at the gel, we see two to four lanes per treatment. Each lane contains a variable number of bands, each at varying intensity. In theory, each lane represents a (dominant) microbial community derived from the rhizosphere soil of the GM versus the non-GM plant. In the lanes, each band may theoretically represent one species in this bacterial community, although there are exceptions to this rule.

The analysis used a molecular fingerprinting method based on amplified soil DNA. This DNA was isolated using a standard soil DNA extraction method, and PCR was used to produce amplicons. Finally, the fingerprinting analysis was done by SSCP - similar types of fingerprints are commonly generated using a technique called DGGE.

Interpretation of the gel (Fig. 1) can be a daunting task. What do the results tell us? Does each band indeed represent a species that is different from the other organisms? Does the band intensity report on the relative abundance of the underlying organisms? What is the detection limit of the method? Is there any bias that is caused by unrepresentative soil sampling, DNA extraction or PCR? What are, in general, the potential problems and caveats with the performed technique? How trustworthy is the technique and what are its strengths and weaknesses?

These questions can be broadly divided into two main parts:

1. What is the quality of the analyses that were performed? This answers questions on, for instance, the quality of the statistics (e.g., the use of a sufficient number of replicates and

the quality of the sampling strategy), of the DNA extraction method (representativeness), of the PCR (possibly preferential amplification) and of the molecular fingerprinting (quality) method.

2. What is the true meaning of the results? This answers any questions on potential biases in any step of the analysis, on how sensitive and specific the used method is and on how the results are to be interpreted. Here, the risk assessor may ask the "so what" question in case of discernable effects. However, in reassuring cases - in which no differences are detected - it is also important to interpret the data in respect of their true meaning.

Eventually, the answers to both questions will tell us to what extent the data relate to the functioning and health of the soil studied.

Returning to the study of Figure 1, the researchers concluded that there were detectable differences between the soil microbial communities in the GM maize rhizosphere versus the non-GM maize rhizosphere. These differences were seen at the level of phylogeny, that is, the community make-up revealed differences. How this relates to soil functioning is unknown, but it can be predicted that, as a result of the commonly high functional redundancy in the living soil, such effects may have been negligible. The changes seen were minor and the authors concluded that the rhizosphere communities were more affected by the age of the plants or by field heterogeneity than by the presence of the transgene.

However, questions arise as to what extent these conclusions deserve merit. Those questions relate to the quality of all steps of the analytical procedure, as well as to data interpretation, and include the following:

1. The protocol selected for soil sampling, the choice of the sample size, compositing and sample transport and storage. Sampling may, for instance, be unrepresentative. See section III.1.

2. The DNA extraction method used. Soil-extracted DNA easily provides a somewhat biased picture, as DNA extraction from soil is seldom representative and unbiased. The isolation, lysis and cleaning methods can, for instance, bias the DNA in favour of, or against, particular microorganisms. For instance, Gram-positive bacteria often lyse poorly if the method applied is not harsh enough. Moreover, there are questions as to how the small

soil samples analyzed can be representative for much larger volumes representing e.g. fields. See section III.2.

3. The primers that target, for instance, the marker gene (often the 16S ribosomal RNA [rRNA] gene). The (here chosen) primers that target actinobacteria, alphaproteobacteria and/or pseudomonads may introduce biases and thus potentially omit organisms that belong to the respective group. See section III.3.

4. Techniques like DGGE or SSCP only reveal bands of microorganisms that are relatively abundant (down to about 0.1% of the total community). Less abundant species can also have important functions and these can be detected by group-specific primers. However, with exceptions (for instance, the ammonia oxidizers), there is no *a priori* link between diversity/community composition and function. See sections IV.4-IV.7.

5. One band in a DGGE (SSCP) gel might not represent one species. In fact, but several species may coincide in the band. See sections IV.4-IV.7.

6. One microbial species might harbor multiple copies of the 16S rRNA gene, thus giving rise to several bands. This leads to an overestimation of the species richness in a specific sample if band numbers are used for this estimation. See section IV.4-IV.7.

7. As a result of the funcional redundancy in microbial communities in soil systems, a change of the community at the phylogenetic level might not have an impact on soil functioning and health. Hence, the implication of a changed phylogenetic composition for functioning may remain unclear. See section IV.4-IV.7.

This list is not exhaustive, as other questions may arise when viewing a single gel and assessing the interpretations thereof. This document gives an overview of the advantages and disadvantages of the techniques that are commonly used in the analyses of GMP effects on the living soil. Section IV gives an easy-to-read overview of the most commonly used techniques, their potential and their caveats. The document continues hereafter with section III, which gives a quality guide to the initial preparatory steps involved in most of the techniques used in soil microbial community analyses. These encompass soil sampling, soil DNA extraction and the polymerase chain reaction (PCR).

Section III: An overview of the procedural steps involved in soil microbial community analysis.

The route to a proper soil microbial community analysis requires some procedural steps which need to be performed independently from which technique will be used for the final analyses. Of necessity, the first step in every analysis is soil sampling, in which particular care needs to be taken to warrant statistical representativeness. The investigators thus should examine particular protocols that define the sampling regime that is indicated for each type of study. Mindless or careless sampling (i.e. sampling without prior consideration of the objectives of the study and the level of heterogeneity that is expected) will undermine the value of any results, as statistical rigor may not have been achieved. Furthermore, the DNA-based methods inherently include a soil DNA extraction protocol, which will involve several technically demanding steps performed on small soil samples and may introduce biases (Ikeda et al., 2006). Hence, given such biases, it is primordial that techniques are at least standardized in order to allow cross-comparisons between samples (treatments) within an experiment (for instance, GM plant versus non-GM plant). Finally, as several of the molecular methods rely on the PCR (e.g. the fingerprinting techniques and clone library analyses), the amplification step needs scrutiny. In general, PCR is known to be prone to biases that may result from, for instance, preferential amplification (Wintzingerode et al., 1997). The PCR systems that are applied most frequently to soil DNA all use primers specific for a particular group - that thus amplify a mixed community within this group. Biases in amplification rates between members of the groups are well possible (Ikeda et al., 2006). Moreover, PCR performed on soil DNA targets can be technically demanding, especially when relatively impure soil DNA is used as the template (Wintzingerode et al., 1997).

In this section, a checklist of the intricacies of the three steps involved in the molecular analysis of the living soil, i.e. soil sampling, soil DNA extraction and PCR, is provided. This checklist should serve as a guide for regulators to check the quality, and the level of bias, of a given study.

Soil sampling

The first step in every analysis of the soil microbiota, be it culture-dependent or culture-independent, is representative sampling of the soil, which needs to follow particular statistical rules such as those laid out in standard manuals (e.g. Methods of Soil Analysis). The objective of the study, the expected level of variability and the statistical rules dictate the number, size and distribution of the samples over the field , as well as the processing of these (i.e., whether samples will be composited or used as single samples). Ignorance of the rules may lead to inadequate sampling, which, in the light of the statistical requirements of the study, may undermine the value of the data obtained . Thus, important prior consideration or estimation of the expected variability is needed. This is crucial, as most soil systems are heterogeneous and a too limited sampling strategy would yield insufficient statistical depth (Van Elsas and Smalla, 2006).

Checklist for soil sampling

- In dependency of the purpose of the study and the expected variability over the field, samples of soil should be taken from a pre-established number of independent subplots within a field. Within each plot, samples may be taken at various places across the plot, after which they may be composited. This is to flatten out differences at the miniscale level and thus to provide a meaningful average over the plot (Sliwinski and Goodman, 2004a; Sliwinski and Goodman, 2004b).

- Sampling of rhizosphere soil will also depend on the aim of the study. It can be performed in different ways, e.g. total, deep or shallow root parts can be separately sampled in the soil, or the root base versus root tip parts can be sampled. Interpretation of the results should include considerations on either a dilution effect due to the presence of excess bulk soil or contamination of the sample with endophytes (Kent and Triplett, 2002); (Ikeda et al., 2006). Rhizosphere sampling should be performed in a rigorous manner, to allow cross-comparison of samples. For instance, samples of rhizosphere soil are taken from similar parts of the root, as the microbial community may differ from the root tip to the basal area (Yang and Crowley, 2000).

- As for most molecular analyses small soil samples are used (<1 g), it is advised that these are taken from homogenized composite soil samples of at least 20-50 g. This is to overcome the influence of the heterogeneity of soil (Nicol et al., 2003). Naturally, this is not needed if one wants to examine differences at the microscale level.

- Over a growing season, it is important to collect samples from plants that are in similar growth phases, as rhizosphere microbial communities can shift over time with plant development (Baudoin et al., 2002). Time course experiments *a priori* take these shifts into account and are preferred.

- To analyze fungal community structures, larger soil samples (e.g. 10 instead of 0.5-1 g) may be needed for the analyses, as data have suggested that the variability of fungal communities is higher than that of bacterial ones (Girvan et al., 2004).

- Transport of samples from the field to the laboratory should be performed in shaded, closed boxes at temperatures prohibitive for outgrowth or shifts in the microbial community, thus preferably below 6-7 °C. The soil processing and subsequent analyses should start as fast as possible to prevent changes in the community, preferably on the same day or ultimately on the next day (Ikeda et al., 2006).

DNA EXTRACTION

With the exception of phospholipid fatty acid (PFLA)-based methods, culture-independent techniques rely mostly on the prior extraction of DNA or RNA from the soil. In many cases, DNA is used in the analyses, however RNA offers the advantage of being naturally amplified in the cell (ribosomal RNA). We here focus on the extraction of soil DNA, but virtually all of the assumptions and statements are also valid for soil RNA. The procedure involves several steps which all influence the downstream analyses.

Checklist for soil DNA extraction

- Soil DNA extraction has become largely (commercial) kit-based, although several non-kit based protocol are still in use. The DNA extraction kits all guarantee stability / robustness in their functioning and hence in the final results. However, for each “new” soil, the

performance of an extraction kit is to be tested as the efficiency of soil DNA extraction is soil-dependent. Furthermore, it is considered unwise to compare the results produced with different kit- or non-kit-based protocols, as they all introduce their own biases (Carrigg et al., 2007) (Ikeda et al., 2006). Hence, rigorous standardization within a single experiment is required.

- Lysis of cells is a key step in any DNA extraction protocol, and this step is prone to biases. It is important to recognize the difference between physical (e.g. bead beating based) and enzymatic lysis. For instance, bead beating may result, following break-up of the cells, in enhanced shearing of the DNA of those bacteria with the most fragile cells. On the other hand, enzymatic lysis may not affect those bacteria that are resistant to soft lysis. In both cases, substantial biases can be introduced, and the type of lysis thus determines our view of the microbial community in the sample (Burgmann et al., 2001b; Burgmann et al., 2001a). Lysis should thus be optimized according to the soil type and the bacterial taxon targeted.

- As stated, in different soils the DNA extraction methods will work differently. For instance, nucleic acids may bind differently to soil particles (clay and organic matter) in soils of different texture or mineral composition (Lorenz and Wackernagel, 1987). Comparison of the microbiota in different soil types may thus be hampered by this variable DNA extraction efficiency.

- DNA extraction is often followed by an extra purification step, as the so-called crude DNA sample may still contain a substantial amount of substances (such as humic or fulvic acids), which hamper subsequent PCR-based approaches. This purification step can lead to the loss of substantial DNA (Tsai and Olson, 1992). Comparison of samples that have undergone different purification protocols may be risky in the light of possible biases of these.

PCR (polymerase chain reaction)

Most downstream analyses of soil DNA, such as fingerprinting techniques and clone library analyses, rely on PCR. This technique is routinely used in almost every molecular biology

laboratory. PCR allows for the amplification and subsequent detection of genes from high copy numbers down to only 100 cells per gram of soil (Tebbe and Vahjen, 1993). It is often applied in a generic manner, i.e. amplifying particular genes (for instance, the 16S rRNA gene) present in different organisms all-at-once, thus providing snapshots of the diversity and composition of the microbial community. However, it is also prone to amplification biases, such as preferential amplification of particular templates, and may be subject to various technical difficulties (Wintzingerode et al., 1997).

Checklist for PCR

- It should be recognized that, due to possible mismatches or low numbers of particular sequences present in the sample, any primer set will hardly ever amplify all sequences of a target group (Ikeda et al., 2006). The so-called universal primers are never really universal. In fact, different universal primers have been shown to produce different results. A similar observation can be made for primers that target specific microbial taxa. Moreover, a central PCR dogma has it that any primer is as good as the database is and therefore organisms that are not in the database with aberrant primer annealing sites will be missed. The second issue, i.e. the non-amplification of rare sequences, is a commonality in all PCR-based analyses.

- Primers that are older than about four years should always be rechecked against the novel sequence information that is available in the public database. If possible, such primers should be updated on a regular basis.

- Inhibition of the PCR is often overcome by the use of different additives in the PCR reaction (Tebbe and Vahjen, 1993) (Felske et al., 1996; Henckel et al., 1999; Kageyama et al., 2003; van Elsas et al., 2000). However, these additives can also introduce particular biases in the amplification. Therefore, when comparing samples, the PCR reaction conditions used should be as similar as possible.

- DNA templates should not be differentially diluted to overcome amplification problems, as this will introduce different biases towards the most abundant species. In fact, a standard target DNA quantity (i.e. 1-5 ng of soil DNA) should be used per PCR reaction mix (Ikeda et al., 2006)(van Elsas et al. 2006).

- The efficiency of amplification is not the same for every template DNA and hence differential amplification may occur in soil DNA based amplifications. This is an unavoidable process, and differential amplification will thus introduce biases. For instance, the best amplified gene may not necessarily be the most abundant gene in the sample. Hence, quantification of results on the basis of (multi-template) PCR is inherently difficult (Wintzingerode et al., 1997).

- The temperature cycling program used for PCR on soil-derived DNA should involve a "hot start" of "touchdown program" to overcome the problem of initial mispriming (leading to spurious by-products), which is due to the high amount of different templates, including aspecific ones, in soil DNA samples.

- Long extension times should be avoided in the temperature cycling, as these may introduce enhanced numbers of chimeric amplicons (Ikeda et al., 2006). Also, the use of different extension times (if different samples are to be compared) should be avoided, as these may lead to different fingerprinting patterns (Zinger et al., 2007).

- PCR programs that consist of more than 35 cycles should be avoided, as they introduce enhanced numbers of PCR errors (appearing as mutations and causing problems in the analyses) which hamper subsequent analyses (Ikeda et al., 2006).

Section IV: Methods for the analysis of soil microbial communities - an overview

A number of traditional and advanced methods are commonly used in assessments of the effects of GMP's on the soil microbiota. Often, a combination of these methods is used, in which the overall abundance (microbial biomass), the size and nature of the culturable fraction and a fingerprinting-based assessment of community composition and diversity is shown. We here discuss the intricacies of the most commonly used methods.

Chloroform fumigation

Chloroform fumigation is a simple method that determines the nitrogen or carbon content of a soil microbial community, and thus provides a measure of the biomass size. It consists of the treatment of a soil sample with chloroform vapor. The chloroform destroys the membranes of exposed cells, which results in freed cellular carbon and nitrogen compounds. In one approach, after removal of the chloroform and extraction of the sample with K_2SO_4, the soil extract is filtered. Then, the total amount of carbon or nitrogen is compared to that of an unfumigated control sample.

Interpretation and quantification

The outcome of a chloroform fumigation treatment reveals the amount of carbon or nitrogen present in the soil microbiota (and derived from the organisms), and thus provides a measure of the number of microorganisms present in a soil sample.

Specificity and sensitivity

Chloroform fumigation is a rather rough method, and the sensitivity and specificity are thus low. The technique fails to detect subtle changes in a soil microbial community, including those in community composition (Olfs and Scherer, 1996). Furthermore, large changes in the soil microbial community make-up do not necessarily lead to large changes in carbon or nitrogen content. Thus, the number of microorganisms may not have changed in cases in which the community composition did change.

Reproducibility

The reproducibility of the chloroform fumigation method is quite high, as the procedure is fairly simple and straightforward. It is, however, difficult to compare different soils in respect of their absolute biomass, as the soil clay content influences the amount of organics that are adsorbed to soil particles. Thereby, the carbon content of the biomass may be underestimated.

Claims

Chloroform fumigation is a traditional technique which is not often used in GMP studies. If used, it is always combined with other techniques. Chloroform fumigation (followed by extraction and measurement) quantifies the microbial biomass. In recent papers using the method, the interpretation of the data has been very reserved. Both Sessitsch et al. (2004) and Devare (2007) found no or inconsistent changes in microbial biomass levels in soil under GM or non-GM plants. Furthermore, they found microbial biomass differences between different plant growth stages or even years to exceed the inconsistent differences between GM and non-GM. However, the microbial biomass measurements calculated via chloroform fumigation do not report on the different taxonomical groups or their activities. Thus, although a key soil parameter – microbial biomass - is measured, hardly anything can be said about soil microbial community composition, let alone soil health and functioning, as we ignore how these three groups of parameters correlate. However, given the importance of the soil microbial biomass size, cases in which a major decrease of microbial biomass is measured are important to know.

What can be stated when interpreting results of chloroform fumigation?

Chloroform fumigation of soil gives information on the amount of microbially-borne carbon or nitrogen, which correlates with the microbial biomass (number of microorganisms) present.

Conclusion

Although fairly simple to perform (and providing a key parameter of the living soil), chloroform fumigation data do not provide direct information on soil microbial community composition nor on soil health and functioning. To study the effects of GM crops on soil microbial communities, chloroform fumigation is considered to yield rather crude data. Therefore, the method is complementary to other ones and it should always be combined with higher-resolution techniques that allow a dissection of the soil microbiota.

Substrate induced respiration (SIR)

Using the SIR technique, the total biomass of a soil sample is measured by adding a readily-decomposable respiratory compound (usually glucose) and subsequently measuring the initial maximum respiration rate (CO_2 release). The higher this initial rate, the higher the calculated biomass is (West and Sparling, 1986).

Interpretation and quantification

Results of SIR are simple values describing respiration rates of a given soil sample, which correlate with the estimated microbial biomass in the sample.

Specificity and sensitivity

As with chloroform fumigation, the sensitivity of SIR is adequate for its goal, i.e. the estimation of the respiration-responsive biomass. The data obtained should thus be valued to their merit. For instance, subtle changes in the microbial community, in respect of composition and activity, are not measurable and information on taxonomic groups involved in the response is not retrieved. On the positive side, fungal and bacterial biomass can be separately measured by adding inhibitory compounds for either the bacterial or the fungal community (Lin and Brookes, 1999).

Reproducibility

As the protocol for SIR is fairly robust, simple and straightforward, the reproducibility of SIR measurements is high.

Claims

SIR is a traditional technique, which is almost always used in combination with other techniques. Because of its simplicity and speed (it is considerably faster and easier than chloroform fumigation), it is still very much in use. The SIR data provide an estimate of soil microbial (bacterial and/or fungal) biomass, which is a key facet of the living soil. Information on taxonomical groups and differences between them are to be obtained with additional techniques.

What can be stated when interpreting results of SIR?

Statements that can be made on the basis of a SIR assay involve the amount of respiration performed by the microorganisms in a soil sample when adding a particular C source. It correlates with the amount of microorganisms and their activity (Lin and Brookes, 1999).

Conclusion

Substrate-induced respiration represents a simple and fast way to gather information on the microbial biomass of soil. It inherently does not give additional information on taxonomical placement of the soil microorganisms present. Therefore, SIR is complementary and is ideally combined with molecular fingerprinting techniques.

Colony forming unit counts

Counting and analysis of colony forming units on Petri plates is a traditional method to get insight into the soil microbiota in respect of the size of the culturable community. Although cultivation-based studies have often been surpassed by cultivation- independent analyses, a lot can still be learned from the culturable fraction of the microbial community. Some authors claim that CFU counts tell us about the key components of the soil microbiota, namely those organisms that are able to grow when confronted with available substrate.

What do the cultivation- based studies show?

After dislodging microorganism from the soil matrix, dilution plating on different agar media results in plates with bacterial and/or fungal colonies in different sizes, colors and morphologies. The type of organism captured on the plate depends on the medium used (e.g. either nutrient-rich or nutrient-poor, specific substrates), the additives added (e.g. antibiotics, signaling molecules) and the incubation conditions (e.g. temperature, oxygen concentration and time) (da Rocha et al., 2009). It is safe to state that every single plating condition will result in a changed view of the culturable organisms present in the sample, and hence that an unbiased view of the community is hard to obtain ((da Rocha et al., 2009; Liu et al., 2005). However, the size of the culturable biomass (i.e. the numbers of CFU retrieved per g of soil) represents a loose measure of the potential activity of the soil microbiota, which deserves merit in the light of the above arguments.

Interpretation and quantification.

Interpretation of cultivation studies usually involves counting of the colonies formed, by eye or with the help of a microscope. Often, if the medium is not selective enough for a target bacterial group and distinction based on morphology is impossible, PCR fingerprinting (e.g. BOX, ERIC) is performed on single colonies and countings will thus be based on colonies identified by similarity of fingerprinting profiles. These post-plating applications are laborious.

Specificity and sensitivity.

The detection limit of dilution plating often lays around 10^2 bacteria per g of soil (but this obviously depends on the way the soil sample is processed). To increase the specificity (only target organisms should be present on the plate), several compounds can be added to the agar medium that favor the development of the target organism at the expense of other organisms, or specific growth conditions can be used (as mentioned above) (Liu et al., 2005). If, for instance, nitrogen-fixing bacteria are the group of interest, bound nitrogen is omitted from the medium. To isolate anaerobic microorganisms, plates are incubated under anaerobic conditions. Still, not more than 1-5% of the microorganisms present in a soil system can generally be cultured on a plate, an observation termed the Great Plate Count Anomaly (Sorensen, 1997). Hence, the majority of organisms in soil are so far unculturable, as their specific growth conditions are unknown. Moreover, numerically dominant soil organisms like Acidobacteria still have very few culturable representatives, while bacterial groups like the pseudomonads are overrepresented in culture-based analysis. Conclusions based on cultivation studies are therefore biased towards those organisms that readily form colonies on plates, which provides a skewed view of the microbiota of soil.

Reproducibility

Cultivation-based studies are easy to perform, and therefore easy to standardize. Commonly, replicate (e.g. triplicate) samples are examined, and replicate plates counted per dilution. This often yields values which are reasonably close between replicates, with standard deviations below 20-30% of the mean. However, as the processing and plating in such studies are commonly performed manually (as compared to automatic or computerized techniques), an exact reproduction of results is difficult. Still, if similar plating conditions are applied and overall counts are performed, a comparison between studies and laboratories is warranted and feasible.

On the other hand, comparison between different soils is often fraught with uncertainties, as cell dislodging efficiencies will vary between soils, depending on the attachment of microbial cells to soil particles (Ikedia, 2006).

Further analysis.

As mentioned above, dilution plating is often succeeded by (BOX / ERIC) fingerprinting PCR to get more insight in the nature (type) of the colonies. Other post-plating procedures include the sequencing of the 16S/18S rRNA genes to obtain information on the phylogenetic nature of the isolates. One of the greatest advantages of plating is the fact that the microorganisms are available for further study, which may include an analysis of substrate utilization, growth characteristics or even whole genome sequencing.

Claims

CFU counting is a classical technique which provides an estimate of the abundance of growth-responsive forms in soil. If combined with post-plating analyses, the diversity and nature of these forms can be assessed. Although cultivation biases are well accepted and the technique thus often gives a skewed view of microbial diversity, it is still very much in use in studies on GMP impact. However, other - complementary - techniques are almost always used next to the plating-based assays.

Plating assays thus give an overview of the abundance and diversity of culturable organisms in soil. Possible changes in this abundance and the diversity of the microorganisms may become apparent. However, CFU counts should be taken for their merits. As only a small percentage of the microorganisms in soil is culturable, changes in the culturable fractions (e.g. as related to the presence of GM versus non-GM plants), unless they are drastic and reproducible, may not tell us much about soil microbial community composition nor about soil functioning and health (Liu et al., 2005). Plating data should therefore always be combined with data from other techniques that describe the uncultured organisms (the silent majority). However, plating-based analyses remains useful, especially when specific isolates need further scrutiny (i.e. whole genome sequencing).

What can be stated when interpreting results of colony forming unit assessments?

Statements on the basis of CFU counts involve the culturable microorganisms, which are often below 1-5% of the total microbiota that is present (Sorensen, 1997). Thus, conclusions on the abundance of growth-responsive organisms are obtained. Clear and substantial differences in these (for instance resulting from the presence of a GMP) merit further investigation, e.g. via molecular techniques. Application of molecular fingerprinting techniques (see below) or 16S rRNA analysis on the relevant colonies will yield conclusions on the types of organisms affected. Furthermore, direct testing of physiology or whole-genome sequencing (for fastidious organisms) will give hints to their (potential) in situ function.

Conclusions

Cultivation-based studies are amongst the cheapest and easiest methods to get an estimate of the abundance and, with additional work, diversity of the culturable soil microbial community. However, conclusions as to which organisms and processes are really important in the system are difficult to draw, as the majority of organisms is not culturable under laboratory conditions. Thus, hardly any conclusion on soil community composition, soil health and soil functioning can be drawn. Cultivation-based assessments should therefore be combined with cultivation-independent techniques. Cultivation-based studies can, however, still be valuable as they allow for a thorough subsequent analysis of the bacterial isolates.

Methods based on soil DNA and PCR

Denaturing Gradient Gel Electrophoresis (DGGE)/Temperature Gradient Gel Electrophoresis (TGGE)

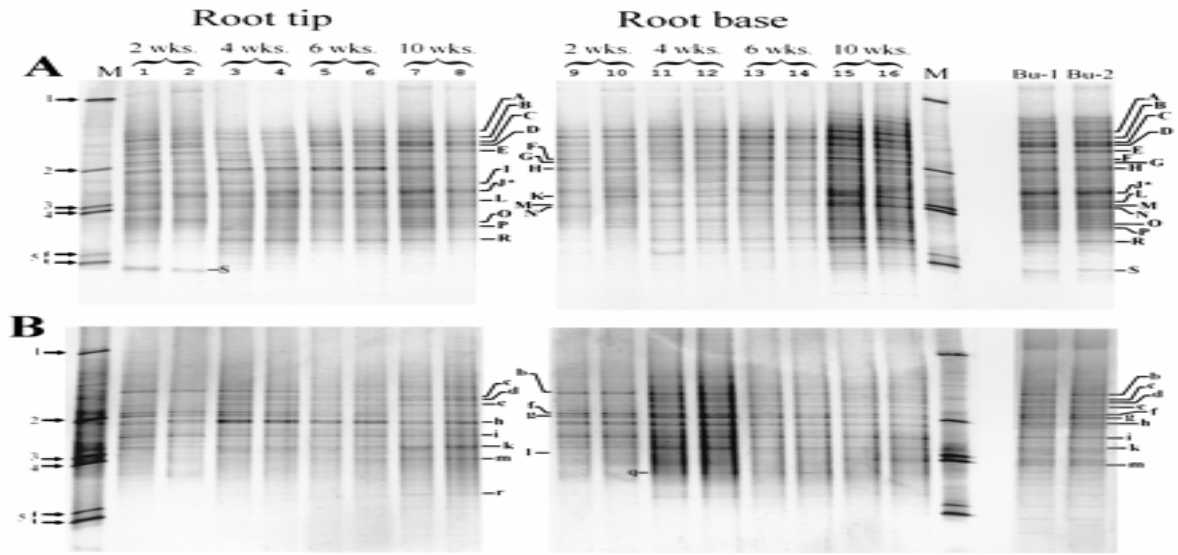
Of all fingerprinting techniques, DGGE and TGGE are probably the most commonly used, although terminal restriction fragment length polymorphism (T-RFLP) also is a popular technique. The SSCP technique mentioned earlier is currently less popular. As DGGE/TGGE (much like T-RFLP and SSCP) is preceded by DNA isolation and PCR, biases introduced in these sample processing steps should be taken into account in the final analysis and subsequent interpretations. In DGGE and TGGE , similar-sized amplicons generated by PCR are separated based on differences in their nucleotide sequences. For this, a polyacrylamide gel with a denaturing or a temperature gradient

is used for DGGE and TGGE, respectively. The technique (TGGE) has originally been developed for mutation detection. Since the nineties (Muyzer and Smalla, 1998), both DGGE and TGGE have been extensively used for microbial community analyses. DGGE/TGGE can be based on phylogenetic markers, e.g. 16S / 18S rRNA genes, or on functional gene markers.

Bacterial phylogenetic DGGE/TGGE

What does a phylogenetic DGGE/TGGE analysis show?

Figure 2a shows a typical example of a phylogenetically-based DGGE (A TGGE gel has a similar appearance). Generally (and much like SSCP), a DGGE gel depicts a number of lanes which contain a varying number of bands, with varying intensity. Every lane depicts a snapshot of a particular bacterial community in the sample at a certain time point.



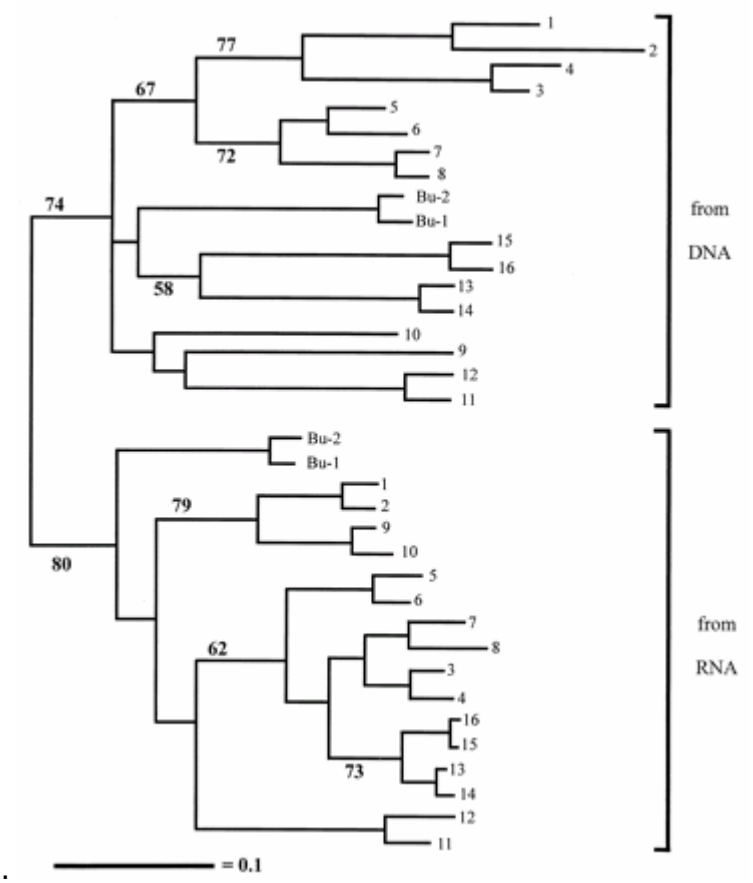


Figure 2: A bacterial phylogenetic DGGE analysis (A) and its phylogenetic tree (B) showing the difference in bacterial community composition of a root tip and root base

Interpretation and quantification of bands on a DGGE/TGGE

Ideally, one band in a DGGE profile represents one bacterial type ("species"). Given the fact that species are difficult to define in Microbial Ecology, experts have agreed to use the term operational taxonomic unit (OTU), and, in the context of this report, the terms species and OTU are to be interpreted with this difficulty/uncertainty in mind. Often, amplicons generated from different species show similar migratory behavior in the gel. Thus, particular bands in the gel may harbor multiple species (Sekiguchi et al., 2001). Other caveats in DGGE/TGGE gel interpretation include the occurrence of fuzzy bands (the question is whether these are bands composed of differentially degraded or melted molecules, or artifacts) (Kisand and Wikner, 2003) and the formation of heteroduplexes (Speksnijder et al., 2001), which overestimates the number of bands and thereby that of OTU. This, together with the previously-mentioned qualitative and potentially biased nature of the PCR, discredits the a priori conclusion that abundant bands are truly and always representative of the most dominant OTU. Another intricacy of DGGE/TGGE is

the fact that only dominant members of the community (>0.1% of the amplified organisms) will yield bands on gel. Hence, DGGE/TGGE provide depictions of the top 3 log units of members of the targeted community, but the relative dominance that is apparent from the gel may have been distorted by the method. The technique can, however, be nicely used in a comparative fashion, thus cross-comparing samples from the same study that were processed identically.

Specificity and sensitivity

Phylogenetically-based DGGE/TGGE using bacterial primers shows bands that are provenient from the most abundant bacterial types present in the sample, i.e. organisms whose abundance exceeds 0.1% of the total target bacteria present in the sample (MacNaughton et al., 1999). Therefore, less abundant, but potentially important bacteria, like for instance the ammonia oxidizers, are often not detected. To overcome this problem, primers targeting a diversity of specific groups or taxons have been developed (Andreote et al., 2008a; Andreote et al., 2008b; Costa et al., 2006; Garbeva et al., 2004; Heuer and Smalla, 1997; Leys et al., 2004). Bacteria belonging to these specific groups and representing much lower percentages of the total bacterial community can be visualized this way. However, the presence of specific bacteria does not necessarily tell us anything about the rates of particular processes, although there are exceptions (e.g. the ammonia oxidizers (Prosser and Nicol, 2008). Moreover, many processes are carried out by more than one bacterial lineage and hence a considerable functional redundancy may exist in the community. Furthermore, the actual activity of the bacteria represented in the bands needs to be assessed by other means. Thus, phylogenetically based DGGE does not a priori describe soil health and functioning. On the contrary, it provides a depiction of the phylogenetic diversity of the dominant community members, and there may be biases in this.

In Figure 2a, the 16S rRNA gene is used as the phylogenetic marker. However, many organisms harbor multiple copies of this gene (which can even differ in sequence and thus end up at different places in the gel) (Klappenbach et al., 2001). This hampers conclusions on the amount of OTU/species present in the community. To surpass this problem, alternative phylogenetic markers are used, like *rpoS* (Case et al., 2007) and *gyrB* (Tacao et al., 2005), which generally only have one copy per genome.

Reproducibility

DGGE/TGGE analyses are technically demanding and can be quite hard to reproduce, mainly because the gel consists of a gradient which is hard to exactly reproduce. It is therefore difficult to compare results from different studies across gels and it is even more difficult to compare results from different laboratories. Comparisons should really be based on data from one gel, or gels should be very rigorously standardized.

Further analysis of bands and statistics

Often, bands that are specific in certain lanes and thus potentially specific for certain environments are excised and sequenced (bands A-S in picture 2). The additional information gathered may be very valuable, as it gives insight in the phylogenetic composition of the community. However, the precision of the analysis can be somewhat hampered by the limited size of the sequence, which can range from 100 to 500 bp.

Additionally, Shannon indices are often calculated to estimate the diversity (richness and evenness) on the basis of the DGGE banding patterns of a given soil sample. One should realize that such indices only report on the dominant members and that there are additional problems. Hence, they do not tell us about the true diversity but rather typify a relative diversity. On the positive side, the calculations and subsequent statistics are easy to perform and make interpretation of the gels more elaborate.

Figure 2b shows a tree calculated from the gel in the picture shown in figure 2a. There are a number of ways the statistical analysis and the algorithms behind it are performed. Regrettably, these are often poorly understood by the researchers. Moreover, comparison of different studies can be hampered by the use of different statistical analyses.

The programs used for the gel analyses and the statistical analyses are often prone to misinterpretation and errors, and all results should, where possible (e.g. manually checking by eye whether bands are rightly attributed), be carefully checked.

Claims

DGGE/TGGE, as well as other fingerprinting techniques such as T-RFLP, are among the most used techniques that assess soil microbial communities in GMP-research. Hence, they are also amongst the most overstretched ones. Overstatement of the results is the most common problem, as changes on DGGE/TGGE are often interpreted as having large influences on soil

health and functioning, while so far this facet is scientifically unknown. Furthermore, some researchers fail to recognize that only the most abundant species are visible on DGGE gel, but that the remaining 'invisible' organisms might be of greater importance for soil well-being (viz. the ammonium oxidizers)(van Elsas, 2006).

An overview of the data shown in GMP research varies between the detection of no changes to that of large changes, depending on the plant studies and the organisms targeted. In general, scientists agree on the fact that the information of such fingerprinting techniques, especially when it comes to phylogenetic fingerprints, is actually quite limited. However, because of the ease, speed and well-developed protocols, phylogenetic fingerprinting techniques are still widely in use. Especially when combined with techniques like 16S rRNA gene based clone libraries and sequencing, they provide fast information on any changes in the dominant species. Hence, they signal differences in the communities at the phylogenetic level when used in a comparative fashion and form a starting point for a more thorough investigation should such differences be persistent and meaningful.

What can be stated when interpreting results of a bacterial DGGE/TGGE?

Bacterial phylogenetic DGGE/TGGE gives information on the diversity and community composition of microorganisms in soil samples. Specific microbial groups can be targeted using specific primers. As the number of bands that are visible on a gel is limited to a maximum of about 100, only the most abundant species are presented. DGGE/TGGE only gives information on their presence, not on their function or activity. Furthermore, no phylogenetic information is provided unless bands are analyzed by sequencing. Also, conclusions on relative abundance are hard to obtain, given the inherent biases in PCR. In a comparison, the only statement which can be made is whether or not there are differences in the phylogenetically defined communities of microorganisms, i.e. by presence/absence of bands. Even the absence of a band will only indicate the respective organism is present below a certain threshold, but it can never indicate its complete absence.

Conclusion

Although phylogenetically-based DGGE/TGGE has developed into a quick, cheap and reliable technique, it is hard to interpret the data when it comes to assessing soil health and functioning. The link between the changes in the bacterial communities and

concomitant changes in soil functioning is still far from known. To this point, however, phylogenetically based DGGE/TGGE can hardly provide any conclusion on health and functioning of the soil system.

Fungal phylogenetic DGGE/TGGE

Fungal phylogenetic DGGE/TGGE works along similar lines as bacterial phylogenetic DGGE/TGGE. Instead of 16S rRNA, either or both the 18S rRNA gene (Oros-Sichler et al., 2006) and the ITS (Anderson et al., 2003) region are used for analysis. Fungal DGGE/TGGE has been less used for soil community analyses than its bacterial counterpart.

What does a fungal phylogenetic DGGE/TGGE show?

The appearance of a fungal phylogenetic DGGE/TGGE is similar to its bacterial counterpart. In principle, each band can represent an OTU, but there are caveats as in the bacterial analysis.

Interpretation and quantification of bands on a fungal DGGE/TGGE

Interpretation of a fungal phylogenetic DGGE/TGGE is similar to that of a bacterial phylogenetic DGGE/TGGE. Quantification and any conclusion on the abundance of particular OTUs underlying bands on the basis of band intensity is, next to problems described for the bacterial analysis, hampered by the fact that fungal spores and hyphae can be multinucleated, while large parts of the hyphae might have no nucleus at all. (Anderson and Cairney, 2004) This can either overestimate or underestimate the abundance of a specific fungus in a community, respectively. Still, comparative assays can be very useful.

Specificity and sensitivity

As there are less fungal species than bacterial species per gram of soil, fungal phylogenetic DGGE/TGGE has, in principle, a higher resolution than the bacterial analysis. Furthermore, some fungal species, like arbuscular mycorrhizal fungi, are important for soil health and are sensitive to disturbance (Kowalchuk, 1999) (Liang et al., 2008).. They might therefore serve as good indicator species for soil health and functioning. On the other hand, the amount of fungal sequences in the public database is low compared to the amount of bacterial sequences, which hampers primer design, i.e. presumably a large part of fungal species remains unseen in

DGGE/TGGE. Another problem is that the 18S rRNA gene sequence is rather conserved across the fungi, which may cause several of the amplicons to coincide on gel (Kowalchuk, 1999).

Reproducibility

The reproducibility of a fungal phylogenetic DGGE/TGGE is in principle similar to that of its bacterial counterpart. The aforementioned concerns regarding the necessary care in respect of standardization and interpretation are also valid for the fungal analysis.

Further analysis of bands and statistics

Further analysis of a fungal phylogenetic DGGE/TGGE is similar to that of the bacterial analysis. Hence, a range of statistical clustering or ordination methods can be applied. When bands are removed for sequence analysis, the analysis of the retrieved sequences can be hampered by the relatively low amount of fungal sequences in public databases, although this is now quickly improving.

Claims

The claims concerning fungal phylogenetic DGGE/TGGE are similar to those pertaining to bacterial DGGE/TGGE. analyses Important to recognize and often overlooked by researchers is the fact that, due to the limited databases and resolving power of the 18S rRNA gene sequence, any knowledge about fungal diversity is still rather limited when compared to that of bacterial diversity (Kowalchuk, 1999). Therefore, primer design is hampered and DGGE/TGGE results have been less informative; i.e. the researcher has a narrowed view of the total diversity due to the limited resolving power. However, it is recognized that certain fungi, like mycorrhizae, are of great importance for soil functioning and that fingerprints of these fungi have great informative value and possibly give information on soil health and functioning, although details have still to be elucidated. Given this importance, primer systems for detection are continuously being improved and hence future refinement of the technique is foreseen.

What can be stated when interpreting the results of a fungal phylogenetic DGGE/TGGE?

Statements and interpretations are similar to those based on bacterial phylogenetic DGGE/TGGE, but the aforementioned caveats (See claims) should be taken into account. Also, utmost care should be taken with fungal DGGE/TGGE, as bands often do not represent single OTUs.

Conclusion

Fungal phylogenetic DGGE/TGGE is as cheap, simple and reliable as bacterial phylogenetic DGG/TGGE. The fungal primer sets have, however, not yet been very well developed. On the basis of well-developed primers, fungal phylogenetic DGGE/TGGE may become potentially more revealing than bacterial DGGE/TGGE, as there are fewer fungal species per gram of soil. This theoretically results in fewer bands on DGGE/TGGE, which would make it easier to observe changes between treatments. Furthermore, there are specific fungi, e.g. certain arbuscular mycorrhizal fungi (AFM), that are highly sensitive to disturbance and are highly relevant for soil health and functioning. DGGE/TGGE targeting those fungi has the capacity to become very important to define indicator species. However, also for fungi, a baseline needs to be developed so as to be able to answer questions as to what extent a soil fungal community is variable and affected by changes.

Functional (function-based) DGGE

As phylogenetic DGGE/TGGE does not provide information on specific functions in the soil, such as nitrogen fixation and ammonium oxidation, the focus of many researchers has been shifting to the direct analysis of functional genes. Reduction or changes in abundance or diversity of genes encoding such environmentally-relevant functions are believed to have a big impact on soil health and functioning (Kowalchuk et al., 2003) (van Elsas et al., 2006).

What does a functional DGGE/TGGE show?

The appearance of a functional DGGE/TGGE gel is similar to a phylogenetically-based analysis. As the abundance and diversity of specific functional genes is generally lower than those of phylogenetic marker genes, the amount of bands on a functional DGGE/TGGE is generally lower.

Interpretation and quantification of bands on a functional DGGE/TGGE

Interpretation of a functional DGGE/TGGE is similar to that of a bacterial phylogenetic DGGE/TGGE, be it that a more direct observation of potential function is obtained, which potentially can more directly indicate important shifts in functioning of the soil system.

Specificity and sensitivity

DGGE/TGGE using functional genes that are involved in sensitive soil processes such as ammonium oxidation, nitrogen fixation or steps of the sulfur cycle, theoretically gives more insight into soil health and functioning than that of phylogenetic maker genes. The observed changes are more likely to have an impact on soil health and functioning. However, the link between functional gene diversity and soil functioning is still far from understood. One of the largest challenges for the forthcoming years will be to understand how functional gene diversity affects soil function and to address the issue of stability of function in the face of stress imposed on the soil. Additionally, the lack of a broad overview of functional genes in public databases – as compared to the amount of, for instance, 16S rRNA gene sequences– still hampers reliable primer design, but this is quickly improving.
As there are few examples of varying copy numbers of functional genes per genome, a functional DGGE/TGGE analysis is, in terms of quantification, more robust than a 16S rRNA DGGE/TGGE (van Elsas et al., 2006).

Reproducibility

The reproducibility of a functional DGGE/TGGE is similar to that of a bacterial phylogenetic DGGE/TGGE.

Further analysis of bands and statistics.

Further analysis of a functional DGGE/TGGE fingerprint is similar to that of a bacterial phylogenetic DGGE/TGGE. That is, a range of clustering and ordination methods can be applied to the fingerprints, resulting in a statistically processed view of the effects of treatments. Furthermore, bands can be identified in respect of their sequence. However, identification of the retrieved sequences (on the basis of bands) can be hampered by the still low number of functional gene sequences in the public databases.

Claims

Claims made concerning functional DGGE/TGGE are similar to those related to phylogenetic analyses. Functional DGGE is heralded as being superior to phylogenetic DGGE. Although this is partly true as a result of its lack of variability and the opportunity of a direct view on genes underpinning soil function, it suffers from overstatement. Furthermore, although changes in functional gene diversity are very well visualized on DGGE/TGGE gels, no knowledge about the

effect of the changes on soil health and functioning is implicitly obtained. Furthermore, researchers need to acknowledge that our knowledge of functional gene diversity is less developed than that of phylogenetic gene diversity and therefore primers may miss large parts of the extant diversity. This skews the results and leads to misinterpretations in respect of the true effects of the factor studied on (potential) soil function. In summary, with functional DGGE/TGGE one is able to get a quick view of functional gene diversity in a relative and comparative fashion, but the picture obtained is often incomplete and may be biased. Further conclusions on the true effects of any changes observed in the patterns are difficult, unless there is a proven relationship with soil function.

What can be stated when interpreting results of a functional DGGE/TGGE?

The conclusions that can be obtained from a functional DGGE/TGGE are, in terms of what the method is able to show, largely similar to those from phylogenetic counterpart analyses. However, in the former method one is looking at the diversity of the abundant functional genes rather than at that of a phylogenetic marker, which in itself is an asset as, although such a relationship has sparsely been proven, it potentially relates to function of the system.

Conclusion

Functional DGGE/TGGE is as cheap, simple, rapid and reliable as phylogenetically-based DGG/TGGE. The current primer sets have, however, not yet been fully developed with respect to their coverage of the full genetic complement of soil function. This is due to a lack of knowledge of a large part of the extant functional diversity in soil. The results gathered are more informative, in respect of function, than those from phylogenetic DGGE/TGGE. In fact, changes, especially decreases, in the abundance of particular functional genes (which may be key under relevant ecological conditions) are believed to considerably influence soil health and functioning. However, the exact link between the diversity and abundance of functional genes and soil health and function is far from understood. Conclusions and interpretations should thus be drawn with great care, and often one will find that more knowledge is needed for a more profound assessment of what a change really means.

SSCP and T-RFLP

Besides the aforementioned DGGE/TGGE, SSCP (single strand conformational polymorphism) and T-RFLP (terminal restriction fragment length polymorphism) are two other important fingerprinting techniques in current microbial ecology that allow a description of the soil microbial community. SSCP (See example treated earlier in this document) is based on the separation of different conformations of single-stranded DNA molecules (generated via PCR and subsequent melting followed by partial reannealing) which is determined by the sequence of the 16S rRNA gene (Orita et al., 1989; Schwieger and Tebbe, 1998). In T-RFLP, separation of fragments is based on the length of the terminal restriction fragment, which is separated via gel electrophoresis and detected via a fluorescent label at the 5' or 3' (or both) termini of the fragment (Tiedje et al., 1999). In a recent study (Smalla et al., 2007), four different soil types were analyzed using DGGE, T-RFLP and SSCP. Interestingly, the authors found that, irrespective of which one of the three techniques was used, clustering of the resulting fingerprints correlated with soil physicochemical properties. In other words, the three techniques were able to cluster the different soils in a similar manner and thus told similar stories about the differences in the community make-up of the soils.

What do SSCP and T-RFLPfingerprints look like?

Figure 3 (a and b) shows the results of an SSCP and a T-RFLP analysis, respectively. SSCP gels look similar to DGGE/TGGE gels, in that several fingerprints (banding patterns) may appear, which consist of variable numbers of bands with varying intensity. Like DGGE/TGGE, the figure shows snapshots of the bacterial communities at particular time points.

T-RFLP patterns look different from those generated by the other fingerprinting techniques, the main reason being that it has been semi-automated. The patterns commonly show a limited number of peaks with varying heights (Osborn et al., 2000). The peaks are dependent on the terminal restriction sites present in the underlying molecules. Given the known sharedness of particular sites in 16S rRNA genes between different species, the number of peaks on a T-RFLP gel is usually lower than the numbers of bands on DGGE/TGGE or SSCP gels (Tiedje et al., 1999). In other words, T-RFLP has a lower resolution than DGGE/TGGE and even than SSCP.

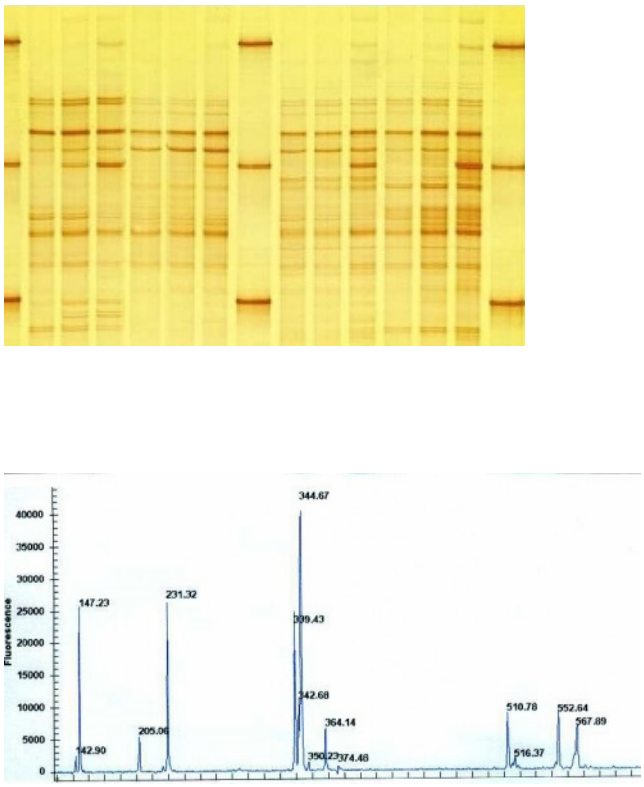


Figure 3: An example of an SSCP (A) and a T-RFLP (B) analysis.

Interpretation and quantification of SSCP and T-RFLP results.

Interpretation and quantification of SCCP data is similar to those of phylogenetic DGGE/TGGE, as both methods are based on the use of the phylogenetic marker. Hence, SSCP has the same drawbacks as DGGE/TGGE. Quantification of T-RFLP data is performed by calculating the area underneath each one of the peaks, and assuming that the greater the area, the greater the relative abundance of the respective group is. However, one should consider that the peaks in T-RFLP gels depict groups of bacteria as opposed to one single species or OTU, as the terminal restriction sites are often not species-specific, but rather specific for broader groups.

Specificity and sensitivity.

The specificities as well as sensitivities of SCCP and DGGE/TGGE data are similar, as these depend on the intricacies of both PCR (similar for both techniques) and separation on gel (although based on different principles, roughly similar between the two techniques). Hence considerations of DGGE are equally valid for SSCP. The sensitivity of T-RFLP is also in a range similar to DGGE, however the technique is less specific than the other three techniques. Single mutations falling outside restriction sites are not visible, and T-RFLP is therefore considered to be broader group-specific instead of species-specific. Therefore, terminal restriction fragments of rather unrelated organisms can form single peaks in the pattern. This is why T-RFLP fingerprints often reveal lower numbers of peaks than those present in other fingerprinting techniques. In very complex systems, this is sometimes considered to represent even an advantage, as the apparent complexity of the community is reduced. T-RFLP allows to obtain different community fingerprints from one and the same sample, as the samples can be digested by multiple restriction enzymes. This feature is unique for this type of fingerprinting, and potentially enhances its resolving power (Tiedje et al., 1999).

Reproducibility

SSCP data (fingerprints) are generally quite reproducible. The technique is fairly straightforward, as just a simple electrophoresis apparatus is needed without the need to prepare a gradient. This enables easier gel-to-gel comparison than DGGE/TGGE.

As T-RFLP is automated, it is robust. Gel-to-gel comparison is thus easy and reliable, in principle enabling good comparison between studies and laboratories. Obviously, when comparing studies, similar amplification and restriction enzymes need to be used, and one often finds that difficulties arise due to differences in the quality of such enzymes.

Further analysis.

Further analysis of SSCP data is similar to that of DGGE/TGGE data. This includes the excision of bands and the statistical aftermath.

T-RFLP does not easily allow for excision of bands (Ikeda et al., 2006), so information cannot be readily gathered with respect to the phylogenetic placements of the peaks. As T-RFLP is

automated, the method is more reproducible, making statistical analysis more reliable and less prone to errors.

Claims

Claims made on the basis of SSCP and T-RFLP data are similar to those based on DGGE/TGGE data. This implies that robust relative statements about apparent community make-ups can be safely made, but that one needs to realize that such make-ups can be far away from the true community make-ups. Hence, the power of SSCP and T-RFLP lies in the comparative manner the techniques can be used, be it that T-RFLP is often less discriminative than SSCP, and both techn iques may be less so than DGGE/TGGE.

What can be stated when interpreting results of SSCP or T-RFLP gels?

The conclusions drawn from SSCP data can be largely similar to those from DGGE/TGGE, as the techniques are quite comparable in respect of their resolving power. With T-RFLP analysis, the conclusions involve the presence and relative abundance of groups of microorganisms rather than species.

Conclusion

Both SSCP and T-RFLP fingerprintings are well developed techniques, which have been used in various soil studies. Both techniques enable better reproduction than DGGE/TGGE, which are plagued by gel-to-gel variation. However, much like in DGGE/TGGE, any conclusions on the relevance of SSCP or T-RFLP data for soil health and functioning remain elusive as long as we do not have a clear vision of the link between the thus described community structure and function. T-RFLP is different from the other fingerprinting techniques, as it is broadly group-specific rather than species-specific.

Clone libraries

Analyses based on fingerprinting techniques are often accompanied or even replaced by those based on clone libraries. In these, 16S (or 18S) rRNA gene specific PCR is run on soil DNA, after which the similar-sized PCR fragments are separated by ligating them into a vector plasmid and subsequently bringing them into *E. coli* by transformation. After a growth step of single colonies that captured the vectors with insert, the cloned PCR fragments can be isolated, sequenced and analyzed.

What do clone libraries look like?

The sequences obtained from the analysis of the clone libraries are often shown using dendograms (phylogenetic trees), in which the evolutionary distance of all sequences is calculated and visualized. Depending on the primer set used, the phylogenetic tree can consist of fragments of broadly amplified16S rRNA genes or of specific subgroups. Also, clone libraries of functional genes can be presented in phylogenetic trees. An average tree consists of at least thirty sequences, and often many more. However, for most soil systems and using phylogenetic markers, there is hardly ever high coverage of the total extant diversity. Hence, much like in the fingerprinting techniques, the data will only report on the dominant members of the community. Regularly, previously isolated sequences taken from the database are incorporated in the tree as reference points.

Interpretation and quantification of clone library results.

Interpretation of clone libraries often consists of the comparison of the sequences from one library with those of another library, which is often accompanied by statistical analyses (see further analysis). Using varying sophisticated statistical tools (e.g. UniFrac or LIBSHUFF), this allows an assessment of the degree to which one library resembles or overlaps with another library. With 16S rRNA gene analyses, the sequences are further compared to those of public databases, in order to elucidate the identity of the different bacterial (or fungal) species present. Sequences with less than three percent difference between them are generally agreed to belong to the same species (van Elsas, 2006). Sequences generated from different environments or treatments can be placed in the same tree, which enables an easy comparison between samples.

Specificity and sensitivity.

The sensitivity of clone library analysis – provided large enough libraries are analyzed - can be higher than that of fingerprinting techniques, mainly because sequences are analyzed separately, so single sequences from abundant or even less abundant species (given a large enough sample size) are well detectable. Specificity issues are similar to those of fingerprinting techniques, as especially the choice of the primer set determines the specificity. Clone library analysis however adds, next to the DNA extraction and PCR bias, another bias into the analysis, i.e. the cloning bias (van Elsas, 2006). DNA fragments are ligated into the vector plasmid with possibly differential efficiency. The bias can be severe, as was observed when non-cloning-dependent (thus, direct) sequencing of soil DNA was developed (see pyrosequencing).

A major advantage of, in particular 16S rRNA gene based, clone libraries is the ability to directly obtain and analyze novel sequences, which increases our understanding of soil microbial diversity. With current high-throughput facilities, the data obtained in a clone library can be overwhelming, including the generation of many novel sequences.

Reproducibility

Clone libraries are very reproducible, and the comparability between laboratories and studies is high. Due to the inherent cloning bias, it is recommended to solely compare libraries produced with similar cloning vectors and transformation hosts.

Further analysis.

The sequences retrieved from a clone library analysis are often subjected to statistical analyses. These include the use of non-parametric estimators like Chao1 and ACE, which calculate the richness of a soil microbial community (Hardoim et al., 2009). Similarly, rarefaction curves are calculated to obtain a glimpse on the diversity of a given soil sample and to visualize how many more clones need to be checked before no novel sequence will be retrieved. The correct interpretation and usefulness of these statistics are still under debate.

Claims

Clone libraries suffer from the same overstatement issues as do the molecular fingerprinting techniques, although clone libraries are less used in comparative studies. This is mainly due to the enormous amount of information which is to be analyzed, and the rather time-consuming nature of the method. Overstatements include the lack of attention for the fact that any differences between communities at GM and non-GM plants do not exceed differences between sample locations or sampling season. Furthermore, some researchers fail to recognize the cloning bias, overstating the importance of particular sequences. Very recently, the development of techniques that lack a cloning step (pyrosequencing) revealed the cloning bias to severely skew the final results of the analyses. Other issues include researchers assigning specific functions to the sequences (often 16S rRNA gene sequences), because the first hit organism in the database harbors that specific function. Similar sequences (especially when small) may come from different organisms, which may have very different functions.

What can be stated when interpreting the results of clone library analyses?

Similarly to data from molecular fingerprints, those from clone library analyses will only report on the presence of particular microorganisms in a specific soil sample. Additionally, one gets information on the phylogenetic placements of the respective microorganisms. The greatest asset of clone library analyses lies in the comparative power at the phylogenetic level. That is, it offers the possibility that a strong reduction of the abundance of a particular member of the community as a result of a treatment or GM plant is detected at the phylogenetic level.

Conclusion

Clone library analysis currently is an easy-to-perform, somewhat laborious technique, which, with current ultra-high-throughput sequencing facilities, allow for in-depth analysis of microorganisms or functional genes present in a soil microbial community. Clone libraries have high resolution but do not allow for a quick view (as large samples are needed to detect less abundant species) on the diversity or difference between samples, as is the case with fingerprinting techniques. Therefore, it is useful to combine these two types of approaches. The link between diversity and function of the microbiota in a soil system, however, remains unknown. Furthermore, it is important to recognize the

cloning bias that is inherent to the technique, which severely hampers the interpretation of the real microbial diversity.

PLFA analysis

An alternative to DNA/RNA fingerprintings is the analysis of lipids that are present in the membrane of microorganisms via phospholipid fatty acid (PLFA) analysis. In PLFA analysis, total lipids are extracted from a soil community via a fairly laborious isolation protocol and these are subsequently analyzed via chromatography of the extracts (White et al., 1979a; White et al., 1979b). PLFA are broadly specific for particular microbial groups (extending beyond the Prokarya, e.g. fungi and protozoa can be detected to some extent) and so a rather broad overview of the community can be obtained. Particular signature PLFA allow the detection of the abundance of particular microbial groups.

What does PLFA analysis look like?

The chromatography is computerized and thus results of a PLFA analysis look like computerized peak patterns. The peak patterns serve as phospholipid fatty acid based fingerprints of the soil community, similar to DNA/RNA based fingerprints. Peaks can be of various heights which correspond with the abundance of the underlying organisms.

Interpretation and quantification of PLFA analysis results.

In respect of assessing community make-up (richness, evennes and community structure), the analysis and interpretation of PLFA data is similar to that of the DNA-based fingerprinting techniques. It is important to realize that PLFA analysis is group-specific rather then species-specific. In fact, different species from one group often have similar phospholipids and thus would yield the same peaks in the PLFA chromatogram. Much like in T-RFLP, quantification of the peaks is performed by calculating the area underneath a peak.

PLFA analysis also allows for an estimation of the total biomass, by summing up all peak areas (White et al., 1979a).

Specificity and sensitivity.

PLFA is a fairly sensitive technique, as only 150 picomole of each PLFA is necessary to obtain a signal. This corresponds with approximately 10^3 cells of a particular organismal type per gram

soil. The specificity of PLFA analysis is rather low, and it is not species-specific (Green and Scow, 2000). Even between microbial groups, PLFAs can be similar. Unrelated organisms therefore may appear under similar peaks. However, as with T-RFLP, this low specificity is sometimes considered to be an advantage when dealing with complex samples, as the complexity of the apparent community is reduced.

An advantage of PFLA analysis is the fact that PFLAs break down faster than DNA in natural environments. Therefore, less material from dead cells is taken into analysis when using PFLAs than when DNA-based methods are used (Harvey et al., 1986).

Further analysis.

Comparison of soil-derived PLFA profiles is usually performed by multivariate statistics like Principal Components Analysis (PCA). Although these statistics allow for an easy analysis of soil community structure and diversity, the underlying calculations are often hard to follow.

PFLA analysis of soil samples may allow for an identification of bands, and hence information on the phylogenetic placement of the peaks can be obtained. Some PLFA typical for particular groups have been described. In other cases, such an identification is, however, impossible.

Claims

Claims based on PFLA analyses are similar to those based on DNA based fingerprints, in particular those from T-RFLP analyses. Broad impressions of apparent community make-up are obtained, and data are to be used in a comparative fashion. Caveats may be the lack of completeness of the analysis (due to incomplete sampling or extraction) as well as the sharedness of particular PLFA across organisms, making these rather useless as markers.

What can be stated when interpreting results of a PLFA analysis?

As argued in the above, the statements based on PLFA analysis are similar to those of T-RFLP analyses. See further claims.

Conclusions

PLFA analysis is a well-developed (now almost routine) technique which is used for comparing soil microbial communities. However, the protocols can be fairly laborious and the equipment is more expensive than that of DNA based techniques. Conclusions drawn from PLFA data are, in terms of what the data can tell us, similar to those from DNA-based other fingerprinting techniques, in particular T-RFLP. Thus, whereas a broad overview of relative community make-up is obtained, hardly anything can be concluded about soil health and functioning. Also, the level of resolution (discriminating power) of particular PLFA markers is below that of DNA sequences such as used in, for instance, DGGE.

FISH

Fluorescence in situ hybridisation (FISH) is a highly specialized technique, in which fluorescently-labelled DNA probes (ca. 20 bp in length) target specific organisms (usually the 16S rRNA molecule) in its natural or simulated setting (Amann et al., 1990a). FISH is mostly used in studies on the interactions between organisms, e.g. those between prokaryotes and eukaryotic hosts. It has also been used in studies on GMP effects, in particular those studies that address the plant-associated microbial communities.

What does FISH look like?

Figure 4 shows a typical result of a FISH analysis in soil (van Elsas, 2006). The large 'branches' show fungal hyphae, while the colored 'rods' are bacteria. The figure typically shows how the bacteria interact with the fungal hyphae, but it also allows to count and thus quantify the bacteria present. So, with FISH, both the location of microorganisms in a system and the actual interactions of microorganisms with each other or with higher organisms like plant roots can be visualized. One asset might be the study of the localization of key members of the microbial community of soil, in particular concerning the possible effects of GM plants.

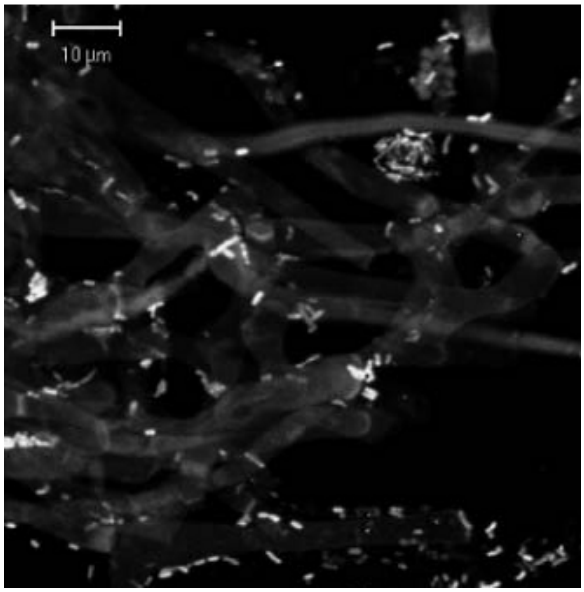


Figure 4: The interaction between a fungus and various bacteria, visualized by FISH.

Interpretation and quantification of FISH results.

Interpretation of FISH results in GMP studies usually implies the counting of particular microbial cells that were specifically stained in the system. Cell counts using FISH are fairly reliable, and robotization/automation of the counting process has been developed. However, especially in soil there is a particularly high background due to some soil particles exhibiting autofluorescence, which leads to a possible overestimation of the number of cells present.

Specificity and sensitivity

The sensitivity of FISH is rather low, as several thousands of rRNA target molecules are necessary to obtain a detectable FISH signal (Lynch et al., 2004). Therefore, only identification of active organisms, with high ribosome content, is possible, whereas dormant organisms or organisms with low activity – which presumably have lower ribosome numbers - will not be visualized. Also, FISH often shows low detection compared to DAPI (total count) staining (only 35-40% is visible when using a probe targeting all bacteria)(van Elsas, 2006).

The specificity of FISH relies on the specificity of the probes used. Many specific probes have been developed (currently >1200). Probes older than 4 years should always be rechecked for their specificity against the sequences present in public databases. An advantage of FISH is the possibility of using multiple probes in the same sample, enhancing our ability to study the interaction or to calculate relative abundances (Amann et al., 1990; Wagner et al., 2006)).

The FISH technique is not dependent on DNA extraction and downstream applications like PCR on beforehand. This rules out any biases introduced with those steps.

Reproducibility

FISH is not often used for comparing samples across treatments. In those rare cases in which it was used, however, the comparability seems to be rather good, at least if similar protocols are followed between treatments.

Further analysis

Since FISH involves direct counting and calculating (relative) abundances of particular target organisms in the system, the results are straightforward and no complicated (multivariate) statistics are needed.

Claims

FISH is not often used in GMP studies, and thus not many claims have been made by researchers involved in such studies. When FISH is applied to study the difference between GM and non-GM plants, it is used through simply counting and comparing the numbers of organisms. Hence, the interpretations are simple and solid as long as similar protocols are used. When different probes are used together on one sample, the relative abundances of the different targets can be calculated.

What can be stated when interpreting the results of FISH?

Statements that are warranted when performing FISH involve inferences of the presence and abundance of particular microorganisms. Furthermore, the interactions between different FISH detected microorganisms may become visible when the localization of the target organisms is taken into account (Amann et al., 1990).

Conclusion

Although attractive as a result of its ability to *in situ* observe the interactions between organisms, FISH is not a priori considered to be very suitable for GMP effect studies. It is a laborious technique which allows only a limited analysis of the soil microbiota. This makes it in general unsuitable for comparing large numbers of samples, as usually is the

case in GMP effect research. If used, it should be combined with higher throughput techniques like soil DNA-based fingerprintings.

SIP – Stable isotope probing

Stable isotope probing is a technique that allows to link the structure of a microbial community to its function. Stable (heavy) isotopes, generally ^{13}C or ^{15}N, are incorporated in molecules of which the effect on the soil microbial community is to be followed. For instance, when plant compounds are labeled with ^{13}C, and the label is, following soil or rhizosphere processes, found in the nucleic acids of bacterial species thriving in the soil, then we have evidence that the respective species was an active utilizer of the compound. The destination of the heavy C or N atoms is thus tracked in cellular macromolecules like DNA, RNA or FA (Fatty Acids), by fractionating in soil extracts, the $^{13}C/^{15}N$ – containing fractions from the $^{12}C/^{14}N$ fractions, based on e.g. buoyant density. The active microbes, which specifically incorporated the heavy atoms, can thus be teased out and analyzed. The tracking of the stable isotopes (DNA- , RNA- or FA-based) utilizes a range of fractionation and detection techniques (Van Elsas, 2006). This may include DGGE (SIP-DGGE) or PLFA (SIP-PFLA).

What do SIP data look like?

Incorporation of stable (heavy) isotopes into cellular macromolecules and subsequent separation of the heavy fraction is always followed by molecular analyses. These may include (when based on DNA or RNA) various fingerprinting methods like DGGE, cloning followed by sequencing or microarray-based analyses. With these techniques, the ^{13}C or ^{15}N fractions are (following their singling out) fractionated. He resulting fingerprints can be compared to those of the light (assumedly inactive) fraction (Dumont and Murrell, 2005).

Interpretation and quantification of SIP results.

As described, after separation the heavy fractions are analyzed via various techniques. For instance, if DGGE is used as the analysis method, the heavy fraction may be compared to the light one by assessing if certain bands have higher intensities, which indicates activity by the underlying organisms in uptake of the labeled molecule of interest. As SIP relies on such additional techniques to reveal the data, all possible considerations on those techniques (described in the preceding and forthcoming sections) also count for this technique. In addition,

minor changes between the heavy and the light fractions might be hard to track, which hampers the analysis of subtle changes in substrate utilization.

Specificity and sensitivity

SIP relies on isotopic enrichments, which in turn relies on the activity of particular microorganisms involved in the process studied. As the replication rate of microorganisms in soil may be rather low, efficient DNA-based detection of the 13C enrichment in the cell can be severely limited. Hence, only very active bacteria that incorporated sufficient amounts of label are often detected (Dumont and Murrell, 2005). In contrast, detection based on soil-extracted RNA is often more sensitive, as this molecule is more abundant in the cell, and is not related to cell division. However, RNA-based analysis is more demanding due to the inherent instability of the molecule and the more difficult fractionation (Dumont and Murrell, 2005).

Other specificity and sensitivity issues are described in the section of the corresponding techniques used after SIP.

Reproducibility

No reproducibility issues have been described for SIP itself (that is, the incorporation of label and the subsequent separation of the heavy fraction). Assumedly, such incorporation is similar in similar ecological conditions. The questions about reproducibility lay mainly in the techniques used for subsequent analyses.

Further analysis

SIP relies on adequate further analyses (i.e. following incorporation of the heavy isotope and fractionation of the heavy fraction). As stated, these may be fingerprintings, clone libraries or PLFA profiles and allow the SIP method to be of use in soil microbiota assessments. All analysis techniques used afterwards have been described elsewhere in this section.

What can be stated when interpreting results of SIP?

The conclusions obtained by SIP involve statements in respect of which bacteria use a particular C source. In risk assessment of GM plants, the method will allow a shift in this utilization to become visible.

Conclusion

SIP is an attractive technique, as it allows for linking the structure of a soil community and its (defined) function to be analyzed. SIP can be of great use in GMP risk assessment to elucidate if a genetic modification alters, for instance, the nature and quantity of plant root exudates and thereby the utilizer microbial communities. However, SIP is laborious and does not allow for high-throughput analyses. It has therefore hardly ever been used in GMP effect research. Furthermore, the changes in root exudates and subsequent changes in populations might be very subtle, and therefore undetectable by SIP. Furthermore, if changes are found, the altered microbial communities not necessarily imply that soil health and functioning are altered, so the linkage to these parameters is obscure.

DNA microarrays

A DNA microarray is an array consisting of thousands of microscopic spots of DNA oligonucleotides, each containing a specific DNA sequence. Fluorescently-labeled target DNA obtained from a soil sample is hybridized to the arrayed probes and subsequently the hybridization signal is detected and quantified (Li et al., 2005; Yergeau et al., 2007)).

What do microarrays show?

A microarray result consists of thousands of colored dots indicating the presence, absence or relative abundance of particular gene sequences in the DNA from the sample. One colored dot theoretically represents one bacterial or fungal species (phylogenetic oligonucleotide array; POA - phylochip) or functional gene (functional gene array; FGA - geochip). The chips that have been developed currently contain up to 500.000 oligonucleotides of almost 10.000 different operational taxonomic units for the POA (phylochip, G. Andersen, USA) ((Brodie et al., 2006)), or 37,000 gene sequences of 290 functional groups involved in for instance nitrogen, carbon, sulfur and phosphorus cycling, metal reduction and resistance, and organic contaminant degradation for the FGA (geochip, Zhou, USA) (Zhou, 2003).

Interpretation and quantification of microarray results.

Interpretation of microarray results occurs via the analysis of the absence or presence of specific colored dots on the microarray, analyzed via a computer. Quantification of the intensity of the colored dots is also possible: good linear relationships between the amount of target DNA and the signal strength have been described (Yergeau et al., 2007). However, problems occur with

cross-hybridizations, especially with the phylogenetic microarray. The Andersen laboratory (Brodie et al., 2006)has developed a very well-wrought DNA microarray with plentiful controls to diminish this cross-hybridization problem. Sophisticated software is available that takes this into account and establishes "normalized" values (sequences from different organisms can be very similar, and subsequently hybridize to the wrong probe, overestimating that particular signal). Interpretation is also hampered by the enormous amount of information gathered, which needs data processing to yield tangible data.

Specificity and sensitivity

The sensitivity of the microarray technique is high provided sufficient DNA of good quality is obtained from a sample. For the functional microarray (the so-called geochip microarrays), 10-80 ng of DNA (approximately 100 cells) is necessary to yield a signal from soil DNA . For its phylogenetic counterpart (the so-called phylochip microarrays) , this is approximately 250-1000ng. Still, either the functional or the phylogenetic marker genes can occur in low abundance, i.e. below the detection limit. Therefore a PCR targeting the specific group of interest is sometimes performed before microarray analysis, but this introduces biases as described before. Pre-amplification, however, elevates the sensitivity of microarray detection to DNA levels of 1 ng. The sensitivity of a microarray is also affected by the size of the probes used. In general, the smaller the probe the more specific, but the less sensitive, microarray detection is (Zhou, 2003). This implies that researchers need to optimize microarrays in such a way that an optimum between sensitivity and specificity is achieved.

Next to the length of the probes used, the specificity of microarray analysis depends on the specificity of all probes in the array, which are optimized continuously. Specificity can be hampered by the aforementioned cross-hybridizations with non-target sequences. The observed signals thus are overestimations of the presence of certain microorganisms. Vice versa, the sometimes low sequence similarity of particular functional genes that encode proteins performing similar functions causes not all functional genes within a class to be detected. Functional genes generally have to have at least 85 percent similarity to the probe on the array slide to be detected. Therefore, evolutionarily-distant genes with similar function inherently are hard to detect with single probes (Zhou , 2003).

The specificity of a microarray is also hampered by the intrinsic characteristic that the slide used is fixed in the sense that no new sequences can be picked up. Therefore, important micro-organisms of which the functional complement is unknown can be totally missed in the analyses (Yergeau et al., 2007). This immediately touches upon another problem. Due to the enormous numbers of targets present in any soil, delicate choices need to be made as to which probes should be placed on the chip, and inevitably particular sequences will not be present on the chip.

An interesting feature of microarray analyses is that the chip specificity can be altered by altering the hybridization conditions from highly stringent to less stringent. However, less stringent conditions also incite more cross-hybridizations.

Reproducibility

The reproducibility and therefore comparability of microarray analyses is regarded as problematic, especially between laboratories (Zhou, 2003). This lack of reproducibility is mainly due to observed differences in labeling and hybridization efficiencies between runs, and to the use of different protocols in different labs.

Further analysis and statistics

Microarrays lead to an enormous amount of data which are difficult to analyze and integrate into a limited set of numbers or values (Zhou, 2003). Statistical methods are being developed but are often not appropriate for dealing with the complex data sets produced by the microarrays, although improvements are continuously developed.

Claims

As microarray analysis of soil microbial communities is a fairly recent technique, not many papers describe it in GMP risk assessment research. However, the technique has been used in other areas of microbial ecology and claims can be extrapolated to the field of GMP effect research. Researchers should, for instance, be cautious in attempting to link the results from an FGA analysis to the capacity for a specific biogeochemical function. The presence of one gene from a specific cycle does not necessarily indicate the presence of the whole cycle. Furthermore, one needs to recognize that, although the number of genes present on a microarray is huge, it never covers the entire genetic diversity in a sample, especially when dealing with the complex soil environment. Lastly, it is important to recognize the difficulty of analyzing the

overwhelming amount of information, which might lead to arbitrary choices in respect of which results are important. Within the next couple of years, when microarray analysis becomes a more standard technique in GMP risk assessment research, pitfalls and possibilities of claims regarding this technique will be more clear.

What can be stated when interpreting the results of microarrays?

Data obtained from microarray analyses describe the relative presence and abundance of particular microorganisms (phylochip) or functional genes (geochip) in a particular environment. In GMP risk assessment, such data are to be used in a comparative manner.

Conclusion

Microarray analysis is a very promising technique in GMP risk assessment research, as it allows for the screening of thousands of genes simultaneously. Of course, the specific link between microbial diversity and soil functioning remains the challenge of the near future. An additional challenge is how to cope with the enormous amount of data gathered. The data produced are often too overwhelming but statistical analysis tools that can deal with these data are continuously being improved.

Quantitative PCR - qPCR

The basis of qPCR is soil DNA. In qPCR, the formation of PCR products on the template DNA is monitored during the amplification as opposed to the end point detection which is used in normal PCR. By monitoring a fluorescent signal which is proportional to the formation of the product, the exact amount of the gene of interest can be calculated from a calibration curve, in which the starting point of detectable fluorescence is plotted against gene copy numbers (Lee et al., 1993; Wittwer et al., 1997)).

What does qPCR show?

The most commonly used qPCR protocol involves the use of so-called Taqman probes, which specifically bind to the gene of interest. These probes contain a so-called reporter and a quencher dye in such a way that when the probe is intact (i.e. when it is bound to the target), no fluorescent signal is released. When the probe is released due to the activity of the polymerase during the PCR reaction, a fluorescent signal is released. This signal is monitored on-line in the

thermal cycler, and the cycle in which the signal strength exceeds a certain (arbitrarily chosen) threshold value (often ten times the standard deviation of the baseline), it is used to calculate the number of target genes by comparing it to a calibration curve. The output, therefore, is no more than a simple cycle number which is then converted (using the calibration curve) to the inferred concentration of the respective target molecule (Wittwer et al., 1997).

Interpretation and quantification of qPCR results.

Interpretation of the qPCR is fairly straightforward, as concentrations of target molecules between different samples are compared to each other.

qPCR analysis can be hampered by spurious fluorescent signals that are released by non-target products. Primer and probe specificity, therefore, is of great importance in the qPCR. This, in turn, impinges on the fact that most primers often do not capture all sequence-divergent species, especially in sequence-divergent soil samples (Schena et al., 2004).

Another potential problem is that direct conversion of the amount of target molecules to cell densities may be hampered by the fact that sometimes multiple copies of a particular gene might be present in a bacterial species (Klappenbach et al., 2001).

Specificity and sensitivity

Sensitivity of the qPCR is very high, and often only 100 copies of the target molecules per gram of soil need to be present for a qPCR to readily detect it. The sensitivity of the qPCR depends, however, on amplicon length. To get good sensitivity, amplicon length should not exceed 250 bp, which makes most of the existing primer sets used in non-qPCR-based diversity studies non-usable. New primer sets have thus to be developed (Sharma et al., 2007). Furthermore, the necessity of short amplicon sizes narrows down the possibilities for primer design, making it hard to design primers which target the whole sequence diversity of a target gene.

As stated above, the specificity of a qPCR reaction highly depends on the probe and primer sets used, and non-target binding can severely skew the analysis.

Reproducibility

In general, qPCR is highly reproducible. The caveats are in the DNA extraction and purification (see before), and not so much in the qPCR technique.

Further analysis and statistics

qPCR normally does not involve specific further analysis (except for a check on the melting behaviour of the amplicons) or statistical analysis.

Claims

The interpretation of qPCR data is normally fairly simple and straightforward, and the claims of investigators therefore normally do not involve any misinterpretation or overstatement of the data. Most important to recognize is that the presence of particular genes does not imply any activity of those genes (Schena et al., 2004). Furthermore, changes in gene abundance do not necessarily affect soil health and functioning, although linear relationships between functional gene numbers (e.g. amoA, the gene involved in ammonium oxidation) and activity have been described.

What can be stated when interpreting results of qPCR?

Statements based on qPCR encompass the abundance of particular target genes or species in a soil sample. In a comparative fashion, qPCR can tell us about the effects of treatments on such gene abundances. If key functional genes are targeted (like amoA ((Hermansson and Lindgren, 2001)), the analysis can point to any effects of treatment on the abundance of these key functional genes, which may relate to activity.

Conclusion

qPCR is a powerful, simple and high-throughput technique, which allows to acquire specific knowledge on the quantities of functional or other genes of interest. The protocols used in qPCR have mostly been standardized and the outcomes are easily interpretable. The technique therefore has high potential for GMP risk assessment studies, even though inherent problems, mainly involving specificity, should be handled with care. It should furthermore be kept in mind that changes in the quantities of specific genes do not necessarily relate to soil health, implying that to make claims on soil health/functioning, such a relationship should always be established.

Pyrosequencing

Pyrosequencing encompasses the direct sequencing of DNA isolated from the environment.

What does pyrosequencing show?

The result of a typical pyrosequencing analysis encompasses thousands of sequences of limited sizes, e.g. between 100 and 250 (advanced machines go up to 400-450) bp each (Liu et al., 2007). These sequences, which collectively describe the soil metagenome, are of enormous variety and can be compared to those of public databases and the sequences of interest can be filtered out (i.e. 16S rRNA or functional genes). Visualization of the results is similar to that of clone library visualization. That is, the genes found can be placed in phylogenetic trees, which report on the relatedness to each other and to database entries.

Interpretation and quantification of pyrosequencing results.

The interpretation and quantification of pyrosequencing results is performed similar to that of clone libraries. Thus, the relative abundances in the soil DNA of selected target genes can be quantified and an impression of the diversity (richness and evenness) can be obtained.

Specificity and sensitivity

The sensitivity of pyrosequencing is strongly determined by the efficiency of soil DNA isolation. Further, although thousands of sequences can be produced, the ability to analyze the data forms a bottleneck affecting the sensitivity. The sensitivity is among the highest of all discussed techniques, because a direct high-throughput analysis of gene abundance and diversity is achieved.

The sensitivity of pyrosequencing is thus high. As no PCR or cloning biases are involved, the results of pyrosequencing in principle represent the true gene abundance and diversity in the DNA sample, and – if that is representative for the soil – the soil sample. Sensitivity is only limited by our capability to analyze the immense amount of data obtained and by the ability of the database to filter out the genes of interest. Evolutionarily-distant genes with similar function from previously unknown sources might be left out of the analyses as databases fail to identify such sequences. Given current analytical power, direct pyrosequencing will allow to dissect the system from top to bottom, i.e. starting with the most abundant species going down into the so-called rare biosphere (Liu et al., 2007; (Roesch et al., 2007).

A major advantage of pyrosequencing is that, through its ultra-high-throughput character and the lack of biases, many new sequences will be discovered. Thereby, novel insight into soil microbial diversity will be gained (Elshahed et al., 2008).

Reproducibility

So far, not much research has been performed towards assessing the reproducibility of pyrosequencing results. The possible sources of variation lie primarily in the sampling and the expected heterogeneity of the soil sampled.

Further analysis and statistics

Further analysis and statistics to be used are similar to those of clone libraries. The analyses are limited by our (bioinformatics) capabilities to analyze and deal with the enormous amount of novel data.

Claims

Pyrosequencing is a novel technique and hence no studies on GMP risk assessment have used it. In fact, only very few studies in other areas have applied it. Therefore, only a few things can be said about the claims regarding this technique. It is important to recognize that, although several biases are excluded with this technique, there still is the bias inherent in the soil DNA extraction technique used. Thus, certain sequences may be missed as a result of such a bias, and these potentially have key effects on soil health and quality. Furthermore, the relatively small fragments that are produced and the overwhelming amount of sequence data, which make arbitrary choices in the analyses insuperable, will hamper the analyses and thus influence claims (Elshahed et al., 2008).

What can be stated when interpreting results of pyrosequencing?

Statements are similar to those made on the basis of clone libraries. However, it is expected that, given the current rate with which sequencing throughput increases, pyrosequencing will eventually be able to cope with the overwhelming soil microbial diversity, thus finally providing a complete description of such diversity.

Conclusion

Pyrosequencing is a very promising technique in GMP effect research, which is at the moment very limited through its high costs. The high sensitivity and specificity of pyrosequencing, and the inherent exclusion of key biases in PCR and fingerprinting will make this technique one of the most important analysis methods based on soil DNA in the near future. When analysis of the enormous amount of data is more accessible, one might eventually get the best view of the true diversity in a soil sample by pyrosequencing. Still, the link between the observed gene abundance and diversity and the functioning and health of the soil needs to be elucidated.

Concluding remarks

This report describes the variety of techniques that are available for determining the changes in the soil microbial community that may come about by the presence of GM plants (Table 1, page 65). A few commonly used traditional and a range of advanced techniques are listed and their merits discussed. Over the years, the advanced techniques have gained considerable resolving power, specificity and sensitivity. Also, as-a-whole, most of the techniques have increasingly enhanced throughput, which is mainly attributed to the greatly enhanced DNA sequencing capabilities.

However, although progress has been made, any conclusions on the link between the data obtained with any method and soil health and functioning are still hard to make. This is mainly due to our lack of understanding as to whether such a link exists and how is is shaped. Furthermore, the biases which are inevitable present when it comes to soil DNA isolation, PCR and/or cloning play a role. The lack of conclusive power is also attributable to our lack of knowledge about soil resistance and resilience, i.e. on how a soil responds to stress.

Therefore measurements of diversity and (more importantly) community structure should be combined with measurements of already established parameters which are indicative of soil functioning, starting with soils where we know that they do differ in functioning. First we have to decide which aspects of soil functioning we are aiming for: carbon and nutrient cycling, nutrient supply to the crops, maintenance of soil structure, disease suppression, or as many aspects as possible. Nutrient cycling and supply is reflected in total microbial biomass and activity, in potential carbon and nitrogen mineralization, but also in amounts of mycorrhizal fungi and nitrogen-fixing bacteria. Soil structure is correlated with amounts of fungal hyphae and microbial exudates (polysaccharides, labile carbon). Disease suppression is related to total microbial biomass and activity and the presence of specific antagonists. If changes in these indicators coincide with changes in community composition, then relationships between community structure and soil ecosystem functions (or services) can be resolved.

Despite the methodological progress, no single method or combination of different techniques can be stated as superior and standardize the analyses. Combination of novel techniques such as pyrosequencing, which gives enormous amounts of information on both functional as phylogenetic diversity, and phylogenetic microarrays, which give information on the presence

and diversity of essential microorganisms, is preferable. This then can be combined with biomass and activity measurement using for instance SIR. However, various other combinations can be as informative as the proposed combination.

To be able to compare results and eventually understand the effect of changes on soil health and functioning, a baseline to compare any results to is necessary. One of the possible solutions for this issue involves the establishment of a normal operating range (NOR) of a certain soil. This NOR will describe the minimum community structure, diversity and function, that a given soil has to meet to be considered healthy. To this baseline, future results of GMP research can be compared. However, considering the large number of different soils and the enormous diversity of microorganisms, it remains unknown if NOR will be a usable concept.

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Table 1: Overview of characteristics of techniques for analysis of the living soil

Technique		Type	Sensitivity	Technical requirements	Costs	Level of throughput	Automation	Processing time	Reproducibility	Specificity	Quantifiability
Chloroform fumigation		Chemical	Low	Low	Low	Low	Not possible	Medium	High	Low	Medium
SIR		Metabolic	Low	Low	Low	Low	Not possible	Low	High	Low	Medium
Colony forming units		Culture dependent	Medium	Low	Low	Low	Not possible	Medium	High	Low	High
SIP		In situ	Low	Medium	Medium	Low	Not possible	Medium	High	N/a	N/a
DGGE/TGGE	Phylogenetic	Fingerprint	Medium	Medium	Low	Low	Not possible	Medium	Medium	Medium	Medium
	functional	Fingerprint	Medium	Medium	Low	Low	Not possible	Medium	Medium	Medium	Medium
SSCP		Fingerprint	Medium	Low	Low	Low	possible	High	Medium	Medium	Medium
T-RFLP		Fingerprint	Medium	Medium	Medium	Medium	Possible	Medium	High	Low	Medium- High
Clone library		Molecular	High	High	Medium	High	Possible	High	High	High	High
PLFA		Fingerprint	Medium	Medium	Medium	Medium	Possible	Medium	High	Low	Medium-High
FISH		In situ	Medium	Medium	Low	Low	Not possible	High	n/a	Low	High
Microarrays		Molecular	High	High	High	high	possible	High	Medium	Very High	High
qPCR		Molecular	High	High	Medium	High	possible	Medium	High	High	High
Pyrosequencing		Molecular	Very high	High	Very high	High	possible	High	High	Very high	High

Table 1 (continued)

technique		Specific for (species/community/system)	Major pitfall (interpretational)	Interpretation of results	Advantages (technical)	Disadvantages (technical)
Chloroform fumigation		Community	No information on community changes.	Very limited information on microbial community. No information on functional or phylogenetic changes	Cheap and fast.	Low sensitivity and specificity.
SIR		Community	Only information on microorganisms which are able to use added compound, no information on community changes	Similar to chloroform fumigation	Cheap and fast, information on active community.	Low sensitivity and specificity.
Colony forming units		Species/community	Only culturable microorganisms visible (only 1% of community).	Limited information, no information on soil health and functioning.	Easy and cheap, ability to further analyze colonies including metabolic characteristics or whole genome sequence	Low resolution. Not representative. Morphological differences hard to distinguish makes laborious further analysis necessary.
SIP		Species	Relies on activity of microorganisms, which can be very low	Direct information on incorporation on Carbon or Nitrogen atoms in the community is attractive, but changes in exudates should be fairly big to detect changes.	Gives information on the active community. Relation between structure and function can be elucidated.	Necessity for performing additional techniques before obtaining information. Low sensitivity. Low throughput.
DGGE/TGGE	Phylogenetic	Species/community	Only species >1% abundance are visible.	No information on soil health and functioning	Well optimized and easy, bands can be excised.	Intergel comparison difficult. Artifacts
	Functional	Species/community	Few information on functional genes hampers primer design	Information on functional genes, but not on activity of those genes. Relationship between functional gene shift and soil functioning remains unknown	Same as above; Higher resolution than phylogenetic DGGE/TGGE	Same as above
SSCP		Species/community	Similar to DGGE/TGGE	Similar to DGGE/TGGE	No need for gradient or GC-clamp	Laborious preparation of sample.

T-RFLP	Species/community	Terminal restriction fragment not specie specific.	Similar to DGGE/TGGE	Easy comparisons between samples, possibility of obtaining different fingerprints from same sample	No identification possible
Clone library	Species/community	Large cloning bias.	Lots of information on diversity, but no information on activity. No information of soil health an functioning.	Currently very high throughput, direct information on sequences. Sensitive.	Manual checking of sequences laborious.
PLFA	Species/community	Fatty acids not specie specific	Similar to DGGE/TGGE	Easy comparison between samples.	Laborious protocols, expensive equipment.
FISH	Species	Only very active organism are visible.	Information on active, dominant community	*in situ* technique: interactions and location visible	Large background in soil. Low resolution and low-throughput.
Microarrays	Species/community	Cross-hybridizations, difficult data analysis	Large amounts of information but no information on soil health and functioning	All-in-once analysis in high-throughput. High potential for comparative studies	Costly, not possible to detect novel sequences
qPCR	Species/community	Misinterpretation through non target signals	High sensitivity, real numbers on abundance of genes, but no info on cell numbers.	Easy to interpret results, technically well developed	Novel primer sets needed with limited possibilities on amplicon length. Calibration curves needed
Pyrosequencing	Species/community	Enormous amount of data makes data analysis difficult	Non biased view on diversity, many novel sequences detectable, large amount of data	Very sensitive technique, very high throughput and direct information on sequences.	Short sequence length are obtained, very costly.